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REVIEW ARTICLE

Dunaliella biotechnology: methods and applications

A. Hosseini Tafreshi and M. Shariati

Mansour Shariati, Department of Biology, University of Isfahan, Isfahan, Iran

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Correspondence
Mansour Shariati, Department of Biology, University of Isfahan, Hezarjarib St., Isfahan, Iran. E-mail: mansour@sci.ui.ac.ir, mansour_shariati@yahoo.com

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Summary

The microalga *Dunaliella salina* is the best commercial source of natural *β*-carotene. Additionally, different species of *Dunaliella* can accumulate significant amounts of valuable fine chemicals such as carotenoids, glycerol, lipids, vitamins, minerals and proteins. They also have a large potential for biotechnological processes such as expressing of foreign proteins and treatment of wastewater. In this review, we discussed several biotechnological aspects of the mass cultivation of *D. salina* like strain selection, carotenoid induction, culture conditions, culture systems and downstream processes. We also discuss several traditional and new applications of the genus.

Introduction

Dunaliella species belong to the phylum Chlorophyta, order Volvocales and family *Polyblepharidaceae*, *and are* unicellular, photosynthetic and motile biflagellate microalgae morphologically distinguished by the lack of a rigid cell wall (Ben-Amotz and Avron 1987). The best-known species of *Dunaliella* are *Dunaliella salina*, *Dunaliella tertiolecta*, *Dunaliella primolecta*, *Dunaliella viridis*, *Dunaliella bioculata*, *Dunaliella acidophyla*, *Dunaliella parva* and *Dunaliella media*. A recent review has assessed current issues of the taxonomy of *Dunaliella* (Borowitzka and Siva 2007).

Dunaliella cells are ovoid, spherical, pyriform, fusiform or ellipsoid with size varying from 5 to 25 *μ*m in length and from 3 to 13 *μ*m in width. The cells also contain a single cup-shaped chloroplast which mostly has a central pyrenoid surrounded by starch granules (Ben-Amotz 1980). Besides chlorophylls a and b, the members of *Dunaliella* contain valuable carotenoid pigments such as *α*- and *β*-carotene, violaxanthin, neoxanthin, zeaxanthin and lutein. *Dunaliella* cells have other organelles typical of the green algae: membrane-bound nucleus, mitochondria, vacuoles, Golgi apparatus and an eyespot (Ben-Amotz and Avron 1989a). The cell is enclosed only by an elastic plasma membrane covered by a mucus surface coat and shrinks or swells rapidly when exposed to hypertonic and hypotonic conditions, respectively (Ben-Amotz and Avron 1990; Ben-Amotz 1993). *Dunaliella* multiplies by lengthwise division, but sexual reproduction does occur rarely by isogamy with a conjugation process.

Species in the genus are isolated from aquatic marine habitats like sea and inland salt lakes with a wide range of chemical compositions and salt concentrations ranging from 0·5% to saturation (around 35%), which makes this alga the most halotolerant eukaryote known. The mechanism by which *Dunaliella* cells can adapt to this wide range of salt concentrations was shown to be based on the ability of the alga to change its intracellular concentration of glycerol (Avron 1992; Shariati and Lilley 1994). When *Dunaliella* is grown at high salinity, the intracellular glycerol content exceeds 50%, which is sufficient to account for most of the required osmotic pressures. In this condition, glycerol acts as a 'compatible solute' that protects enzymes against both inactivation and inhibition (Brown and Borowitzka 1979). It was also shown that both the glycerol synthesis under hypertonic conditions and its elimination under hypotonic condition are independent of protein synthesis and occur in the light or dark (Ben-Amotz and Avron 1989a). On the other hand, more recent data suggested that *Dunaliella* has an exceptional ability to remove Na^+ ions in hypersaline environments by using a novel redox-driven sodium pump (Katz

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and Pick 2001). The results from proteomics approaches also indicated that in addition to the means above, *Dunaliella* responds to transfer to a high salinity by enhancement of photosynthetic CO_2 assimilation (Oren 2005).

In addition to this exceptional halotolerance of most *Dunaliella* species, *Dunaliella acidophila* can grow in very acidic environment (pH 0–1). *Dunaliella antarctica* thrives at subzero temperatures and some strains of *D. salina* can tolerate high light intensities. Furthermore, *Dunaliella* is more tolerant to fuel oil contamination compared with other planktonic algae (Brown and Borowitzka 1979). Thus, these organisms are unique in their abilities to adapt to some of the most severe conditions of the global habitats.

Dunaliella salina was first recognized by Teodoresco (1905). This alga is often found in natural marine habitats where it colours the water red. For the first time, Milko (1963) reported that *Dunaliella* contains high concentrations of β-carotene and later it was also recommended as a commercial source of glycerol (Ben-Amotz 1980). Nowadays, we know that *Dunaliella salina* is the best commercial source of natural β-carotene among all organisms in the world (Borowitzka 1995). This alga accumulates large amounts of β-carotene as droplets in the chloroplast to prevent chlorophyll photo-damage, when culture conditions include high light intensities, high temperature, high salinity and deficiency of nutrient (Ben-Amotz and Avron 1983; Ben-Amotz and Shaish 1992). The β-carotene contents of up to 14% of dry weight have been reported for *D. salina* (Aasen *et al.* 1969). Mass cultivation of *D. salina* for β-carotene production has being accomplished in several countries including Australia, USA and China (Borowitzka and Borowitzka 1989; Borowitzka 1999). Pilot-scale projects have also being attempted in Spain, Kuwait, Chile, Iran, etc. (Borowitzka 1990; Garcia-Gonzalez *et al.* 2003; Hosseini Tafreshi and Shariati 2006). Some other aspects of *Dunaliella* biotechnology have also been reviewed elsewhere (Del Campo *et al.* 2007; Raja *et al.* 2007; Ye *et al.* 2008).

Different issues for mass culturing of *Dunaliella* in different culture systems were addressed below.

Strain selection, improvement and laboratory maintenance

The first step prior to mass culture of *Dunaliella salina* in open ponds or bioreactors is to select the strains most suitable for mass culturing in terms of β-carotene content and rate of growth. Only some strains of *D. salina* have the ability to accumulate high amounts of β-carotene (up to 10–14% of dry weight) (Aasen *et al.* 1969). *Dunaliella parva* may also produce significant contents of β-carotene (up to nearly 4% of dry weight) but none of the other species can synthesize such large concentrations of β-carotene (Massyuk 1973). According to some authors, several strains of *Dunaliella* species reported in the literature have been misnamed (Borowitzka 1990). For example, strain UTEX 200 = CCAP19/3 of *D. salina* is probably *D. viridis*, which does not turn red at salinities up to 25%. There are also some confusing differences in nomenclature of the strains between different authors. Ben-Amotz and Avron (1990) stated that under appropriate conditions where β-carotene nonaccumulating strains of *Dunaliella* contain about 0·3% β-carotene, only *D. salina* Teod. and *Dunaliella bardawil* (=UTEX 2538) are able to produce large amounts of β-carotene more than 10% of dry weight. These two strains are morphologically similar to other strains but their cell volume is larger than that of other strains. Ben-Amotz and Shaish (1992) have shown several differences between these two strains in terms of the presence and the number of eyespot or lipid globules. There are also some reports of major differences in the regulation of carotene synthesis in response to high irradiances and various salinities between them (Gomez *et al.* 2003). In contrast, Borowitzka (1990) believed that *D. bardawil* is an incorrect name for *D. salina*.

