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Carotenoids from Microalgae: A Review of Recent Developments

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Abstract

Carotenoids have been receiving increasing attention due to their potential health benefits. Microalgae are recognized as a natural source of carotenoids and other beneficial byproducts. However, the production of micro-algal carotenoids is not yet sufficiently cost-effective to compete with traditional chemical synthetic methods and other technologies such as extraction from plant based sources. This review presents the recent biotechnological developments in microalgal carotenoid production. The current technologies involved in their bioprocessing including cultivation, harvesting, extraction, and purification are discussed with a specific focus on downstream processing. The recent advances in chemical and biochemical synthesis of carotenoids are also reviewed for a better understanding of suitable and economically feasible biotechnological strategies. Some possible future directions are also proposed.

Keywords: microalgae, carotenoids, extraction, astaxanthin, lutein, β-carotene, harvesting, downstream processing, cell disruption

1. Introduction

Microalgae play a fundamental role in ecosystems (Guedes et al. 2011). Recently, microalgae are gaining attention as a source of useful products such as carotenoid pigments, polyunsaturated fatty acids, vitamins, lipids and proteins. Some of the specific advantages microalgae cultivation offers, compared to traditional plant-based sources, include a faster cultivation, processing and harvesting cycle and the ability to be cultured on waste materials. The anti-oxidants astaxanthin, β-carotene, lutein, lycopene, and canthaxanthin are the major carotenoids of commercial value found in microalgae. In spite of the perceived advantages, the large scale and cost-effective manufacture of the carotenoids from algae is currently quite challenging both in terms of production and downstream extraction and purification. An integrated bioprocessing approach using microalgae thus needs to consider both the upstream production of microalgae and the downstream harvesting and extraction of carotenoids. The existence of rigid cell walls in many algal species poses difficulties as this prevents full recovery of bioactive compounds. This is, therefore, a significant bottleneck in the overall bioprocess.

Many recent reviews have previously discussed microalgae and their products and applications (A Catarina Guedes et al., 2011; Markou and Nerantzis, 2013; Mata et al., 2010); however, there has been less focus on the downstream processing aspects. In this review, an attempt has been made to emphasize the extraction and downstream processing steps as a critical component for the overall bioprocessing. First the chemistry and biochemistry is described for a better understanding of the carotenoid production. Next the biotechnology, engineering and downstream approaches are discussed.

2. Chemistry and biochemistry of carotenoids

Carotenoids are lipophilic compounds and are usually colored yellow, orange or red and are the most diverse and wide spread pigments found in nature (Sasso et al., 2012; Varela et al., 2015). Most carotenoids share a common C40 backbone structure of isoprene units (termed terpenoid), and are divided into two groups: carotenes and xanthophylls. Some common carotenoids structure are shown in Figure 1. Each of the carotenoids consists of different *trans* and *cis* isomers. Xanthophylls, the oxygenated derivatives of carotenes (which are hydrocarbon only), are relatively hydrophilic compounds due to the presence of hydroxyl groups and keto-groups at the end rings. As antioxidants, carotenoids are in general sensitive to light, oxygen and heat, which can lead to difficulties in storage and handling.

In spite of the diversity of the carotenoid family, less than 30 carotenoids play important roles in photosynthesis (Varela et al., 2015). Most of these are located in the thylakoid membranes, and are bound with the Light Harvesting Complexes (LHCs) (Nisar et al., 2015). The carotenoids function to absorb light and quench excess energy in photosynthetic metabolism. Some primary carotenoids like lutein serve as accessory pigments which can transfer absorbed energy to chlorophylls (Ye et al., 2008), therefore expanding the light absorbing spectrum of

algae or plants.

Secondary carotenoids like astaxanthin and canthaxanthin play a role in cell protective mechanisms. Unlike primary carotenoids which are tightly associated with structural and functional components in the cellular photosynthetic apparatus, the secondary carotenoids are produced to high levels and are dispensed in oily droplets. They function to form a protective layer when the cells are exposed to stressed conditions, and provide the characteristic pink/red color of some stressed algae (Begum et al., 2015; Wang et al., 2015). Most carotenoids are found in ester or di-ester form, therefore saponification is needed after the extraction of pigments (Rebecca et al., 2011).

Due to their anti-oxidant property, carotenoids can protect cells from reactive radicals, prevent lipid peroxidation, and promote the stability and functionality of the photosynthetic apparatus (Grossman et al., 2004). The integrity of membranes, which is essential for cell survival, can also be promoted by carotenoids. In particular, they improve the cell membrane fluidity under high temperature or high light conditions (Camejo et al., 2006). Similar stabilization effects were reported for low temperature as well when the lipids became more unsaturated (Ramel et al., 2012). In addition, the excess energy generated inside the cell can be dissipated as heat by non-photosynthetic quenching (NPQ). The energy dissipation is to protect cell damage from chemical reactive species ($^1O_2^+$, ^{3}Chl*), and is achieved by intersystem crossing from triplet state carotenoids to the ground state, (Musser et al., 2015; Niyogi et al., 1997; Velikova et al., 2005).

2.1 Biosynthesis of carotenoids

The biosynthesis of carotenoids differs from species to species; however, almost all photosynthetic microalgae or plant species share the common primary metabolic pathway as shown in figure 2. All pathways initiate from the same C5 building block, isopentenyl pyrophosphate (IPP) or its isomer, dimethylallyl diphosphate (DMAPP), produced from either Acetyl-CoA (the cytosolic mevalonic acid pathway (MVA) pathway) or pyruvate and G3P (the plastidic methylerythritol 4-phosphate (MEP) pathway). Although both pathways lead to the same end-product, it was suggested that the carotenoid synthesis uses IPP or DMAPP derived from MEP pathway (Barredo, 2012). Then the intermediate C15 farnesyl diphosphate (FPP) or C20 geranylgeranyl diphosphate (GGPP) is synthesized by successive chain elongation in the head to tail style in the presence of enzymes. This step is followed by head to head condensation as in Figure 2, which forms the C40 carotenoid, phytoene. In the presence of desaturase, ζ-carotene can be formed in algae or higher plants (the metabolic pathways in bacteria or fungi would slightly differ). Then the first colored carotenoid, lycopene is formed (Varela et al., 2015). Further, by two types of cyclization reactions, the commonly recognized α-carotene or β-carotene structures are produced. Additional chain transformations, including hydroxylation, epoxidation, ketolation, glycosylation and oxygen cleavage then can lead to the highly diverse carotenoids family (Barredo, 2012). Astaxanthin however is not found in many higher plants, it is more commonly synthesized from canthaxanthin or zeaxanthin by photosynthetic microalgae

(Mann et al., 2000).

The biosynthesis of carotenoids takes place in the chloroplast, with some specific steps located in the cytoplasm. Phytoene synthase (*PSY*) is among the key enzymes for carotenoid biosynthesis in photosynthetic organisms since it carries out a rate limiting step. The expression for *PSY* or other synthase genes can be up-regulated by environmental stresses. Several reviews on enzymes in biosynthesis pathway of carotenoids are available (Bertrand 2010; Nisar et al. 2015).

2.2 Chemical synthesis of carotenoids

The total chemical syntheses of carotenoids (starting with β-carotene synthesis), was developed by three teams (Karrer and Eugster; Inhoffen et al., and Milas et al.) independently in 1950 (Britton et al., 1996). Two examples of the total synthesis approach are illustrated in Figure 3. Currently, many synthesis pathways are available. The first scaled up method was the Roche synthesis (C19+C2+C19, Grignard coupling, elimination, then partial hydrogenation) by F. Hoffman-La Roche & Co. Ltd in 1954. Later in 1960 the higher yield Badische Anilin & Soda-Fabrik (BASF) pathway emerged based on the Wittig condensation from the original synthesis of Inhoffen et al., C20+C20; and many other pathways emerged later (Ribeiro et al., 2011). Synthetic large scale production of astaxanthin became available in 1990s, also from the Roche group (Higuera-Ciapara et al., 2006). More recently, the fermentative reduction method for industrial-scale total synthesis of (3R,3'R)-zeaxanthin led to a new direction of carotenoid synthesis (Ito et al., 2009).

Although the chemical synthesis of carotenoids is a well-established market, the use of these products in direct human consumption is limited due to potential safety concerns. The natural carotenoids are usually complex mixture of various isomers, and are usually found mixed with other bioactive compounds. Synthetic carotenoids, however, are dominantly all-*trans* compounds. Due to the competitive inhibition among carotenoids for human absorption, the intake of certain synthetic carotenoid isomers is considered not as safe as the intake of the natural occurring mixtures (Patrick, 2000). Thus the applications of synthetic carotenoids are limited to animal feed, colorants, preservants etc. The natural carotenoids have the advantage of lower toxicity and higher customer preference for medicine or as supplements (Praveenkumar et al., 2015). Therefore, with the high cost of chemical synthesis, natural carotenoids are gaining more attention. However, due to current production technology limitations, only ca. 2% beta-carotene of the global market is from natural sources (Dufossé et al., 2005).

3. Significance of carotenoids to human health

Even though carotenoids have widespread applications as food colorants, cosmetics and feed additives (Ye et al., 2008); it was not until recently that the benefits of carotenoids for human health were better understood. Similar to the protective roles carotenoids played in microalgae and plants, these pigments provide a protective role for humans. Many studies have

reviewed the health benefits of carotenoids, which are usually related to their anti-oxidant activities (Britton, 1995; Chuyen and Eun, 2015; Fiedor and Burda, 2014; Manayi et al., 2015; J. Zhang et al., 2014). The anti-oxidant property in general mediates the harmful effects of free radicals, hence can potentially protect humans from compromised immune response, premature aging, certain cancers, cardiovascular diseases, and/or arthritis. Anti-oxidant pigments are also frequently reported to reduce the risks of AIDS, diabetes, cataract, macular degeneration, and neurodegeneration (Dufossé et al., 2005; Varela et al., 2015). Deficiency in these pigments may result in exophthalmia, night blindness, and in severe case keratinization of the conjunctiva and cornea (Britton, 1995).

At the present time, the major carotenoids of market interest are β-carotene, astaxanthin, lutein (with zeaxanthin), lycopene, and canthaxanthin. Fucoxanthin is another carotenoid that can be produced by microalgae. Although not a major market sharer, fucoxanthin has been marketed an anti-obesity functional food, anti-cancer and potential anti-inflammatory activities (Heo et al., 2010; Nanba and Toyooka, 2008). The health benefits of these six common carotenoids to human health are summarized in Table 1.

