Biotechnology Advances 43 (2020) 107602

Contents lists available at ScienceDirect

Biotechnology Advances

journal homepage: www.elsevier.com/locate/biotechadv

Research review paper

Biotechnological production of astaxanthin from the microalga *Haematococcus pluvialis*

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ARTICLE INFO

Keywords:
Astaxanthin
Microalgae
Haematococcus

ABSTRACT

Although biotechnologies for astaxanthin production from *Haematococcus pluvialis* have been developed for decades and many production facilities have been established throughout the world, the production cost is still high. This paper is to evaluate the current production processes and production facilities, to analyze the R&D strategies for process improvement, and to review the recent research advances shedding light on production cost reduction. With these efforts being made, we intend to conclude that the production cost of astaxanthin from *Haematococcus* might be substantially reduced to the levels comparable to that of chemical astaxanthin through further R&D and the future research might need to focus on strain selection and improvement, cultivation process optimization, innovation of cultivation methodologies, and revolution of extraction technologies.

1. Introduction

Astaxanthin (3,3'-dihydroxy-β,β-1-carotene-4,4'-dione) is one of naturally occurring xanthophylls, which, together with carotenes, constitute a class of more than 600 molecules called carotenoids. All carotenoids have a structure of 40 carbon unsaturated backbone and are biologically synthesized from a basic isoprene structure. The difference between carotenes and xanthophylls is that carotenes consist only of hydrocarbons but xanthophylls contain one or more oxygen bearing functional groups such as hydroxyl and ketone (Ambati et al., 2014; Gong and Bassi, 2016). Astaxanthin contains both a hydroxyl and a ketone group at each of its molecular ends, and its chemical structure is shown in Fig. 1 (Dey and Harborne, 1997; Lohr, 2009). Although almost all carotenoids have antioxidant properties which primarily derive from conjugated double bonds and thus have a range of functions in human health, the antioxidant activities of astaxanthin is found to be superior than most, if not all, other carotenoids probably because its hydroxyl and ketone groups can be easily oxidized (Terao, 1989; Kobayashi and Sakamoto, 1999; Naguib, 2000; Dose et al., 2016). Also, due to its bearing hydrophilic groups on both ends and lipophilic backbone structure in the middle, the molecule can easily traverse the cell membrane without affecting the structural integrity or the electron density of the double layered membrane, and thus astaxanthin may exert antioxidant function both inside and outside of tissue cells (Tripathi et al., 2002; Ambati et al., 2014). These features mechanistically make astaxanthin one of the most valuable carotenoids in human health and animal nutrition (Eggersdorfer and Wyss, 2018; Galasso et al., 2018; Hu et al., 2018; Rodriguez-Concepcion et al., 2018).

Astaxanthin has very high market value due to its applications in human and animal nutrition. With more than a dozen of health benefit claims, astaxanthin is currently marketed as a food supplements, but the primary application of astaxanthin is still for aquaculture feed for the time being (Lorenz and Cysewski, 2000; Guerin et al., 2003; Yuan et al., 2011; Shah et al., 2016; Eggersdorfer and Wyss, 2018). The total market value of astaxanthin is reported to be over US$550 million in 2017 and is expected to reach US$800 million by 2022 with a CAGR (Compound annual growth rate) of 8.0% , according to recently published market research reports (ReportLinker, 2017; Global Market Insights, 2018; Grand View Research, 2019). The market share of aquaculture feed segment was still the largest, followed by the sector of human dietary supplement, cosmetics and others. The market value of astaxanthin for human nutrition was estimated to be over US$200M in 2017 and might hit US$1 billion in 2025 due to continuous extremely

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https://doi.org/10.1016/j.biotechadv.2020.107602
Received 14 January 2020; Received in revised form 5 July 2020; Accepted 13 July 2020
Available online 22 July 2020

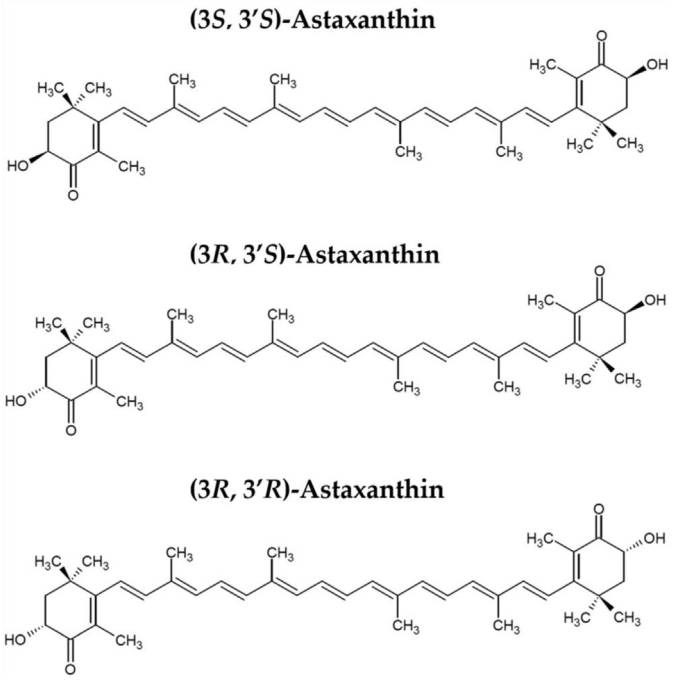

Fig. 1. Structural formula of astaxanthin

high sales growth, according to the information from major manufacturers such as Cyanotech Inc., USA and recently published market research reports (ReportLinker, 2017; Grand View Research, 2019).

Astaxanthin can be produced either by chemical synthesis at a lower cost or biosynthesis at a higher cost (Li et al., 2011). Chemical synthesis can be realized through the Wittig reaction of 3-methyl-5-(2,6,6-trimethyl-3-oxo-4-hydroxy-1-cyclohexenyl)-2,4-pentadienyltriarylphosphonium and 2,7-dimethyl-2,4,6-octatrienedial (Ernst et al., 1991; Krause et al., 1997). Biosynthesis of astaxanthin can be achieved by cultivation of microalgae, yeast, bacteria and plants. Chemical and biological astaxanthin have different structures. Astaxanthin has two chiral centers at the 3- and 3′-positions of the β-ionone ring with a hydroxyl group at each end of the molecule, and therefore, there are three stereoisomers: (3R,3′R), (3R,3′S) (meso), and (3S,3′S) (Please refer to Fig. 1). According to the extent of hydroxyl group esterification with fatty acids, astaxanthin can exist in free, mono-ester, and di-ester forms. Chemical astaxanthin on the other hand contains a mixture of isomers (3S, 3′S), (3R, 3S), and (3R, 3′R) with a ratio 1:2:1 respectively and is not esterified. Astaxanthin from microalgae and plants consists mostly of (3S, 3′S) isomer and is mostly esterified, astaxanthin from bacteria is also (3S, 3′S) isomer but not esterified, yet astaxanthin from yeast is exclusively (3R, 3′R) isomer and also not esterified (Higuera-Ciapara et al., 2006; Huang et al., 2013; Ambati et al., 2014; Katsumata et al., 2014). Chemical astaxanthin is not approved for human direct consumption due to concerns about the safety issues arising from structural differences between chemical and biological (natural) astaxanthin and from possible residues of chemical synthesis intermediates although it is approved for aquaculture feed applications (Capelli et al., 2013). Natural astaxanthin has been approved not only for aquaculture feed but also for dietary supplements, cosmetic ingredients and other applications by regulatory agencies in the USA, China, Japan and several European countries (Shah et al., 2016; García et al., 2017). Currently 95% of astaxanthin is produced chemically and meets the market demand of aquaculture because of lower production costs and lower prices, whereas natural astaxanthin, although produced in much smaller quantities, commands much higher prices (roughly at $5000 per kg, not extracted in biomass) and is mostly marketed as dietary supplements, cosmetic ingredients, and food additives (Lim et al., 2017).

Replacing of chemical astaxanthin for feed application with biological astaxanthin has been very attractive although current production of biological astaxanthin is not as cost effective as chemical astaxanthin (Nguyen, 2013). The marketing prices of chemical astaxanthin are pretty high, above $2000 per kg, compared with other biologically produced carotenoids such as lutein and zeaxanthin (Gong and Bassi, 2016). The production cost of natural astaxanthin has been reducing rapidly due to technical improvements and is getting close to and even potentially lower than the marketing prices of chemical astaxanthin (Li et al., 2011; Benemann et al., 2018). Biological astaxanthin is in fact found to be superior to chemical astaxanthin in terms of coloring effects and antioxidant activities, and besides the structural and functional differences with natural astaxanthin, chemically derived astaxanthin also raises concerns in human health and the environment (Tejera et al., 2007; Capelli et al., 2013; Capelli et al., 2019). Today's consumer calls for safe and ecofriendly food production, and thus, reducing the production cost of biological astaxanthin to replace chemical astaxanthin for animal feed becomes interestingly favorable (Gómez et al., 2013; ABC News, 2016).

Several biotechnological processes are promising to replace chemical synthesis as methods for biological astaxanthin production. As mentioned before, in nature, astaxanthin can be synthesized by microalgae, fungi, bacteria and plants either constitutively or through metabolic engineering (Higuera-Ciapara et al., 2006; Ambati et al., 2014; Rodriguez-Concepcion et al., 2018). Processes based on these organisms have been under development to produce astaxanthin (Mann et al., 2000; Lee et al., 2004; Ip and Chen, 2005; Rodríguez-Sáiz et al., 2010; Huang et al., 2013). However, large scale production of biological astaxanthin has so far only been carried out through cultivation of one green microalgal species, *Haematococcus pluvialis* (Schmidt et al., 2011; Lim et al., 2017). This work intends to evaluate the current production processes and facilities, to analyze R&D research strategies for process improvement, and to review important research advances that might immediately facilitate the technical improvement for production cost reduction, and to propose valuable ideas for process optimization.

2. Current production

After about four decades of industrial development, dozens of *Haematococcus* astaxanthin production facilities have already been established throughout the world. The facilities vary not only in scales of production but also in cultivation methodologies, and thus vary in production costs.