Numerous strains of *D. salina* were isolated from various marine habitats such as inland salt lakes, salt marshes and artificial salt ponds around the world where the water contains more than 10% salt. One approach for identification of *D. salina* is the method described by Butcher (1959) and Loeblich (1982) who proposed that *D. salina* strains should contain a carotenoid to chlorophyll ratio higher than 6 : 1. Various *D. salina* strains isolated or studied by different authors contained different values of carotenoids per cell and per unit volume. In one study accomplished by Cifuentes *et al.* (1992), total carotenoid contents of eight strains of *D. salina* isolated from salt ponds in Chile ranged from 4 to 42 pg cell^{-1} and from 7·2 to 38·2 mg l^{-1}. In another study, specific growth rate and carotenoid content of five commercial strains of *D. salina* were determined in outdoor batch culture (Garcia-Gonzalez *et al.* 2003). In that experiment, the growth rate ranged from 0·16 to 0·20 day^{-1}, the highest value being for strain UTEX 2538; the carotenoid content ranged from 8·1 to 15·1 mg l^{-1}, the maximum again being for UTEX 2538. Interestingly, Powtongsook *et al.* (1995) isolated a clone named DS91008 that produced the carotenoid content up to 80·3 pg cell^{-1} at 30% salinity under continuous illumination at 270 μmol m^{-2} s^{-1}. Because of the different carotenogenic conditions used in different experiments, comparing their data with each other is not easy and this should be done only at similar conditions. Logically, from a commercial point of view, the best strains of *D. salina* for use in mass cultivation should

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have the maximum specific growth rate and the highest β-carotene content per unit time and culture volume under optimized conditions.

Besides the study of physiological differences of the strains, some more recent researchers worked on genetic diversity of *Dunaliella* strains. Such studies may allow the detection of unknown genotypes, which could have the genes with beneficial potential and hence can be used to create an excellent procedure for strain selection. Gomez and Gonzalez (2001) genetically analysed eight Chilean strains of *D. salina* by using random amplified polymorphic DNA (RAPD) technique and showed, e.g. that strain CONC-006 isolated from an alpine salt lake had more genetic similarity to CONC-001 from a coastal lagoon than other alpine strains. Gomez and Gonzalez (2004) used RAPD band patterns and nuclear ribosomal DNA internal transcribed spacer (ITS-1 and ITS-2) sequences for studying the genetic variability of seven strains (three from Chile and four from other locations). Their results showed some degree of correlation between the differences in physiological traits of the strains, like carotenogenic capacity and the genetic diversity among them. By these kinds of studies in the future, we will be able to efficiently reduce the risk of misassumptions about the biotechnological traits of the strains, which mainly result from plasticity in phenotype.

Several attempts have been carried out to mutagenize different strains of *Dunaliella* and to isolate mutants with special traits by using chemical mutagens and UV. Some of these variants might be used in basic science as tools for elicitation of physiological or biochemical pathways in the alga. For example, several mutants defect in salt tolerance have been reported (Zamir 1992). More recently, Vismara *et al.* (2004) described and isolated a spontaneous mutant of *D. salina* with very short flagella. They ultimately introduced a possible model to explain the mutant motion pattern. On the other hand, mutagenesis procedure has been exploited to create mutants with improved qualitative or quantitative biotechnological traits. Using UV irradiation, mutants of *D. bardawil* with higher potential for synthesizing β-carotene under relatively low light intensities have been isolated (Shaish *et al.* 1991). Recently, a novel mutant of *D. salina* named *zea1* was isolated by ethyl methyl sulfonate mutagenesis treatment (Jin *et al.* 2003a). *Zea1* carries a defect in zeaxanthin epoxidation reaction, and as a result, lacks a number of the β-branch xanthophylls neoxanthin, violaxanthin and antheraxanthin, while retaining significant amounts of zeaxanthin, providing a potential commercial source of this high value bio-product.

One of the most commonly used media for laboratory culturing of *Dunaliella* species is the modified Johnson medium (Borowitzka and Borowitzka 1988a). Other media such as modified ASP medium (Mclachlan and Yentsch 1959), f/2 Guillars' seawater medium (Guillard and Ryther 1962), Parvasoli-enriched seawater medium (PES) (Mclachlan 1973) and artificial medium (ART) (Ben-Amotz *et al.* 1988) have also been used. The PES medium, which has the low contents of nitrate and phosphate, has been effective to induce carotenogenesis (Gomez *et al.* 2003). ART medium also contains a limiting nitrogen contribution but not inorganic phosphate and similar to PES medium could be inductive of carotenoid syntheses (Gomez *et al.* 2003). Cultures are usually maintained on agar solid or liquid medium with subculturing every 1–2 months. Borowitzka and Borowitzka (1989) also reported successful maintenance of *Dunaliella* alga in liquid nitrogen for up to 12 months. In addition, the methodology of cryopreservation of eukaryotic algae such as *Dunaliella* reviewed by Taylor and Fletcher (1999). By such a way, biological samples can be maintained in a state of 'suspended animation' at low temperatures.

Carotenoid induction

It has been suggested that β-carotene accumulation protects cells against the deleterious effects of high intensity irradiation by absorbing light in the blue region of the spectrum (Ben-Amotz *et al.* 1987; Ben-Amotz 1993). *Dunaliella* β-carotene is composed of a mixture of the *cis*- and *trans*-isomers with a typical composition of 9-*cis* (41%), all-*trans* (42%), 15-*cis* (10%) and other isomers (6%) (Borowitzka and Borowitzka 1989). In contrast to all-*trans* isomer, which is insoluble in oil and easy to crystallize, the 9-*cis* β-carotene (Fig. 1) has higher oil solubility and is more difficult to crystallize (Ben-Amotz 1993).

Several strategies have been used to maximize the production of β-carotene per unit time and per culture volume (Borowitzka and Borowitzka 1990; Ben-Amotz 1995; Garcia-Gonzalez *et al.* 2003). These strategies are based on the observations that severe conditions, such as high salinities, low nutrient levels and high temperatures combined with high irradiance, retard growth and at the same time, induce β-carotene production in the cell. Indeed, the higher the stress intensity and as a result the slower the growth rate of the alga, the greater is the total amount of the light absorbed by the cell during one division cycle. This situation can lead to higher accumulation of β-carotene per cell. However, these conditions at the same time decrease the cell number per unit culture volume by affecting cell viability. Ben-Amotz and Avron (1983) showed that the salinity and irradiance have an additive or synergistic effect on carotenogenesis in *D. bardawil*. In contrast, prolonged nutrient starvation can lead to high mortality of the algae.