Astaxanthin and β-carotene are the two most recognized carotenoids in the global market, and make-up almost half of the carotenoid market (Business Communications Company, 2015). Astaxanthin is best recognized for the pinkish color in aquatic fish and shrimps. Being the strongest anti-oxidant in carotenoids, astaxanthin exhibits several-fold stronger anti-oxidant activity than vitamin E and β-carotene. As reported by some authors, it has the potential to enhance antibody production, anti-aging, sun-proofing, and it also demonstrates anti-inflammatory effects when administered with aspirin (Li et al., 2011). Guerin et al. (2003) have reviewed the benefits of astaxanthin for human health. Another carotenoid, β-carotene, is responsible for the prevention of toxin build-up in liver, potentially improves the immune system, and may have a preventative role in eye diseases like night blindness and cataract (Dufossé et al., 2005). However, some recent studies have related long-term β-carotene intake with increased risk of cancer as well as increased cancer death rate (Liu, 2013; Virtamo et al., 2014).

Two other bio-products, i.e., lutein and zeaxanthin are also becoming increasingly important in the nutraceutical market since they are now understood to play a significant role in eye health (Manayi et al., 2015). As the predominant pigments in the macula, lutein is clinical proven to prevent cataract and macular degeneration. These compounds also may function as strong anti-oxidants to decrease around 60 chronic disease risks (Ye et al., 2008). In general, these two xanthophylls are not considered toxic, and are relatively safe for human consumption.

Lycopene was marketed as an anti-oxidant and was proposed for treatment of cardiovascular diseases and prostate cancer; however, insufficient scientific evidence is present at this time to support this claim. Canthaxanthin may protect people from some blood disorder diseases. However, it was reported to be possibly unsafe in daily consumption, and may potentially cause blindness or aplastic anemia when consumed in large quantities for the purpose of creating skin tan color (Clinton, 1998; J. Zhang et al., 2014). Fucoxanthin is another carotenoid that can be produced by microalgae, and it is attracting increasing attention for its potential anti-obesity, anti-cancer and anti-inflammatory activities (Heo et al., 2010; Nanba and

Toyooka, 2008). It is also considered a safe compound for human health and some authors reported that it did not exhibit toxicity and mutagenicity at low dosage (Beppu et al., 2009; Peng et al., 2011).

4. Advantages and Disadvantages of microalgae as a carotenoid source

Microalgae have potential to serve as a natural pool of biochemicals with various health potential. Compared to higher plants, microalgae have a faster growth rate. Lin *et al.* (2015) reviewed the technological aspects and productivity of lutein using microalgae vs. Marigold flowers. Microalgae, especially those strains belong to chlorophyta, such as *Dunaliella salina*, *Haematococcus pluvialis*, *Chlorella vulgaris*, *Chlorella zofingiensis* and *Chlorella pyrenoidosa* have been successfully developed in the mass production of β-carotene, astaxanthin, canthaxanthin, lutein and other carotenoids (Kyriakopoulou et al., 2015; Mendes-Pinto et al., 2001; Prommuak et al., 2013; J. Zhang et al., 2014).

Compared to higher plants, microalgae usually have a greater specific carotenoid content (mg/g). The lutein content in marigold flowers is commonly reported to be 0.3 mg/g, while for microalgae, the content is usually over 4 mg/g (Ho et al., 2014). Both astaxanthin and β-carotene were reported to be over 50 mg/g under specific stress conditions of cultivation for the microalgae (Kyriakopoulou et al., 2015; Suh et al., 2006). Microalgae also have higher carotenoid content than macro-algae as the alternative source. For example, some microalgae have up to 15 times higher fucoxanthin (18.23 mg/g) than the predominant producer seaweeds (Gómez-Loredo et al., 2015; Kim et al., 2012).

Due to their versatility in adapting to a wide range of growth conditions and climates, (e.g., glacial to tropic, fresh water to hyper-saline), and varied pH, microalgae display a clear advantage over higher plants. These microalgae can be produced year-round, this eliminate the requirements of long-term storage and subsequent, potential degradation of the stored carotenoids. Meanwhile, wastewater can be used as nutrient source. Therefore, the micro-algal process helps to reduce the pressure on both the carbon and the water footprint.

Microalgae production of carotenoids is less labor-intensive compared to higher plants as it does not require cutting, drying and many other common farming operations. In addition, some pigments like astaxanthin are rarely found in higher plants, makes microalgae a more versatile source for carotenoids.

Chemically synthesized carotenoids are generally cheaper than natural pigments, but many undefined diseases have been related to the use of synthetic products (Göçer et al., 2006), e.g. synthetic β-carotene has been related with some increasing risk of lung cancer and cardiovascular diseases in smokers or asbestos workers (Omenn et al., 1996). The biological functions and food safety concerns have increased the recent market on natural pigments in particular for human consumption (Li et al., 2011). However, although many studies have been carried out, the cost of production of most carotenoids using microalgae is still prohibitive. Many challenges still exist in downstream processing, especially the harvesting and extraction processes. These aspects are considered below.

5. Current technologies for carotenoid production

Synthetic astaxanthin and β-carotene have occupied the majority of the market. While the production of carotenoids from microalgae is of increasing interest, only natural astaxanthin is approved by FDA for direct human consumption. The synthetic astaxanthin costs ca. $1000~2000/kg, and sells ca. $2500/kg; while the estimated cost of production of natural astaxanthin can be reduced to $700 using microalgae as a source, or $250 using fungi (Li et al., 2011; Nguyen, 2013).

In the photosynthetic metabolism of secondary carotenoids, mainly astaxanthin and canthaxanthin, the pigment content can be increased by introducing environmental stresses such as elevated light, low nitrogen, and / or salt-stress. *Haematococcus pluvialis* is able to accumulate over 50 mg/g astaxanthin, therefore it is recognized as one of the major source of astaxanthin since the late 1990s. Genetic modification for higher astaxanthin content has been successfully developed for this strain. Some companies have been established based on this production line, e.g. Cyanotech (Hawaii, USA), Alga-technologies (Israel), and Astareal (Japan). The switch of some companies like Algacan (Canada) from biofuel production to carotenoids production also showed the feasibility of this process.

One other mature bio-product line is β-carotene from microalgae *Dunaliella salina,* which shares similar bioprocessing operations as for astaxanthin production. The largest production processes are reported to be in Austria (Curtain, 2000) and Israel (Ben-Amotz, 2004). A two-stage cultivation strategy is commonly applied due to the contradiction of growth and pigmentation. The first stage is a "greening" phase where the most suitable conditions for micro-algae growth are provided. When the cell concentration reaches a certain level, stress conditions (such as low nutrients or high light) are applied to force cells accumulate more carotenoids, this is called the "reddening" phase since the cells turn a red or pink color (Wichuk et al., 2014).

Lutein produced from Marigold flowers (*Tagetes erecta* and *Tagetes patula*) has a more competitive price than synthetic methods. Therefore, the biological process is dominant in this industry. Marigold flowers are rich in xanthophylls and have the advantage of their simple xanthophyll component in the petals: no significance level of other pigments exist other than lutein and zeaxanthin esters. Therefore, the extract is easier for further separation and purification processes. Usually, the milled dry flower petals undergo a solvent extraction process (typically n-hexane) for the oleo-resin, and if necessary, potassium hydroxide (KOH) can be added for the release of free lutein. The production areas are mainly located at developing countries like China, India and some African countries due to the labor-intensive process. Companies like Super Lutein (Japan) sells lutein as eye health promoting products. Microalgae have been proposed as an alternative lutein source, extensive research has been conducted on process development due to the higher lutein content. A comparison of the two processes can be seen in figure 4. The processing of lutein from microalgae requires fewer operational steps than that from Marigold flowers to produce crystalline form of lutein.

Apart from the processes discussed above, a yeast (*Phaffia rhodozyma*) has been reported

to have potential to produce astaxanthin, yielding higher biomass concentration and less heavy metal content. The microalgae *C. zofingiensis* is also able to produce canthaxanthin at a level of 8.5 mg/g under salt stress and light limiting conditions. Bacteria such as *Xanthophyllomyces dendrorhous, Saccharomyces cerevisiae, Blakeslea trispora and E. coli* have been genetically modified to produce astaxanthin, β-carotene, lycopene, and canthaxanthin (Nanou and Roukas, 2016; Scaife et al., 2012). Although satisfactory carotenoids content can be achieved (over 10 mg/g) (Alper et al., 2005; Li et al., 2013), the cost of production still remains high in both biomass production and for downstream processing.

6. Technologies of microalgae cultivation for carotenoid production

In this section, the technologies used to cultivate microalgae are discussed briefly. To acquire high productivity of carotenoids, both the microalgae production rate and carotenoid content in microalgae need to be optimized. First the strategies for microalgae production are described.

6.1 Cultivation systems

At this time, the two most commonly applied technologies of the microalgae cultivation for carotenoids production are the open pond system or closed photo-bioreactors (PBRs). The cost of open ponds is reported to be much lower than for closed PBRs. Raceway ponds are the most commercially employed method as they are cheapest to construct and maintain (Borowitzka and Mohemani, 2012). Paddle-wheels usually gives a flow rate and suspend cells more uniformly, provide better mass transfer (Singh and Sharma, 2012). The liner is typically the most expensive capital cost, with mechanical mixing as the major operational cost. Other open pond systems including shallow lagoons and ponds, inclined systems, circular central-pivot ponds, and mixed ponds were also available, but much less attention has been paid to them due to the low productivity. Rogers et al. (2013) have estimated the economic requirements for open ponds, concluding that water loss, CO_2 and nutrient requirements would be the major concerns for large scale algae production. The drawbacks of using open pond systems are obvious: uneven light intensity, and poor mass transfer; bad weather resistance aside from tropical areas, and contamination from other algal/bacterial strains (Singh and Sharma, 2012). Therefore, the closed PBRs seem preferred.

Wichuk et al. (2014) stated that the efficiency of light-driven photosynthesis is the bottleneck for large scale microalgal carotenoids production. PBRs represent the most successful approach in harvesting light, optimizing fluid dynamics, mass transfer and mixing, and minimizing water loss. Flat or tubular PBRs are the basic design structures; various modification and extensions can be added based on them. As PBRs are much more expensive to build and operate, inexpensive approaches to scale up are the major challenges now facing the researchers and industry. However, due to the better control of operating conditions in a PBR, higher biomass productivity quality is possible (Gupta et al., 2015). Olivieri et al. (2014) have

summarized the advances in PBR design for microalgae production and modeling. An alternative approach using an immobilized film method (attached cultivation) was studied by Zhang et al. (2014), however, it was found not as efficient as suspended algal cultures in PBRs due to poor light penetration (Zhang et al., 2016).

Heterotrophic microalgae cultivation typically employs stirred tank bioreactors since light penetration is not an issue and increased mass transfer is possible due to enhanced mixing. For autotrophic growth, vertical tubular or airlift PBRs are easy to build, and have relatively satisfactory biomass production considering the cost. One limitation for long tubular PBRs is O_2 inhibition due to O_2 accumulation in the tubes. Flat panel PBRs (FBR) are available for high-density algal production under autotrophic growth conditions. However, the FBR is difficult to scale up owning to its configuration. Acién-Fernández et al. (2013) concluded that most current PBR technologies are available for large-scale production, with companies established in Europe and all over the world. The cost of cultivating microalgae may be reduced to $5/kg in a horizontal PBR when operated in a 100-hectare scale, cheaper than raceway ponds at commercial scale (Kleinegris et al., 2011). In another study, Li et al. (2011) evaluated the economics of a two-stage large scale microalgae production in (a) 1000 to 8000 L airlift PBR, (b) 100 m^2 raceway pond, getting an estimated astaxanthin cost of $718/kg, and an algae production cost of $18/kg.

