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Research review paper

Biosynthesis and regulation of carotenoids in *Dunaliella*: Progresses and prospects

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ABSTRACT

Natural carotenoids are high in demand in global market owing to their widespread applications in nutrition, medicine, food coloring agent and cosmetic, as well as to the natural and healthy preference of consumers today. Some strains of *Dunaliella* are well known for their talent of massive β-carotene accumulation. Content of the high bioavailability stereoisomer of β-carotene, the 9-*cis* stereoisomer, is highest in *Dunaliella* among all the natural carotenoids sources. These valuable algae have been exploited commercially for β-carotene-rich *Dunaliella* powder and natural β-carotene in many countries since 1980s. However, drawbacks of traditional production methods have hampered the worldwide promotion of carotenoids production with *Dunaliella*. To shake off the dilemma, complete understanding of carotenogenic mechanism is urgent. Carotenogenic mechanism in *Dunaliella* is described in present paper, including carotenogenic pathway and its regulation. Generally, it seems that carotenogenic pathway in *Dunaliella* is close to the one of higher plants. It is known that reactive oxygen species (ROS) were involved in signal transduction for gene activation. Induction of ROS is in parallel with the enhanced β-carotene accumulation in *Dunaliella*. It is suggested that ROS trigger massive carotenoids accumulation in *Dunaliella*. It also revealed that relation may exist between enhanced β-carotene accumulation and lipid metabolism. For the talent of β-carotene synthesis, it is possible that *Dunaliella* massively accumulates β-carotene and other high-value carotenoids by genetic technologies.

Contents

Abbreviations: Apx, ascorbate peroxidase; Cgp, carotene globule protein; crt genes, carotenogenic genes; crt enzymes, carotenogenic enzymes; DMAPP, dimethylallyl pyrophosphate; DPME, 4-diphosphocytidyl-2-C-methyl-D-erythritol; DXP, 1-deoxy-D-xylulose 5-phosphate; *dxs*, gene for DXS; DXS, 1-deoxy-D-xylulose 5-phosphate synthase; GGPP, geranylgeranyl pyrophosphate; GGPS, geranylgeranyl pyrophosphate synthase; GHS, glutathione; Ipi, IPP isomerase; IPP, isopentenyl pyrophosphate; LYC, lycopene cyclase; LYC-B, lycopene β-cyclase; LYC-E, lycopene ε-cyclase; MEP, 2-C-methyl-D-erythritol 4-phosphate; *pds*, gene for PDS; PDS, phytoene desaturase; *psy*, gene for PSY; PSY, phytoene synthase; ROS, reactive oxygen species; RT-PCR, reverse transcription polymerase chain reaction; SOD, superoxide dismutase; ZDS, ζ-carotene desaturase.

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1. Introduction

First described in saltern evaporation ponds on the Mediterranean coast of France by Michel Fliex Dunal in 1838, the genus of unicellular biflagellate green algae is named *Dunaliella* in honor of Dunal by Teodoresco in 1905 (Oren, 2005). Belonging to the empire Eukaryota, the kingdom Plantae, the phylum Prasinophyta, the class Prasinophyceae, the order Dunaliellales and the family Polyblepharidaceae, genus *Dunaliella* is composed of 24 species temporarily, including *Dunaliella salina*, *Dunaliella bardawil*, *Dunaliella tertiolecta* and so on, basing on the data of algaebase (http://www.algaebase.org) up to October 2007. It is known today, the green microalga firstly reported by Dunal in saltern is *D. salina*. Oren (2005) has perfectly described the story of *Dunaliella*. This unicellular and uninucleate alga is devoid of a rigid polysaccharide wall. However, it could grow in salinities as different as 0.05–5.0 M NaCl with constant and low intracellular sodium concentration by regulating the metabolism of osmolyte, glycerol, to balance external diverse salinities (Ben-Amotz and Avron, 1981). Inside the biflagellate and motile cell, there is a large cup-shaped chloroplast (Fig. 1), in which β-carotene accumulation takes place under stress conditions. *Dunaliella* undergoes complex life cycle, including vegetative and sexual reproduction. Capacity of massive β-carotene accumulation induced by diverse stress factors has made *Dunaliella* a potentially useful model in understanding the greatly interesting mechanism of carotenoids formation.

Some species of *Dunaliella*, especially *D. salina* and *D. bardawil*, process the ability to accumulate large amounts of carotenoids in appropriate growth conditions (or carotenogenic conditions). Basing on the resolution of reversed phase high performance liquid chromatography (RPHPLC), *Dunaliella*-yielded carotenoids are dominated by β-carotene, and the rest includes α-carotene, lutein, zeaxanthin, cryptoxanthin and neoxanthin (Ben-Amotz et al., 1982). These appropriate growth conditions are not the optimal condition for algae growth, in contrast they are adverse growth conditions with high salinity, high light intensity, stress temperature, privative nutrients (deficiency in nitrogen, sulfur or phosphorus) and so on (Ben-Amotz et al., 1982; Raja et al., 2007). Under suitable conditions, some strains of *Dunaliella* could accumulate 10% or more β-carotene of the total dry organic matter in weight. Aasen et al. (1969) reported that the percentage of β-carotene in *D. salina* even comes up to 13.8% under natural conditions. However, the percentage falls to only about 0.3% in unsuitable conditions (Johnson and Schroeder, 1995). High β-carotene content makes the cell display bright red coloration owing to the characteristic absorption spectrum of β-carotene, and Oren (2005) has showed this bright red cell clearly. Electron microscope observation showed that

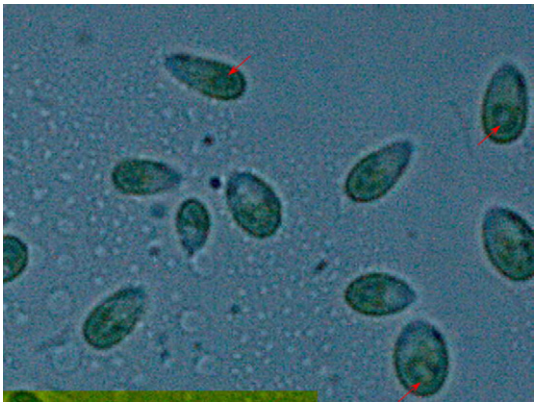

Fig. 1. *D. salina* cells are showed in photograph. Cell's single cup-shaped chloroplast is designated by the red arrow. Experiencing carotenogenic conditions β-carotene will be massively accumulated in chloroplast in the form of lipid globules, and at this time chloroplast and even the whole cell will display bright red coloration. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