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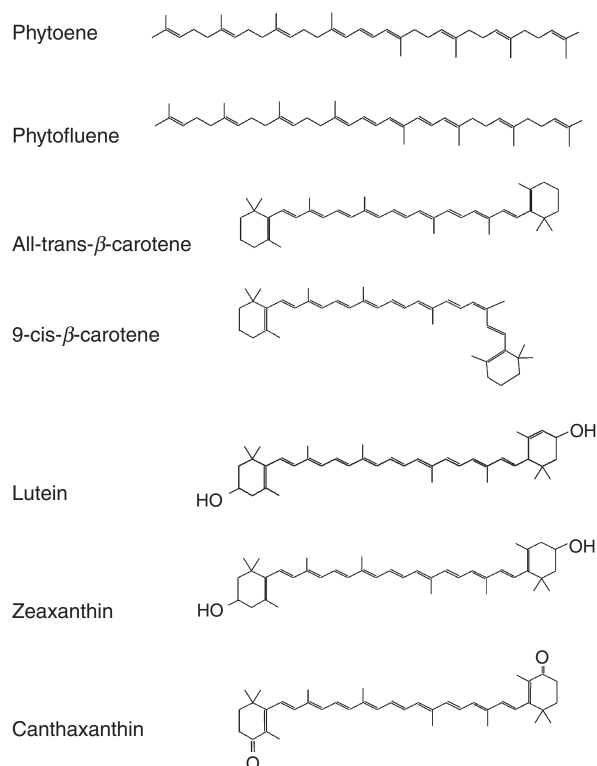

Figure 1 Structures of common carotenoids in *Dunaliella*.

Therefore, it is recommended by one group of authors that adjusting light and salinity likely is one of the best strategies to achieve optimal β-carotene production in mass cultures of *D. salina* (Marin *et al.* 1998). In addition, increasing salinity can inhibit the growth of *Dunaliella* predators and of noncarotenogenic *Dunaliella* species that naturally occur in open ponds (Borowitzka and Borowitzka 1990). In contrast, other authors commented that the salinity does not have a clear effect on β-carotene accumulation per cell (Gomez *et al.* 2003). The explanation for these controversial reports probably is related to the fact that the effect of salinity, like other inducing factors on β-carotene production in *D. salina*, is strain dependent. Chen and Chi (1981) proposed a two-stage method for *Dunaliella* cultivation in which cells are grown in a medium rich in nutrient and 18% NaCl in stage one and then transferred to the carotene-induction medium of the second stage in which nutrient is poor and salt concentration is about 27%. Borowitzka and Borowitzka (1990) claimed that this procedure was not feasible because at lower salinity of the first stage, noncarotenogenic *Dunaliella* species and protozoan predators of *Dunaliella* could exist in the ponds which can lead to a rapid decrease in algal biomass. Therefore, some authors proposed a semi-continuous mode in which cells are cultivated in ponds in a medium of intermediate salinity (Borowitzka and Borowitzka 1990). Ben-Amotz (1995) cultivated *D. salina* by a new two-phase growth strategy for β-carotene production. In this mode, the cells were firstly cultivated in small nursery ponds to attain optimal biomass and then transferred to large production ponds and diluted by adding medium deficient in nitrate and/or higher concentration of salt to approximately one third for carotenoid induction (Ben-Amotz 1995; Hosseini Tafreshi and Shariati 2006). Using this strategy, productivities up to 450 mg m^{-2} day^{-1} in stage one and to 300 mg m^{-2} day^{-1} in stage two have been reported, compared with the productivity of below 200 mg m^{-2} day^{-1}, using a one-stage method of cultivation (Ben-Amotz 1995).

This effectiveness of dilution on carotenoid induction is in accordance with the fact that over and above light intensity and stress, a more important factor for carotenogenesis in *D. salina* is the 'aerial density' or the residence time of the cells in ponds (Grobbelaar 1995). At much higher aerial density the amount of light absorbed by a cell is low (due to the shading effect) and hence the resident time of the cell in ponds required to reach the maximal β-carotene content is longer. Consequently, optimization of the aerial density in which the maximal biomass and carotenoid content would be obtained is an important step both in ponds and photobioreactors. Garcia-Gonzalez *et al.* (2003) reported that the optimal values of population density, which yield the highest output rate in semi-continuous regime, were between 0·7 and $0{\cdot}9 \times 10^6$ cell ml^{-1} which was similar to those observed by Ben-Amotz (1995) in two-phase mode. The operation parameters of culture systems like mixing rate, depth of the culture, etc. can also affect the output rate and will be considered later.

From a biotechnological point of view, it is desirable to increase the 9-*cis* to all-*trans* β-carotene ratio in the cell because 9-*cis* isomer has shown to be a better antioxidative and cancer-preventive than another (Chidambara Murthy *et al.* 2005). The information about the conditions that trigger synthesis of 9-*cis* isomer as well as β-carotene accumulation is also controversial. Studies on *D. bardawil* have shown that high irradiation (up to 2000 μE m^{-2} s^{-1}) favour the accumulation of 9-*cis* isomer (Ben-Amotz *et al.* 1988). Garcia-Gonzalez *et al.* (2005) also found that a suitable approach for the production of high quality β-carotene with high 9-*cis* isomer content is the cultivation of *Dunaliella* in closed tubular photobioreactors, which have low mutual shading. In contrast, other authors commented that decreasing light intensities could promote a higher ratio of 9-*cis* to all-*trans* in different strains of *D. salina* than high irradiances (Orset and Young 2000). Gomez *et al.* (2003) represented that in

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contrast to *D. bardawil*, Chilean CONC-007 strain of *D. salina* did not increase the 9-cis to all-*trans* ratio when grown in higher salinities. Exposure to low temperature in the range of 10–15°C could also induce the 9-*cis isomer* synthesis in *D. bardawil* (Ben-Amotz 1996). Consequently, there is a great physiological variability in response to different carotene induction factors among different strains of *D. salina*. The intrinsic response of each strain to each inductive factor alongside the complex interactions among various environmental conditions demonstrate that there is no predictable unique condition for reaching the maximum carotenoid and 9-*cis* β-carotene contents per unit time and per unit volume. The optimization procedure should be done by testing the best strains and the most effective strategies under optimal conditions.

Recently, Mojaat *et al.* (2008b) studied the effects of Fe^{2+} ions and organic carbon source on growth and carotenogenesis of *Dunaliella salina*. In their study, a significant increase in β-carotene contents per cell was observed, with a maximum value of 70 pg $cell^{-1}$ when the culture was supplemented with acetate and $FeSO_4$. The approach might be a good alternative method for production of carotenoids by alga in photobioreactors after optimization.

Requirements

Dunaliella has advantage of being capable of thriving in environments with high concentrations of NaCl, which makes it feasible to culture in open ponds as well as closed bioreactors. However, in all *D. salina* culture systems it is required to add some nutrients and to adjust some limiting physiological factors for optimal growth and carotenogenesis.