6.2 Cultivation strategies

Among the microalgae species, the following were most frequently documented for carotenoids production: *Chlorella vulgaris, Chlorella protothecoides, Scenedesmus almeriensis, Dunaliella salina, Haematococcus pluvialis, Porphyridium cruentum* (Rhodophyta), and *Haslea ostrearia* (Diatom) (Pignolet et al. 2013). Stress conditions are often applied for high carotenoid content in microalgae, but different carotenoids have varied responses to stress conditions (Hodgson et al., 2016). The pigment content of some carotenoids like astaxanthin can be elevated from a few mg/g to over 50 mg/g, while the contents of carotenoids like lutein changes in much smaller scale and the microalgae growth rate is more essential in this case.

6.2.1 Stress-driven adaptive evolution

Unfavorable environmental conditions can be used for adaptive evolution. This represents a most adopted growth strategy to enhance carotenoids production. Investigation of the parameters involved in this process can help to obtain high carotenoid productivity. The limited production of biomass under stress could be countered by applying multi-stage growth strategy (Hodgson et al., 2016).

6.2.1.1 Primary carotenoids

Primary carotenoids are growth-coupled metabolites. Lutein is a typical primary carotenoid that suffers degradation under stress. Located in the chloroplast and mitochondria membranes

(Collins et al., 2011), the pigmentation of lutein occurs at the center region of the algal cell. Many variables affect lutein productivity, the most common being the type of algal species, temperature, light intensity, photoperiod, pH, nutrient availability, and salinity.

Temperature controls the enzymes involved in carotenoids biosynthesis, and it also controls the growth rate. Low temperature decreases the nutrient uptake rate, and slows lutein accumulation (Bhosale, 2004). Higher temperatures are favorable for cell growth and lutein accumulation. Fernández-Sevilla et al. (2010) showed that 28^{o}C is the optimum temperature for the lutein production considering the cell growth rate, while the inhibition from temperature starts at 32^{o}C where the cellular lutein content decreases to half of that at 28^{o}C.

Light is a critical factor affecting carotenoid pigmentation. High light intensity increases the lutein fraction among the pigment pool (Maxwell D.P. et al., 1994), while decreasing the cellular lutein content. However, due to the benefits of abundant light to the microalgae growth (Xie et al., 2013), the lutein productivity increases as light intensity increases from 186 to 460 µmol photon m^{-2} s^{-1} (Cordero et al., 2010). Solovchenko *et al.* (2008) stated that the irradiance tolerance is a strain specific characteristic as a 6-8% decrease in total lutein content were observed for *Parietochloris incise*, whereas *S. almeriensis* exhibited good light tolerance till 1625 µmol photon m^{-2} s^{-1}. In addition, the full white light spectrum is more favorable than a monochromatic LED light sources for lutein production (Ho et al., 2014). Microalgae also accumulate lutein under heterotrophic conditions. With 40 g/L glucose, a lutein productivity of 83.8 mg/L can be reached (Shi et al., 2000).

Nutrients also influences the lutein accumulation with the nitrogen source being most essential for lutein production, however conflicting results have been reported previously. When present in sufficient quantity, nitrate does not show significant effect on the lutein content, but as nitrate content decreases, lutein content also decreased dramatically in *C. zofingiensis* (Cordero et al., 2010). The interaction between nitrate concentration and salinity is most significant for lutein biosynthesis from *D. salina* (Fu et al., 2014). Therefore nutrient rich conditions favor the growth (Xie et al., 2013), while the nitrogen source does not influence the lutein production in *Muriellopsis sp.* (Jin et al., 2003).

The addition of oxidizing substances can slightly introduce oxidative stress hence increase the cellular lutein content (A Catarina Guedes et al. 2011). Salinity itself also does not influence the lutein content, while combined with light or proper trace metals, it can improve the lutein production rate by 80% to 260 mg/L in *C. vulgaris* and remain stable when scaled up to a 25,000 L fermenter (Jeon et al., 2014).

The pH is important as it influences the CO_2 availability via the chemical conversions between CO_2, HCO_3^- and H_2CO_3. The shifting of *C. onubensis* growth from air to CO_2 provides increased cell growth; however, the accumulation of lutein at high cell density (5-6 g/L) does not depend on CO_2 concentration (Vaquero et al., 2014). Unlike the carotenoids that could be over-produced by stress conditions, natural over-production of primary carotenoids like lutein is much more difficult. Genetic modification might be the potential solution for this challenge (Mulders et al., 2014).

6.2.1.2 Secondary carotenoids

Some of the carotenoids, namely secondary carotenoids like astaxanthin, can be produced under extreme conditions of microalgal stress to achieve greater cellular content levels. However, several primary carotenoids like β-carotene, can also act as secondary metabolites under stress conditions and therefore are discussed here (Hodgson et al., 2016). The secondary carotenoids can be found in cytoplasmic lipid globules under stress conditions rather than in the chloroplast (Collins et al., 2011). Two-stage cultivation has been successfully adopted for these types of carotenoids' production (Wan et al., 2014). In the first stage, optimal conditions for cell growth allowed for cell accumulation (green phase, flagellate cell), while in the following stage (red phase, cysts), stress conditions are introduced for the pigmentation. This strategy is most commonly used for astaxanthin production. Continuous growth in the single stage under limited stress is easier in terms of operation, but is applied less nowadays due to poor astaxanthin accumulation rates. Aflalo et al. (2007) compared the difference between the two strategies for astaxanthin production, and they concluded that the two-stage operation was easier to scale up as well. Suh et al. (2006) developed a double layer reactor combining both green and red growth phases in a single reactor for simultaneous cell growth and astaxanthin production, and obtained an astaxanthin content of 57.9 mg/g in this system.

The reduction of nitrogen, phosphate, and the introduction of salts such as NaCl, especially under strong light conditions are also effective strategies for astaxanthin accumulation (Harker et al., 1996; Orosa et al., 2000). The stress condition requirement is similar for β-carotene (Bhosale, 2004).

Light also plays an important role in carotenoids pigmentation at a wide range of intensity, from 50 to over 1250 µmol photon m^{-2} s^{-1}. Under strong light, cell division slows down and cell lysis increases (Bhosale, 2004). An increase in β-carotene content can be observed in *D. salina* under strong light (Ribeiro et al., 2011). Continuous light might also be favorable in terms of stressing the microalgae (Bhosale, 2004). Zhang et al. (2016) modeled the light attenuation, temperature, and nitrogen sources and concluded that 27 ^{o}C, 4.4 mM NO_3^- would be optimal for astaxanthin production. Similarly, through another modeling study, a pH = 9 and 20% NaCl, were found to benefit β-carotene production (Çelekli et al., 2014). The fucoxanthin accumulation by *Cyclotella cryptica* (diatom) is promoted by controlling light and nitrate (Guo et al., 2016). However, the best fucoxanthin production is not at the best growth conditions for *Isochrysis galbana* (diatom). More studies are required to reveal the effect of culture conditions on fucoxanthin production (Gómez-Loredo et al., 2015). Heterotrophic growth is not desirable for the carotenoid production, as the highest β-carotene content was around 1.01 mg/g (Ip and Chen, 2005), much less than that of autotrophic conditions where it can reach 40mg/g (Aflalo et al., 2007). Therefore insufficient work has been done for heterotrophic algal carotenoids production (Lowrey et al., 2015).

Temperature has strong effect on carotenoids accumulation. Considering the growth rate and cell density, 24 to 29^{o}C would be a suitable range for carotene production from *D. salina* (Bhosale, 2004). Ras et al. (2013) studied the ability of microalgae to endure temperatures above

the optimal range, which would of particular interest in tubular PBRs since heat might accumulate as light is focused in the center, especially during outdoor cultivation. The effects of day and night time temperature were studied by Wan et al. (2014), to improve outdoor astaxanthin production rate. Daytime temperatures at 28^{o}C and slightly lower temperatures in the night were found to be optimal for both growth and astaxanthin accumulation. Employing this method, 40- 45 mg/g astaxanthin can be obtained (Margalith, 1999).

Ferrous salts also generate oxidative stress through the formation of hydroxyl radicals, thus can be used as an alternative energy saving strategy in place of strong light. This has been investigated for canthaxanthin production in *C. zofingiensis* but not for astaxanthin production (Pelah et al., 2004). The combination effect of strong light and low nitrate was examined by Cordero et al. (2010). Other heavy metals can also introduce oxidative stresses, but may not be suitable for human consumption. Wang et al. (2013) indicated that initial cell density has an impact on growth, and 0.8 g L^{-1} was best for astaxanthin production. Astaxanthin productivity of 38 mg/g or 16 mg L^{-1} d^{-1} was possible under outdoor cultivation conditions.

6.2.2 Metabolic engineering

Microalgae serve as an excellent model host for metabolic pathway regulation or genetic engineering since they present the advantage of simplicity of culture and fast growth rates compared with plants. In addition, microalgae physiological and genetic analogies with plant cells, therefore could potentially reveal the genes coding for carotenoids biosynthesis in plant (Gimpel et al., 2015; Leu and Boussiba, 2014; Shah et al., 2016; Varela et al., 2015).

Mutagenesis using UV radiation or other methods were applied to wild strains of microalgae for strain improvement. Jin et al. (2001) used mutagenesis to enhance zeaxanthin production in *D. salina*, and successfully generated two zeaxanthin-overproducing strains. A zeaxanthin epoxidase mutant was recognized in the study (Jin and Melis, 2003); analogous mutations exist in other strains like *S. obliquus* and *C. reinhardtii* (Ghosh et al., 2016). The zeaxanthin content (per cell) is 15-fold higher than the wild type under non-stressed conditions (Polle et al., 2003). Site-directed mutagenesis of enzyme phytoene desaturase was also reported for *H. pluvialis* astaxanthin production (Steinbrenner and Sandmann, 2006).

Extensive studies have been conducted to transform model microalgae, like *C. reinhardtii*, *N. gaditana* and *P. tricornutum* (diatom) (Jinkerson et al., 2013; Varela et al., 2015), while other strains are beginning to be understood, like *Nannochloropsis sp*. (Kilian et al., 2011), *S. obliquus* (Guo et al., 2013), or β-carotene-producing *D. salina* (Feng et al., 2009) and astaxanthin-producing *H. pluvialis* (Kathiresan et al., 2009). Ghosh et al. (2016) have summarized the strains for which genomic projecst and transformation has been successfully completed.

