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Microalgal Biotechnology: Carotenoid Production by the Green Algae *Dunaliella salina*

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Abstract Unicellular green algae of the genus *Dunaliella* thrive in extreme environmental conditions such as high salinity, low pH, high irradiance and subzero temperatures. Species of *Dunaliella* are well known in the alga biotechnological industry and are employed widely for the production of valuable biochemicals, such as carotenoids. Some strains of *Dunaliella* are cultivated commercially in large outdoor ponds and are harvested to produce dry algal meals, such as polyunsaturated fatty acids and oils for the health food industry, and coloring agents for the food and cosmetic industries. During the past decade, the advances in molecular biology and biochemistry of microalgae, along with the advances in biotechnology of microalgal mass cultivation, enabled this microalga to become a staple of commercial exploitation. In particular, the advent of molecular biology and mutagenesis in *Dunaliella* has permitted enhancements in the carotenoids content of this green alga, making it more attractive for biotechnological applications. Accordingly, the present review summarizes the recent developments and advances in biotechnology of carotenoid production in *Dunaliella*.

Keywords: biotechnology, carotenoids, *Dunaliella*, microalgae

INTRODUCTION

Microalgae constitute a largely unexplored and unexploited renewable natural resource [1]. Microalgae are very attractive for commercial exploitation, because they are fast growing. In commercial conditions, microalgae, growing in mass cultures, duplicate once a day. This phenomenal rate of growth and doubling of the biomass is achieved solely through the supply of inorganic nutrients and sunlight, both of which are abundant in nature. Microalgae can produce a variety of natural products: pigments, enzymes, fatty acids, vitamins, and other bioproduct commodities. However, only a few microalgae are currently being employed in the production of high-value compounds, such as astaxanthin from *Haematococcus pluvialis*, β-carotene from *Dunaliella bardawil* and DHA from *Cryptothecacodinium* [2-8]. In addition to these high-value bioproducts, lutein from *Muriellipsis* sp. and zeaxanthin from *Dunaliella salina* have been under consideration for commercial application [9-11].

Dunaliella is one of approximately 30,000 microalgal species that is being grown commercially in countries such as Australia, India, and Israel. Currently, due to their high market value, carotenoids are the major products in the field of microalgal biotechnology.

Recently, *Dunaliella* has been the subject of mutagenesis and genetic manipulation to enhance the quantity of carotenoids some of its natural high-value bioproducts. In this article, an overview is presented of recent advances in the area of carotenoid biotechnology with the green microalga *Dunaliella salina*.

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FUNCTIONS OF THE MICROALGAL CAROTENOIDS

In microalgae, carotenoids function as accessory pigments in the light-harvesting photosystem during photosynthesis. They serve as structural components of chlorophyll-carotenoid-protein light-harvesting complexes in the chloroplast photosystem, act as photoprotective agents, and may also be involved in phototaxis [1,12]. Currently, carotenoids, including β-carotene, lycopene, cantaxanthin, lutein, zeaxanthin and astaxanthin are used in the pharmaceutical and neutraceutical industries as agents against macular degeneration. Lutein and zeaxanthin are known to play a critical role in maintaining normal function in animal vision [13]. These polar compounds are the predominant carotenoids in the macula, while other non-polar carotenoids, including β-carotene and lycopene (the principal circulating carotenoids) are absent [14-18]. Carotenoids are important in the pigmentation of fish and poultry, and in addition, they are

used in the coloration of food, drugs and cosmetics. Regulations on the use of synthetic dyes in the food industry are very strict. As such, this stringency has stimulated the research and development of microalgal carotenoid production and use as natural food additives. It has also been proposed that carotenoids act as an effective preventive agent for a variety of human diseases. Lutein, for example, was reported to have cancer-preventing properties [19-21]. In addition, the intake of lutein was strongly correlated with a decreased risk of cataracts and age-related retinal degeneration [20,22-24]. In general, carotenoid species that contain a β-ring can be converted to retinol, and thus, serve as precursors to vitamin A. The role of vitamin A in human nutrition is thought to be of primary importance in relation to human health. However, additional health benefits may accrue due to carotenoid antioxidant activity *in vivo* [25-27].

Astaxanthin is another carotenoid that is ubiquitous in nature, especially in the marine environment, and is probably best known for creating the pinkish-red hue in the flesh of salmonids, shrimp, lobster and crayfish. Because these organisms are not able to synthesize astaxanthin *de novo*, it must be acquired through their diet. In the marine environment, astaxanthin is generated in the primary production level of the food chain through *de novo* biosynthesis in microalgae or phytoplankton. Zooplankton, insects, or crustaceans accumulate astaxanthin through the consumption of microalgae, and these secondary feeders are, in turn, ingested by larger organisms that will then acquire the pinkish-red coloration [28,29]. There is growing commercial interest in the biotechnological production of astaxanthin, due to its antioxidant properties and the increasing demand from the aquaculture of salmonoids and other seafood industry, which uses it as a natural coloring agent and a feed supplement for these marine organisms [30,31].