Light

Because *Dunaliella* is an obligate photoautotroph, light is the unique energy source for its metabolism (Borowitzka and Borowitzka 1989). In open ponds, the only source of light is sunlight, whereas in photobioreactors the light may be provided by using either white fluorescent lamps or sunlight. Growth and carotenoid synthesis respond differently to different quality and quantity of light. It was found that carotenoid induction is independent of wavelength, however, strongly depends on light intensity (Ben-Amotz and Avron 1989b). Jahnke (1999) also observed that unlike UV-B, UV-A radiation caused in massive accumulation of carotenoid in *D. bardawil* without slowing growth.

Temperature

Dunaliella has the ability to thrive in a wide range of temperature from below 0 to around 45°C. In laboratory cultures, the optimal temperature for *Dunaliella* growth is about 32°C with a broad optimum between 25 and 35°C (Ben-Amotz 1995). Because of technical limitations, temperature in open ponds is not controllable. Borowitzka and Borowitzka (1987) showed that the low night temperatures like those found at Hutt lagoon (located in Western Australia, Australia) pilot plant decrease the growth rate, depressing the cell yield. On the other hand, temperatures around 40°C and higher promote carotenoid induction but at the same time slow down the growth rate (Borowitzka and Borowitzka 1989). In addition, temperatures higher than 40°C cause dramatic leakage of glycerol into the medium (Wegmann *et al.* 1980), which can serve as an organic carbon source for bacteria and filamentous fungi which then become dominant (Ben-Amotz 1995). This may be one of the major problems in outdoor culture of *D. salina* particularly in nonarid areas with hot summers. Compared with humid areas, much higher evaporation rates in arid zones can significantly lead to further reduction in the pond temperature and consequently, these areas are the most suitable for outdoor culture of *Dunaliella* and other microalgae. Unlike open ponds, the temperature in photobioreactors is exactly controlled by using a thermostat unit.

pH control

Dunaliella species have a wide rage of pH tolerance from 0 to 11 but the optimum pH for *D. salina* is between 9 and 11. In autotrophic algal cultures, the pH rises because the photosynthetic fixation of CO_2 with NO_3^- uptake contributes to further release in OH^- (Ben-Amotz and Avron 1989a). There is a risk of precipitation by several calcium salts and flocculation of the algal biomass at higher pH, especially when the concentration of Ca^{2+} is high, the conditions usually found in many natural sources of water. This can lead to reduction in algal growth and hence it is necessary to avoid an increase of the pH above 8 in cultures (Ben-Amotz and Avron 1989a). In intensive mode operated open ponds, the pH usually is maintained at $7{\cdot}5 \pm 0{\cdot}2$ by double control of both CO_2 gas and HCl where the first is flowed routinely to the culture and the second occasionally is added as upper threshold control (Ben-Amotz 1995). In some open ponds and photobioreactors where the major source of inorganic carbon is bicarbonate ion, the pH is controlled only by the addition of HCl.

Nutrient requirements

Because *Dunaliella* is a photoautotroph, it can use only carbon dioxide and bicarbonate as inorganic carbon sources. The lack of an appropriate inorganic carbon source is the most common growth limiting factor under the conditions present in *D. salina* cultures such as the

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high salinity, increased pH and high temperature (Borowitzka and Borowitzka 1989). A variety of gas-bubbling devices inject small CO_2 bubbles into the cultures. In one approach, the gas was passed through porous plastic pipes fixed at the bottom of the ponds and digital mass flow meter used to achieve a gas flow rate of 0·4 l ml^{-1} (Garcia-Gonzalez *et al.* 2003). Alternatively, 10 mmol l^{-1} $NaHCO_3$ may be used as carbon source for good growth between pH 7·5 and 9·5; however, the algae will grow well up to pH 11 when a higher initial bicarbonate concentration is provided because at the higher pH, the supply of soluble carbon dioxide becomes restricted (Ben-Amotz and Avron 1989a). As an approach, 5 mmol l^{-1} $NaHCO_3$ was added daily to the bioreactors after sterilization of solid sodium bicarbonate at 120°C in an oven under the dark and then mixing with sterilized water (Hejazi *et al.* 2003).

The best source of nitrogen for *D. salina* is nitrate. In practice, 5 mmol l^{-1} $NaNO_3$ or KNO_3 is added to the medium for optimal growth of the algae. On the other hand, limiting the nitrate is one of the most common ways for the reduction of growth rate leading to the induction of carotenoid production. However, prolonged nitrogen limitation in the culture can eventually lead to high mortality of cells as well as to serious reduction of carotenoids per unit culture volume. Other nitrogen sources such as ammonium salts and urea are not appropriate because they can result in the death of the algae under certain conditions (Borowitzka 1990). It has been also shown that the use of NH_4NO_3 or $(NH4)_2CO_3$ as source of nitrogen has toxic effects on rapidly growing *D. salina* (Borowitzka and Borowitzka 1987).

Phosphorus in the forms of KH_2PO_4 or NaH_2PO_4 gives the best results. It was claimed that the optimum phosphate content for growth is about 0·2 $\mu g\ l^{-1}$ KH_2PO_4 (Gibor 1956). In open ponds, higher concentrations can inhibit growth because concomitant presence of phosphate and calcium especially at pH higher than 8, can lead to Ca_3PO_4 precipitation and algal flocculation (Sukenik and Shelef 1984).

Dunaliella also needs high concentrations (approximately 2 mmol l^{-1}) of sulfate for maximal growth, but this is rarely needed to be added in commercial pond medium because natural water sources such as seawater or tapwater contain much higher contents of sulfate, around 30 mmol l^{-1} (Ben-Amotz and Avron 1989a).

Other elements required for good *D. salina* growth include K^+, Ca^{2+}, Mg^{2+}, Cl^-, Na^+, chelated iron and trace elements. The ratios of $Mg^{2+} : Ca^{2+}$ and $Cl^- : SO_4^{2-}$ in the medium may also affect both growth and carotene synthesis (Ben-Amotz and Avron 1983). *Dunaliella* can tolerate a wide range of $Mg^{2+} : Ca^{2+}$ ratio from 0·8 to 20·0 (Borowitzka 1990). The best $Cl^- : SO_4^{2-}$ ratio for optimal *D. salina* growth has found to be around 3·2 whereas the optimum ratio for carotenogenesis was about 8·6 (Massyuk 1956). Chelated iron usually is added to the cultures in the form of $FeCl_3$–EDTA or ferric citrate–EDTA. Borowitzka and Borowitzka (1987) showed that, compared with ferric citrate, $FeCl_3$ increased more rapidly the initial growth rate of *D. salina*; however, the Fe-citrate finally gave a higher cell yield than $FeCl_3$. Four microelements, including manganese, zinc, cobalt and copper are also necessary for optimal growth of *Dunaliella*; however, for most the time, there is no need to add these elements to the medium if it is composed of technical salt or seawater (Ben-Amotz and Avron 1989a).