The key metabolic steps controlling carotenogenesis are discussed by Giuliano (2014) and the vector construction and gene selection strategies are reviewed by Qin et al. (2012). The genetic engineering of microalgae toward carotenoid production requires sufficient isoprenoid precursor supply, which represents one of the major approaches and may be realized by the

overexpression of the important enzymes in the pathway or silencing/suppressing branch pathways via RNA interference (RNAi) (Varela et al., 2015). The enzymes to be highlighted are PSY, PDS, BKT in conventional genetic engineering strategies to increase carotenoids production. Extensive attempts by single or multi-gene overexpression of these proteins have been conducted and summarized by Gimpel et al. (2015). The coding gene is different for the main target PSY in different strains (Ye et al., 2008), by overexpression of the corresponding gene in *Chlamydomonas*, a 2-fold increase in carotenoid level was displayed (Cordero et al., 2011). By *PDS* gene mutation, the astaxanthin levels were increased in *H. pluvialis* (Steinbrenner and Sandmann, 2006). Additionally, expression of BKT in *C. reinhardtii* can lead to the synthesize of keto-carotenoids not present in the wild strain (León et al., 2007), and hydroxylase (CHYb) genes are also associated with astaxanthin overproduction as revealed in a study with *C. zofingiensis* while the PDS gene is the dominant factor (Liu et al., 2014). For fucoxanthin from *P. tricornutum* (diatom), the DXS transformants reached 2.8 fold higher fucoxanthin content, while PSY transformants reached up to 1.8 fold than wild type (Eilers et al., 2016). When in combination with the central carbon metabolism transformation, additional carotenoids increase can be achieved (Heider et al., 2014). Since a nitrogen source is essential for protein synthesis and cell division, the deprivation of nitrogen source would enhance LCYb enzyme synthesis, hence increase the pigmentation rate (Cordero et al., 2010). The approaches for sufficient supply of IPP and DMAPP was reviewed previously (Harada and Misawa 2009). Besides, balanced expression of the target genes and the creation of sufficient storage space for overproduced carotenoids are also necessary (Heider et al., 2014; Mulders et al., 2014; Varela et al., 2015). Addition of the transport route for the biosynthesized pigments out of the photosystem to cytoplasm may help increase the carotenoids productivity as well (Mulders et al., 2014).

The complex multi-enzyme pathways of carotenoid biosynthesis have hindered classical approaches on genetic improvement based on random mutagenesis or multiple transgenes overexpression (Daboussi et al., 2014). By the manipulation of the central regulatory carotenoid transcription factors (TF), ideally changing only one central regulator of a pathway to activate multiple components in the pathway, the emerging transcriptional engineering (TE) may provide a better solution (Bajhaiya et al., 2016). Identification and characterization is vital for the success of TE; the possible methods include mapping target genes and determining *cis* elements, importing foreign TFs from other biologically relevant organisms, and generating synthetic TFs by *in silico* design. Emerged genome editing tools during the past decade include zinc-finger nucleases, meganucleases (MNs), transcription activator-like effector nucleases (TALEN) and clustered regularly interspaced short palindromic repeats (CRISPR/Cas9) (Bajhaiya et al., 2016; Daboussi et al., 2014; Scranton et al., 2015).

To date, the modification of the genome of microalgae is reported in only a few studies. The feasibility of TE is suggested by studies of *Dunaliella bardawil* (Lao et al., 2014). and *Chlamydomonas* (Gargouri et al., 2015). Baek et al. (2016) successfully used DNA-free CRISPR/Cas9 to knock out genes and enhanced zeaxanthin production. *P. tricornutum* (diatom) genome is stably modified by target editing tools of meganucleases and TALEN; while zinc-finger nuclease technology is used for *Nannochloropsis* and *C. reinhardtii* genome

modification (Daboussi et al., 2014; Kilian et al., 2011). Therefore, genetic engineering including TE is a promising biotechnological approach for carotenoids production, while much work is needed to achieve high productivity and stability of the transformants (Bajhaiya et al., 2016; Varela et al., 2015).

7. Downstream processing for carotenoids

Kim (2013) stated that harvesting and extraction are the two most expensive steps in microalgal carotenoids production. Cost reduction in downstream processing hence needs to be emphasized (Park, 2015). These various downstream strategies are discussed below.

7.1 Harvesting

Harvesting of suspended microalgae is a major challenge, and the difficulty increases as the cell size decreases. The harvesting process accounts for 20- 30% of the total cost to produce microalgae (Georgianna and Mayfield, 2012). Currently no suitable method is present for microalgae harvesting, especially for the carotenoid production process as the latter usually requires non-toxicity and minimizing carotenoid degradation. Large cell size and auto-flocculation might be of interest, and the experiences from water treatment can be borrowed owing to comparable techniques (Uduman et al., 2010).

7.1.1 Physical methods

Centrifugation is a reliable, fast and efficient method, most widely applied for harvesting microalgae in both lab scale and small industrial applications especially for astaxanthin production. It is suitable for most of algal strains. Over 80% microalgae were reported to be recovered from a suspension within 2-5 minutes (Chen et al., 2011). However, the high capital cost and continuous energy investment during the operation has largely limited further scaling-up. In addition, there is potential to damage the cell structure during high speed centrifugation. Grima et al. (2003) has reviewed the technical aspects of microalgae harvesting. Sedimentation was considered as an economical approach.

Filtration or screening is greatly dependent on the particle size. For small size microalgae, this process may be extremely time and energy consuming. Counter-current technologies or turbulent flow can be used to reduce the fouling or clogging of the filter or membrane.

Gravity sedimentation is an inexpensive method, but it requires a very long time for small uniformly suspended culture when no additional flocculants are present. Around 15% cell density can be achieved by this means, but this is very species specific and usually is therefore more suitable for large, dense, and non-motile cells such as some diatoms. Currently, sedimentation is sometimes used as a first stage treatment to reduce the energy consumption when combined with other methods, most commonly centrifugation to get less moisture content.

7.1.2 Chemical method for cell harvesting

Comparing with mechanical methods, the chemical harvesting method consumes much less energy, and requires lower capital investment. The major cost is for the use of flocculant chemicals. Although not as efficient as mechanical methods; and with a higher final water content in the slurry, flocculation has received much attention due to the possibility to treat large scale microalgae suspensions at a lower cost. This method is also widely applied in industry, in particular, for water and wastewater treatment (Gorin et al., 2015). Flocculation works by adding coagulants to neutralize the surface charge on suspended particles and/or increase the particle size to accelerate the sedimentation process. The type and dose of flocculent is species dependent; the required concentration of flocculent may range from 10-50 mg/L or more, while the types can vary from inorganic salts, mainly aluminum or ferric based, polymer based or nanoparticles and magnetic particles (Hu et al., 2013). Polymer based flocculation poses less of an environmental burden, and may be potentially non-toxic. Some literature however has reported decreased quality of the subsequent product and reduced quantity of carotenoids, mainly due to the covalent bonding through the coagulant to the polar functional groups of the pigments (Utomo et al., 2013). Meanwhile, the addition of chemicals also adds a complexity to the subsequent treatment (Hu et al., 2013), and the technology may not work well with marine micro-algae.

Flotation is another method in contrast to with sedimentation/flocculation. The solids float to the surface of the liquid assisted by gas bubbles. In dissolved air flotation, the pressure of the gas is essential as it is associated with both bubble size and the prevention of back flow. Dispersed air flotation is slightly different, interaction of air bubbles with the negatively charged surfaces of algal cells is important for effective harvesting (Pragya et al., 2013). In general, flotation is species specific and involves high capital cost and operational requirements, therefore it is not the best recommended method at present (Uduman et al., 2010).

More advanced harvesting methods usually involves electromagnetic techniques. Since no additional chemicals are added, this makes them more environmental compatible. However, the fouling in the electro-cathodes may cause problems in large-scale operation (Chen et al., 2011). Generally, development of an efficient and cheap harvesting method is an urgent need at this time in order to produce micro-algal carotenoids.

7.2 Cell disruption

Cell disruption is often suggested as a necessary step to increase the carotenoids or lipid recovery yield by several fold. Therefore, although it introduces additional processing cost, the pre-treatment step is still considered obligatory. It has been pointed out that the selection of a suitable cell disruption method is algae species specific (McMillan et al., 2013). Cold soaking with solvents is enough for some frustule absent species; for example, *C. reinhardtii* is a good carotenoid source without cell disruption, or diatoms are good candidates as well. Kim et al. (2012) reported that approximately 95% fucoxanthin in *Isochrysis galbana* can be released by a single solvent extraction. For many other algae, the thick rigid cell wall requires cell disruption

to release the inner contents. Without cell disruption, the extraction results can be very inefficient (Chan et al., 2013; Gille et al., 2016). Michalak and Chojnacka (2014) summarized the advantages and disadvantages of some cell disruption techniques to extract biologically active compounds from algae without their degradation. However, most previous studies on cell disruption have been specifically for lipid recovery, not for carotenoids recovery, but the basic approaches are similar. The efficiency and advantages/disadvantages for different cell disruption methods as well as extraction methods are also compared in Table 2.

7.2.1 Mechanical disruption methods

Grinding, bead-milling and high pressure homogenizers are the most commonly adopted mechanical cell disruption techniques for microalgae for both laboratory and pilot scale. They are easier to scale up than other methods (Taucher et al., 2016). However, the high energy requirement for these approaches is still a significant limitation during bioprocess scale-up and careful control is needed to remove excessive heat generated to avoid carotenoid degradation.

7.2.1.1 Grinding

Manual grinding with wet biomass was reported to be able to efficiently extract pigments from microalgae (Hu et al., 2013). However, it is time-consuming and almost impossible to scale up (Utomo et al., 2013). Cryogenic grinding, or grinding with liquid nitrogen, is reported to be extremely efficient, but it also has limitations due to its high cost which makes it impractical for industrial applications (Grima et al., 2003; Zheng et al., 2011). A review on extraction methods for bioactive compounds including carotenoids, using both traditional and novel approaches is available by Kadam et al. (2013).

7.2.1.2 Bead milling

Bead milling offers a potentially attractive approach for larger scale operations in comparison to grinding. Previous studies demonstrated that bead-beating is most efficient for carotenoids extraction among various approaches such as autoclaving, bead-beating, microwaves, sonication, osmosis shock, French press, freeze and thaw, and lyophilization (Chan et al., 2013; Taucher et al., 2016). The mechanical solid shear in bead mills offers a non-specific and highly effective cell disruption. Two types of bead mill are currently available, one category involves shakers (bead beating) for laboratory use, and the other involves agitated beads for large-scale. In both types, high speed spinning of beads leads to collision or friction of the cells. This has the advantage of generally high efficiency and minimized contamination risk from the environment, hence this method is often adopted for DNA extraction (Mercer and Armenta, 2011). One challenge with this approach is that the efficiency is low when treating the small micron size microalgae like *C. vulgaris* (J. Y. Lee et al., 2010). Extensive heat can as well be generated during the extraction, which can result in degradation of functional compounds (Kim et al., 2015).