2.1. Current production processes

Most companies employ a two-step process to cultivate *Haematococcus* for astaxanthin production (Harker et al., 1996; Olaizola, 2000; Fábregas et al., 2001; Olaizola, 2003; Han et al., 2013; Liu et al., 2017). This process has been developed based on the life cycle and cell biology of *Haematococcus*, which mainly consists of primarily at least two stages, the green stage when replete-nutrient medium is supplied under favorable growth conditions, and the red stage when nutrients are depleted under conditions of stress. During the green stage, the cells can reproduce and accumulate biomass but not astaxanthin, while during the red stage, the cells lose the ability of reproduction and motility but are capable of accumulating as high as 5% of astaxanthin on dry biomass weight (Kobayashi et al., 1997; Fábregas et al., 2001; Kang et al., 2005). Thus for the two step approach, first *Haematococcus* cells are cultivated usually in photobioreactors and in normal growth conditions for cell proliferation and biomass accumulation, and then cells are transferred into either larger scale photobioreactors or raceway ponds and are steered towards astaxanthin accumulation and encystment under stress and nutrient-deficient

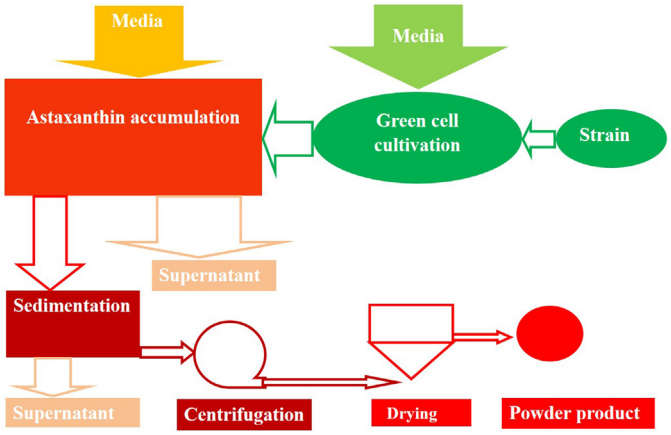

Fig. 2. Diagrammatic representation of two-step cultivation processes for Haematococcus

conditions. The biomass accumulation step, also called green phase, usually is performed in enclosed photobioreactor systems phototrophically or mixotrophically. The astaxanthin accumulation step, also called red phase, is usually carried out phototrophically in raceway ponds or large scale outdoor tubular photobioreactors. In very rare cases, the red phase has been performed in enclosed stainless steel fermenters either heterotrophically or mixotrophically even though research results have indicated that phototrophic induction of astaxanthin is more effective (Kang et al., 2005). A schematic diagram representing the current production processes is shown in Fig. 2.

After being encysted, the *Haematococcus* cells are further processed for harvest of biomass and extraction of astaxanthin. Most companies employ gravity sedimentation as the first measure to harvest the biomass, and then the collected slurry is dewatered again by centrifugation to get algae paste. The paste cells are usually cracked by high pressure homogenizers or bead millers and then dehydrated by either spray or belt dryers. For astaxanthin extraction from cracked biomass, edible oil or organic solvents, such as ethanol, propanol, and hexane, were used at the early stage of industrial development of astaxanthin production in 1990s. But currently most companies use supercritical fluid extraction with CO_2 as the only solvent (Li et al., 2011; Han et al., 2013; Liu et al., 2017; Zgheib et al., 2018).

2.2. Current production facilities

More than ten commercial plants have been established for astaxanthin production from *Haematococcus* since past decades. Among those, Cyanotech Inc., USA, Mera Pharmaceuticals Inc., USA, Alga Technologies Inc., Israel, Fuji Chemical Industries Co. Ltd., Japan, and Parry Pharmaceuticals Inc., India, have established astaxanthin production facilities in the late 1990s and early 2000s (Jin et al., 2006; Del Campo et al., 2007). More recently more than ten plants have been constructed, and more pilot scale plants might be under development. Table 1 shows a list of *Haematococcus pluvialis*/astaxanthin production companies. Fig. 3 and Fig. 4 are pictures of production facility courtesy of two of these companies.

Mera Pharmaceuticals Inc. (reorganized from Aquasearch Inc.) was among the first a few companies which established large scale astaxanthin production facilities globally. The company was located in Kona, Hawaii, USA, where weather conditions are extremely suitable for outdoor *Haematococcus* cultivation (Leonard et al., 1999a; Leonard et al., 1999b; Olaizola, 2000; Olaizola, 2003). The company employed a two-step autotrophic cultivation approach. The green phase of microalgae growth was conducted in 25000L enclosed and computerized outdoor photobioreactor systems, and the red phase in raceway ponds. A similar approach but with a different type of outdoor photobioreactors, so called covered raceways, for green phase cultivation, was used by Cyanotech Inc., which set up the production facility at the same location as Mera Pharmaceuticals Inc., but on a much larger scale in the late 1990s. AlgaTechnologies Inc., located in Eilat, Israel, also established a *Haematococcus* cultivation facility back in the late 1990s. Quite differently from its American competitors, the company used glass tubular photobioreactors for both green and red phase to phototrophically cultivate microalgae (Boussiba et al., 2000). Fuji Chemical Industries Co. Ltd. claimed the world's first industrial *Haematococcus* plant, operated by its subsidiary, AstaReal AB in Sweden. Instead of cultivating microalgae phototrophically outdoors using sunlight, they cultivated both green phase and red phase microalgae mixotrophically in closed stainless steel fermenters with light conducting tubes submerged in the media (Olaizola and Huntley, 2003). Parry Pharmaceuticals Co. Ltd., located in Chennai, India, patented its own way of green phase cultivation although using conventional raceway pond technology for red phase cultivation (Thomas et al., 2004). Instead of cultivating the green phase cell culture in expensive photobioreactor systems, the company conducted green phase in raceway ponds by germination of haematocyst cells from red phase which could reproduce ten-fold faster than green phase cells, hence reaching high biomass levels in a short period of time (Kobayashi et al., 1997; Triki et al., 1997; Hagen et al., 2001). In this way, expensive photobioreactor systems could be avoided. However, probably due to complexity of the biology of *Haematococcus* and inefficacy of process development, their production processes suffered frequent failures.

Recently, several production facilities, which might be equivalent to Cyanotech's scale, have been established in Asia and South America. Among them, AstaBiotec LLC, located in Shilin, China, and Yunnan Alphy Biotech Co., Ltd., located in Chuxiong, China, both employ technologies similar to AlgaTechnologies Inc., and glass tubular outdoor photobioreactors are used to cultivate both green and red phase microalgae (Liu et al., 2017). Yunnan LvA Bio-Tech. Co. Ltd., located in Lijiang, China, has established red phase cultivation in raceway ponds, which is similar to that of Cyanotech, with minimal amount of microalgae cultivated in small scale flat panel photobioreactors (Zhang et al., 2009). Algatec S.A., located in Santiago, Chile, and Atacama Bio Natural Product S.A., located in Atacama, Chile, have also used raceway ponds for red phase cultivation, but it is not very clear how they cultivate green phase microalgae.

2.3. Current production cost estimation

A detailed production cost analysis of astaxanthin from *Haematococcus* was reported for the first time by Li et al. (Li et al., 2011). These researchers conducted outdoor investigations in Shenzhen, China, and developed the optimized astaxanthin production processes using *H. pluvialis* at pilot scales with a two-step approach. For the first step, microalgae were cultivated in airlift tubular photobioreactors, and for the second step, microalgae were stressed in raceway ponds for astaxanthin production. Based on the pilot process parameters developed, a conceptual plant with virtual production capacity of 36 tons of biomass or 900 kg astaxanthin per year was designed. Then the production cost was analyzed and estimated based on capital and operation cost of the conceptual plant. It was concluded that the production cost of microalgae biomass and astaxanthin could be as low as US$18 per kg and US$718 per kg (in biomass, not extracted) respectively. The economics of astaxanthin production based on outdoor flat panel photobioreactors was evaluated in Thailand back in 2011 (Issarapayup et al., 2011). Although the definite values of production cost of biomass and astaxanthin were not given, the authors concluded that as much as US$614 per kg of biomass could be saved by adopting outdoor cultivation compared with indoor operation, and scaling up flat panel photobioreactors from 17L to 200L could further decrease biomass production cost from US$394 per kg to US$242 per kg biomass. Another case of thorough cost analysis has been performed

Table 1
Major haematococcus pluvialis/astaxanthin production companies

Company	Locations	Methodologies	Plant size / Capacity[a]
Algaetech International Sdn. Bhd.	Lumpur, Malaysia	Plastic bag PBR	< 5 ha / < 10 t/y
Alga Technologies Inc.	Eilat, Israel	Glass tubular PBR + Glass tubular PBR	< 10 ha / < 50 t/y
Algalif AS	Reykjanesbaer, Iceland	Glass tubular PBR	< 5 ha / < 10 t/y
Alimtec S.A.	Santiago, Chile	Raceway pond	< 5 ha / < 10 t/y
AstaBiotec LLC (BGG)	Shilin, China	Glass tubular PBR + Glass tubular PBR	< 20 ha / < 100 t/y
Atacama Bio Natural Product S.A.	Atacama, Chile	Raceway pond	< 10 ha / < 50 t/y
ZionBio Algae Health Co.	Baoshan, China	Fermenter + Glass tubular PBR	< 5 ha / < 25 t/y
Cyanotech Inc.	Kailua-Kona, USA	Covered Raceway pond + Raceway pond	< 20 ha / < 100t/y
Kunming Biogentic Co. Ltd.	Kunming, China	Bubble column PBR + Column PBR	< 10 ha / < 1 t/y
Mera Pharmaceuticals Inc.	Kailua-Kona, USA	Tubular PBR + Raceway pond	< 5 ha / < 1 t/y
Ningbo Honglong Biotech. Co., Ltd	Ningbo, China	Raceway pond	< 10 ha / < 5 t/y
Parry Nutraceuticals Co., Ltd.	Chennai, India	Raceway pond	< 10 ha / < 1 t/y
Minghui Biotech Co. Ltd.	Weihai, China	Bubble column PBR + Glass tubular PBR	< 10 ha / < 5 t/y
MC Biotech Sdn Bhd	Gadong, Brunei	Glass tubular PBR + Glass tubular PBR	< 10 ha / < 25 t/y
Supreme Health NZ Ltd.	Nelson, New Zealand	Indoor LED PBR + Indoor LED PBR	< 2ha / < 2 t/y
Sweden AstaReal AB.	Gustavsberg, Sweden	Indoor PBR + Indoor PBR	< 5 ha / < 25t/y
U.S.A-AstaReal, Inc.	Moses Lake, USA	Indoor PBR + Indoor PBR	< 10 ha / < 50 t/y
Yunnan Alphy Biotech Co., Ltd.	Chuxiong, China	Glass tubular PBR + Glass tubular PBR	< 10 ha / < 50 t/y
Yunnan LvA Bio-Tech. Co. Ltd.	Yongding, China	Flat panel PBR + Raceway pond	< 5ha / < 25 t/y

[a] Plant size/Capacity information is roughly estimated based on personal communication and pubic news.