GENERAL BIOLOGY OF THE ORGANISM

Cells of the green alga *Dunaliella* (*Chlorophyta*, Volvocales) are ovoid biflalgellates with cell volumes in the range of 100-1,000 μm^3 [32,33]. Unlike other unicellular algae, *Dunaliella* lacks a rigid polysaccharide cell wall. Instead, the cell is enclosed by a thin elastic plasma membrane covered by a mucus coating. The lack of a rigid cell wall permits rapid cell volume changes in response to extracellular changes in osmotic pressure. Under favorable growth conditions, *Dunaliella* is green, and contains only those pigments (carotenoids and chlorophyll) that are necessary for photosynthesis. In contrast, under environmental stress conditions, some species of *Dunaliella* produce and accumulate high amounts of β-carotene, with a concomitant change in coloration of the cells from green to orange [34-36]. β-Carotene, which can account for up to about 10% of the dry cell weight, can accumulate in oily globules located in the interthylakoid space of the chloroplast [35,37]. The globules have a small diameter and are exclusively composed of β-carotene, neutral lipid, and small amounts of protein. Stress conditions such as high salinity, extreme temperature and deprivation of mineral nutrients, including nitrate, sulfate and phosphate can enhance the concentration of β-carotene in *Dunaliella* [6,32,37-41]. The process of β-carotene accumulation in *Dunaliella* cells upon exposure to environmental stress conditions is referred to as carotenogenesis.

Dunaliella occurs in a wide range of marine habitats, such as oceans, salt lakes, and salt marshes near the sea. *Dunaliella* is probably the most halotolerant eukaryotic organism known. It shows a remarkable degree of acclimation to salinity, being able to acclimate to concentrations as low as 0.08 M and as high as the point of salt saturation in water (about 5 M). Therefore, it can be found in various salt lakes such as the Dead Sea in Israel, Great Salt Lake in Utah, USA, and Pink Lake in Western Australia. Since *Dunaliella* lacks a rigid cell wall, the algae shrink or swell rapidly in hypertonic or hypotonic conditions, respectively. The halophytic properties of *Dunaliella* confer to this green alga an important advantage for outdoor cultivation. Currently, mass culturing of *Dunaliella* in open ponds has the objective of β-carotene production rather than biomass [32]. Two main species of *Dunaliella*, *Dunaliella bardawil* and *Dunaliella salina*, are currently being exploited commercially. Because β-carotene accumulation in *D. bardawil* and *D. salina* requires high irradiance stress, production facilities are located in areas where solar insulation is maximal.

BIOSYNTHETIC PATHWAY OF CAROTENOIDS IN *DUNALIELLA*

In the past 10 years, the then state of the art techniques in the biochemistry of carotenogenesis, especially the cloning of the carotenoid biosynthesis enzymes genes has advanced considerably. Availability of these genes has been helpful in characterizing the enzymes of the carotenoid biosynthesis pathway and their regulation. A summary of the chemical structures and carotenoid biosynthetic pathways of microalgae is shown in Fig. 1.

The carotenoid biosynthesis pathway begins with geranylgeranyl pyrophosphate. Phytoene synthase (PSY) catalyzes the first committed step in carotenoid biosynthesis by condensing two 20-carbon geranylgeranyl pyrophosphate molecules to form a 40-carbon phytoene molecule, which is the precursor to all other carotenoids. Two structurally and functionally similar enzymes, phytoene desaturase (PDS) and ζ-carotene desaturase (ZDS), convert phytoene to lycopene via ζ-carotene. Lycopene undergoes cyclization at each end of the linear molecule, which is a reaction catalyzed by the enzyme lycopene β-cyclase (LCYB), to form β-carotene. The two beta rings of β-carotene are subjected to identical hydroxylation reactions to yield zeaxanthin, which, in turn, undergoes epoxidation, once to form antheraxanthin and twice to form violaxanthin. Then, neoxanthin is derived from violaxanthin by an additional molecular rearrangement [1,42]. Higher plants and green algae contain additional carotenoids, α-carotene derivatives (β,ε-carotenoids), which

Table 1. Varieties of vectors that have been employed in microalgal transformation