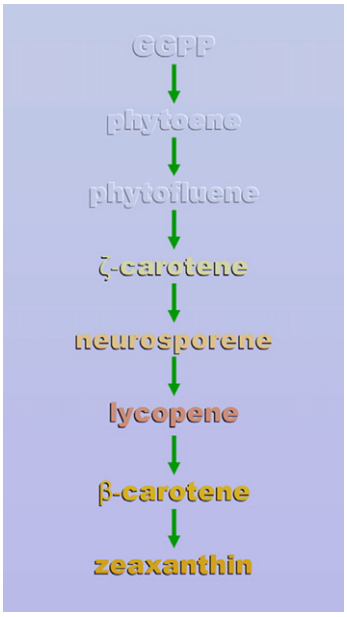

Fig. 2. Specific coloration of geranylgeranyl pyrophosphate (GGPP) and some carotenoids. GGPP, phytoene and phytofluene are colorless, and colors become deep gradually from ζ-carotene (pale yellow) to zeaxanthin (dark yellow). As showed, the metabolic route in *Dunaliella* from GGPP to zeaxanthin is common in plants and other organisms. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

β-carotene is concentrated in the interthylakoid spaces of the cell's single chloroplast in the form of lipid globules under carotenogenic conditions, and few or even no globule exists when the cell was cultivated under non-induced conditions (Ben-Amotz et al., 1982). It is detected that this membrane-free lipid globule exclusively contained β-carotene (more than half), neutral lipids (mainly with triacylglycerols) and a small amount of protein with an average diameter of 150 nm (Fried et al., 1982; Ben-Amotz et al., 1982). Katz et al. (1995) detected that a 38-kD protein (carotene globule protein, Cgp), which was located in the periphery of lipid globule, was induced in parallel with β-carotene accumulation, or rather its occurrence was dependent on the appropriate adverse conditions. They suggested that the function of Cgp is to stabilize the β-carotene-contained lipid globule.

2. Carotenoids: natural, healthy and medicative pigments

Synthesized universally in photosynthetic organisms, natural carotenoids are diversiform in structure as well as great variation in function. At least 700 types of carotenoids have been detected from the natural sources (Feltl et al., 2005; Hornero-Méndez and Britton, 2002). Most of carotenoids are C_{40} isoprenoids, which are composed of eight isoprene units (Naik et al., 2003). Diversification of conjugated double bonds of the polyene chain results in the various characteristic absorption spectra and photochemical properties of carotenoids. Specific coloration of some carotenoids has been shown in Fig. 2.

Functions of carotenoids in photosynthetic organisms have been investigated in previous works. Due to the conjugated polyene structure, carotenoids play an important role in photosynthesis in terms of energy and oxygen transport as accessory pigments (Blass et al., 1959; Siefermann-Harms et al., 1978), and in protecting the photosynthetic organisms against the injure caused by photooxidation. In addition, the valuable function that carotenoids quench singlet oxygen ($^1O_2$) and free radicals, which ware generated from metabolism and would damage metabolizing tissues, is vital for living. In medical science, carotenoids possess prevention and treatment properties against many kinds of diseases, such as cardiovascular disease (Kohlmeier et al., 1997; Shaish et al., 2006), cataract (Gupta et al., 2003) and

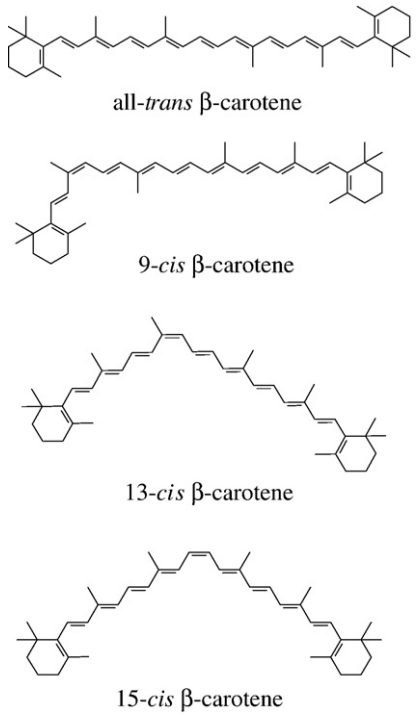

Fig. 3. Chemical structures of all-*tran*, 9-*cis*, 13-*cis* and 15-*cis* β-carotene.

certain cancer (Michaud et al., 2000; van Poppel, 1993; Mayne et al., 1994). Tapiero et al. (2004) have elaborately reviewed the functions of carotenoids in preventing human pathologies. As carotenoids are the pigments accounting for the showy color of animal and their eggs, and most of animals cannot synthesize these pigments naturally, carotenoids are widely used in animal feeds as colorant and nutrition. Moreover, natural, healthy and colorful preferences of consumers today result in the universal use of carotenoids daily as natural additives for food and cosmetic. According to a report by BCC (Business Communications Company, http://www.bccresearch.com), the worldwide market value of commercially-used carotenoids was US $886.9 million in 2004, and it was expected to break the billion dollar barrier by 2009.

3. Current application of *Dunaliella* for β-carotene accumulation

As a member of carotenoids, β-carotene is found in various structures as the configuration of each double-bond in β-carotene can exist in *trans* or *cis* naturally. The all-*trans*, 9-*cis*, 13-*cis* and 15-*cis* stereoisomers of β-carotene have been identified in natural sources (Patrick, 2000) (Fig. 3). Stereoisomers of β-carotene display various biokinetics and biological activities. β-carotene biosynthesized by *Dunaliella* consists of 9-*cis* and all-*trans* stereoisomers, representing 40% and 50% respectively (Ben-Amotz et al., 1988). The 9-*cis* to all-*trans* ratio is proportional to the integral light intensity during cell's division cycle (Ben-Amotz et al., 1988). To date, the content of 9-*cis* β-carotene is highest in *D. bardawil* among natural sources (Shaish et al., 2006). Culture of *Dunaliella* is the most important process for natural production of β-carotene (Del Campo et al., 2007). Like other carotenoids β-carotene plays an important role in medicine, nutrition and coloring agent, and is high in demand in global market. The worldwide market value of β-carotene was US$242 million in 2004, and will reach US$253 million by 2009 (a report by BCC). More and more attentions are being attracted to the biotechnological investigations and applications in *Dunaliella* for massive accumulation of β-carotene and other carotenoids.