Controlling predators

At high concentrations of NaCl similar to that present in the media of *Dunaliella* ponds, only a few micro-organisms have the ability to thrive including halotolerant and halophilic bacteria, a few ciliates, a few amoebae, *Artemia salina* and certain fungi (Post *et al.* 1983; Butinar *et al.* 2005). Of these, some amoeba and zooplankton ciliates are dangerous predators of *Dunaliella* mainly at temperatures higher than 38°C (Ben-Amotz and Avron 1989a). For example, one taxonomically nonidentified ciliate predator found in outdoor cultures of *Dunaliella* located in Spain and Portugal has been characterized by having a very high growth rate, a great grazing capacity and a very high ingestion rate for *Dunaliella* (Moreno-Garrido and Canavate 2001). Such a tremendous grazing capacity can results in serious reduction in the number of *Dunaliella* cells during a few days. Therefore, it is desirable to find chemical compounds with the capacity for killing ciliates but exhibiting a very low toxicity to the algae. Moreno-Garrido and Canavate (2001) found that unlike several compounds such as formaldehyde, ammonia, hydrogen peroxide or metronidazole, treatment of outdoor algal mass cultures with 10 mg l^{-1} quinine sulfate completely eliminated ciliates and at the same time allowed the algal cells to continue growing.

Culture systems

Mass culture of microalgae have been developed in various types of culture systems including large open ponds, circular ponds, raceway ponds, cascade ponds, large bags, tanks, heterotrophic fermenters and several kinds of closed photobioreactors (Borowitzka 1999; Pulz 2001). The intrinsic properties of the algae are one of the most important factors to be considered for the selection of a practical culture system. *Dunaliella*, like other two commercially used microalgae, *Clorella* and *Spirulina*, can grow well in a highly selective medium and hence can be easily cultivated at high concentrations

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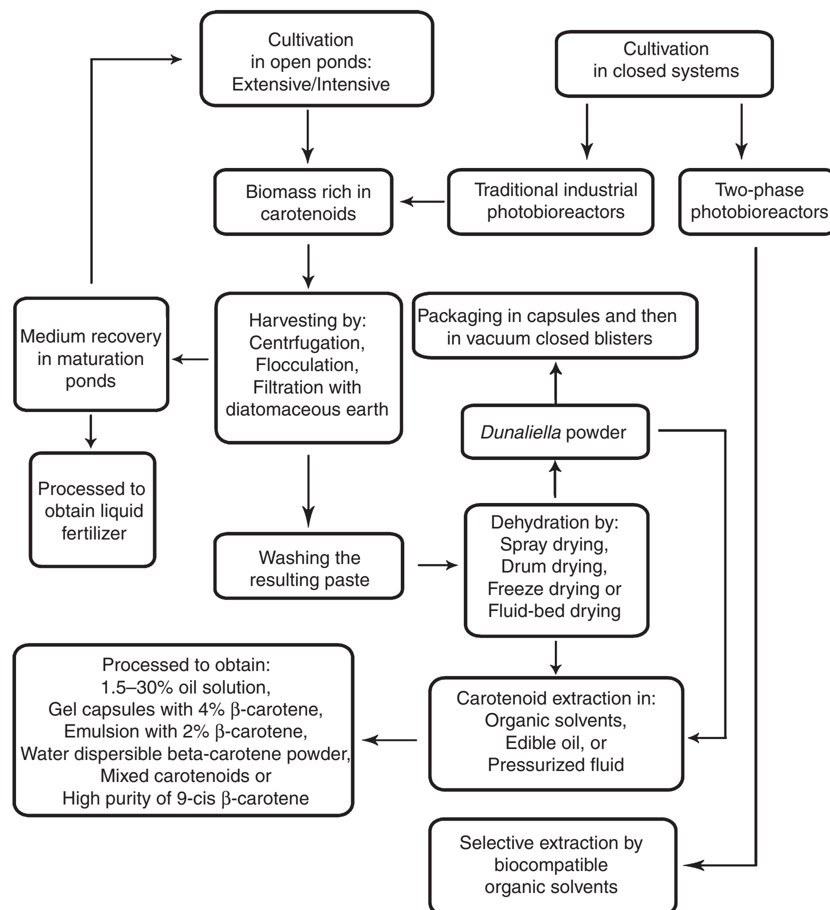

Figure 2 Flow chart of culturing and processing of *Dunaliella salina* in different culture systems.

of salt in open-air ponds (Borowitzka and Borowitzka 1988a). These cultures are predominated by *Dunaliella* and relatively free of contamination by protozoan predators of *Dunaliella* and/or other algae (Borowitzka and Borowitzka 1990). Superintensive modes of *Dunaliella* species culturing have been developed in various types of photobioreactors (Ben-Amotz 1993; Su *et al.* 2003). Compared with open ponds, such systems are much cleaner and yield a higher biomass and carotenoid concentration. The steps commonly followed for processing of *D. salina* in different culture systems are summarized in Fig. 2. Here, we consider different aspects of *Dunaliella* cultivation systems.

Open ponds

Nowadays, the most commercial and common systems for mass culturing of *Dunaliella* are open-air ponds (Borowitzka 1999). *Dunaliella* production plants are located in areas having a hot and dry climate with minimal cloudiness and commonly situated at, or near a suitable source of brine. These sites should also be remote from pollution that affect the quality or quantity of cell growth or of final products. There are two major types of open-air systems currently utilized for culturing of this alga: first, very large ponds (extensive mode) of up 250 ha which are unmixed other than by wind and/or convection and are located in Australia (Borowitzka and Borowitzka 1990). Betatene Ltd (now Henkel-Cognis) and Western Biotechnology Ltd were the first Australian companies to attempt the mass culture of *Dunaliella salina* for β-carotene production in 1986, using the extensive mode. Henkel-Cognis, the largest producer of *D. salina* in the world, constructed a very large algal pond in Whyalla, South Australia with a depth of approximately 20 cm by modifying a natural salt lake. Western Biotech also constructed the Hutt River pond that was only recently taken over by Cognis (Curtain 2000). Both sites are based on natural salt lakes that already had dense natural populations of *D. salina*. In a typical manner, the algae are grown at high salinities occurring in these natural saturated-salt lakes. To control salinity, seawater with lower salt concentration is pumped from seaward site of the lagoon into the lakes and then appropriate amounts of nutrients are added for optimal growth. After the ponds

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reach the appropriate β-carotene content, the culture is then transferred to the harvesting plant on the shore and the remaining medium is returned to the ponds (Borowitzka 1990).