Fig. 3. Haematococcus cultivation in glass tubular photobioreactor systems. Picture courtesy by AstaBiotech

Fig. 4. Haematococcus cultivation in open raceway ponds. Picture courtesy by Acatama Bio

through a theoretical process model approach for two European cities (Livadeia, Greece and Amsterdam, the Netherlands) (Panis and Carreon, 2016). The model assumed to use a hybrid system for photoautotrophic cultivation, fence type photobioreactors for green phase cultivation and raceway ponds for red phase cultivation, on a piece of land of 1 ha. The model simulations showed that 426 kg per year and 143 kg per year astaxanthin production capacity could be reached in Livadeia and Amsterdam respectively, and for the best scenarios, the production costs of natural astaxanthin (in biomass, not extracted) for facilities at Livadeia and Amsterdam were predicted at €1536 per kg and €6403 per kg respectively (in biomass, not extracted). A production process economic feasibility study which was primarily focused on downstream processing has been recently reported by Zgheib et al. (Zgheib et al., 2018). The authors assumed that *Haematococcus* biomass with roughly 2.5% of astaxanthin were available at US$36.16 per kg and proposed a downstream processing facility with a production capacity of 2592 kg of astaxanthin per year theoretically located in Bekaa, Lebanon, a place with plenty of sunshine and appropriate temperature for microalgae cultivation. By considering raw materials cost, process cost and capital cost, they reached the conclusion that the proposed process would be economically feasible if the price of extracted astaxanthin was higher than US$1500 per kg.

3. Production cost reduction

Although astaxanthin production processes from *Haematococcus* have been under intensive development since the 1970s and more than dozens of production facilities have been established globally, the technology is still in its infancy and far from being mature (Jin et al., 2006; Del Campo et al., 2007; Cysewski, 2017). Astaxanthin production from *Haematococcus* is a very intricate process because of the complicated biology and physiology of the microalgal cells and biochemistry of astaxanthin accumulation, and even after more than 20 years of operation, companies like Cyanotech Inc., still face the challenge to maintain consistent and reliable production (Cysewski, 2017). The current production processes are different from each other in terms of cultivation modes including phototrophic, heterotrophic, mixotrophic and combination of those, of cultivation environment including indoor and outdoor conditions, and of cultivation apparatus ranging from totally enclosed photobioreactors to totally open raceway ponds. Thus the current production cost of different companies also varies substantially.

The production cost of astaxanthin from microalgae might be substantially reduced with the further rational development of appropriate microalgal technologies. The current production cost of microalgae biomass such as *Spirulina* (*Arthrospira platensis*, used mainly as nutritional supplement) can be as low as $4 per kg (Benemann et al., 2018).

From a production facility point of view, the only major difference between *Spirulina* and *Haematococcus* is that expensive photobioreactor systems are not used for *Spirulina* production and only raceway ponds are employed. If the high cost of photobioreactor cultivation of *Haematococcus* green phase can be reduced to the level of that of raceway pond cultivation, the production cost of *Haematococcus* biomass might be reduced to the level of that of *Spirulina*. If that were the case, the production cost of astaxanthin from *Haematococcus* could be reduced to as low as $130 per kg which is comparable to the cost level of chemical astaxanthin, assuming 3% of astaxanthin content in dry biomass (Li et al., 2011).

Although, at least in theory, the production economics could be substantially improved if low cost photobioreactor systems were developed for *Haematococcus* cultivation, unfortunately, in practice, low cost industrial photobioreactor systems developed for microalgal suspension cell culture have only achieved marginal success over the last three decades and might not constitute an area of R&D with sufficient promise in the future (Carvalho et al., 2010; [illegible] et al., 2015; Acién et al., 2017). Instead, the R&D strategies to reduce production cost may need to focus on strain selection and improvement, optimizing current production processes, applying recent research breakthroughs in cell biology, physiology and biochemistry of *Haematococcus* for production process improvement, and developing innovative cultivation approaches specifically geared towards avoiding the use of expensive photobioreactor systems.

3.1.1. Strain selection and improvement

Strain selection and improvement might be the most important step for the process development of industrial microbial productions. *H. pluvialis* is a globally distributed species, and numerous strains are collected and identified almost every year worldwide. The wildtype strains vary substantially in phenotypes, and selecting the best performance strains might be essential to initiate a successful process development project. Further genetic modification of wildtype strains might also be worth the effort to cut down production cost.

3.1.2. Strain selection

Strain selection is a vital step for industrial cultivation of microalgae just like that of other industrial microorganisms. Fortunately, *H. pluvialis* is a globally distributed species, and thus it might differ considerably in phylogeny and physiology from location to location. The existing biological diversity would provide the opportunity for selecting good performance strains for astaxanthin production without the need for genetic manipulations. Although the idea is fairly straightforward, only recently these assumptions have been tested experimentally and specifically examined, with some interesting observations reported (Allewaert et al., 2015; Gómez et al., 2016; Allewaert et al., 2017). With the intent to clarify the relation between the genotype and phenotype of some European *Haematococcus* isolates, Allewaert et al. studied their phylogenetic diversity based on internal transcribed spacer (ITS) rDNA and the chloroplast gene rbcL (ribulose-1,5-biphosphate carboxylase/oxygenase large subunit), morphology and cell growth rates. Three strains of *H. pluvialis* were identified and described by Allewaert et al. in 2015, and the optimal growth temperature was found to be between 17°C and 23°C, which was considerably lower than previously reported 25°C (Cifuentes et al., 2003; Dalay et al., 2007; Hanan et al., 2013). This result indicates the importance of selecting strains of cultivation for specific facility locations under different weather conditions. Interestingly, Allewaert et al. (2017) found that industrially cultivated strains had shown lower astaxanthin productivity than their natural isolates, indicating it might be a good strategy to use natural isolates instead of long-term laboratory cultivated stains for industrial application. Another observation was that the difference in overall astaxanthin productivity among strains was mostly determined in the stress phase or red phase, and the growth rates of the strains in the green phase were roughly similar. This suggests further R&D to reduce production cost through strain selection might need to focus on selecting strains with higher astaxanthin accumulation capacity at red stage instead of cell biomass growth rates at green stage and thus on optimizing specifically red phase cultivation conditions.

3.1.3. Strain improvement by mutagenesis

Mutagenesis might be an effective way to modify the genotype and phenotype of interesting microalgal strains for industrial exploitation as is the case for classical industrial microorganisms. Mutagenesis occurs naturally and can also be easily achieved in laboratories by physical or chemical mutagens even without any prior genomic knowledge of the microalgal species. It has also been proven that the hereditary traits of microalgae can be easily improved by artificial mutagenesis. Since the laboratory-based mutagenesis processes of microalgae by physical or chemical mutagens are not substantially different from the natural mutation processes, the resulting mutants with desired traits are not considered as Genetically Modified Organisms (GMOs) and can be employed for industrial application without special concern on regulation. While generation of a large population of mutants can be easy, the screening of desired phenotypes can be very laborious and challenging. Desired phenotypes can be multiple and complex, each of which might require a specific and not easily manageable screening protocol (Hlavova et al., 2015).

The desired phenotypes of *Haematococcus* for astaxanthin production include mostly high growth rates at the vegetative stage, high astaxanthin accumulation rates at the encystment stage, and high astaxanthin titre in the final biomass. Most research work on mutagenesis of *Haematococcus* has been focusing on developing protocols of mutating the microalgae and screening of mutants for the above phenotypes. UV radiation and chemical mutagens, such as ethyl methanesulphonate (EMS) and diethyl sulfate (DES), were first used to mutate the cells, and herbicides (also as carotenoid synthesis inhibitors), such as diphenylamine (DPA) and nicotine, were used as selecting agents for mutants. The resulting mutants were proved to be better than wildtype strains in terms of astaxanthin content, and the activity of lycopene cyclase, an enzyme involved in astaxanthin synthesis, was found to be higher in mutants under laboratory conditions (Tripathi et al., 2001; Chen et al., 2003). With a similar mutating techniques, a new herbicide, glufosinate, was used as screening agent by Kamath et al. in 2008, and mutants with up to 3.8% astaxanthin content, which was 77% more than that of the wild type strain in laboratory conditions, were obtained (Kamath et al., 2008). N-methyl-N-nitro-nitrosoguanidine (MNNG) was also shown to be an effective mutagen for *Haematococcus*, leading to the acquisition of some cell wall deficient and astaxanthin overproducing mutants (Wang et al., 2005; Hu et al., 2008). A chemical mutagenesis and herbicide selection strategy was first demonstrated to be successful in improving strain properties at industrial scales by Gomez et al. in 2013. They started with a wildtype Chilean strain and developed several mutants with interesting traits, one of which was cultivated in 25000-L outdoor raceway ponds. The productivity of the tested mutant showed about 30% of productivity enhancement compared with the wildtype (Gómez et al., 2013). A three step mutation by UV, EMS and DES and further screening on agar plate with DPA addition were adopted by Wang at al. in order to acquire mutants with more than one desired traits, and some resulting mutants did exhibit all the desired traits for astaxanthin production although the experiments were performed only in the laboratory (Wang et al., 2016).

Recent research works on mutagenesis of *Haematococcus* have been focused on applying new mutating techniques and developing innovative screening procedures in order to obtain mutants with desired traits. Both γ-ray irradiation and dielectric barrier discharge plasma technologies were used to mutate *Haematococcus*, and similar results as UV or chemical mutagens were achieved (Cheng et al., 2016; Liu et al., 2016). From the populations of EMS mutants, the cell colonies which

grew slower under mixotrophic conditions than heterotrophic conditions on microplates were selected out as photosensitive mutants by Hong et al. in 2012. These mutants were found to be more prone to light induction of astaxanthin accumulation than the wildtype although the cell growth and proliferation were partially reduced during the vegetative stage (Hong et al., 2012). This enhanced light inducibility of astaxanthin accumulation in the cells was presumably attributed to the generation of reactive oxygen species (ROS) from the inefficient operation of photosystem II, and with this assumption, a new class of chemicals that could stimulate oxidative stress *in vivo*, such as azide, were proposed and tried as agents for screening astaxanthin hyper-producing mutants. The mutants generated by the new screening methodology were proved to be stable in the absence of selection pressure and conferred improved phenotypic traits for astaxanthin production (Hong et al., 2018). The use of Fourier transform infrared (FT-IR) and Raman microspectroscopy as screening methods was studied by Liu et al. in 2016, and non-invasive and quantitative analysis of astaxanthin were achieved for both single cells and bulk cells. They found out that the FT-IR absorbance band area ratio I(1740)/I(1156) of single cells were correlated well with the astaxanthin content, based on which an astaxanthin hyper-producing mutant screening strategy was proposed (Liu and Huang, 2016). Current methods of transcriptomic and metabolic analysis have been recently used to study the reasons for the superior astaxanthin productivity of a *Haematococcus* mutant obtained by low-temperature plasma (LTP) mutagenesis under high light irradiation (Chen et al., 2020). The authors found that the mutant strain had higher CO_2 utilization efficiency to channel the precursors of astaxanthin biosynthesis by increasing the expression levels of several enzymes, including phosphoenolpyruvate carboxylase (PEPC), malate dehydrogenase (MDH), and ribulose bisphosphate carboxylase/oxygenase (Rubisco) activase (RCA) while decreasing the expression levels of fructose-1,6-bisphosphatase (FBP). In addition, the mutant maintained higher photosynthetic activity by regulating the chlororespiration pathway and elevating the content of the non-photosynthetic pigments lutein, β-carotene, and astaxanthin to alleviate photooxidative damage (Chen et al., 2020).For a complete list of reports and methodologies used for mutagenesis of *Haematococcus* with improved astaxanthin productivity, please refer to Table 2.