Microalgae	Vectors	Remark	References
Chlamydomonas reinhardtii	pS108	Rbcs gene promoter directs *ble* gene expression. *ble* gene is resistant to zeocin	[51]
Phaedactylum tricornutum	pfcpA/ble, pfcpB/ble, pfcpE/ble	Fcp (fucoxanthin chlorophyll binding protein) gene promoter with *ble* cassette	[54]
	pPha-T1	Fcp (fucoxanthin chlorophyll binding protein) gene promoter with *ble* cassette	[58]
	pPTEGfp,	pPha-T1 is inserted by GFP gene	[57]
Thalassiosira weissflogii	pFCPFp-ble, pFCPBp-ble, pFCPBp- GUS	pFCBp-*ble* is replaced by GUS gene in ble cassette	[56]
Dunaliella tertiolecta	DbleFLAG1.2	*D. tertiolecta* rbcs gene promoter with *ble* gene	[60]
Chlorella ellipsoidea	pCVT	*Chlamydomonas* rbcs gene promoter direct *ble* gene expression	[53]

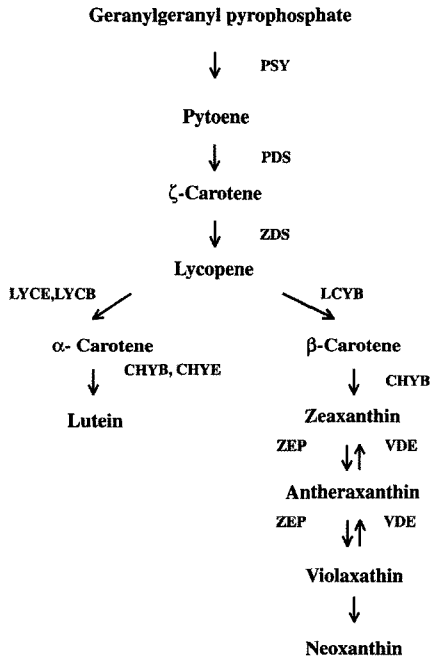

Fig. 1. Schematic diagram of the pathway of carotenoid biosynthesis in microalgae (green algae). PSY : Phytoen Synthase, PDS: Phytoen Desaturase, ZDS: ζ-Carotene Desaturase, LCYE: Lycopene ε-Cyclase, LCYB: Lycopene β-Cyclase, CHYE: ε-ring Hydroxylase, CHYB: β-ring Hydroxylase, ZEP: Zeaxanthin Epoxidase, VDE: Violaxanthin Deepoxidase.

are derived from lycopene through the cyclization of two structurally related enzymes, lycopene β-cyclase and lycopene ε-cyclase (LCYE). The hydroxylation of the β and ε rings of the α-carotene molecule yields lutein [43,44].

CULTURE SYSTEMS

The microalga aquaculture industry is now over 30 years old where the main microalga species being cultivated are *Chlorella* and *Spirulina* for the neutriceutical industry, *Dunaliella* for β-carotene production and *Haematococcus* as a source of astaxanthin. The majority of the culture systems currently in use for *Dunaliella* cultivation are open ponds. There are four major types of open-air pond systems: shallow large surface area ponds, tank-type containers, circular ponds and racetrack ponds. The intrinsic properties of the algae, as well as the local climatic conditions and the costs of land and water are all deciding factors in the selection of a particular system. For example, Australian producers employ large shallow ponds of up to 250 ha in surface area as their growth facilities. This large employment is possible in Australia because land costs are low, water is free and the climate is close to optimal so that production is possible throughout the whole year. On the other hand, producers in Israel use paddlewheel driven racetrack ponds to achieve higher cell densities in a smaller surface area [45]. In this case, the determining factor is the higher cost of land in Israel, and the need to supply NaCl to the ponds. To make the process economically feasible in Israel, pond area and volume size must be minimized, and cell density in the culture must be maximized [39].

MOLECULAR BIOLOGY

The recent genetic elucidation of bacterial and plant carotenoid biosynthetic pathways, leading to the accumulation of zeaxanthin, canthaxanthin and astaxanthin, may offer interesting alternatives for their *in vivo* production [18,46-49]. For example, cyanobacteria can be readily transformed with autonomously replicating plasmids, while endogenous genes can be disrupted by homologous recombination. A number of commercial alternatives have been proposed for recombinant cyanobacteria. For example, *Synechocystis* sp. strain PCC 6803 was used as a transformation host to over-produce zeaxanthin *in vivo* [50]. The unicellular green alga, *Chlamydomonas reinhardtii*, was developed into a sophisticated molecular system, and a useful model organism, which has contributed to the understanding of photosynthetic and other cellular processes [51]. Although recombinant *Chlamydomonas* does not, at this time, have direct commercial application,

Table 2. Zeaxanthin formation in *Dunaliella, E. coli and cyanobacteria*

	Zeaxanthin content	References
Dunaliella	Unit (mg/g dw)	
WT	0.23	[11]
zea1	5.9	
Metabolically engineered *E. coli*	0.28-1.6	[66, 67]
Synechocystis sp.	Unit (fold)	
WT	1	[50]
mutant	2.5	

the molecular genetics technology developed for *Chlamydomonas* has provided direction for the development of transformation techniques in other algae. Applications of transformation technology in *Chlorella* [52,53], diatoms [54-58], and *Dunaliella* [59,60] have been established (Table 1). These microalgal transformation technologies may find direct commercial application. The recent recombinant technology in the economically valuable algae can be utilized to confer heterotrophy to otherwise obligate autotrophic algae [57], thereby, permitting an increase in the cell mass per liter culture and enhancing the economic value of algae. Table 1 summarizes the transformation vectors that have been employed with many of the microalgae. Beyond the successes of microalgal transformation, it is also important to specifically improve the promoters of vectors that direct the selection of transformed cells and to better define selection methods, which will enhance the efficiency of algal transformation.