The container shape, mixing/agitation rate, bead amount, sample amount, bead size and bead type all have effects on the final cell disruption efficiency. Wet milling of *S. dimorphus* or *C. protothecoides* was shown to provide improved results than other extraction methods (Mercer and Armenta, 2011). In another study for *Botryococcus sp*., bead beater and MAE showed the best results (Pragya et al., 2013). However, the extraction may be strain specific, since for some cyanobacteria bead milling was reported to be not as efficient as some other treatments like ultrasound assisted extraction (UAE), microwave assisted extraction (MAE) or autoclaving (Prabakaran and Ravindran, 2011). The addition of beads also adds complexity to the system, which may require further separation of the beads. Continuous bead milling may be potentially used to recycle beads as well as increase extraction efficiency (Ho et al., 2008).

7.2.1.3 High pressure homogenizer

The high pressure homogenizer (HPH) is a continuous system that can deal with slurry algal suspension and allows for easy scale-up. The cell suspension is forced through a narrow nozzle outlet by high pressure pumping. A rotor-stator homogenizer is also available, but only suitable for low viscosity liquid treatment. The working principle of high pressure homogenizer is not well understood, but it is generally believed to be caused by the high shear force and cavitation in the liquid coupled with the sudden pressure drop between the nozzle and the outer environment (Ho et al., 2008).

The applied pressure, cell size, and nozzle diameter are the main factors for high pressure homogenizer system control. Cooling is also essential to prevent carotenoid degradation (Lee et al., 2012). High pressure homogenizers showed superior results than traditional pre-treatment methods like osmotic shock or enzymatic hydrolysis (Grima et al., 2003). This was also reported to be particularly suitable for *Chlorococcum* cells compared with bead milling and ultrasound assisted extraction (UAE) (Halim et al., 2012b; Kim et al., 2015) while the results were found to be contrary for *Botryococcus braunii*: the high pressure homogenizer was not as efficient (Pragya et al., 2013).

7.2.1.4 Autoclave

An autoclave involves high temperature steam and can efficiently break the microbial cell wall and extract lipids. This approach showed good results in a study by Lee et al. (2010) for lipid extraction from *C. vulgaris*. However, due to the temperature sensitive nature of carotenoids, such methods may not work for carotenoids extraction. This conclusion has been supported by the study of Chan et al. (2013) where lutein from autoclave treated algae was only 25% of that from bead milling.

7.2.2 Non-mechanical disruption methods

7.2.2.1 Microwave

Microwave assisted extraction (MAE) is a relatively new method (developed within the last 30 years) for cell disruption and currently employed in vegetable oil and animal oil extraction (Lee et al. 2010). The MAE generates high frequency waves with wavelength 0.001 m to 1 m. The electromagentic radiation is transmitted to the medium and can be absorbed to homogeneously heat up the mixture, and lyse the microalgae via rapid heat shock. The moisture content inside the cell is vaporized, producing a high pressure inside the cell towards the cell wall (Gil-Chávez et al., 2013). This allows better disruption of the cells and can be particularly effective for those algae with strong mechanical resistance (Barba et al., 2015). In addition to the operational factors of MAE (such as power, working volume, temperature), the dissipation factor, heat capacity of the solvent, and the polarity of solvent, are the other important factors affecting the extraction (Zheng et al., 2011). High heat dissipation factors coupled with a high dielectric constant would in general facilitate the extraction process (Gil-Chávez et al., 2013).

Less energy is required for MAE than for the mechanical methods mentioned earlier. Lee et al. (2012) and McMillan et al. (2013) demonstrated that MAE has similar efficiency as for bead milling operations, and MAE could break 94.92% *N. oculata* cells with 13 fold less energy consumption. It was reported that MAE can accelerate lipid extraction when combined with grinding (Soštarič et al., 2012). In addition, the thermal gradients in MAE are eliminated, which is a concern to other treatment methods especially ultrasonication. However, degradation of carotenoids usually starts from 60^{o}C (Pasquet et al., 2011), so additional temperature control for carotenoids extraction is needed. This may be the major obstacle for applying MAE for carotenoids extraction (Kadam et al., 2013).

Overall, MAE is a simple and efficient method for carotenoids extraction, since it requires less solvents and has demonstrated potential for further scale up making it potentially more economical. The reaction mechanism need to be further investigated, and MAE reactor design of large scale systems is still needed. Li et al. (2015) suggested that microwave reflection tank may be applied, while more efficient microwave absorbent need to be developed. It is also very important to monitor the temperature change to minimum carotenoids loss.

7.2.2.2 Ultrasonication

Ultrasound assisted extraction (UAE) was widely studied for the extraction of proteins, sugars and lipids. Cavitation is considered the underlying mechanism for UAE, where micro-bubbles form and collapse near the cells, creating micro turbulence, high liquid shear and pressure shock. All of these factors help to break the cell wall. UAE has the benefits of higher efficiency, reduced extraction time, low to moderate cost, negligible toxicity, and simple handling. Ultrasound frequency and working power play important roles in the performance of UAE systems (Wang et al., 2014). The sample volume is also particularly important for this

method as the energy dissipates easily through transmittance (Zheng et al., 2011). UAE is a scalable process owning to the recent developments of installations by arranging multiple devices with flowing fluid. OriginOil has applied this technology with electromagnetic pulses to disrupt cells (Mercer and Armenta, 2011).

When coupled with Soxhlet extraxtion, UAE was the most efficient in extracting lipids from *Crypthecodinium cohnii* (dinoflagellate) (Cravotto et al., 2008; Mercer and Armenta, 2011). For *Nostoc sp*. and *Chlorella sp*. cell disruption, UAE also showed the best results (Grima et al., 2003; Plaza et al., 2012). However, despite reported to be efficient in some studies, some conflicting results have been reported. In one study, UAE was reported to be insufficient to break microalgae cell wall (Pasquet et al., 2011). McMillan et al. (2013) concluded that UAE was not as efficient as heating in a water bath, Halim et al. (2012b) showed that high pressure homogenizer and bead milling gives much better results than UAE. However, since the comparisons were usually not conducted at the same energy output, the insufficiency of UAE may result from its low power input compared with other methods, (e.g. Ultrasonicator 40-130 W; bead beater 850 W; MAE 1000 W) (Halim et al., 2012b); The sample volume vary with each other (350 mL for UAE versus 10 mL in MAE in McMillan et al.'s study (2013)), which indicates another possible reason for the inconsistent results (McMillan et al., 2013). Therefore, based on the reported studies, it is difficult to conclude definitely whether UAE is suitable to treat microalgae.

7.2.2.3 Enzymatic hydrolysis

Enzymatic hydrolysis is an expensive process due to the high cost of enzymes. The major advantage of enzymatic hydrolysis is it decreases the activation energy of the chemical reaction, providing milder conditions for the process. Moreover, the selectivity is higher, leading to less by-product formation; no corrosion issue is involved and higher yield may be achieved. These benefits make this approach appealing for carotenoids extraction (Deenu et al., 2013). However, the high price of the enzymes and the requirement to maintain stable condition largely limits applications, and the longer hydrolysis time offers less potential for processing microalgae for larger scale industrial applications (Kadam et al., 2013; Zheng et al., 2011). Chen et al. (2013) suggested the co-cultivation of bacteria with algae to lyse cell walls, which would be an interesting alternative to traditional enzymatic hydrolysis (Kim et al., 2015).

7.2.2.4 Pulsed electric field

The pulsed electric field approach for cell disruption was developed since the 1990s. This is a non-thermal treatment that requires much lower energy input. By applying high intensity intermittent electric fields on the cells for periods of time in the order of micro-seconds, pulsed electric field assisted extraction improves the membrane permeability in cell membranes by electroporation (Luengo et al., 2014). The pores formed can be controlled to be reversible or irreversible by adjusting the intensity of the electric field. In the last decade, pulsed electric field

has been shown to be a efficient method to extract lipids and bioactive compounds from microalgae and plant tissues (Barba et al., 2015; Mercer and Armenta, 2011); this highly selective extraction method shown good ability in retaining the bioactivity of carotenoids (Grimi et al., 2014; Sánchez-Moreno et al., 2005). The short treatment time and smaller solvent requirements (Yu et al., 2015) makes it extremely attractive for carotenoids extraction. However, although this method can improve the extraction efficiency, it does not perform as well as HPH (Lai et al., 2014); and for the extraction of carotenoids by this method, organic solvent requirements remain essential (Luengo et al., 2014).

7.2.2.5 Osmotic shock, acid/ alkaline treatment, ionic liquids

The advantages of easy scale-up, low energy input, and modest capital cost made the physical or chemical treatment methods such as osmotic shock, acid/alkaline treatment methods attractive particularly in the operation aspects (Kim et al., 2015). However, osmotic shock is not capable to extract pigments for cells with rigid cell wall; on the contrary, and although cell breakage was achieved at high efficiency by acid/alkaline treatment, the carotenoids were destroyed (Halim et al., 2012a).

Ionic liquids have superior solubility of biomass and recently are became studied extensively. Ionic liquids acts as cell destabilizers in algae suspensions (Park et al., 2015), but the high price of ionic liquid based solvents, high energy requirement, and toxicity currently has prevented their industrial applications. In addition, the ionic liquids would cause carotenoids to degrade since they are not inert solvents. Therefore, ionic liquids are not suitable for carotenoids production.

7.3 Extraction

Carotenoids extraction is the vital step for pigment production. In addition to the conventional solvent extraction approaches, the development of super-/sub-critical extraction offers a new concept of operation while the cost greatly limited the use.

7.3.1 Conventional solvent extraction

Since “like dissolves like”, a similar polarity is vital for efficient recovery of target compounds from a mixture. Chloroform/methanol, hexane/isopropanol, ethanol and other solvents were widely used for efficient extraction (Sicaire et al., 2014). The Bligh & Dyer method and Soxhlet were most commonly adopted in the small scale chemical engineering processing as mature protocols (Dejoye Tanzi et al., 2013; Hita Peña et al., 2015). The principles and operational considerations of solvent extraction of lipids or bioactive products from microalgae have been widely reviewed, but few focused specifically on the extraction of carotenoids (Gil-Chávez et al., 2013; Reverchon and De Marco, 2006). In Taucher et al.'s study (2016), dichloromethane is the optimal solvent among the six tested solvents. The best extraction temperature is at 60°C. Apart from single solvent extraction, binary extraction systems are also reported. Dichloromethane and

methanol, chloroform and methanol, acetone and petroleum ether, and hexane and ethanol all presented better results than single solvent for carotenoids extraction (Soares et al., 2016).

The conventional solvent extraction is cheap and easy to scale up, the concern is these processes may take long time, require further treatment like evaporation to concentrate the extract, and cost large amount of solvents which brings environmental burden, so other more advanced extraction method were developed (Halim et al., 2012a).