3.1.4. Strain improvement by metabolic engineering

Metabolic engineering is a powerful tool to improve the productivity of endogenous compounds or new compounds through genetically adapting enzymes involved in metabolic pathways. Unlike physical or chemical mutagenesis, the strains improved by most metabolic engineering techniques are considered as GMOs and only allowed to be cultivated in contained environments, which is the major reason that no transgenic algae have been reported in outdoor cultivation trials. However, genetically modified microalgae might be used without strict regulation if pieces of DNA sequence are removed from the genome or pieces of DNA sequences or genes of endogenous origin are inserted back into the genome, according to the guidelines of EU regulation (Hlavova et al., 2015; Sharon-Gojman et al., 2015). From this perspective, the possibility does exist that *Haematococcus* strains improved by metabolic engineering could be commercially cultivated for astaxanthin production in the future. Besides the commercial drives, the metabolic engineering is also a useful tool to study the physiology of *Haematococcus* cells and to clarify the metabolic network of astaxanthin synthesis in this species of microalgae.

Most reported research work on metabolic engineering of *Haematococcus* has been focusing on establishing transformation protocols and developing appropriate vectors. The first reported stable gene transformation of *Haematococcus* was accomplished by Steinbrenner and Sandmann back in 2006. The gene of phytoene desaturase (pds), an enzyme in the biochemical pathways of carotenoid synthesis, was isolated from *H. pluvialis* and modified for improved herbicide norflurazon resistance, based on which the transformation vector was constructed. Upon biolistic transformation with the vector, the integration of the gene, the gene expression and protein function were confirmed by Southern, Northern and Western blotting in 11 transformants with enhanced norflurazon resistance (Steinbrenner and Sandmann, 2006). Also using pds as the selection marker gene, Sharon-Gojman et al. further developed this transformation method by introducing two gene insertion sites in upstream and downstream regions of pds coding sequence. The new vectors were proved to be more efficient and convenient in transforming *Haematococcus* (Sharon-Gojman et al., 2015). Arguing that transformants that developed by so called functional self-cloning approach might not be considered as GMOs, Sharon-Gojman et al. introduced two endogenous genes into the nuclear genome of *Haematococcus,* employing the vectors reported in their previous research, and the cell physiology and carotenoid synthesis of *Haematococcus* were found to be improved in terms of astaxanthin production (Sharon-Gojman et al., 2017). With a similar approach, the hexose uptake protein gene was integrated into the nuclear genome of *Haematococcus*, and the resulting transformants acquired the capacity of

Table 2
Genetically Modified Strains of *Haematococcus pluvialis*

Strain	Strategy	Cultivation	Biomass increase	Astaxnathin content increase	Final astaxanthin content	Reference
Not specified	UV and EMS mutagenesis + Diphenylamine	Autotrophic in flask	0%	275%	1.5%	Tripathi et al., 2001
EU3	EMS and UV mutagesis + Nicotine	Mixotrophic in flask	0%	105%	2.5%	Chen et al., 2003
N5	NTG mutagenesis + Glufosinate	Autotrophic in flask	-5%	80%	4.0%	Kamath et al., 2008
MT2877	MNNG mutagenesis	Mixotrophic in flask	0%	95%	3.9%	Hu et al., 2008
PP-PS#160	EMS mutagesis	Autotrophic in flask	14%	30%	5.8%	Hong et al., 2012
B24	EMS mutagenesis + Diphenylamine	Autotrophic in ponds	30%	32%	2.6%	Gómez et al., 2013
DPA12-1	UV EMS and DES + Diphenylamine	Autotrophic in flask	40%	170%	4.7%	Wang et al., 2016
M3	DBD mutagenesis	Autotrophic in flask	50%	50%	3.3%	Liu et al., 2016
Not specified	γ-ray radiation mutagenesis	Autotrophic in 0.5L PBR	15%	50%	1.8%	Cheng et al., 2016
M13	UV mutagenesis + Azide	Autotrophic in 5L PBR	0%	50%	4.6%	Hong et al., 2018
P3	Biolistic nuclear tranformation + Norflurazon	Mixotrophic in flask	NA	26%	1.1%	Steinbrenner and Sandmann, 2006
G111	Biolistic nuclear tranformation + Norflurazon	Autotrophic in 0.5L PBR	0%	16%	2.1%	Sharon-Gojman et al., 2017
PDS-1	Biolistic chloroplast tranformation + Norflurazon	Mixotrophic in flask	0%	38%	2.6%	Galarza et al., 2018

EMS Ethyl methanesulfonate NTG N-methyl-N′-nitro-N-nitroguanidine MNNG N-methy-N-nitro-nitrosoguanidine
DES Diethyl sulfate DBD Dielectric-barrier discharge UV Ultraviolet

utilizing glucose under dark or low light conditions (Wassman-Levy et al., 2019). Using newly identified endogenous promoter and terminator of the constitutively expressed alpha tubulin gene (tub) and biolisitic bombardment approach, Yuan et al. reported another case of successful transformation of *Haematococcus*. Three antibiotic, spectinomycin, hygromycin and zeocin resistance genes were integrated into the transformation vectors, and stable spectinomycin resistance colonies were obtained after transformation. Not only were the details of transformation procedures and tricks disclosed, but also the mechanisms of foreign DNA integration were discussed (Yuan et al., 2019). The agrobacterium-mediated transformation protocol for *Haematococcus* modification was first developed by Kathirsan et al. in 2009, using hygromycin as selection agent and GFP or β-glucuronidase(gus) as reporter marker, and the expression of exogenous genes and proteins was confirmed in hygromycin resistant transformants by southern blots, PCR, fluorescence microscopy and enzyme assays (Kathiresan et al., 2009). Following on this established protocol, these researchers reconstructed a new vector, replacing the reporter gene (gus or GFP) with an endogenous carotenoid synthesis gene, bkt, the DNA sequence of β-carotene ketolase, and transformed *Haematococcus* cells, expecting to obtain transformants with enhanced astaxanthin productivity. The transcript levels of carotenogenic genes and the content of carotenoids were observed to be much higher in transformants than wild type strain under various stress conditions for astaxanthin synthesis(Kathiresan et al., 2015). A chloroplast transformation approach for genetic engineering of *Haematococcus* was firstly reported by Gutierrez et al. in 2012 and was recently proved to be also helpful in getting astaxanthin hyper-producing transformants (Gutiérrez et al., 2012; Galarza et al., 2018). With the whole genome being sequenced recently and transformation protocols developed, there might be more metabolic engineering research work of *Haematococcus* undergoing (Luo et al., 2019). In this vein, metabolic engineering could be used to enable astaxanthin overproduction in more robust and easier to cultivate microalgal strains than *Haematococcus*. As an example, *Dunaliella viridis*, a producer of β-carotene, which is the precursor of astaxanthin, was metabolically engineered recently to produce astaxanthin through the integration of two key enzymes of *H. pluvialis*, β-carotene hydroxylase (CRTR-B) and β-carotenoid ketolase (BKT), by homologous recombination into the chloroplast genome of *Dunaliella* (Lin et al., 2019). Similar efforts have been recently directed towards the cyanobacterium *Synechocystis sp.* PCC6803 whose carotenoid biosynthesis pathway was metabolically engineered to produce astaxanthin. The β-carotene ketolase gene (bkt) and carotenoid hydroxylase gene (crtR-B) from *H. pluvialis*, which are absent in wild-type *Synechocystis sp.* PCC6803, were codon optimized and heterologously expressed in this cyanobacterium leading to a strain capable of overproducing astaxanthin (Liu et al., 2019). For a complete list of reports and methodologies used for transgenesis of *Haematococcus* with improved astaxanthin productivity, please refer to Table 2.

3.2. Process optimization and improvement

Early development of astaxanthin production process from *Haematococcus* has been mostly focused on optimizing biomass growth conditions and stressing factors for astaxanthin production, targeting to optimize the two-stage production processes (Fábregas et al., 2001; Jin et al., 2006). Recent investigations also address identifying chemical regulators to stimulate astaxanthin accumulation and applying research breakthroughs of algal biology to improve process efficiency.

3.2.1. Optimizing biomass growth conditions

The first stage of the astaxanthin production processes is to cultivate green cells of *Haematococcus* to build up biomass. Major cell culture conditions, such as media composition, temperature, light intensity, pH value, superficial gas velocity, and CO_2 supply, have been extensively investigated to optimize the vegetative growth of *Haematococcus*. Various conventional media, such as BBM, Z8, BAR, FAB, BG-11,KM1, MM1, and MM2 were tested for cultivation of *Haematococcus*, and among those tested, the BBM media was found to be the best for growth of green cells (Tripathi et al., 1999; Domınguez-Bocanegra et al., 2004). The thorough optimization of medium mostly based on BBM media composition for *Haematococcus* vegetative growth was firstly reported by Fábregas et al. back in 2000 (Fábregas et al., 2000). They conducted semi-continuous culture experiments and tested up to 18 nutrient components with a single variable optimization strategy. Among those tested, zinc, boron, iodine and vanadium were proved to be non-essential and even detrimental elements, and other elements, including nitrogen and phosphorous, were further optimized for maintaining highest cell density. The improved formulation of optimal *Haematococcus* medium (OHM) was shown to be three-fold more productive than traditional BBM media for green phase growth. Though their research work was quite thorough, there might still be rooms for improvement. The effects of thiamine, cobalamin, selenium, and copper addition on growth were still rather illusive, and the purity of chemicals and water might have significant influence on experimental results. For industrial production, it might be worthwhile to optimize the medium with local water supply. The above analysis was found to be reasonable by a later research (Dalay et al., 2007), and the novel media recipe developed only containing inexpensive agricultural fertilizers even had better growth performance than previously reported media including OHM (Fábregas et al., 2000) and Modified BG-11 (Boussiba et al., 2000). Following this effort, Tocquin et al. also reported a new medium totally based on commercial fertilizers which was proved to be not only efficient but also capable of promoting vegetative growth of *Haematococcus* (Tocquin, 2012). In an earlier investigation from a very interesting angle, nitrogen sources were optimized among urea, sodium nitrate and ammonium chloride, and it was founded that ammonium served better than nitrate and urea for growth of cells at a concentration level of 2.9 mmol L^{-1} N (Cifuentes et al., 2003). More recent research on green phase nutritional formulations reported high growth rates with EG:JM medium which contains tryptone and yeast extracts, indicating *Haematococcus* might be able to utilize amino acids or other organic sources besides acetate and glucose under mixotrophic growth conditions (Butler et al., 2017).