CAROTENOID ABERRANT MUTANTS OF *D. SALINA*

Despite the fact that pigment mutants of cyanobacteria and green algae have been used extensively to study the biogenesis and function of photosynthetic complexes [61-63], so far, only a few mutants from *Dunaliella* have been reported. Jin *et al.* [11,59] employed mutagenesis of *Dunaliella salina* to manipulate the composition and quantity of carotenoids, especially that of zeaxanthin. In general, the level of irradiance regulates the zeaxanthin content of the microalgae. Under photosynthetically active conditions photosynthetic organisms, including *D. salina,* contain only trace amounts of zeaxanthin. Two-different zeaxanthin-overproducing strains of *D. salina,* termed *dcd1* and *zea1*, were generated upon mutagenesis [11,59]. The *dcd1* mutant was selected on the basis of its yellow-green coloration under moderate illumination. In the *dcd1* mutant, zeaxanthin content was slightly greater than in the wild type [59]. The *zea1* mutant showed a constitutive zeaxanthin accumulation under all growth conditions and lacked all zeaxanthin derivatives [11]. Thus, the *zea1* mutant lacked neoxanthin, violaxanthin and antheraxanthin, and constitutively accumulated zeaxanthin in its thylakoid membranes. Under normal growth conditions (low-light), the *zea1* mutant had a zeaxanthin content that was twenty times larger than the wild type (Table 2). Biochemical analyses strongly suggested that *zea1* is a 'zeaxanthin epoxidase' mutant. Table 2 compares the zeaxanthin content in the wild type and *zea1* mutant that was grown under low light conditions. It was seen that the wild type contained 0.23 mg zeaxanthin per g dry weight, whereas the *zea1* strain contained about 6 mg zeaxanthin per g dry weight (Table 2). This value (0.6% zeaxanthin per dry weight) is close to the 1% value (*i.e.,* 10 mg zeaxanthin per g dry weight), and is thought to represent the minimum cellular content of zeaxanthin for a cost-effective commercial exploitation of this product [11,32]. A mutant of *Chlamydomonas* [64], which is similar to *zea1,* and a mutant containing a knockout of zeaxanthin epoxidase in potato tuber [65] also constitutively accumulated zeaxanthin while other β-branched xanthophylls were missing.

Efforts to generate zeaxanthin overproducing *E. coli* strains using metabolic engineering [66] have resulted in the maximum accumulation of about 1.6 mg zeaxanthin/g dry weight in this bacterium (Table 2). However, this yield is only about 27% of that yielded by the *zea1* strain of *D. salina* (6 mg zeaxanthin/g dry weight) (Table 2). Furthermore, efforts to increase the yield of zeaxanthin in *Synechocystis* sp. (cyanobacteria) produced a 2.5-fold increase in zeaxanthin accumulation in these mutant strains [50]. These successes have encouraged a growing, worldwide interest in the manipulation of the carotenoid biosynthetic pathways in plants and microalgae.

CONCLUSIONS AND FUTURE DIRECTIONS

Currently, applications of algal biotechnology in the area of high value bioproducts are limited to β-carotene and astaxanthin. Processes for the commercial extraction of zeaxanthin and lutein from microalgae are also being considered. Industrial application of carotenoids includes their use as coloring agents for human consumption, feed additives to enhance the pigmentation of fish, poultry and eggs, and also color enhancement in cosmetics and pharmaceutical products. To date, most of the β-carotene used commercially is chemically synthesized. However, there is an important market for xanthophylls (lutein, zeaxanthin, astaxanthin *etc.*), provided that suitable microalga production systems can be developed. For a cost-effective production of naturally occurring carotenoids from microalgae, it is necessary to develop strains whose carotenoid content is much higher than that of their wild type counterparts. Promising in this respect is the recent advent of transformation technologies, which have been established in some of the microalgae. However, at this point, algal strains generated through new recombinant techniques are not yet being applied commercially in mass cultures. Therefore, further R&D of microalga transformation technologies must be undertaken. Such efforts should be paralleled by the development of methods for the mass-cultivation and bio-processing of genetically engineered microalgae. Such advances will un-

doubtedly bolster the industry of algal biotechnology and broaden the commercial application of microalgae.

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