Alternatively, smaller paddle wheel stirred raceway ponds have been used in several areas of the world (Ben-Amotz and Avron 1990; Ben-Amotz 1995). The ponds usually are constructed with brick, concrete, earth or fibreglass berms and with a surface area of approximately 1000–4000 m^2. Gaseous CO_2 is usually used for both providing the source of inorganic carbon and controlling the pH. Details of these systems and their technical considerations can be found elsewhere (Oswald 1988; Ben-Amotz and Avron 1989a). The outdoor culture may be operated either in batch or semi-continuous modes. The mixing of the cultures has several advantages. It can effectively prevent cell sedimentation and thermal stratification and aid nutrient and gas distribution and excess oxygen removal. Mixing the culture reduces photo-damage (photoinhibition) usually caused by exposing the algae to the high intensities and high concentrations of dissolved oxygen.

The parameters affecting the productivities of the algae in ponds such as mixing rate, depth of the culture, size of inoculate, etc. need to be optimized in any case study because the result can be variable from one experimental condition to another. For example, Ben-Amotz (1999) commented that a linear medium velocity of at least 15 cm s^{-1} in commercial *Dunaliella* production units is most efficient whereas, the highest productivities in 3m^2 ponds reported by Garcia-Gonzalez *et al.* (2003) occurred at a mixing rate of 55 cm s^{-1}. The depth of the culture is other important factor. It was claimed by Ben-Amotz and Avron (1989a) that there is no difference between the productivities of *D. bardawil* in small ponds in depths ranged from 5 to 30 cm because the light fully absorbed by the cells in less than 5 cm of medium. These results and those reported by Garcia-Gonzalez *et al.* (2003) are different. These later authors observed that the best results were obtained for 6 cm depth and in summer, when the carotenoid productivity and carotenoid content were maximal. Nevertheless, because of technical restriction in construction of *Dunaliella* ponds with uniformly smooth bottom, the operation at depth less than 10 cm is practically impossible and most commercial *Dunaliella* have a depth of between 10 and 20 cm (Ben-Amotz 1999).

Extensive culture of *Dunaliella* achieves a typical β-carotene concentration of 0·1–1·0 mg l^{-1} from a productivity of about 0·05–0·1 g *Dunaliella* per m^2 per day with maximal β-carotene productivity of 10 mg m^{-2} day^{-1}. In comparison, intensive cultures in raceway ponds yield 10–20 mg β-carotene per litre, 5–10 g *Dunaliella* per m^2 per day and a maximum productivity of β-carotene 400–750 mg m^{-2} day^{-1} (Ben-Amotz 1993, 1995). The selection of an extensive *vs* intensive mode of cultivation ultimately depends on the costs of land and water as well as the climatic condition of the area. For example, in Australia, land costs are low, free seawaters are available, solar irradiation is close to optimum level nearly all year round and thus the companies like Henkel-Cognis grow *Dunaliella* in world's largest algal farms with a total capacity of 800 ha. Although the productivity is lower in such systems compared with that in intensive mode, the process is economically more attractive for the company because it has an effective and cheap harvesting method to process high volumes of brine (Borowitzka 1990). Alternatively, other producers such as those in USA use extensive mode of cultivation to achieve higher cell densities because the costs of land and site preparation in such an area are significantly high (Borowitzka 1999).

Closed systems

Industrial photobioreactors are excellent systems for controlling multiple culture parameters and hence for culturing photosynthetic micro-organisms such as microalgae, cyanobacteria and plant cells. The microalgal biomass obtained by this way can be processed to produce valuable nutraceutical-type products such as polyunsaturated fatty acids and carotenoids, as well as specialty chemicals such as radio-labelled compounds or alternatively be used as feed in aquacultural industries. Generally, three basic designs of these closed systems developed for commercial cultivation of microalga are used: flat plate bioreactors, tubular photobioreactors and ultrathin immobilized configurations (Borowitzka 1996, 1999; Tredici and Zitelli 1997; Pulz 2001). All of these reactors attempt to distribute the amount of light and nutrients uniformly to each cell and to enhance gas exchange by optimal mixing of the medium and reducing the light path. Other important considerations are the control of the growth parameters. Therefore, process sensing is a necessity for optimizing photobioreactor productivity and hence in *Dunaliella* closed photobioreactors; pH, dissolved oxygen, temperature and light intensity are commonly monitored (Li *et al.* 2002; Su *et al.* 2003). Recently, Zhu and Jiang (2008) designed and optimized their helix tube photo bioreactor (PBR) with a volume of 10 l for indoor cultivation of *D. salina*.

Recently, production of carotenoids by *Dunaliella* in two-phase photobioreactors has been studied (Hejazi *et al.* 2002; Leon *et al.* 2003). In this new approach, carotenoids which are produced by the algae in aqueous phase continuously are extracted into the upper organic phase, composed of a biocompatible organic solvent with a log $P_{octanol}$ (an indicator of the hydrophobicity of organic

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solvents) value of >6. Hejazi *et al.* (2003) designed a system for recycling the organic phase through the aqueous phase to enhance β-carotene extraction. In a similar approach, Leon *et al.* (2005) also enhanced the production of phytoene and its selective extraction into the organic phase by combined use of carotenogenic pathway inhibitors and biphasic aqueous/organic systems. More recently, Mojaat *et al.* (2008a) reported that mixing of a biocompatible solvent (decane) with a toxic solvent like CH_2Cl_2 could improve β-carotene extraction by six times compared with using pure decane.

Immobilization of *D. bardawil* cells in Ca-alginate capsules followed by cultivation in the bubble column bioreactor has also been claimed to be an effective method for high-density cultivation (Joo *et al.* 2001).

Unfortunately, in spite of higher biomass concentration and better control of the culture parameters as well as all other beneficial aspects of closed systems, such as reduced contamination risk, lack of CO_2 losses, flexible technical design and reproducible cultivation condition, until recently data have indicated that volumetric productivity and cost of production in these systems are not better than those obtainable in open ponds (Lee 2001). Compared with open ponds, the design of photobioreactors is more complex, expensive and difficult to scale up. However, for future applications, open ponds for large-scale production seem to have a lower innovative potential than photobioreactors (Pulz 2001).

Downstream processes

Recycling the medium

Because the addition of salt and nutrient to the medium is a significant additional cost, it is desirable to reutilize the algal medium remains after biomass removal (Fig. 2). This medium contains ion concentrations higher than seawater as well as organic matter (mainly glycerol) resulting from cellular lysis and hence has a high turbidity. Ben-Amotz (1995) claimed that *Dunaliella* growth medium could be recycled biologically by treating the medium with bacteria that are naturally present in medium because of the high concentrations of glycerol, amino acids and other organic compounds. Later, Santos *et al.* (2001) described the optimization of conditions for biological removal of organic matter from *Dunaliella* wastewater at the laboratory level, using halophilic bacteria endemic in the hypersaline medium. They found that the combined addition of Mg^{2+}, K^{+}, PO_4^{3-} and NH_4^{+} to the wastewater enhanced the growth of these bacteria, increasing glycerol removal from the medium to the extent that glycerol was undetectable in cultures after two days incubation. This approach can be used for removing glycerol from *Dunaliella* wastewater to allow recycling of the medium. Additionally, halophilic bacteria contain bacterioruberin, a red pigment that has been attractive in biotechnology because of its antioxidant activities (Saito *et al.* 1997).