Although the culture growth conditions of the green phases of *Haematococcus* have been extensively investigated and reported, the results of those research works might not be easily applied to practical production since most experiments were done in small volume flasks under indoor conditions (Del Río et al., 2005; Jin et al., 2006; Del Campo et al., 2007). The optimal temperature for autotrophic green phase growth under laboratory conditions were mostly fixed at roughly 25 ± 2°C regardless of the origins of various strains of *Haematococcus* for early research works (Fábregas et al., 2001; Sarada et al., 2002a; Tripathi et al., 2002; Cifuentes et al., 2003; Dalay et al., 2007; Evens et al., 2008). A four factor two level factorial design experiment optimizing the green cell growth revealed that 30°C might be the optimal growth temperature for the strain used in the investigation, as indicated by ANOVA analysis (Hanan et al., 2013). In a more recent effort aimed at optimizing *Haematococcus* growth temperature for industrial production (Giannelli et al., 2015), the authors found that temperature had profound effects on the photosynthesis complex of *Haematococcus* cells and that both the cell division rates and cell sizes were affected by the temperature conditions. It was proved that though the strain of *Haematococcus* studied had the quickest division rates at 20 °C, the quickest biomass growth occurred at much higher temperatures up to 30.5 °C. The light intensity employed in reports of green growth of *Haematococcus* have ranged from tens of μmol m^{-2} s^{-1} up to about 200 μmol m^{-2} s^{-1}, and optimal levels of growth varied with experimental settings were obtained at around 70 μmol m^{-2} s^{-1} (Fábregas et al., 2001; Park and Lee, 2001; Cifuentes et al., 2003; Evens et al., 2008). An interesting finding here was that the cell size of *Haematococcus* was also influenced by light intensity as in the case of temperature , and both the green and red

phase cells were enlarged two- to three-fold with increasing light intensity (Park and Lee, 2001). The effects of light intensity on green cell cultivation of *Haematococcus* were carefully examined by Torzillo et al. (Torzillo et al., 2005).The biomass growth, chlorophyll content dynamics, photochemical parameters, oxygen revolution rates, and respiration rates were investigated under light conditions ranging from 50 μmol m^{-2} s^{-1} up to 600 μmol m^{-2} s^{-1}, and no light saturation was observed under 200 μmol m^{-2} s^{-1} for vegetative growth. In an effort to develop growth models, very high levels of irradiation were used to mimic outdoor sunlight conditions, and surprisingly light inhibition and even light saturation were not observed even under very high levels of light irradiation up to 2500 μmol m^{-2} s^{-1}(García-Malea et al., 2005). The effect of pH on green cell growth was still inconclusive maybe due to intraspecific variations of different *Haematococcus* strains. An early investigation had reported that the cell growth was significantly affected by pH, and that the growth rates were substantially reduced if pH values were outside of the optimal range of pH 7.0-8.0 (Sarada et al., 2002a). However, more recent research works have shown unmistakable results that *Haematococcus* could grow well on a wide range of pH between pH 4.0-9.0 (Hanan et al., 2013; Hwang et al., 2019).

Photobioreactor systems have provided better controlled condition than flasks, and the effects of superficial velocity on green cell growth of *Haematococcus* were investigated in various indoor photobioreactors with artificial light supply(Vega-Estrada et al., 2005; Suh et al., 2006; Kaewpintong et al., 2007; Ranjbar et al., 2008). It was reported that high level aeration had some detrimental effects on green growth of *Haematococcus* in both airlift and bubble column photobioreactors, but the optimal superficial velocities of air flow seemed to differ in different photobioreactor settings. High level aeration intended to transform motile green cells with flagella into non-motile green cells without flagella and thus to hinder cell growth. In the split-cylinder internal-loop airlift photobioreactor reported (Vega-Estrada et al., 2005), the optimal superficial velocity was found to be about 12 mm s^{-1}, and either increase or decrease of this level would result in decreased cell growth rates. In the later reports that using bubble column and draft airlift photobioreactor systems, it was concluded that the upper levels of superficial gas velocity for optimal growth should be under 4 mm s^{-1} (Kaewpintong et al., 2007; Ranjbar et al., 2008). The CO_2 enriched air-gas mixture aeration had long been proven to be essential for high productivity of large scale microalgal cultures, and in most research work the concentration levels of CO_2 in the air-gas supply stream ranged from 1% to 5% for autotrophic cultivation experiments of *Haematococcus* in photobioreactors systems (Vega-Estrada et al., 2005; Suh et al., 2006; Kaewpintong et al., 2007; Lee et al., 2015), with some exceptions where the CO_2 was supplied by on demand control of pH (Del Río et al., 2005; Ranjbar et al., 2008). The beneficial effect of moderately high CO_2 levels up to 5% were confirmed by a recent research through examining chlorophyll fluorescence and maximum photochemical quantum yields under different cultivation conditions, but higher CO_2 levels were found to be detrimental for cell culture growth (Chekanov et al., 2017).

The research results of outdoor investigations of *Haematococcus* cell culture in photobioreactor systems could be more valuable for optimization and development of current astaxanthin production process than those of indoor investigations because the commercially viable production processes up to date might still rely on natural sunlight resources, although the relevant research reports were much fewer than those on indoor cultivation (Olaizola, 2000; García-Malea et al., 2006; López et al., 2006). The design and operation of a 25000-L airlift tubular photobioreactor system used for commercial production by Aquasearch Inc. (now reorganized as Mera Pharmaceuticals Inc.) was reported by Miguel Olaizola in 2000, and a green biomass productivity of 0.052 g L^{-1} day^{-1} was achieved at Kailua Kona, Hawaii, USA. Two major types of outdoor photobioreactors, airlift tubular and bubble column reactors, were comparatively studied using colorimetric and digital analysis methods, and the conclusion was that the airlift tubular photobioreactor performed better than the bubble column reactor in terms of green cell productivity(López et al., 2006). An empirical mathematical model for autotrophic growth of *Haematococcus* was developed first in indoor reactors mimicking outdoor irradiation conditions and then was tested in outdoor airlift tubular photobioreactors (García-Malea et al., 2006). Very high levels of biomass productivity up to 0.68 g L^{-1} day^{-1} were achieved in the outdoor reactor upon optimizing operation conditions at Almeria, Spain, and the bioprocess model developed was able to predict experimental data adequately and could serve as a powerful tool for optimizing the design and operation of outdoor photobioreactor systems for *Haematococcus* green cell cultivation.

3.2.2. Optimizing stress conditions for astaxanthin accumulation

The second stage of the production processes is based on stressing *Haematococcus* cells to accumulate astaxanthin. *Haematococcus* could initiate astaxanthin synthesis in response to various abiotic stress factors that inhibit cell reproduction although they might have the same or different impacting mechanisms on cell physiology (Boussiba, 2000; Fábregas et al., 2003). In order to optimize the second stage of astaxanthin production processes, major environmental factors, such as light intensity, temperature, nutrient level, pH, salinity and acetate concentration, either singly or combined, have been intensively investigated regarding their influences on astaxanthin accumulation in *Haematococcus* over the last decades(Boussiba, 2000; Jin et al., 2006; Del Campo et al., 2007; Han et al., 2013).

Although astaxanthin accumulation could occur under heterotrophic cultivation conditions without light supply, phototrophic induction might be more cost-effective than heterotrophic or mixotrophic cultivation (Droop, 1955; Kang et al., 2005; Han et al., 2013; Shah et al., 2016). First the cell growth rates under heterotrophic or mixotrophic conditions were proved to be lower or not substantially higher than those of phototrophic conditions and to be much lower than those of typical microorganisms in industrial fermentations (Hata et al., 2001; Jeon, You Chul et al., 2006; Sun et al., 2015; Wan et al., 2015; Sun et al., 2017); Secondly the final astaxanthin content in dry weight biomass produced heterotrophically or even mixotrophically was much lower than that induced phototrophically (Kang et al., 2005; Orosa et al., 2005). Thirdly, due to slow growth rates, low cell culture density, and easy contamination, heterotrophic and mixotrophic cultivation of *Haematococcus* required very large volumes of fermentation equipment of highly demanding hygiene, which could be very and even forbiddingly expensive especially in the case of mixotrophic cultivation(Hu et al., 2018). Thus it might be concluded that outdoor phototrophic induction utilizing sunlight would still be the major method for large scale cultivation of *Haematococcus* for astaxanthin production for the next decade.

Since light is the sole energy provider for phototrophic cultivation, light supply is indispensable for both cell growth and astaxanthin accumulation. It has been clearly concluded that *Haematococcus* could maintain high levels of photosynthetic activity even after cell encystment, and even a transient increase of photosynthetic oxygen evolution has been reported by some researchers (Wang et al., 2003; Gu et al., 2013; Scibilia et al., 2015; Huang et al., 2019). It can also be concluded that the biomass and astaxanthin productivity have been positively related to levels of irradiance and duration of light supply, and the longer the light supply and the higher the light intensity, the more astaxanthin accumulation would occur especially under outdoor cultivation conditions(García-Malea et al., 2009; Zhang et al., 2009). Despite being observed in laboratory and small scale outdoor cell culture conditions, the light inhibition and even light saturation were not commonly experienced under large scale outdoor cultivation conditions especially at the red stage probably due to the light scattering and mutual light shading by high cell density culture and light acclimation of *Haematococcus* cells(Li et al., 2010; Wang et al., 2013; Wang et al., 2014). Under these circumstances, high levels of irradiance might

mostly serve as energy source to sustain astaxanthin accumulation instead of major inducers of carotenogenesis, given the fact that the average levels of irradiance inside of a dense outdoor cell culture could be fairly low due to mutual light shading of cells (García-Malea et al., 2009). From the perspective of microalgal biomass production, operating the large scale cell culture under light limiting growth conditions and maximizing utilization efficiency of natural light available at certain production facility sites might be an effective strategy to reduce production cost not only for *Haematococcus* but also for other microalgal species since artificial light supply is too expensive for most commercial microalgal cell culture operations (Li , J et al., 2015; Benemann et al., 2018).