7.3.2 Super-/sub-critical solvent extraction

Supercritical fluid extraction (SFE) has been well documented in literature for valuable compounds recovery from microalgae (Liau et al., 2010; Patil et al., 2013). Supercritical fluids have a similar density as fluid, but a similar viscosity as gas. The high pressure forces supercritical liquid into the cells, as the supercritical liquid have a diffusion rate similar to gas, the mass transfer is greatly enhanced, thus the extraction time can be much shorter than for conventional solvent extraction. Mature processes have been developed to use SFE for decaffeination and essential oil extraction (Gil-Chávez et al., 2013). Also, as the polarity of solvent is tunable, SFE can more selectively used to extract the target product than conventional solvent extraction (Guedes et al., 2013). Yen et al. (2015) have recently reviewed the advantages and challenges facing SFE extraction from microalgae biomass. Halim et al. (2012a) reported that eight minutes SFE treatment would have a better extraction result than 5.5 hours conventional solvent extraction.

In SFE, chemical solvent usage is minimized or eliminated. CO_2, for its relatively cheap price, safety, non-toxicity, and chemical inertness, and suitable critical temperature, became the most popular solvent (Daintree et al., 2008; Wang et al., 2008). In this case, the extractant can be separated easily from solvent if no co-solvent is added since CO_2 is gaseous under normal condition. The drawback of CO_2 as supercritical solvent is its low polarity is not suitable for polar compound extraction. To compensate for this, polar co-solvents can be added. Ethanol is a good choice for carotenoids extraction due to its suitable polarity and non-toxic nature; moreover, it is miscible with CO_2 and is approved for nutraceutical and pharmaceutical use (Reverchon and De Marco, 2006). The addition of co-solvents may not be attractive in terms of extractant separation since ethanol is a liquid at room temperature. Meanwhile, the addition would alter the mixture's critical point, requiring harsher conditions (Reverchon and De Marco, 2006).

Sub-critical CO_2 extraction is more practical due to less strict environment control requirement than SFE. Sub-critical refers to a condition at which the temperature ranges from boiling point to critical point, and pressure sufficient to maintain the fluid state. Lutein, β-carotene, and astaxanthin extraction by sub-critical CO_2 have been extensively studied and numerous investigations have focussed on the optimization of extraction conditions (Mendes et al., 2003; Herrero et al., 2006). Pressure, temperature, fluid flow rate, and the addition of co-solvents are the critical factors affect the extraction yield (Chen et al., 2012). Pressure up to 35 MPa, temperature from 40-45 ^{o}C, with 5% ethanol addition are the common conditions for sub-critical CO_2 extraction (Du et al., 2015). 1,1,1,2-tetrafluoroethane was also studied for the

extraction of carotenoids for it has more reasonable critical points (Lu et al., 2014). Water is another environmental-friendly solvent for SFE use. However, it may not work as well with carotenoids, since high temperature may destroy the functional activity of the carotenoids (Gil-Chávez et al., 2013).

A two-stage operation of SFE is also suggested for more selective outcome, with the first stage using low density CO_2 (300 bar) for non-polar or volatile lipid compounds, and the second stage with high density CO_2 with co-solvent (500 bar) for more polar carotenoids and other products (Reverchon and De Marco, 2006). The pilot plant design is available in Soto's review (Rosello-Soto et al., 2016).

7.3.3 Other extraction methods

Other extraction processes have been considered previously. Switchable solvent extraction systems refer to a solvent that can switch its polarity under different atmospheres, it was also proposed for the lipid extraction from algae. However, similar to ionic liquids discussed in section 7.2.2.5, it may not suitable for carotenoids extraction (Boyd et al., 2012; Du et al., 2015). OriginOil has developed a single-step electromagnetic field process for dewatering, cell disruption, and lipid recovery. *In situ* extraction has recently gained more attention, it aims to obtain the target carotenoid without killing the cells. For review, refer to Kleinegris et al. (2011). However, to date, the solvent used for extraction was either not capable to efficiently extract out carotenoids.

7.3.4 Wet extraction

Similar to cell disruption, drying is another energy-consuming step. Attempts are focused on elimination of this step by using wet algae for direct extraction. Freeze drying is a more preferred drying method due to its mild conditions comparing with spray or oven drying, which often lead to degradation of thermal-liable products and loss of volatile lipids coupled with the nonuniform particle size (Park et al., 2015). Wet extraction methods has been developed to overcome the bottleneck of high energy consumption (Park et al., 2015).

The effects of wet algae on extraction efficiency is still unclear. On the one side, the hypothesis is that the presence of residual water will adversely affect the extraction efficiency as water forms a barrier that prohibits the solvent mass transfer from the inside the cell to the outside. Although wet algae was successfully used for carotenoid extraction by SFE in the presence of co-solvent (Chen et al., 2012), the presence of water may cause many problems, since super-/subcritical CO_2 may degrade carotenoids by its potential catalytic effects for the hydrolytic-based reactions in the presence of water. The presence of water may result in flow impedance and restrictor plugging and channeling, and formation of highly compacted bed within the vessel (Barbosa-cánovas, 2015).

An alternative hypothesis is that residual water in the biomass will improve the carotenoids extraction, as the presence of water swells the cell and facilitates the lysis of cell wall, allowing better chance of solvent to access the inner cell content and enhances mass

transfer. Du et al. (2015) suggested that the existence of water may swell the center of cell matrix and act as a polar co-solvent to facilitate extraction.

Soh and Zimmerman (2011) studied the effect of moisture content (up to 20%) on the extraction efficiency; no obvious change was observed in their study. Unlike SFE, mechanical cell disruption methods and solvent extraction are highly effective with wet extraction. Jiménez Callejón et al. (2014) harvested tripled amount of lipids than the conventional Bligh & Dyer method. The study by Halim et al. (2012b) showed reduced efficiency for lipid extraction using non-polar system, while in the polar system, the extraction efficiency was enhanced with moisture. Sarada et al. (1999) also concluded that the usage of fresh biomass may reduce up to 50% pigment loss during the drying process. Therefore, wet processing have more potential for future carotenoids production studies.

7.4 Purification

The current carotenoids purification method was developed based on the Willstatter method (Burdick, 1956). Organic solvents are used for separation of carotenoids after saponification for the solution containing crude carotenoids. The detailed method is described in Figure 5, after microalgae extraction, NaOH or KOH are usually added to the microalgae extract as saponification agents to release the carotenoids from their naturally occurring ester form. In this step, temperature is usually kept below 60^{o}C to prevent carotenoids degradation (Yuan and Chen, 2000). Saponification conditions such as time, temperature, and alkali concentration, as factors affecting yield, were investigated in many studies (Chan et al., 2013; Palumpitag et al., 2011). KOH concentration of 2.5-40% are most commonly used below 60^{o}C, reacting for hours to overnight.

Solvent extraction is then applied to obtain the non-water-soluble compounds from the saponified microalgae extract. In the past, hexane was often used, while EtOH-Water-CH_2Cl_2 solvent system was used to treat saponified solution with better efficiency (Li et al., 2006). Chlorophyll is removed by alkaline hydrolysis, and converted to salts that are soluble in water. The unsaponifiable compounds will appear in the organic phase in the following extraction step, including carotenes, xanthophylls, waxes, phospholipids, sterols and phytol split from chlorophylls (Burdick, 1956). Relative solubility and intermolecular attractions of different binary solvent systems were also studied for the better separation of carotenoids (Dineshkumar et al., 2015).

The solvents are then concentrated for subsequent purification, recrystallization or chromatography can be used to further purify the crude product. Due to the presence of residual water in the organic solvent, Na_2SO_4 can be added then filtered out as $Na_2SO_4 \cdot (H_2O)_2$ to remove trace water (Nobre et al., 2006).

The described multi-step process is time-consuming and requires large amount of solvents. More advanced purification methods were also reported such as selective absorption of lutein on solid phase (Shen et al., 2011); expanded bed coupled column chromatography method (Fernández-Sevilla et al., 2010); and reversed phase HPLC or high speed counter current

chromatography for a small quantity of high purity carotenoids (Chen et al., 2005; Li et al., 2006; Li and Chen, 2001). Supercritical anti-solvent precipitation can generate solid carotenoids within a few minutes (Liau et al., 2010; Shen et al., 2011). However, the cost of these methods is higher than the conventional multi-step process. Therefore, a cost effective simple process is desirable for high purity carotenoids production.

8. Storage stability

Carotenoid degradation is catalyzed by oxygen and light and also accelerated by heat (Shen & Quek, 2014). Carotenoids were shown to be more stable than chlorophylls under short time (5 min) high temperature and high pressure treatments up to 117 ^{o}C and 625 MPa (Sánchez et al., 2014). Dias et al. (2014) studied carotenoids stability over a long term. An inert atmosphere, lower temperature (-20^{o}C), low light and the presence of anti-oxidant butylated hydroxytoluene (BHT) were suitable conditions for carotenoids storage up to 6 months (Chan et al., 2013). During degradation, the all *trans* form of the pigment may be transformed into *cis* forms. The rate is reported to fit a first order model (Tang and Chen, 2000). Crystalline lutein is easy to degrade (Fernández-Sevilla et al., 2010), so it was more commonly sold in ester form or suspended in vegetable oil. Sunflower oil is reported to be a better choice than olive oil since the tocopherols from it can act as an antioxidant (C. Y. Chen et al., 2016). Microencapsulation can better protect carotenoids as well (L. Chen et al., 2016). Similiarly, the complexion of astaxanthin with hydroxypropyl-beta-cyclodextrin may slightly increase the stability (Yuan et al., 2013). To prevent astaxanthin degradation, 180/110 °C is the best temperature to spray dry *H. pluvialis* biomass while a temperature of -20 °C under nitrogen atmosphere can preserve astaxanthin for nine weeks (Raposo et al., 2012). In addition, the lycopene stability is also higher in unextracted indicating free form of carotenoids may alleviate the degradation (Sharma and Le Maguer, 1996). More studies would benefit the understanding of storage conditions of carotenoids.

9. Market and economics

Due to the vibrant color and anti-oxidant related health boost properties, strong demands on carotenoids raised in recent years. The overall carotenoid market was $1.5 billion, with β-carotene, lutein, and astaxanthin occupied over 60% market share, and the global market was estimated to reach $1.8 billion by 2019 (Business Communications Company, 2015). Among the ten carotenoids best marketed, β-carotene, lutein, astaxanthin, and canthaxanthin could be efficiently produced by microalgae (Zaghdoudi et al., 2015). Their applications include food supplements, food colorant, feed additives, cosmetics, and drugs (Borowitzka and Mohemani, 2012).

To date, two successful stories of commercialized microalgal carotenoids production are astaxanthin from *H. pluvialis* and β-carotene from *D. salina*. The cost of synthetic astaxanthin is over $1000/kg, while the sales price over double of that (Li et al., 2011). Li et al. estmated

$718/kg cost to product astaxanthin from microalgae, while it may be further decreased by lower microalgae cost according to Kleinegris et al. (2011), (see also section 6.1). Apart from raising the production rate and pigment content, a few attempts were also conducted to reduce the cost. Tran et al. (2014) tried to reduce the cost by recycling the cultivation medium. Currently, the bottleneck to further reduce production cost is in the harvesting and extraction steps (Barba et al., 2015).