Harvesting

Isolating the cells from the brine medium at the end of the culturing process is one of the most critical and difficult steps in mass cultivation of *Dunaliella.* Cell harvesting needs special considerations for several reasons. First, *Dunaliella* lacks a rigid cell wall and is protected only by a plasma membrane with mucous materials; second, the salinity of the cultures is high; and third, *Dunaliella* cultures generally have low cell density. These factors, alongside the small cell size of *Dunaliella*, exclude many routine harvesting methods used frequently in microalgal biotechnology. For example, it has been shown that many different types of filtration methods through sand filters, cellulose fibres and other filter materials is unreliable mainly because the cells clog the filter pores rapidly by forming a layer of mucous material and prevent further filtration (Ben-Amotz and Avron 1989a). In addition, attempts to remove this limitation by repeated backwashing (Naghavi and Malone 1986) or pressure filtration (Mohn 1980) have also failed. The only successful filtration has been patented by Ruane (1977) who passed the *Dunaliella* cultures through diatomaceous earth and then used the filtrated algae directly for extraction of β-carotene with an organic solvent.

Promotion of the formation of microalgal aggregates followed by sedimentation or floatation using chemical flocculants like multivalent metal salts such as ferric chloride ($FeCl_3$), aluminum sulfate [$Al_2(SO_4)_3$, alum] and ferric sulfate [$Fe_2(SO_4)_3$], prepolymerized metal salts, polyelectrolytes and polymeric flocculants like Chitosan have given positive results (Grima *et al.* 2003), but in most cases the presence of the flocculating chemicals is not considered a safe impurity. Further process would be required to release the flocculant from the final product. Changing the pH of the algal broth by adding NaOH solution has been proven to be an effective method for flocculating *Dunaliella tertiolecta* (Horiuchi *et al.* 2003), but has yet not tested for *D. salina*.

Despite the need for a higher initial investment, and high ongoing costs for energy, labour and maintenance, because of the high volumes that must be treated and discharged, centrifugation using continuous-flow and automatic discharge has been claimed to be one of the most effective harvesting methods for recovering *D. salina* from mature cultures (Ben-Amotz and Avron 1989a).

Salinity-dependent hydrophobic binding of the *Dunaliella* cells (Curtain and Snook 1983) is the technique used by Betatene/Cognis at Whyalla and Western Australia. It

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is the only proven large-scale industrial procedure that is effective for harvesting *Dunaliella* for recovering β-carotene. In practice, the company harvests the cells from dilute culture by either flocculating them with a hydrophobic flocculant or by passing the culture through hydrophobic columns. The cells are absorbed by columns and the β-carotene is recovered by eluting with a solvent like hexane (Curtain 2000).

Other methods based on exploitation of salinity dependent buoyancy properties in stationary and moving gradients (Bloch *et al.* 1982), exploitation of the phototactic and gyrotactic responses of the algae (Kessler 1985), absorptive bubble separation (Guelcher and Kanel 1999) and migration of the cells to an added, less saline layer (Fernandes *et al.* 1997) have also been patented.

Drying

Algal paste remains after harvesting can be dehydrated or dried to extend the shelf-life of the biomass. Dehydration methods used for microalga include sun drying; drum drying, spray drying and freeze-drying. Sun drying of the samples of *Dunaliella* may results in rapid degradation of β-carotene. In contrast, drum drying, spray drying and freeze-drying give satisfactory results in terms of uniformity of the powder and stability of the carotenoid (Ben-Amotz and Avron 1989a). Freeze-drying is most suitable for laboratory scale purposes and, compared with spray drying, generally yields higher recovery of carotenoids. However, in large-scale commercial production of biomass, spray drying is the method of choice because it is less expensive than freeze-drying. As an interesting new approach, the entrapment of *Dunaliella* cells in calcium alginate beads followed by drying in a fluid-bed dryer has also been demonstrated (Leach *et al.* 1998). Finally, several kinds of *Dunaliella* meal products have been manufactured by processing of spray-dried algae (Tanaka 1990).

Extraction

Several methods have been patented for the extraction of carotenoids from algal paste or dry powder; including extraction in conventional organic solvents such as hexane, ethanol, chloroform and diethyl ether (Ruane 1977), direct extraction of the carotene from the algae in an edible oil (Nonomura 1987), separation of β-carotene isomers via CO_2 supercritical fluid extraction (Bonshtein *et al.* 2002), and selective extraction by biocompatible organic solvents in two-phase bioreactors (Hejazi *et al.* 2002). Other protocols for obtaining high purity of 9-*cis* β-carotene have also been provided (Suzuki *et al.* 2000). This valuable product may be used in some pharmaceutical purposes. Pressurized fluid extraction of carotenoids from microalgae such as *Haematococcus pluvialis* and *D. salina* in organic solvents has been examined as an alternative technology which has showed higher or equal extraction efficiencies as compared with traditional solvent extraction methods while maintaining the integrity of chemical components (Denery *et al.* 2004; Herrero *et al.* 2006b).

From the customer preference point of view, utilization of carotene/oil compositions is superior to organic solvents for extraction, because of the possibility of carry-over of minute amounts of petrochemical residues to the carotene final product.

Applications

Although the production of carotenoids is the major application for *Dunaliella*, a number of other applications

Table 1 Applications proposed for *Dunaliella* species by different authors

Species	Proposed application	References
Dunaliella spp.	Source of glycerol-liquid biofuel	Ben-Amotz and Avron (1980); Ginzburg and Ginzburg (1993); Grizeau and Navarro (1986); Tsukahara and Sawayama (2005)
	Removing of heavy metal, treating wastewater	Takimura *et al.* (1996); Thakur and Kumar (1999); Hirata *et al.* (2001); Tsuji *et al.* 2002
	Food and supplement	Kay (1991)
D. salina	Source of carotinoids, vitamins and antioxidants	Chidambara Murthy *et al.* (2005); Milko (1963)
	Source of PUFA(ω_3 polyunsaturated fatty acids)	Abd El-Baky *et al.* (2004)
	Biological reactors	Geng *et al.* (2003)
	Single cell protein (SCP), minerals	Supamattaya *et al.* (2005)
	Enzymes	Ben-Amotz and Avron (1990)
D. tertiolecta	SCP, minerals	Fabregas and Herrero (1985)
	Ecological indicators	Hall and Golding (1998)
	Source of bioactive compounds	Borowitzka(1995)
D. primolecta	Source of antibiotic substances	Chang *et al.* (1993)
D. viridis	Source of oxygenated carotenoids	Moulton and Burford (1990)

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have been proposed and practiced for other species of this genus. These are elaborated in the following (also see summary in Table 1).

β-carotene

It is well known that β-carotene is oxidized by liver enzymes to produce vitamin A, which is necessary for the proper function of vision and of the epithelial tissues. Hence, β-carotene sourced from *Dunaliella* is used in human and animal dietary supplements (Borowitzka and Borowitzka 1989; Pulz and Gross 2004). In addition, this carotenoid has been used in food, cosmetic and pharmaceutical products as colourant and/or antioxidant (Borowitzka 1995; Dufosse *et al.* 2005). Supplementation of animal feed by addition of this pigment enhanced the colour of the flesh of fish and the yolk of eggs and improved the health and fertility of grain-fed cattle (Borowitzka and Borowitzka 1988b).