Appropriate temperature conditions might be the second most important factor for outdoor astaxanthin production because of the high cost associated with the temperature control of large scale outdoor *Haematococcus* cell culture. The optimal temperature for astaxanthin accumulation was found to be around 27-28°C (Kang et al., 2007; Evens et al., 2008; Wan et al., 2014b; Giannelli et al., 2015), somewhat higher than the generally believed optimal temperature for vegetative growth of most strains of *H.* pluvialis. At lower temperatures, rates of astaxanthin accumulation were decreased, and at temperature lower than 15°C, both astaxanthin accumulation and cell growth even became marginal for most reported strains of *Haematococcus*(Evens et al., 2008; Wan et al., 2014b; Giannelli et al., 2015), although low temperature tolerant strains were seldom reported(Chekanov et al., 2014). It was also noted that temperatures higher than 30°C had negative effect on astaxanthin accumulation and even were lethal for some stains of *Haematococcus*(Wan et al., 2014b; Giannelli et al., 2015). Recent reports claimed that this negative effect of high temperature on astaxanthin synthesis could be overcome by adding some ferrous sulfate into the stress media (Hong et al., 2015), and this method was proven to be a successful way to enhance astaxanthin production under outdoor summer high temperature cultivation conditions (Hong et al., 2016).

Under the scenario of outdoor phototrophic cultivation for astaxanthin production with natural light, the control of light supply and temperature could be very limited once the location of production facility was set. For the purpose of production, facility site selection might be crucial to enhance productivity and thus reduce production cost. The more sunshine hours and the more appropriate temperature on sites, the higher the unit area productivity. Other resources, such as quality of the water supply, flatland availability and convenient access to electricity, might also be important consideration for site selection to minimize production cost. Selection and improvement of suitable strains and process optimization for local weather conditions might be necessary steps to establish cost-effective production since reported research efforts optimizing stress conditions performed in laboratories could not be applied to practical operations in a straightforward manner.

Although nutrient deprivation and high light intensity are both effective factors inducing astaxanthin accumulation in *Haematococcus* cells, nutrient starvation might be a more appropriate option for outdoor cultivation since continuous high levels of light supply is not easily available for large scale cell culture operation (Han et al., 2013; Solovchenko, 2015). Either nitrogen or phosphorous depletion can initiate quick accumulation of astaxanthin and encyst cells although nitrogen was reportedly more effective than phosphorous (Boussiba et al., 1999; Scibilia et al., 2015). In fact, it was indicated and proved that the deprivation of all nutrients except CO_2 supply might be the most effective and least expensive strategy for autotrophic production of astaxanthin since the rate difference of astaxanthin accumulation between nitrogen depletion and total nutrient depletion was marginal (Boussiba et al., 2000; Imamoglu et al., 2009). It was also confirmed that it was nutrient depletion instead of C/N ratio was the key factor affecting astaxanthin synthesis, and CO_2 supply might only serve as carbon source under autotrophic conditions (Kang et al., 2007). Although the molecular signal transduction pathways of nutrient depletion activating astaxanthin synthesis were still not fully clarified, it was demonstrated that nitrogen starvation might induce astaxanthin accumulation through degrading chlorophylls, promoting chlororespiration, destabilizing photosystems and favouring cyclic electron transport, which differed from high light stress (Scibilia et al., 2015). Interestingly but not surprisingly, the maximal oxygen production rates of cells upon nitrogen starvation were increased if calculated on a chlorophyll basis, indicating that light supply but not necessarily high light supply might be beneficial for sustained accumulation of astaxanthin under nutrient starvation conditions(Scibilia et al., 2015). Comparative transcriptomic analysis revealed that as many as 25480 genes were differentially expressed upon nitrogen starvation, and all the key and rate limiting genes in astaxanthin synthesis pathways were upregulated. At the same time, biochemical pathways associated with cyclic electron transportation and chloroplast protein degradation were also upregulated, but the photosynthesis pathways were attenuated. These results depicted a picture of nitrogen redistribution from chloroplast for photosynthesis to cytoplasm for lipid synthesis, indicating that management of nitrogen supply might be still the key for high level astaxanthin accumulation(Huang et al., 2019).

Salinity levels also affect astaxanthin accumulation. Moderate levels of NaCl seemed to induce and promote astaxanthin synthesis although high salinity levels would bleach the cells under both phototrophic and mixotrophic conditions(Sarada et al., 2002b; Tripathi et al., 2002; Cifuentes et al., 2003; Domınguez-Bocanegra et al., 2004). Similar to nitrogen starvation, salinity stress was also shown to be able to upregulate the expression of astaxanthin synthesis genes although the molecular signal transduction pathways were not identified(Gao et al., 2015).

It is not surprising that appropriate levels of CO_2 supply are required for astaxanthin synthesis since it is the only carbon source under phototrophic cultivation. In common practice, aeration with CO_2 ranging from 1% to 5% were applied to supply inorganic carbon for phototrophic cultivation of *Haematococcus* at the red stage (Suh et al., 2006; Lee et al., 2015). The effect of CO_2 levels on biomass growth and astaxanthin production were studied via photochemical approach, and the benefit of using moderately high levels of CO_2 up to 10% were confirmed(Chekanov et al., 2017). Elevated levels of CO_2 supply up to 15% seemed to be able to promote astaxanthin accumulation to high extents in a much shorter period of time than low levels of CO_2 (Christian et al., 2018).

Evidence is building up to support the assumption that any factors inhibiting cell division and growth might lead to encystment and accumulation of lipid and astaxanthin in *Haematococcus* cells(Droop, 1955; Boussiba and Vonshak, 1991; Fábregas et al., 2003). From this perspective, the synthesis of lipid and astaxanthin might be an energy storage mechanism to ensure the survival and prospering of this species. It was argued and presumed that different environmental stresses had different signal transduction pathways to induce carotenoid synthesis and might have synergistic effect on astaxanthin synthesis (Solovchenko, 2015). Recent research demonstrated that initially applying nitrogen stress followed by incrementally increasing light supply yielded highest levels of astaxanthin content and productivity(Niizawa et al., 2018). This result might be explained by the fact that non-motile *Haematococcus* cells formed at late vegetative phase were more resistant to photoinhibition and more inductive for astaxanthin synthesis (Wang et al., 2014; Li et al., 2019). Thus, the principle to employ various stress factors together should be to regulate carbon metabolic flow totally targeting to astaxanthin and lipid synthesis while at the same time to maintain photosynthetic activity as much as possible for carbon assimilation.

3.2.3. Stimulating astaxanthin accumulation by small-molecule chemicals

In the past few years, small chemical regulators were intensively studied for their effects on microalgal physiology and bioproduct synthesis (Yu et al., 2015). Plant hormones, such as methyl jasmonate,

giberellins, salicylic acid, abcisic acid, and brassinosteroids, were found to substantially affect cell physiology and astaxanthin synthesis of *Haematococcus* (Lu et al., 2010; Raman and Ravi, 2011; Gao et al., 2012a; Gao et al., 2012b; Gao et al., 2013a; Gao et al., 2013c). At moderate levels, i.e. 20 mg/L or 100 μM, methyl jasonate and salicylic acid were able to stimulate astaxanthin accumulation (Lu et al., 2010), while at high concentrations, i.e. 500 μM, inhibited astaxanthin synthesis (Raman and Ravi, 2011). The up regulation of carotenogenic gene expression was observed if these substances were added to cell culture even though gene expression was enhanced at quite different levels (Gao et al., 2012a; Gao et al., 2012b; Gao et al., 2013b; Gao et al., 2013c). 23 chemicals from 8 groups were screened for their effect on astaxanthin accumulation in *Haematococcus*, and phytohormones were confirmed to be effective in stimulating astaxanthin synthesis(Yu et al., 2015b). Other plant hormones such as fulvic acid, melatonin and diethyl aminoethyl hexanoate were further examined for effects of carotenogenesis more recently, and almost similar results were achieved in terms of promoting astaxanthin accumulation(Zhao et al., 2015; Ding et al., 2018a; Ding et al., 2018b; Ding et al., 2019b). Supplementation of low levels of 1-aminocyclopropane-1-carboxylic acid, a precursor of natural plant hormone ethylene, to *Haematococcus* cell culture resulted in promoting both biomass growth and astaxanthin accumulation, but at high concentrations, the stimulating effects became marginal(Lee et al., 2016; Vo et al., 2016). Interestingly, some antioxidants, such as butylated hydroxyanisole and butylated hydroxytoluene, were also identified as effective inducer of astaxanthin in *Haematococcus*(Shang et al., 2016; Zhao et al., 2018). Following supplementation of *H. pluvialis* cultures with BHA, a metabolomic analysis showed that this chemical upregulated pathways of basal metabolism and signaling, such as glycolysis, the TCA cycle, amino acid metabolism and the phosphatidylinositol signaling system, resulting in enhanced astaxanthin accumulation(Ding et al., 2019a).

A novel approach was recently proposed to enhance astaxanthin accumulation in *H. pluvialis* cells by the addition of a synthetic cationic polymer, polyethyleneimine (PEI), into the cell culture. PEI was found to be internalized in the cells through the negatively-charged cell walls, leading to excessive production of reactive oxygen species (ROS) in the cells (Yoshitomi et al., 2019). The authors concluded that the increased oxidative stress by cellular uptake of PEI resulted in increased astaxanthin accumulation in *H. pluvialis*.

Although numerous chemicals were proven to be able to induce astaxanthin accumulation and even promote cell growth, the potential application of these chemicals on production process improvement might still be under question. First, there have been no reports available indicating these chemicals could enhance astaxanthin content level higher than 4% in *Haematococcus* biomass, which was the level of routine industrial operation. Secondly although supplementation of these chemicals to cell culture was reported to induce carotenogenic gene expression and astaxanthin synthesis, the stimulating effect seemed to become marginal when combined with traditional stress factors(Vo et al., 2016). Based on evidences reported so far, it might not be sufficient enough to claim that applying these chemicals to production processes would be able to achieve better process economics than traditional stress factors.

3.2.4. Applying recent biological research breakthroughs

Astaxanthin production processes have been developed based on cell biology, physiology and biochemistry of *Haematococcus*, and thus there should be no surprises that the research breakthroughs in the field would have fundamental impacts in improving and optimizing astaxanthin production processes. The recent research advances in cell biology, physiology and biochemistry of *Haematococcus* have been thoroughly reviewed recently by other researchers (Lemoine and Schoefs, 2010; Han et al., 2013; Solovchenko, 2015; Shah et al., 2016), and here we only highlight the important research findings that we believe might have an immediate and direct impact on improving astaxanthin production processes and thus on reducing production costs.