Lutein is another major market component. Currently produced from Marigold flowers with a price of $500/kg, the natural product has a cost even lower than synthetic products. Growth rate, nutrient requirements, pigment content, tolerance to the environmental fluctuations are all significant for the strain selection and economic considerations. Additionally, consumer acceptance of new functional food may as well affect the microalgal carotenoids market development (Freitas et al., 2012).

Apart from the carotenoids, microalgae can potentially be used for valuable by-products producing, like biodiesel, EPA, DHA, vitamins, proteins, and enzymes. Therefore, considering other compounds like biofuels as a by-product in the microalgal carotenoids production may provide a route to lower production costs. Dineshkumar et al. (2015) extracted lutein and biodiesel using same algae in one process, and got satisfactory yield for both products (6 mg/g lutein and 94 mg/g FAME). A few studies have been conducted in this area for integrated one-step biodiesel and lutein production (Araya et al., 2014; Dineshkumar et al., 2015; Hodgson et al., 2016). Fucoxanthin and lipids from hexane- ethanol system is also established (Kim et al., 2012). Other products were not reported to be produced simultaneously with carotenoids, but since microalgae is known to produce sterols, protein, sugars, vitamins, and moreover, the biomass itself is an edible product that is common in Korea and Japan. Hence the potential to obtain multiple products from microalgae exists, given better separation technology is developed.

10. Conclusions

In this review, the entire process starting from carotenoid synthesis, cultivation of microalgae, harvesting, extraction, purification and storage is discussed. The chemical total synthesis of carotenoids is a well established process, contributing to the majority of the global market, but its safety to human direct consumption is questionable (Ye et al., 2008). Natural carotenoids from biosynthesis are gaining market preference due to the health effect of carotenoids are nowadays better understood. Microalgae are excellent hosts for the mass production of carotenoids since these uni-cellular microorganisms have high carotenoid content, fast growth and many other advantages. The biotechnologies including high efficiency photobioreactors and optimized growth conditions are applied in the cultivation of carotenoid rich microalgae. However, a cheaper and more scalable cultivation strategy is still under investigation. Great challenges remain in the downstream processing especially the harvesting and cell disruption. While centrifugation is one of the most popular harvesting method, appropriate energy reduction approaches are necessary. Flocculation can be an alternative, but

the efficiency of this method remains to be improved. Among the recent developments and including scale-up efficiencies of various cell disruption methods, microwave assisted extraction and pulsed electric field are the most promising methods considering both efficiency and cost.

Further research should emphasize productivity improvement in microalgal bioprocessing and cost reduction. Advanced metabolic engineering tools such as CRISPR/Cas9 can be applied for high through-put strain development. For the production of high quality carotenoids, the storage stability should not be ignored. The linkage between physiological mechanisms of carotenogenesis should be considered to develop the species-specific growth strategy and the cell wall disruption method. Novel methods like *in situ* extraction and switchable solvents may offer interesting directions for further investigation. Switchable solvent extraction may have good potential if non-toxic solvents can be screened out and the bioactivity can be retained. To save on the drying cost, wet extraction methods present another positive new direction of research. In addition, as microalgae biotechnologies generate a variety of valuable products, research attempts should integrate the simultaneous production of multiple products to develop a more economically attractive and sustainable microalgae industry. Microalgal carotenoids production have excellent potential with major challenges to be overcome. There is still a need for better engineering design and innovation to make the processes more cost competitive. In summary, carotenoid production from microalgae is an attractive and potentially rapidly growing field.

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Tables

Table 1. Health benefits of six carotenoids confirmed by human studies

	Health benefits	Reference
Astaxanthin	Strong anti-oxidant property	(Fasano et al., 2014) (Chew et al., 1999)
	Anti-inflammatory effects	(Li et al., 2011)
	Anti-cancer	(Park et al., 2010)
	Cardiovascular health	(Pashkow et al., 2008)
Lutein	Prevents cataract and age-related macular degeneration	(Manayi et al., 2015) (Granado et al., 2003) (Bone and Landrum, 2003)
	Anti-oxidant property	(Cha et al., 2008)
	Anti-cancer	(Vijayapadma et al., 2014)
	Prevents cardiovascular diseases	(Alves-Rodrigues and Shao, 2004)
β-carotene	Prevent night blindness	(Dufossé et al., 2005)
	Anti-oxidant property	(Virtamo et al., 2014)
	Prevents liver fibrosis	(Shaish et al., 2006) (Virtamo et al., 2014)
Lycopene	Anti-cancer	(Viuda-Martos et al., 2014)
	Prevents cardiovascular diseases	(Srinivasan et al., 2009) (Devasagayam et al., 2004)
	Radiation protection	
	Anti-oxidant property	
Canthaxanthin	Creates tan color	(Zhang et al., 2014)
	Anti-oxidant property	
Fucoxanthin	Anti-obesity	(Abidov et al., 2010)
	Anti-oxidant property	(Nanba and Toyooka, 2008)

Table 2. Summary of carotenoids extraction technologies from microalgae

Step	Methodology	Efficiency	Advantages and disadvantages	References
Cell disruption	Grinding	++	Time-consuming	(Hu et al., 2013)
	Cryogenic grinding	+++	Expensive	(Grima et al., 2003; Zheng et al., 2011)
	Bead milling	+++	Most efficient in some studies; not as efficient in several studies; the treatment is strain specific; generates heat	(Chan et al., 2013; Halim et al., 2012a; J. Y. Lee et al., 2010; Prabakaran and Ravindran, 2011; Taucher et al., 2016)
	High pressure homogenizer	+++	Comparable with bead milling and ultrasound assisted extraction	(Grima et al., 2003; Halim et al., 2012b; Kim et al., 2015)
	Autoclave	-	Damage to carotenoids occurs	(Chan et al., 2013)
	Microwave	+++	Comparable efficiency with bead milling; Low energy consumption; Simple method; Generates heat	(McMillan et al. 2013) Lee et al. (2012) Li et al. (2015)
	Ultrasonication	+++ / +	Most efficient in some studies; Not efficient in other studies	(Cravotto et al., 2008; Mercer and Armenta, 2011) (Halim et al., 2012b; McMillan et al., 2013; Pasquet et al., 2011)
	Enzymatic	+	Highly selective; mild	(Deenu et al.,

	hydrolysis		condition; Expensive, Strict condition maintaince; Long treatment time	2013; Kadam et al., 2013; Zheng et al., 2011)
	Pulsed electric field	++	Highly selective; Retain bioactivity of carotenoids Short treatment time; Small solvent requirements	(Grimi et al., 2014; Lai et al., 2014; Sánchez-Moreno et al., 2005; Yu et al., 2015);
	Osmotic shock, Acid/ alkaline treatment,	+ -	Not efficient; Cause carotenoids to degrade	(Halim et al., 2012a) (Halim et al., 2012a)
	Ionic liquids/ Switchable solvent	-	High price; Toxicity; Cause carotenoids to degrade	(Park et al., 2015)
Solvent extraction	Conventional solvent extraction	++	Cheap and easy to scale up; Long extraction time; Multi-step operation; Use large amount of solvents	(Gil-Chávez et al., 2013; Reverchon and De Marco, 2006; Taucher et al., 2016)
	Super-/sub-critical solvent extraction	+++	Polarity of solvent is tunable; Fast; Safe; Easy separation of carotenoids; Expensive	(Du et al., 2015; Halim et al., 2012a; Hong-Wei Yen, Sheng-Chung Yang, Chi-Hui Chen, Jesisca, 2015; Reverchon and De Marco, 2006)

The symbol "-" represents carotenoids degradation; "+", slightly efficient; "++", efficient; "+++",highly efficient.

Captions

Figure 1 Chemical structure of some common carotenoids found in microalgae

Figure 2 Primary steps of biosynthetic pathway of carotenoids in most green microalgae species, and higher plants share almost the same steps except the biosynthesis of astaxanthin which is only found in limited species of microalgae. IPP and DMAPP are the building block of all carotenoids, the oxygenated xanthophylls are derived from α- or β-carotene. The enzymes involved are shown: β-LCY, β-cyclase; β-OHase, β-carotene hydroxylase; CRTISO, carotenoid isomerase; DMAPP, dimethylallyl diphosphate; DXP, deoxy-D-xylulose 5-phosphate; DXS, DXP synthase; ε-LCY, ε-cyclase; ε-OHase, ε-carotene hydroxylase; G3P, glyceraldehyde-3-phosphate; GGPP, geranylgeranyl diphosphate; GGPPS, GGPP synthase; IPP, isopentenyl pyrophosphate; MEP, methylerythritol 4-phosphate; PDS, phytoene desaturase; PSY, phytoene synthase; ZDS, ζ-carotene desaturase; Z-ISO, ζ- carotene isomerase. (adapted from Nisar et al. 2015)

Figure 3 Typical pathways of carotenoids synthesis

Figure 4 The process diagram of carotenoids from microalgae and from marigold flowers. The hollowed arrow implies the production of xanthophylls like lutein. The filled arrow indicates the process of commercialized astaxanthin production, the same process also applies to β-carotene.