Dunaliella have been exploited commercially to yield dried biomass and natural β-carotene in Israel, China, USA, Australia, Mexico and so on since 1980s (Borowitzka, 1999; Raja et al., 2007; Johnson and Schroeder, 1995). However, due to the regional specificity (subtropical regions) or high cost and difficult operation of artificial carotenogenic conditions (high salinity, high light intensities and etc.) in the outdoor open cultures (Raja et al., 2007), 84.8% of the total global β-carotene consumption is supplied in the form of chemical synthesis while the β-carotene yielded by algae only account for 8.5% of the total approximated in 2004 (data from BCC), though many efforts have been concentrated on improving the productivity of *Dunaliella* for β-carotene. Unfortunately, the β-carotene yielded in chemosynthetic pathway is dominated by all-*trans* stereoisomer (Del Campo et al., 2007; von Laar et al., 1996). With high level in *Dunaliella*, the 9-*cis* stereoisomer has a good bioavailability and is more efficient than all-*trans* stereoisomer in scavenging of reactive oxygen species (ROS) (Shaish et al., 2006; Jiménez and Pick, 1993; Levin and Mokady, 1994). The predominance of 9-*cis* stereoisomer is in parallel with the quality of β-carotene (Jiménez and Pick, 1993). In addition, it is detected that chemosynthetic β-carotene caused an increase in the risk of lung cancer and cardiovascular disease in smokers and asbestos workers, though it hasn't been definitely confirmed (Omenn et al., 1996). Restricted number of carotenoids could be produced through chemosynthetic pathway (Schmidt-Dannert, 2000) and functions of carotenoids are dependent on the multiformity of structures. Therefore, interest is boosting in biosynthetic pathway for β-carotene production. Producing carotenoids through the biosynthetic pathway, researchers usually use engineer microorganisms (such as *E. coli* and yeast) transgenic plants (such as *Oryza sativa*, in region with prevalent vitamin A deficiency) (Paine et al., 2005; Al-Babili and Beyer, 2005) employing genetic transformation with carotenogenic (crt) genes. Currently, technologies of gene combination and molecular breeding are universally used in producing carotenoids, especially the rare and novel ones (Schmidt-Dannert, 2000).

4. Present progress on carotenogenic pathway of *Dunaliella*

Massive accumulation of various carotenoids with diverse crt genes in new carotenogenic pathway within genetic transformation organisms boosts interest in investigation of carotenogenic pathway. Identification and clone of crt genes make carotenoids biosynthesis genetically in non-carotenogenic organisms possible. Carotenogenic pathway has been investigated since the mid-1960s (Cunningham and Gantt, 1998), genes for almost all the enzymes resulting in the carotenoids biosynthesis have been cloned and analyzed well in various organisms, such as bacteria, cyanobacteria, fungi, algae and plants (Misawa et al., 1990; Sieiro et al., 2003; León et al., 2007; Hirschberg et al., 1997). Taking advantage of genetic technologies' investigation of *Dunaliella* genes for carotenogenic (crt) enzymes has been carried out since a decade ago, but sequences are restricted in

Table 1
Cloned genes for carotenoids biosynthesis enzymes in genus *Dunaliella*

Enzyme	Species of *Dunaliella*	Gene	Clone type	GenBank accession number
Phytoene synthase (PSY)	*Dunaliella salina*	psy	cDNA	AY601075
	D. salina	psy	genomic	AY547325
	Dunaliella bardawil	psy	cDNA	U91900
	D. bardawil	psy	genomic	DQ057342
	N/A	psy	cDNA	DQ463305
	N/A	psy	genomic	DQ463306
Phytoene desaturase(PDS)	*D. salina*	pds	cDNA	AY954517
	D. salina	pds	cDNA	DQ243892
	D. bardawil	pds	cDNA	Y14807

Basing on the data of National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov) up to October 2007, published sequences for carotenogenic (crt) enzymes in genus *Dunaliella* are showed in table. As showed, both sequences of phytoene synthase (PSY) and phytoene desaturase (PDS) of *D. salina* and *D. bardawil* have been cloned by researchers.

public now. So far, only the sequences of phytoene synthase (PSY) and phytoene desaturase (PDS) of some species of *Dunaliella* have been analyzed and published basing on the data of National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov) (Table 1). Recently, our laboratory has been cloned and sequenced the lycopene cyclase (LYC) of *D. salina* and *D. bardawil*. Taking a wide view of carotenogenic pathway, it could be divided into three steps that are the geranylgeranyl pyrophosphate (GGPP) biosynthesis, the lycopene biosynthesis and the generation of carotenoids with cyclohexene rings.

4.1. Non-mevalonate pathway and GGPP formation

Carotenoids are derived from the C_5 isoprenoid precursors, isopentenyl pyrophosphate (IPP) and its isomer dimethylallyl pyrophosphate (DMAPP) (Shewmaker et al., 1999). Thereby, the early step of carotenogenic pathway is the biosynthesis of GGPP, the C_{20} precursor of phytoene, via sequential adding three IPP molecules to a DMAPP molecule under the catalysis of geranylgeranyl pyrophosphate synthase (GGPS) (Hirschberg, 2001).