Astaxanthin content and biomass growth rates might be the most important process performance parameters influencing production cost. Once the production facility is established, the cost of production would be roughly determined by biomass growth rates and final astaxanthin content in the biomass (Li et al., 2011; Panis and Carreon, 2016). Thus the strategy of production process optimization should focus mostly on enhancing biomass growth rates and increasing astaxanthin content. Biomass growth rates of microalgae could be roughly determined by rates of photosynthesis and organic carbon uptake, while the biotic constraints defining the upper limit of astaxanthin content are still elusive. It has been long known that astaxanthin is deposited in lipid bodies of *Haematococcus* cells, and it has also been reported that astaxanthin accumulation is proportional to lipid synthesis under stressed conditions although inhibition of astaxanthin synthesis did not abolish lipid synthesis(Zhekisheva et al., 2002; Zhekisheva et al., 2005). Recently, the mechanism of this correlation between lipid synthesis and astaxanthin accumulation has been carefully examined(Chen et al., 2015). Chen et al. dissected the molecular mechanism of correlation between lipid and astaxanthin synthesis pathways and claimed astaxanthin accumulation was in feedback control with free astaxanthin, and the astaxanthin esterification step might be responsible for this correlation. The above research findings indicated that increasing the titer of lipid in biomass might be able to increase the astaxanthin content and that over expression of relevant esterifying enzymes might be able to promote both lipid and astaxanthin synthesis. Metabolic profiling and modelling research revealed that under nitrogen starvation conditions, *Haematococcus* could divert carbon partition from starch to lipids and astaxanthin via increased activity of tricarboxylic acid cycle(Recht et al., 2014). This conclusion was further corroborated by quantitative proteomic analysis of thylakoids, which was performed by Gu et al. when studying the light acclimation mechanism of *Haematococcus* and *Dunaliella*, and they proposed that tricarboxylic acid cycle, pentose-phosphate pathway, and glycolysis pathway were coordinated to sustain carbon fixation and lipid synthesis under high light conditions when photosynthesis of *Haematococcus* were undermined (Gu et al., 2014). These findings and proposals reinforce our assertion that a successful strategy to optimize astaxanthin production should try to direct carbon flow and partition towards lipids and astaxanthin through applying stress conditions while at the same time maintaining carbon fixation and assimilation as much as possible.

Biochemical pathways of astaxanthin synthesis have long been well studied and extensively reviewed, but there are still some important issues left under proactive investigation (Han et al., 2013; Solovchenko, 2015; Shah et al., 2016). The intermediate precursor of astaxanthin synthesis, β-carotene, might be synthesised exclusively in chloroplasts of *Haematococcus*, while the subcellular storage location of astaxanthin is mostly in lipid bodies outside of the chloroplast (Grünewald and Hagen, 2001; Grünewald et al., 2001; Collins et al., 2011). Thus there must be a way to transport β-carotene from chloroplast to cytosol, but the exact mechanism of this transport is still unclear (Grünewald and Hagen, 2001; Grünewald et al., 2001; Solovchenko, 2015). Early investigations reported lines of evidences supporting the claim that β-carotene was converted to astaxanthin in lipid bodies, but recently Chen et al. (2015) showed that both free astaxanthin synthesis and esterification might also occur in the endoplasmic reticulum(Harker and Young, 1995; Grünewald and Hagen, 2001; Grünewald et al., 2001; Chen et al., 2015).

3.3. Innovation of cultivation methodologies

The two-step (or two-stage) cultivation methodology has long been established and widely adopted for industrial cultivation of *Haematococcus*, as reviewed in previous sections. However, the current production costs of astaxanthin from *Haematococcus* are still much

higher than those of chemical astaxanthin although after decades of process optimization efforts. Thus, scientists have been working on innovating cultivation methodologies to advance the technology. Some novel production approaches recently reported might have great potential to reduce production cost.

3.3.1. Sequential heterotrophic and phototrophic methodology

An interesting idea is to use a sequential heterotrophic and phototrophic strategy to cultivate *Haematococcus* for astaxanthin production. Some Japanese researchers first reported one case of such researches back in early 2000s, and the approach was still being investigated until recently (Hata et al., 2001; Wan et al., 2015). *Haematococcus* was first cultivated in aerobic fermenters instead of photobioreactors to accumulate a sufficient level of green biomass and then transferred to raceway ponds for astaxanthin production. In this way, expensive photobioreactor systems could be avoided and the production cost might be substantially reduced. Although the reported results were not promising enough to be applied to industry, it seems the process could be further improved. There were two major drawbacks in the results of this research. The first was that the standing biomass concentration is still low, i.e. 7.0 g/L. Above this level, the cells of *Haematococcus* would encyst and stop proliferation. According to our own experiences, this could be either caused by shear stress or lack of macro or micro nutrients. We believe this could be improved by replacing the cultivation system with industrial airlift bioreactors, which exert lower shear stress and is even much cheaper in cost. The second was that substantial amount of cell die when transferring from heterotrophic cultivation to phototrophic cultivation(Zhang, Z. et al., 2016). We believed this problem should be able to be resolved by carefully handling cultivation conditions with the assumption that healthy cells should be able to survive this transition.

3.3.2. One-step continuous cultivation

Another interesting strategy is to replace the current widely employed two-step approach with one-step continuous cultivation. It was observed and reported that flagellated and palmelloid cells of *Haematococcus* were also able to accumulate substantial amount of astaxanthin under high light conditions, without cell encystment and losing the capacity of cell proliferation(Chaumont and Thépenier, 1995; Grünewald et al., 1997; Hagen et al., 2001). Inspired by this observation, a Spanish group developed and reported a one-step method, enabling both cell proliferation and astaxanthin accumulation simultaneously in one growth chamber and under the nitrogen limited instead of nitrogen depleted conditions (Del Río et al., 2005; Del Río et al., 2008). The cells produced this way were not encysted, and thus besides the cost reduction by simplifying cell culture operation, the cost of astaxanthin extraction might be also reduced because the energy consuming process of cell cracking could also be saved (Del Río et al., 2010). This methodology were then further developed and tested in outdoor tubular photobioreactors and raceway ponds (García-Malea et al., 2009; Zhang et al., 2009). Although continuous and high level of astaxanthin production was achieved, the efficiency of this approach was still under question (Aflalo et al., 2007; Del Río et al., 2008). Another serious problem of one-step strategy might be to harvest the biomass since flagellates are much more difficult to be precipitated than encysted cells.

3.3.3. Indoor phototrophic cultivation

Another additional important strategy being pursued has been to utilize artificial light supply and cultivate *Haematococcus* at indoor conditions(Schulze et al., 2014). It is obvious that using artificial instead of natural light would entail extra capital and operational cost, but the facility can provide totally controlled and stable conditions for microalgae cultivation. Not only can this methodology accomplish *Haematococcus* cultivation at constant optimal conditions, but also advanced cultivation modes demanding more strict process control than traditional batch cultivation such as continuous or mixotrophic cultivation can be employed. The extra cost of the facility could be compensated by substantially improved biomass and astaxanthin production rates. This methodology has become even more competitive recently due to the availability of high energy efficiency LED lights suitable for plant growth. Not surprisingly, the physiology of microalgae is affected by the monochromatic spectrum of LED lights, which becomes a process design factor being optimized (Jeon et al., 2005; Lababpour et al., 2005; Jeon, Y. C. et al., 2006; Katsuda et al., 2006; Kim et al., 2009; Lee et al., 2018; Ma et al., 2018). It was found out that short wavelength (380nm-470nm) blue LED monochromatic light seemed to promote astaxanthin synthesis but not biomass growth and that the astaxanthin content in dry biomass could be substantially increased by blue LED light. Based on this fact, a strategy of cultivating green phase with red LED light and of cultivating red phase with blue LED light had been proposed back in 2004 and eventually tested in 2016 (Katsuda et al., 2004; Xi et al., 2016). Surprisingly, a recent investigation indicated that the green LED light might have higher light utilization efficiency than red or blue lights because green light penetrates better into the cell culture better (Ooms et al., 2017). This might be a significant finding due to high cost of power consumption by LEDs. Until now, most studies aiming at improving astaxanthin production by *H. pluvialis* have used non-standardized light regimes, the majority of the experiments being conducted under unnatural conditions of continuous illumination. Based on the hypothesis that the lack of a dark period may constitute an additional stress factor, acting differently from light intensity, on astaxanthin production by *H. pluvialis*, Spanish workers recently compared two light regimes in parallel: continuous light and 12h/12h light-dark circadian regime without modifying incident light intensity (Domínguez et al., 2019). They have found that light-dark cycles are optimal for the production of vegetative green cells of *H. pluvialis*, while continuous illumination is useful as a stressor in enhancing the rate of astaxanthin accumulation once nitrogen is depleted from the culture medium in the red phase. Thus, intermittent and continuous light regimes for the green and the red phase, respectively, may constitute an additional promising tool of process optimization. In the context of enhanced light delivery, a recent demonstration of the power of nanomaterials was offered with the use of highly blue-fluorescent nitrogen-doped carbon dots to boost algal astaxanthin production by at least two-fold (Abu-Ghosh et al., 2017). This process step should be scalable, in principle, and is environmentally responsible as the additives are recyclable. An extension of this innovative idea is the very recent application of biocompatible liquid fluorescent carbon nanodots (C-paints) to improve the productivity of astaxanthin in *H. pluvialis* (Choi et al., 2020). Addition of the liquid C-paints as a stress condition at a concentration of 1–10.0 mg mL^{-1} increased the astaxanthin content up to about 1.8 times higher than that of control cells. Thus, the high light delivery effect of these non-cytotoxic C-paints applied to *H. pluvialis* cell culture was found to be effective in enhancing productivity, and the results could form the basis of a new approach to improve commercial astaxanthin production (Choi et al., 2020).

3.3.4. Attached cultivation

One of the most recent developments in novel cultivation strategies is the investigation of the so-called attached cultivation or biofilm cultivation (Liu et al., 2013; Berner et al., 2015; Schultze et al., 2015). Instead of cultivating *Haematococcus* in suspension cell culture for red phase cultivation, the green cells have been inoculated and grown as biofilms on the surface of a microporous substrate supplying nutrients (Wan et al., 2014a; Zhang et al., 2014). As claimed in the reports, this approach might have some advantages over conventional suspension cell culture methods, such as saving water, preventing contamination by protozoans, easy harvest, saving energy of mixing, and etc. (Yin et al., 2015). However, there are still great challenges to develop an economically viable process for astaxanthin production. First, the experimental biofilm photobioreactor systems for *Haematococcus* so far

have been only tested indoors, and the outdoor productivity could deviate substantially away from predictions based on indoor investigations. Secondly, the scale up of the biofilm photobioreactor system might be a major challenge as in the case of suspension cell culture photobioreactors. Thirdly the quality of *Haematococcus* biomass might be questionable since biofilm microalgae biomass is not as homogeneous as that of a suspension cell culture. Last but not least is issue of the operational cost of biofilm photobioreactors. One can imagine if biofilm was inoculated and harvested manually piece by piece, the labor cost would be forbiddingly high. Although this difficulty might be overcome by a recent tested one-step biofilm cultivation approach, the overall efficiency of attached cultivation might still be lower than that of the traditional approach as indicated by a recent process modelling research (Zhang et al., 2016a, 2016b; Kiperstok et al., 2017).