Figure 5 Schematic description of the general purification of carotenoids

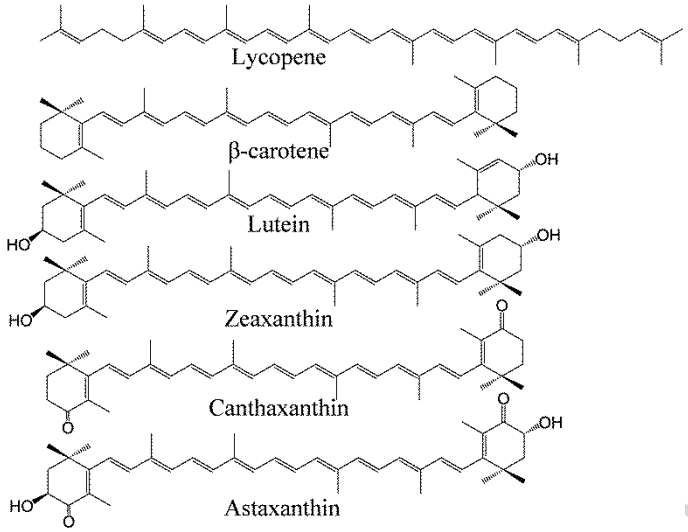

Fig. 1

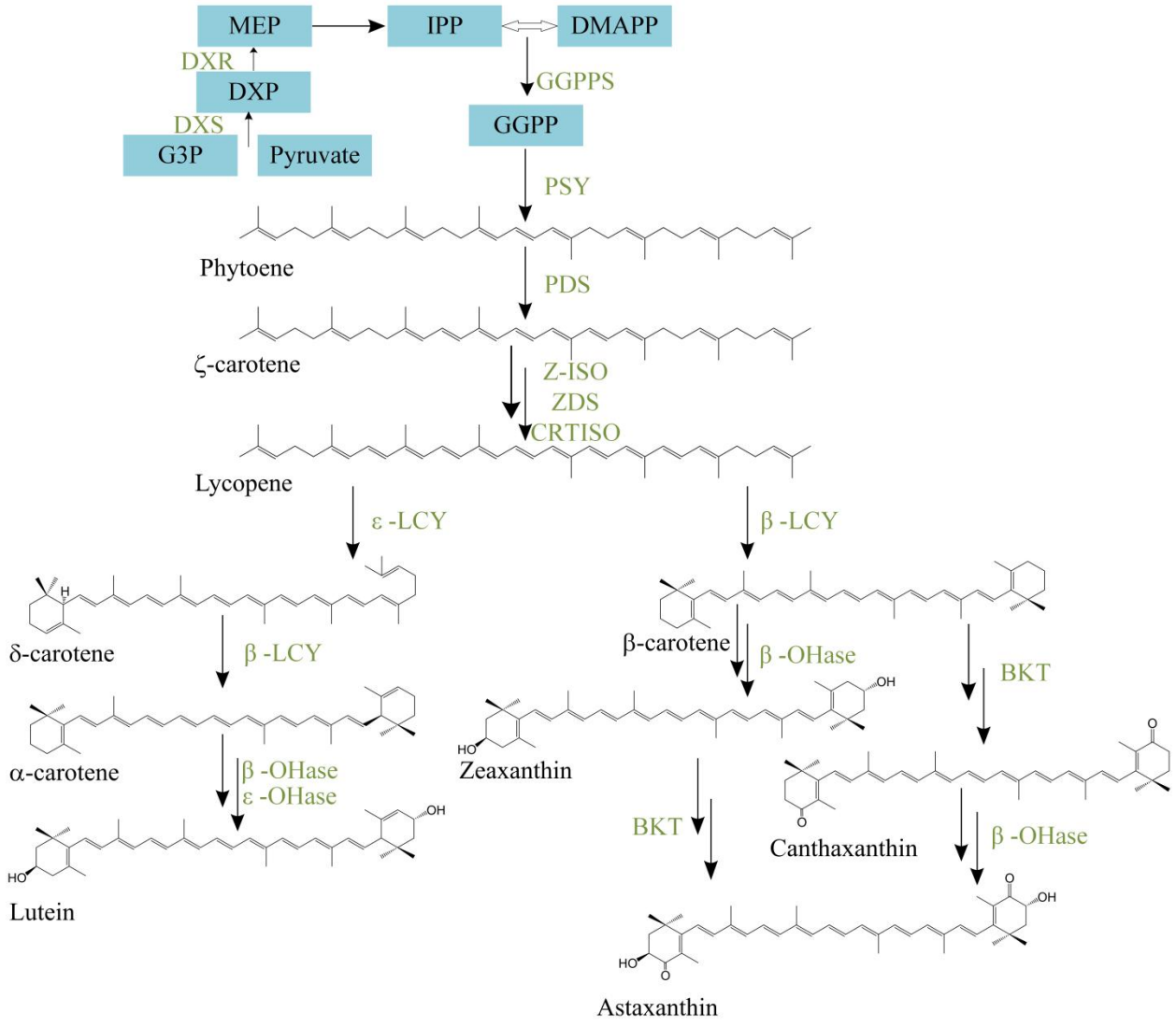

Fig. 2

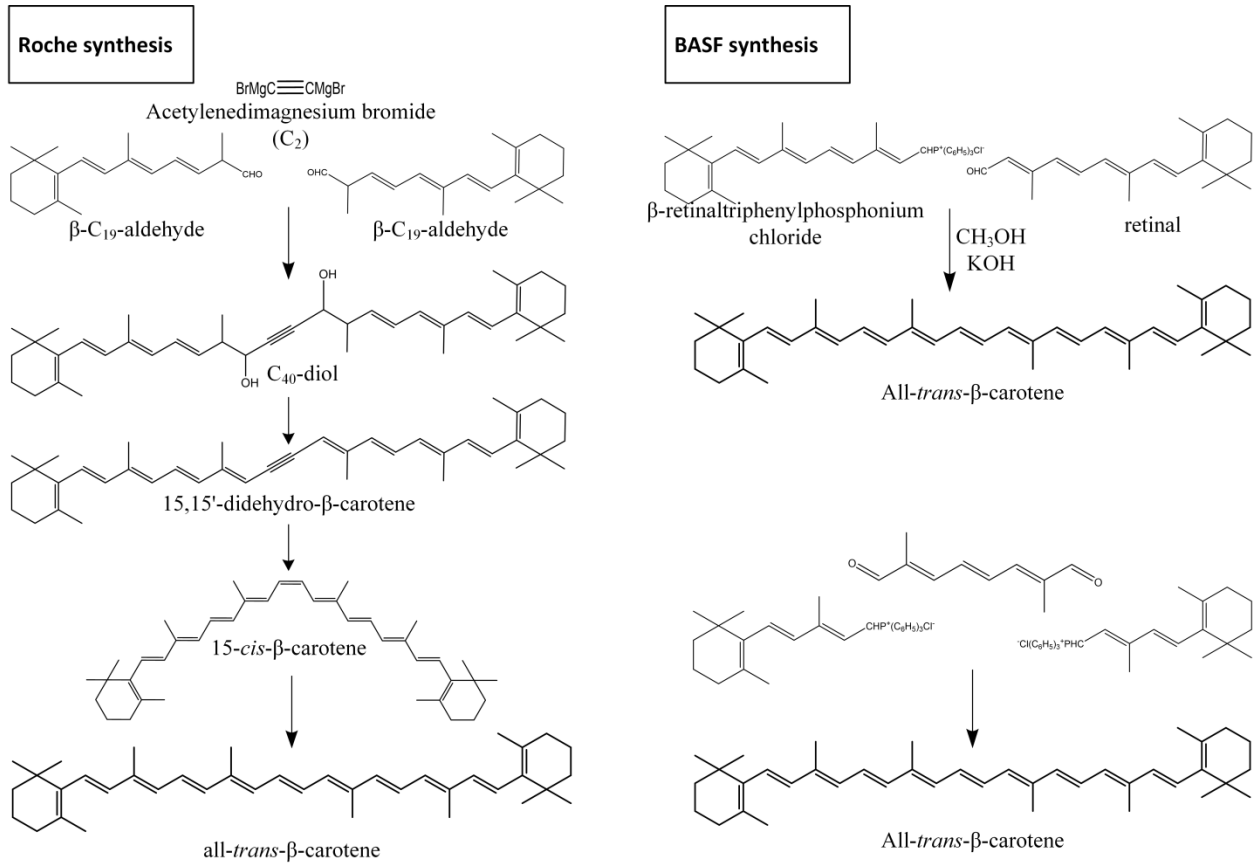

Fig. 3

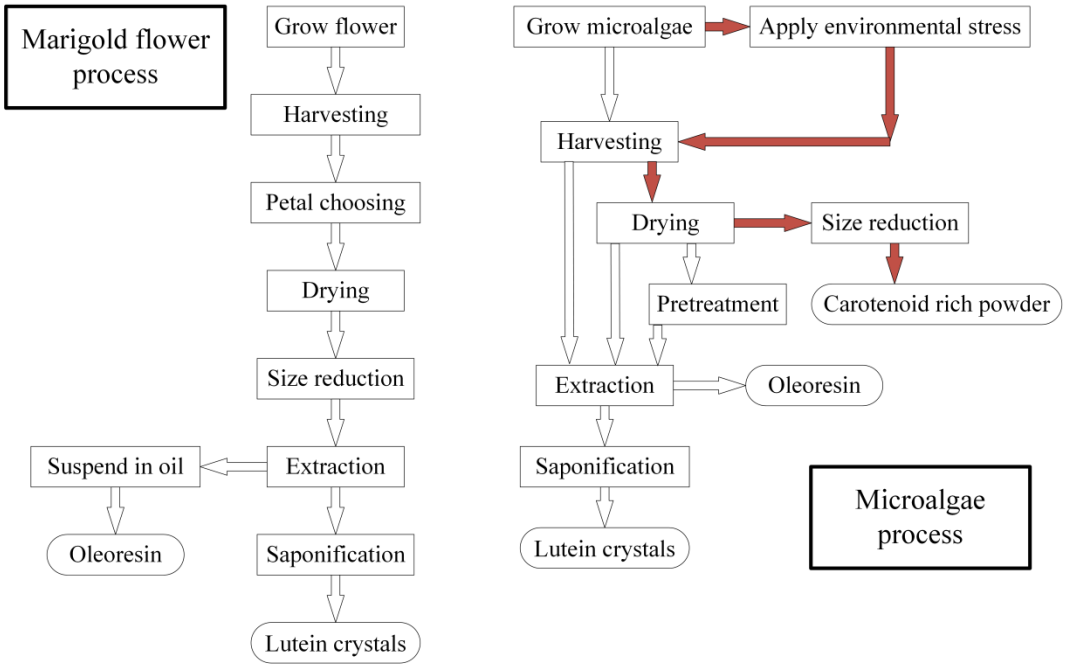

Fig. 4

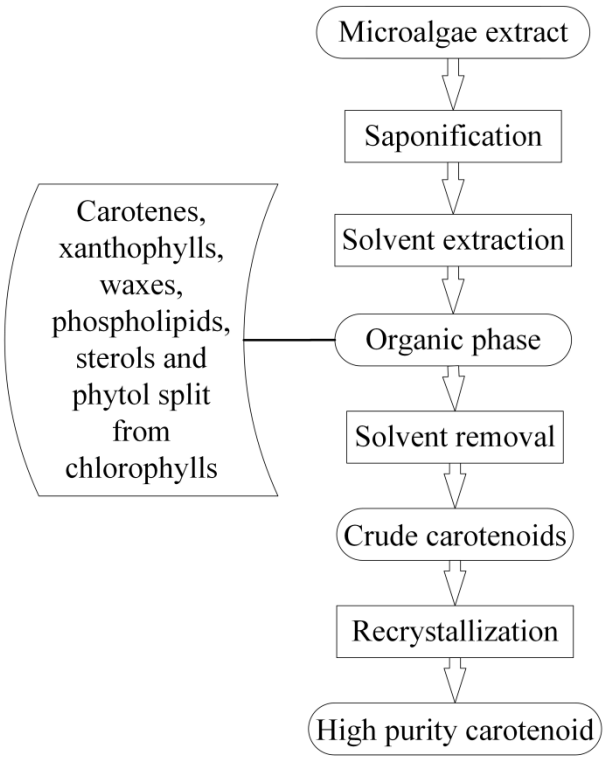

Fig. 5