Until now, two major pathways are responsible for the biosynthesis of IPP in plants and algae, mevalonate (MVA) pathway and non-mevalonate pathway (also known as 1-deoxy-D-xylulose 5-phosphate (DXP) pathway or 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway) (Lange et al., 2000). Discovered in the 1950s, the mevalonate pathway for IPP production starts from acetyl-CoA via MVA (Bloch, 1992). Enzymes in this pathway are localized at the cytosolic compartment, and cytoplasmic IPP is generated in this pathway dominantly (Newman and Chappell, 1999). In plastids, however, IPP is synthesized in a non-mevalonate pathway which was discovered in the late 1980s. It begins with D-glyceraldehyde-3-phosphate (GA3P) and pyruvate via intermediates DXP, MEP, 4-diphosphocytidyl-2-C-methyl-D-erythritol (DPME), 4-diphosphocytidyl-2-C-methyl-D-erythritol 2-phosphate, 2-C-methyl-D-erythritol 2,4-cyclodiphosphate (MEcDP) and 4-hydroxy-2-methylbut-2-enyl pyrophosphate (HMBPP) orderly (Rohmer et al., 1996; Seemann et al., 2006) (Fig. 4). Moreover, IPP given birth through the former pathway is mainly responsible for the biosynthesis of sesquiterpenes (C_{15}) and triterpenes (C_{30}), such as sterols and cadinene respectively, whereas IPP yielded by the latter pathway mostly accounts for the generation of diterpenes (C_{20}), phytyl (C_{20}) conjugates and carotenoids (C_{40}), such as taxol, tocopherols and lycopene respectively (Disch et al. 1998; Shewmaker et al., 1999; Walter et al., 2000). The non-mevalonate pathway has been detected in bacteria (Rohmer et al., 1993), algae (Schwender et al., 1996) and plants (Lichtenthaler, 1998). Genes, enzymes and intermediates in the non-mevalonate pathway have been reviewed particularly by Eisenreich et al. (2004) and Rohdich et al. (2001). In *Dunaliella*, genes involved in IPP generation for the subsequent carotenoids production attract, however, little researchers' attention. Currently, seldom analysis about these genes of *Dunaliella* has been published.

4.2. β-carotene and other carotenoids biosynthesis via the generation of phytoene

The metabolic route for β-carotene biosynthesis from GGPP in *Dunaliella* is common in plants and other organisms, via sequential formations of phytoene, phytofluene, ζ-carotene, neurosporene,

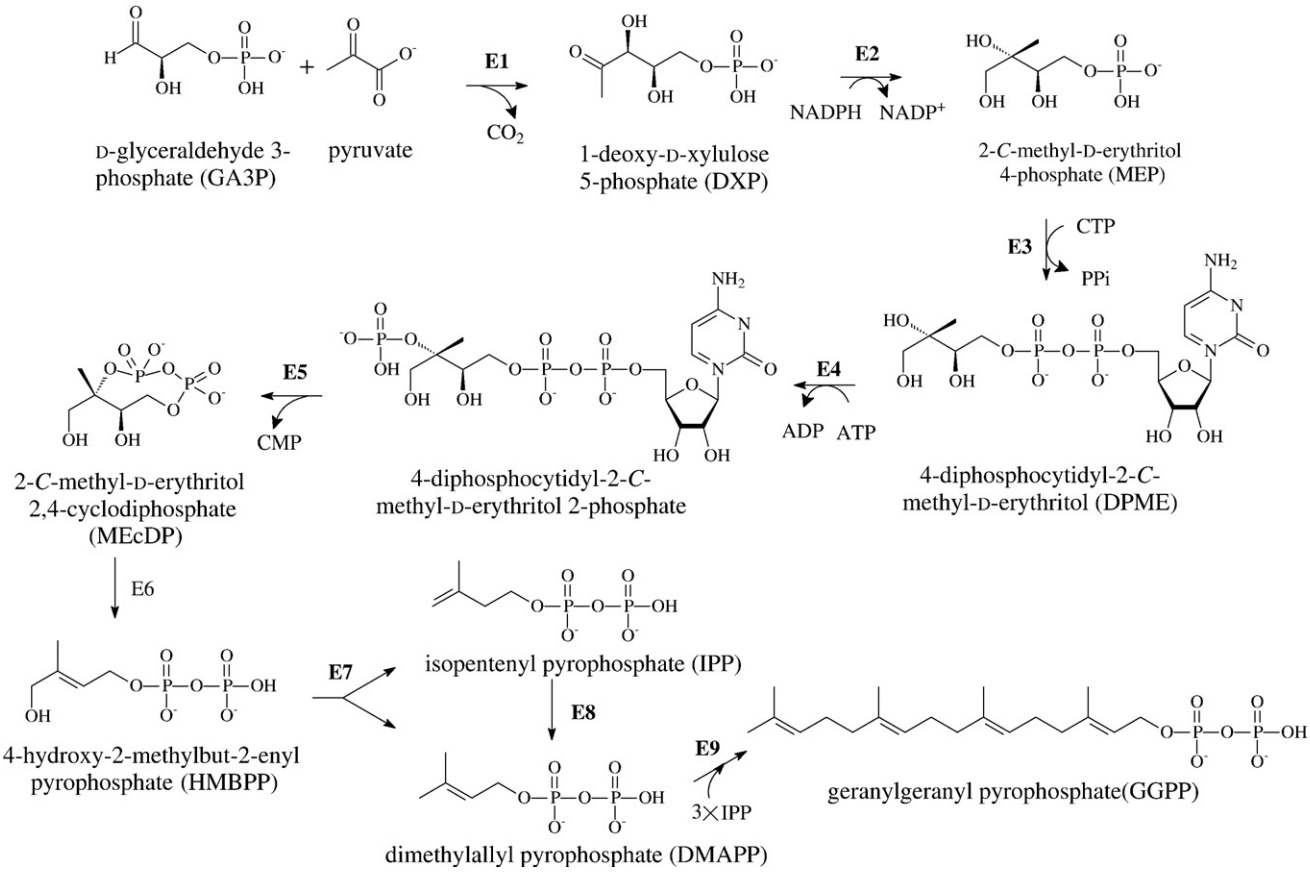

Fig. 4. The non-mevalonate isoprenoid biosynthesis pathway for the generation of geranylgeranyl pyrophosphate (GGPP). E1–E9 are the enzymes for relative reactions. E1:1-deoxy-D-xylulose 5-phosphate synthase, DXS; E2:1-deoxy-D-xylulose 5-phosphate reductoisomerase, DXR; E3:4-diphosphocytidyl-2-C-methyl-D-erythritol (DPME) synthase, CMT; E4: DPME kinase, CMK; E5:2-C-methyl-D-erythritol 2,4-cyclodiphosphate synthase, MEcPS; E6:(*E*)-4-hydroxy-3-methylbut-2-enyl pyrophosphate synthase; E7:(*E*)-4-hydroxy-3-methylbut-2-enyl pyrophosphate reductase; E8:isopentenyl pyrophosphate (IPP) isomerase, Ipi; E9:geranylgeranyl pyrophosphate synthase, GGPS. (Eisenreich et al., 2004; Rohdich et al., 2001; Hirschberg, 2001; Seemann et al., 2006).