3.3.5. Mixotrophic cultivation

Mixotrophic cultivation of *Haematococcus* has long been pursued as an alternative approach for traditional two stage astaxanthin production process (Orosa et al., 2001, 2005; Jeon et al., 2006a, 2006b). In fact the world's first commercial *Haematococcus* astaxanthin production facility appears to have been based on mixotrophic cultivation technologies back in 1995 (Olaizola and Huntley, 2003). However, since then, the phototrophic cultivation approaches have been extensively developed and have become the dominant methodology of natural astaxanthin production. The advantage of mixotrophic cultivation is that the production can be performed under indoor and totally controlled conditions, and the quantity and quality of the production can be well guaranteed. However, the cost associated with the equipment and energy consumption might be too high to compete with phototrophic cultivation utilizing natural light. With the development of inexpensive LED lights, innovation of mixotrophic cultivation systems, evolution of molecular biology tools for strain improvement, the mixotrophic cultivation approach might be worthy of reexamination. A very interesting mixotrophic process was proposed and tested by Goksan et al., 2010. The *Haematococcus* cells were firstly cultivated phototrophically, and then at the stationary phase, the organic carbon source, acetate, was added for mixotrophic cultivation. Both biomass and astaxanthin content were surprisingly boosted by this retarded addition of acetate, compared with traditional mixotrophic approach(Goksan et al., 2010). Mixotrophic cultivation operated in perfusion culture modes was applied for both biomass growth and astaxanthin accumulation with a stepwise increasing light irradiation, and this operation avoided inhibitory effects from both extracellular metabolites and high light, thus enhancing mixotrophic astaxanthin production (Park et al., 2014). Processes developed based on the fed-batch mode operation of mixotrophic reactors were recently reported by Sun et al., and a mixture of blue, red and white LED light was used to maximize cell growth and astaxanthin synthesis (Sun et al., 2015; Sun et al., 2017). Fed-batch operation of heterotrophic cultivation fermenters with increasing C/N ratio media supply could produce non-motile cells of high quantity, which were proved to be more prone to mixotrophic induction of astaxanthin synthesis, and production process based on this novel approach was recently proposed for industrial exploration (Lu et al., 2019). In the research work screening for the alternative carbon sources for mixotrophic cultivation, ribose was singled out as best candidates among nine types of carbohydrates tested with improved performance over acetate which conventionally used in mixotrophic culture (Pang and Chen, 2017; Pang et al., 2019b). Not only the growth rates were increased with ribose as the organic carbon sources compared with sodium acetate, but also the ratio of motile cells to stagnant cells was increased from 7% to 31% under similar mixotrophic cultivation conditions. The improved biomass growth rates were resulted by accelerated photosynthetic rates with ribose added in the medium, and the experimental data suggested that extra metabolites provided by ribose addition could drive Calvin cycle faster and the extra ATP needed for higher rates of dark reaction of photosynthesis were compensated by enhanced cyclic electron flow pathways(Pang et al., 2019b). With this new carbon source, a novel mixotrophic astaxanthin production process was developed with multilevel heuristic LED light regime(Pang et al., 2019a). Polyol alcohols, such as mannitol and glycerol, were also found to be more effective carbon sources than acetate for mixotrophic cultivation of *Haematococcus*, and with these carbon sources, a so called sequential mixotrophy dilution photoinduction strategy (SMDP) was developed for efficient and cost-effective astaxanthin production (Azizi et al., 2019). With this novel mixotrophic cultivation strategy, *Haematococcus* were firstly cultivated mixotrophically with poly alcohols for biomass accumulation, and then the cell culture was diluted to reduce nitrogen levels and further cultivated mixotrophically with increased light intensity and ploy alcohols such as glycerol for astaxanthin accumulation. During the green cultivation stage, the polyol alcohols were able to serve as effective carbon sources, and during the red stage, the glycerol was proved to be better than sodium acetate in boosting astaxanthin accumulation.

3.4. Innovation of extraction technologies

Downstream processing constitutes substantial portion of total production costs, and especially the supercritical fluid extraction of astaxanthin with CO_2 from *Haematococcus*

biomass is costly although it has become prevailing method of astaxanthin extraction for very good reasons(Liu et al., 2017). CO_2 is regarded as a safe solvent almost perfectly suitable for food and feed products without any toxic solvent residues, and extraction can be processed at mild temperatures, preventing sensitive molecules like astaxanthin from degrading. At the same time, very good extraction efficiency can be achieved for astaxanthin extraction from *Haematococcus* biomass at high level pressures. However, the process is expensive because both capital and operational cost are high due to high pressure requirements of the extraction process(Zgheib et al., 2018).

Since downstream processing technologies of *Haematococcus* have been extensively reviewed by other researchers recently (Kim et al., 2016; Khoo et al., 2019; Zhao et al., 2019), here we only highlight some important research advances that we believe might be applicable to industry and that have potential to decrease extraction cost substantially. The established extraction method with supercritical fluid CO_2 demands high operational pressure as high as 50MPa due to the low solubility of astaxanthin in the solvent, and thus require high capital and operational cost. An idea to decrease the cost is to use other Generally Regarded as Safe (GRAS) solvents such as ethanol as a co-solvent to enhance the solubility and to decrease the operation pressure. Reyes et al. reported that with ethanol content up to 50-70%, the extraction pressure could be decreased to 7MPa without compromising extraction efficiency (Reyes et al., 2014). Although the process seems very promising, one must consider the fact that further evaporation of ethanol to concentrate astaxanthin also incur costs. Another idea to cut down the cost is to extract astaxanthin directly from wet biomass without cell disrupting and biomass drying. A novel low toxicity solvent, dimethyl ether (DME), was tried by a Japanese group to extract lipid and astaxanthin from intact cells of wet *Haematococcus* biomass (Boonnoun et al., 2014). It was found that although the astaxanthin extraction efficiency was lower than commonly used solvents maybe due to low polarity of DME, the biomass could be dehydrated by DME extraction step and thus leave remaining biomass with high astaxanthin content more amenable for further solvent extractions. This way, the energy consuming step of drying can be saved. A very interesting recent development was to use switchable hydrophilicity solvent, Dimethylaminocyclohexane (DMCHA), to extract astaxanthin from wet and intact cells of *Haematococcus*, and the maximum extraction yield up to 87.2% has been achieved (Huang et al., 2018). DMCHA extraction method also has other advantages except extraction without cell cracking and biomass drying. The energy consuming step of solvent evaporation is saved because astaxanthin can be separated from the hydrophobic solvents

DMCHA by bubbling CO_2 which reacts with DMCHA to form $DMCHA^+$ and HCO_3^-, and DMCHA can be recovered from water solution by heating CO_2 out of solution. This methodology is almost perfect except that DMCHA is not a food grade or GRAS solvent, and further research work to find a GRAS solvent with similar extraction properties might be very attractive. The idea of milking *Haematococcus*, namely extracting astaxanthin without killing the cells, is more interesting and might even be revolutionary (Samorì et al., 2019). Almond oil was proved to be an effective solvent to extract astaxanthin directly from *Haematococcus* cell culture, and at least most of all if not all the cells was found to be able to survive the astaxanthin extraction process. With this discovery in mind, we can even imagine that a novel continuous process integrating both microalgal cultivation and astaxanthin extraction might be able to be developed if the cells could really milk astaxanthin without dying from extraction. It is also worth mentioning that ionic liquids are tested to disrupt *Haematococcus* cells for astaxanthin extraction, and it seems to be promising to develop a solvent extraction process using ionic liquids to perforate cell walls thus saving the energy consuming step of mechanical cell cracking although further research work is necessary (Desai et al., 2016; Choi et al., 2019).

4. Conclusions

Although biotechnologies of astaxanthin production from *Haematococcus* have been developed for decades and even dozens of production facilities have been established globally, the production cost of biological astaxanthin is still very high. Further R&D might be able to reduce the production cost substantially to the level comparable to that of chemical astaxanthin. In fact, the production technology is still far from mature due to the complexity of biology of *Haematococcus* even after decades of R&D. The production costs of *Haematococcus* are as least 10 times more than those of other industrially cultivated microalgal species, such as *Spirulina*, mostly because currently expensive photobioreactor systems are needed for *Haematococcus* cultivation. If the production costs of *Haematococcus* biomass can be reduced to the levels of those of *Spirulina* biomass, the costs of astaxanthin production can be decreased to the levels comparable to those of chemical astaxanthin.

Since the research works focusing on reducing photobioreactor costs for the last decades have only achieved marginal success, the R&D for cost reduction of astaxanthin production from *H. pluvialis* might need to focus on strain selection and improvement, cultivation process optimization, innovation of cultivation methodologies, and revolution of extraction technologies. The strain properties, such as cell biomass growth rates at green stage, astaxanthin accumulation rates at red stage, and astaxanthin titer in final biomass, are the fundamental factors determining the total production costs, and thus the strain selection and improvement is the primary step to develop the production processes. Further cultivation process optimization efforts on current two-step cultivation might still have great impact on production cost reduction. Diverting the carbon metabolic flow into astaxanthin and lipid synthesis pathways while at the same time maintaining the capacity of carbon assimilation as much as possible in *Haematococcus* cells might be the key for process optimization. However, substantial cost reduction might rely on innovation of cultivation methodologies, and various such methods have been proposed and under intensive research for the last few years. Astaxanthin extraction from biomass constitutes a great portion of total production costs, and in this field of research, some revolutionary technologies, such as swichable hydrophilicity solvent extraction, have been under development.

Acknowledgements

This work was supported by Sichuan Province Science and Technology Support Program in Sichuan, China, with grant number 2019YFN0121 , Joint Science and Technology Support Program of Sichuan University and Panzhihua City, with grant number 2019CDPZH-19, Panzhihua Municipal Science and Technology Support Program in Panzhihua, China, with grant number 2018CY-N-5 , National Key R&D Program of China, with grant number 2018YFD0901501 and the National Natural Science Foundation of China, with grant number 41806171. We are grateful to the anonymous reviewers for their in-depth comments, suggestions and corrections, which have greatly improved this manuscript.

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