lycopene and γ-carotene as the order showed in Fig. 2 from the top down. According to the data of NCBI, sequences for PSY of *D. salina and D. bardawil* in this metabolic route have been detected and analyzed (Table 1). As described in Fig. 5, PSY catalyzes the head to head combination of two molecules of GGPP leading to generation of the first colorless carotenoids phytoene. PSY has been considered to be a key enzyme in carotenoids biosynthesis, and it may be the regulatory point determining the flux of carbon source towards carotenoids (Shewmaker et al., 1999; Bramley et al., 1992). Using genome walking technology and suppression PCR, Yan et al. (2005) have obtained the gene for PSY (*psy*) of *D. salina*, after that the cDNA of PSY has been achieved using reverse transcription polymerase chain reaction (RT-PCR). In addition, Ben-Amotz et al. (1988) suggested that isomerization reaction, which results in the generation of 9-*cis* stereoisomer of carotenoids, occurs at the formation of all-*trans* phytoene or even earlier in carotenogenic pathway. It is a pity that sequences of

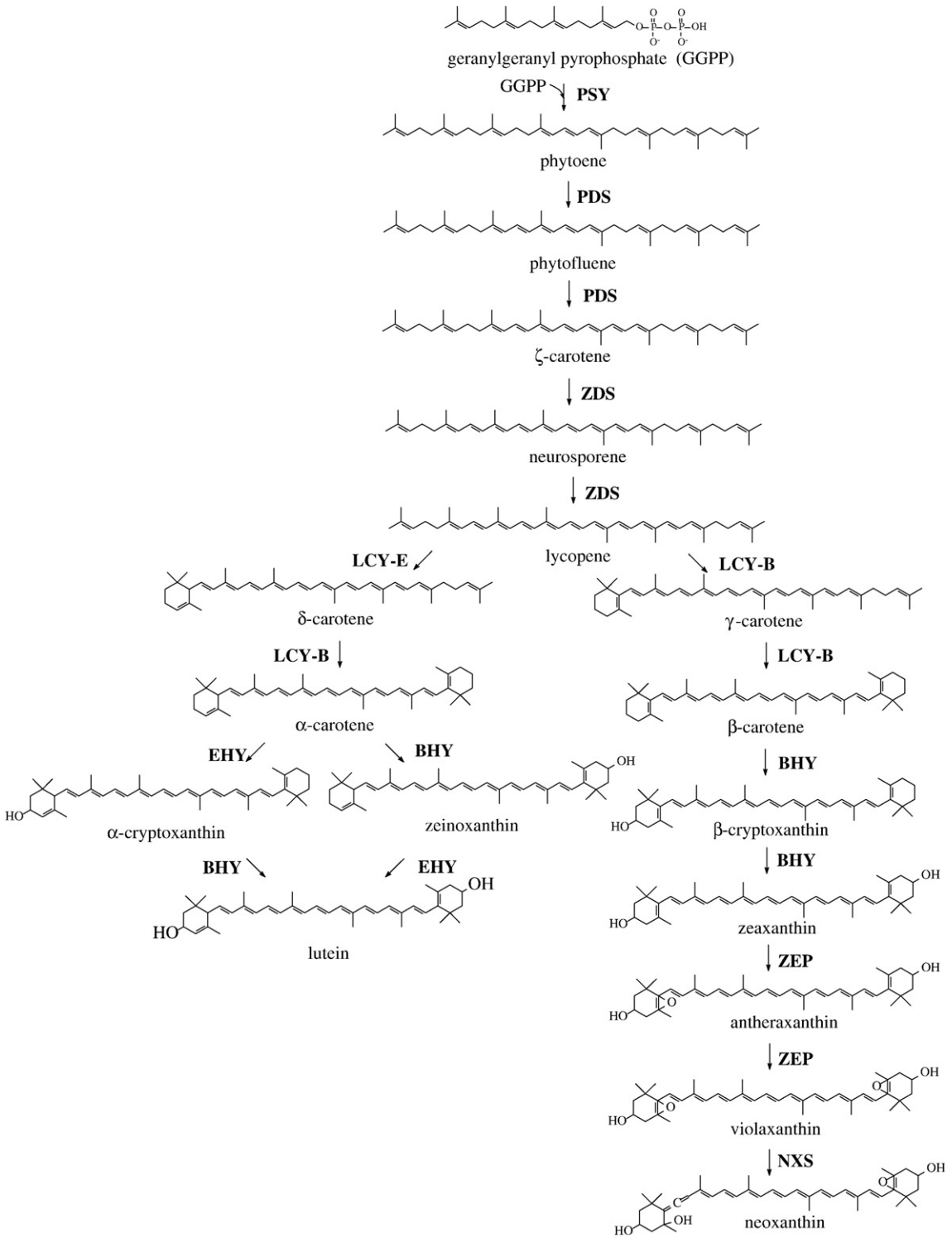

Fig. 5. A hypothetic pathway for carotenogenesis in *Dunaliella*. Enzymes for the relative conversions are in bold. PSY: phytoene synthase; PDS: phytoene desaturase; ZDS: ζ-carotene desaturase; LYC-E: lycopene ε-cyclase; LYC-B: lycopene β-cyclase; EHY: ε-carotene hydroxylase; BHY: β-carotene hydroxylase; ZEP: zeaxanthin epoxydase; NXS: neoxanthin synthase. (Sandmann, 2001; Hirschberg et al., 1997; Hirschberg, 2001; Naik et al., 2003).

enzymes that catalyze the isomerization reaction have not been published until now.

Once the phytoene is biosynthesized, the next steps in carotenogenesis are the stepwise desaturation reactions (or dehydrogenation reactions) resulting in the conversion from phytoene (9 double bonds molecule) to lycopene (has 13 double bonds and a chromophore of 11 conjugated double bonds) via phytofluene, ζ-carotene and neurosporene as intermediates. In bacteria and fungi, these four dehydrogenation reactions are performed by a single enzyme, the CRT-I type PDS, which is coded by the gene *crtI*, while in cyanobacteria and plants, these four dehydrogenation reactions are achieved by two desaturases, PDS-type PDS and ζ-carotene desaturase (ZDS), each of which catalyzes two sequential dehydrogenation reactions and adds two symmetrical double bonds respectively (Albrecht et al., 1995). Basing on the RT-PCR and rapid amplification of cDNA ends polymerase chain reaction (RACE-PCR) methods, cDNA of PDS of *D. salina* has been obtained (Zhu et al., 2005). Phylogenetic analysis indicated that PDS of *D. salina* is close to those of higher plants and cyanobacteria rather than those of bacterial and fungi, or rather is closest to the one of higher plants (Zhu et al., 2005). Therefore, PDS of *D. salina* may be a PDS-type, and as in plants it may exist two desaturases in *Dunaliella*, PDS-type PDS and ZDS. In addition, sequences of PDS in *D. bardawil* and *D. salina* also have been detected and analyzed by other groups (Table 1).

In carotenogenic pathway, there are two downstream routes after the generation of lycopene for the formations of acyclic and cyclic carotenoids respectively (Schmidt-Dannert, 2000). As stated above, the major component of *Dunaliella*-yielded carotenoids is β-carotene, which contains a β cyclohexene ring at each end (Fig. 3). In other words, the metabolic route for cyclic carotenoids is dominant in carotenogenesis in *Dunaliella*. Cyclization that cyclohexene rings are added to either or both ends of lycopene under the catalysis of LYC leads to the formation of δ-, α-, γ- and β-carotene. Two types of LYC have been found in plants, lycopene β-cyclase (LYC-B) and lycopene ε-cyclase (LYC-E). LYC-B can introduce a β ring into each end of lycopene to form γ-carotene (monocyclic) or β-carotene (dicyclic), while LYC-E can only introduce one ε ring into one end of lycopene to produce monocyclic δ-carotene (Cunningham et al., 1996). To produce α-carotene, lycopene should be added a ε ring in one end and a β ring in other, which are catalyzed by LYC-E and LYC-B respectively. It is postulated that these two cyclases may exist in *Dunaliella* basing on the analysis that components of *Dunaliella* carotenoids include β-carotene as well as α-carotene. Recently, our laboratory has obtained the sequences LYC-B of *D. salina* and *D. bardawil*. Downstream steps in carotenogenic pathway involve in addition of oxygen moieties, hydroxyl groups and keto groups, and lead to the formation of a variety of carotenoids (Shewmaker et al., 1999) (Fig. 5). crt enzymes in these steps have been studied little in *Dunaliella*, and few genes for these enzymes of *Dunaliella* has been cloned, analyzed or published.

5. Current progress on carotenogenic regulation in *Dunaliella*

It is known that *Dunaliella* accumulate large amount of β-carotene under adverse environmental conditions, such as high irradiance, high salinity, low temperature, nutrient deprivation and heavy metal stress (Lers et al., 1991; Król et al., 1997; Borowitzka et al., 1990; Ben-Amotz 1996; Nikookar et al., 2005). As a result, *Dunaliella* has been regarded as a valuable model for understanding the interesting regulation of carotenogenesis. In addition, the discovery of expression patterns in response to stress conditions will also provide the basis of effective engineering strategies for massive carotenogenesis in *Dunaliella*. Several sequences, whose transcripts include crt enzymes (see Section 4), carotenoids binding proteins (Lers et al., 1991; Chen et al., 2007) and other products, have been cloned and analyzed in *Dunaliella* owing to the hard works of previous researchers. In *Dunaliella*, regulation of carotenogenesis is, however, inexplicit until now. In this part, current progress on the carotenogenic regulation in *Dunaliella* will be described, including messenger molecules, genes expression and enzymes activation.

5.1. ROS: important messenger molecules for carotenogenesis

Commonly, oxidative stress, or generation of ROS, occurs in biological systems when they experience environmental stress (Elstner et al., 1988; Hideg, 2006; Lesser, 2006). Generally, it is believed that ROS are harmful to living for their oxidative damage to biomacromolecules (Foyer et al., 1994). However, ROS also function as messenger molecules involving in signaling pathways in biological systems under physiological conditions or stress conditions (Hideg, 2006). It is suggested that ROS involve in triggering β-carotene accumulation in *Dunaliella*.

5.1.1. ROS in cells

Photosynthetic organisms, including plants and green algae, have high internal oxygen concentration because of the oxygenic photosynthesis. ROS activated from oxygen threaten photosynthetic organism constantly (Okamoto et al., 2001). ROS are also known as active oxygen species (AOS), they include superoxide radicals, singlet oxygen, hydrogen peroxide and hydroxyl radical (Lesser, 2006). ROS will lead to the damage of biomacromolecules, including lipid, protein and DNA, and this damage is life-threatening. Vital responsibility against the damage caused by ROS is taken by internal antioxidant systems. Commonly, internal antioxidant systems in photosynthetic organisms consist of two protection mechanisms, enzymatic and non-enzymatic (Bouvier et al., 1998). Enzymatic mechanism is that ROS are scavenged by superoxide dismutase (SOD), catalase, glutathion peroxidase and ascorbate peroxidase (Apx) (Foyer et al., 1994), while the non-enzymatic mechanism is that ROS are quenched directly by internal antioxidants such as glutathione (GHS), α-tocopheroland and carotenoids (Fryer, 1992; Bouvier et al., 1998). Besides damage, ROS also play an important role in signal transduction in cellular processes as second messengers for the expression of certain transcription factors and signal transduction molecules (Lesser, 2006). Signaling pathway in response to oxidative stress has been expatiated by Martindale and Holbrook (2002). Lesser (2006) have accomplished an excellent review about the generation and biological functions of ROS. Generally, under the protection of antioxidant systems ROS are controlled at low levels for signal transduction in photosynthetic organisms or rather in all biological systems.

5.1.2. ROS increases in Dunaliella under stress conditions

Like other photosynthetic organisms, *Dunaliella* increases internal ROS when it confronts to adverse environmental conditions (Shaish et al., 1993). Under high light irradiance, ROS rises along with the enhanced excitation of photooxidation. Excessive ROS can damage the photosystem II (PS II) by promoting the degradation of reaction center D1 protein out of the internal repair pace (Richter et al., 1990; Haghjou and Shariati, et al., 2007). Subsequently, ROS generated further owing to the overexcitation of damaged photosystem (Shaish et al., 1993). Low temperature can also disrupt the balance of D1 protein by slowing down repair rate (Haghjou and Shariati, 2007). Moreover, respiration of *Dunaliella* is slightly increased during cold treatment (Haghjou and Shariati, 2007), and it may also account for the increase of ROS. ROS increase may also go with the disorder of photosystem under nutrient deprivation condition (Kolber et al., 1988). Using homology-based proteomics, Liska et al. (2004) displayed that *Dunaliella* enhanced photosynthetic activity for the generation of osmolyte glycerol in high salinity via the upregulation of relative enzymes. As a result, the enhanced photosynthesis leads to the increase of ROS. Subjected to the heavy metals stress, biological systems product ROS through redox

cycling or depletions of GHS and protein-bound sulfhydryl groups (Stohs and Bagchi, 1995; Okamoto et al., 2001).

5.1.3. Effects of ROS in carotenogenesis in Dunaliella under stress conditions

Carotenoids are accumulated in green tissues for the protection against oxidative stress under adverse conditions (Bouvier et al., 1998). Previous studies have been displayed that ROS induce carotenogenesis in some organisms, such as pepper (Bouvier et al., 1998), poplar (Ballach et al., 1992) and Norway spruce (Polle et al., 1992). To overcome the damage of ROS in stress conditions, *Dunaliella* increases the activities of SOD and catalase as well as accumulates large amount of β-carotene (Rabinowitch et al., 1987). Nikookar et al. (2005) found that both accumulation of β-carotene and remarkably increased activity of Apx occurred in *D. salina* and *D. tertiolecta* under the stress of copper. Enhanced β-carotene accumulation in *Dunaliella* under other stress conditions has been also detected by researchers (Lers et al., 1991; Borowitzka et al., 1990; Król et al., 1997). To identify the relationship between β-carotene accumulation and ROS in *Dunaliella*, Shaish et al. (1993) tested and obtained the results as follows. β-carotene accumulated in *Dunaliella* after a partial inhibition of photosynthesic oxygen evolution caused by a transfer from low light irradiance to high irradiance; Activity of catalase increased obviously under carotenogenic conditions with high light irradiance; β-carotene biosynthesis is greatly enhanced after adding promoters of ROS (rose bengal, cumene hydroperoxide and etc.) or respiratory inhibitor (azide, a inhibitor of SOD and catalase) under non-carotenogenic conditions. The high ratios of 9-*cis* to all-*trans* stereoisomer of β-carotene are similar under these conditions. Basing on the results above, it is suggested that ROS are involved in triggering massive β-carotene accumulation in *Dunaliella* when it suffers from stress conditions.

It is suggested that ROS play a critical role in eukaryotic gene expression, but the signal transduction pathway is poorly understood (Ryter and Tyrrell, 1998). Oxidized form of OxyR protein, which is caused by oxidation, can transduce a signal to RNA polymerase. Storz et al. (1990) suggest that OxyR protein positively regulates genes under oxidative stress. Experiencing intensive irradiance, Lers et al. (1990) showed that the increase of β-carotene accumulation in *Dunaliella* required *de novo* transcription and translation. Consequently, experiencing stress conditions *Dunaliella* massively accumulate β-carotene may be involved in ROS-triggered expression of relative genes. More detailed processes are particularly important in understanding of carotenogenic regulation in *Dunaliella*.

5.2. Expression of crt genes and enhanced β-carotene accumulation

Bouvier et al. (1998) have showed that ROS dramatically induce the expression of some crt genes in *Capsicum annuum* (pepper). Correctively, it seems that under adverse conditions enhanced carotenogenesis in *Dunaliella* may undergo a regulatory mechanism closely related to ROS-triggered expression of crt genes. However, due to insufficient study, relation between the expression of crt genes and massive carotenoids accumulation in *Dunaliella* is still obscure.

Unlike the results of Bouvier et al. (1998), Rabbani et al. (1998) found that the amount of either PSY or PDS of *D. bardawil* change indistinguishably, as well as the transcript levels for PSY, during high light treatment. Moreover, they also revealed that intensively increased carotenogenesis typically occurred after acyl lipids biosynthesis by isotopic incubation experiments. Basing on the analysis of carotenogenesis in *D. salina* under nitrogen-sufficient and nitrogen-limited conditions, Sánchez-Estudillo et al. (2006) showed that expression of *dxs* gene, whose product is 1-deoxy-D-xylulose 5-phosphate synthase (DXS), diminished under nitrogen-limited conditions while *psy* is constantly expressed in both conditions. Accordingly, massive β-carotene accumulation is not dependent on increased abundance of PSY or PDS in *Dunaliella* under carotenogenic conditions. It is also suggested that increased carotenogenesis may be closely related with increased activity of crt enzymes. Moreover, acyl lipid biosynthesis may affect the carotenogenesis in *Dunaliella* to some extent because of the anteroposterior relation between acyl lipids biosynthesis and carotenogenesis.

5.3. Non-carotenogenic gene and β-carotene accumulation

As described above, β-carotene is concentrated in chloroplast in the form of lipid globules. Multiple studies imply that the formation and stabilization of lipid globule are the main factors associated with β-carotene accumulation (Raja et al., 2007). There is a 38-kD protein, Cgp, located in the periphery of lipid globule. Katz et al. (1995) demonstrated that Cgp was induced proportional to the β-carotene accumulation. They suggested this protein plays a role in stabilizing the lipid globule. Further, Katz et al. (1995) indicated that induction of Cgp relied on the adverse conditions rather than directly on β-carotene accumulation, because the production of Cgp was not blocked when carotenogenesis was interrupted by PDS inhibitor norflurazon. In view of components of lipid globule, relationship analysis between triacylglycerols biosynthesis and β-carotene accumulation is required.

The lipid components of this globule are mainly composed of triacylglycerols according to the analysis of Fried et al. (1982). Rabbani et al. (1998) revealed that β-carotene accumulation is in parallel with acyl lipids generation. To identify the relationship between acyl lipids generation and β-carotene accumulation, enzyme inhibitors in lipid biosynthetic pathway have been used in *D. bardawil* under high light conditions (Rabbani et al., 1998). These enzyme inhibitors are sethoxydim and cerulenin, which inhibit acetyl-CoA caroboxylase and β-ketoacyl-(acyl carrier protein) synthases respectively, and have no effect on carotenogenic pathway. Consequently, both the inhibitors can result in the absence of lipid globule, and cell loses its bright red coloration (lacking β-carotene-contained lipid globule). Chemical analysis confirmed the relationship again that a perfect parallelism existed between triacylglycerols deposition and β-carotene accumulation. Azachi et al. (2002) displayed that transcriptional activation of a plant-like β-ketoacyl-CoA synthases (Kcs) gene (*kcs*) and enhanced activity of this enzyme both occurred in *Dunaliella* following a hypertonic shock. Kcs is responsible for the first and rate-limiting step in fatty acid elongation by catalyzing the condensation of acyl-CoA with malony-CoA to yield β-ketoacyl-CoA and CO_2. Azachi et al. (2002) suggested that it may be involved in adapting intracellular membrane compartments against the high internal osmolyte glycerol concentration in high salinity. Probably, it may also play a role in producing lipid globule for the accumulation of β-carotene.

Taking one with another, the relation between enhanced expression of crt genes and massive accumulation of β-carotene in *Dunaliella* is unclear now attributing to insufficient study in crt genes. However, it is true that accumulation of β-carotene is proportional to the amount and activity of several enzymes for lipid synthesis. Relation may exist between the generation of lipid globule and the accumulation of β-carotene. Under stress conditions, volume of *Dunaliella* cells experience change (Shaish et al., 1993; Einspahr et al., 1988; Maeda and Thompson, 1986), and membrane composition of cells also modify subsequently. Moreover, membrane lipid fluidity can affect the activity of membrane-associated enzymes (McMurchie and Raison, 1979). It is possible that in *Dunaliella* activity of crt enzymes increases along with the change of lipid metabolism under stress conditions. However, to clear the regulation of β-carotene accumulation in *Dunaliella* under carotenogenic conditions, further work must been carried out.

6. Considerable value of *Dunaliella* in carotenoids production

Molecular analysis showed that crt enzymes are single-gene products, each of which catalyzes its specific reaction. Multi-enzyme

carotenogenic complex is not a prerequisite for the synthesis of zeaxanthin or α-carotene from phytoene (Hirschberg et al., 1997). In addition, different combinations of crt enzymes lead to a variety of carotenoids generation through different carotenogenic pathways. Thus, accumulation of novel and high-value carotenoids could be possible in heterologous expression systems with diverse crt genes combinations through novel carotenogenic pathways. All above are based on a large of crt genes pool, in which the large number and the efficient carotenogenesis of crt genes are both critical for carotenogenesis in heterologous expression systems. Some species of *Dunaliella* accumulate large amount of β-carotene under appropriate growth conditions, which implies that crt enzymes of *Dunaliella* are efficient in carotenogenesis potentially. Therefore, crt genes of *Dunaliella* may promote massive accumulation of carotenoids in heterologous expression systems.

Presently, technologies of gene combination and molecular breeding are diffusely used in carotenoids production. Considerable researches have been carried out on the non-carotenogenic bacteria *E. coli* for carotenoids biosynthesis. However, *E. coli* provides limited isoprenoid precursors, IPP and DMAPP, for the generation of GGPP, and carotenoids subsequently. Namely, carotenogenic competence of *E. coli* (10–500 μg/g cell dry weight) is far lower than that of commercial *Dunaliella* (up to 50 mg/g cell dry weight) (Schmidt-Dannert, 2000; Johnson and Schroeder, 1995). Accumulation of carotenoids in *E. coli* increases noticeable (up to 1.5 mg/g cell dry weight) by overexpression of several enzymes (DXS, IPP isomerase (Ipi), GGPS and so on) so as to supply more precursors for carotenogenesis (Albrecht et al., 1999; Wang et al., 2000). Unfortunately, the further increase of IPP by co-expression of genes for Ipi, DXS and 1-deoxy-D-xylulose 5-phosphate reductoisomerase (DXR) is toxic for *E. coli*, possibly owing to the overloading of cell's membranes with carotenoids (Schmidt-Dannert, 2000). Therefore, the maximum of carotenoids storage in *E. coli* have reached and no additional carotenoids could be stored in this cell. Maybe we could improve it in some degree by means of certain genetic methods, but it more likely to remind us to reconsider the key role of *Dunaliella* in carotenoids accumulation. Inside *Dunaliella*, β-carotene, as well as phytoene, is concentrated in the interthylakoid spaces of chloroplast in the form of lipid globules avoiding overladen carotenoids in membranes (Ben-Amotz et al., 1982). As a result, capacity of carotenoids storage of *Dunaliella* is far larger than that of *E. coli*. In fact, as described above the carotenogenic capacity of commercial Dunaliella (50 mg/g cell dry weight) is obviously larger than that of recombinant *E. coli* (1.5 mg/g cell dry weight). It is possible that carotenoids, including novel and rare ones, are massively accumulated in *Dunaliella* under loose conditions through diverse carotenogenic pathways by use of genetic technologies.

7. Conclusions and future directions

The halotolerant marine microalgae *Dunaliella* has been known as an efficient nature source for β-carotene. It has been used to produce β-carotene all over the world since Masjuk firstly proposed β-carotene production by *Dunaliella* (Borowitzka and Borowitzka, 1988). Previously, a good many researches have been focused on the stress factors and alternative work systems for massive β-carotene accumulation by *Dunaliella*, owing to its talent of massive carotenoids accumulation in outdoor (Del Campo et al., 2007). However, high CO_2 consumption with low efficiency, poor control and high cost of carotenogenic conditions and requirement of land limit the expansion of mass cultures of *Dunaliella* for β-carotene. Globally, algae-produced β-carotene develops slowly and more than 80% of global β-carotene consumption is synthesized chemically, though the latter may be less medicative or even harmful to some extent.

On account of the high performance of *Dunaliella* crt enzymes in carotenogenesis and the competence of *Dunaliella* in carotenoids accumulation, *Dunaliella* is potential and efficient host for biosynthesis of various carotenoids. On the whole, some important efforts should be devoted currently for massive carotenoids accumulation in *Dunaliella*. Firstly, we should exhibit the carotenogenic pathway thoroughly. Secondly, genes for crt enzymes and those associated with massive carotenoids accumulation ought to be cloned and characterized. Thirdly, regulation of carotenogenesis in *Dunaliella* ought to be showed in molecular level completely. Under an appropriate regulation, *Dunaliella* not only accumulates large amount of β-carotene, but also produces plentiful novel and high-value carotenoids by recombinant DNA technology through novel and efficient metabolic routes getting rid of the drawbacks in traditional processes.

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