Furthermore, β-carotene preparations have been shown to inhibit or prevent various types of tumours in humans and animals, including skin cancers such as melanoma (Comstock *et al.* 1991), epidermoid cancers of head and neck (Shklar *et al.* 1989), cancers of the gastrointestinal tract (Stich *et al.* 1988), carcinomas of secretary glands such as pancreas (Woutersen and Garderen-Hoetmer 1988), breast cancer (Basu *et al.* 1989) and other conditions (van Poppel 1993). β-carotene is also effective in controlling cholesterol levels and reducing the risk of cardiovascular diseases, such as myocardial infarction and angina pectoris as well as coronary heart disease (Riemersma *et al.* 1991; Tornwall *et al.* 2004). It seems that these therapeutic effects of β-carotene, like those of other carotenoids, may be related to its protective ability against potentially harmful free radicals and simulative effects on the immune system (Ben-Amotz 1996; Gotz *et al.* 1999).

The β-carotene can be obtained from natural sources like vegetables, fruits, microalgae and the fungus *Blakeslea* (Dufosse *et al.* 2005) or produced by total syntheses (Mayer and Isler 1971). In terms of isomers composition, synthetic β-carotene contains only the all-*trans* isomer (Fig. 1). In contrast, natural β-carotene is a mixture composed mainly of 9-*cis*, the balance being all-*trans* (Ben-Amotz and Shaish 1992). It is evident that the 9-*cis* isomer is a better antioxidant than the all-*trans* and that the higher the 9-*cis* concentration and 9-*cis* to all-*trans* ratio, the higher is antioxidant and anticancer activities (Chidambara Murthy *et al.* 2005; Hu *et al.* 2008). Although, natural β-carotene, like other carotenes, is present in many vegetables and fruits such as citrus, carrots and tomatoes; its concentration in these sources is relatively low. In comparison, a *Dunaliella* cell can accumulate thousands of times more β-carotene than a carrot cell (Klausner 1986). In addition, *Dunaliella* can have the greatest amounts of 9-*cis* isomer among all sources (about 50% of all isomers), depending on the culture conditions. In summary, *D. salina* is the best-known biological source of β-carotene (Ben-Amotz and Avron 1983; Borowitzka and Borowitzka 1988a).

In recent years, several studies have reported that the synthetic all-*trans* β-carotene not only fails to reduce the incidence of cancer, but it may be carcinogenic (Paolini *et al.* 1999; Kelloff 2000; Omenn 2000). These new findings, besides the fact that natural β-carotene has better physical properties (such as its solubility in fat) than the synthetic product, make *Dunaliella* β-carotene much more valuable and attractive for the consumers than before.

A wide range of β-carotene products are now available, including a 4% β-carotene solution used in dietary supplement industries as a fill for soft gelatin capsules, a 1·5–30% oil (e.g. soybean oil) solution used as food colorant (mainly for margarine) and dietary supplement, a 2% emulsion used in beverages, β-carotene-rich *Dunaliella* powder containing 2–5% β-carotene utilized as aquiculture feed for prawns and certain other species or packed in capsules or tablets and labelled as 'natural β-carotene', water dispersible β-carotene powder and mixed carotenoids (Ben-Amotz and Avron 1990; Ben-Amotz 1993).

Other carotenoids

Dunaliella contains a range of carotenoids with wider application. Several investigations have been made into the potential use of related *D. salina* strains as commercial sources of carotenoids such as phytoene, phytofluene, lutein and zeaxanthin (Fig. 1). The colourless carotenoid, phytoene, as one of the early precursors of all carotenoid pigments attracts interest from the cosmetic industry. The cancer-preventing and antioxidant activities of phytoene also have been demonstrated in mammalian cells (Nishino 1998). It has been shown that the treatment of *D. salina* with Norflurazon, a well-known bleaching herbicide, block β-carotene production by inhibiting phytoene desaturase, a thylakoid membrane-bound enzyme which catalyses the oxidation of phytoene (Ben-Amotz *et al.* 1987; Breitenbach *et al.* 2001). Recently, Leon *et al.* (2005) optimized the accumulation of phytoene in *D. bardawil* cultures by the combined use of Norflurazon and two-phase aqueous organic systems. They could obtain about 47 g of phytoene per litre of culture in the organic phase.

Lutein is also an important carotenoid that is a pigmentation factor in fish and poultry, and is used as colourant in the drug and cosmetic industries (Jin *et al.*

2003b). Furthermore, the roles of this carotenoid in decreasing the risk of chronic diseases like cataracts, age-related macular degradation and arteriosclerosis (Krinsky *et al.* 2003) and preventing cancer (Ziegler *et al.* 1996) have been shown. Recently, Garcia-Gonzalez *et al.* (2005) employed the tubular photobioreactor for production of *D. salina* biomass rich in 9-*cis* β-carotene and lutein, but the lutein levels were lower than those for the chlorophycean microalga *Muriellopsis* reported by Del Campo *et al.* 2001.

Zeaxanthin and its stereoisomer lutein (Fig. 1) act as neutraceuticals against macular degradation in the eye (Jin *et al.* 2003b). This compound, like other xanthophylls, is also a potent antioxidant and a valuable bioproduct, but unfortunately is not commercially available via chemical or biological synthesis. Several attempts have been made to produce zeaxanthin from noncarotenogenic bacteria and the cyanobacterium *Synechocystis* (Albrecht *et al.* 1999; Lagarde *et al.* 2000). Microalgae offer an alternative commercial source for zeaxanthin with the advantages of fast and inexpensive growth and the possibility of simultaneous production of other valuable compounds. Recently, a zeaxanthin over-accumulating mutant of *D. salina* (*zea1*) was isolated and recommended for commercial application in biotechnology (Jin *et al.* 2003a). The mutant is impaired in the zeaxanthin epoxidation reaction and can accumulate 15 times more zeaxanthin than that the wild type under low light conditions. The amount of zeaxanthin produced by the *zea1* mutant (6 mg g^{-1} DW) is three times more than that in engineered *Escherichia coli* strains, but 2·5 less than that produced using the bacterium *Synecocystis* sp. (Jin *et al.* 2003b). *Dunaliella viridis* has also been proposed as a source of oxygenated carotenoids (Moulton and Burford 1990).

Production of dried algal meal

The dried algal meal, which remains after removal of glycerol and β-carotene from *Dunaliella*, contains about 40% proteins (Ben-Amotz and Avron 1990). The amino acid composition of *Dunaliella* protein is similar to that of plants like soybean (with low cysteine and methionine and high lysine) but in addition, to some extent low in isoleucine and tryptophan (Mokady 1992). The relatively good protein and fatty acid quality of *Dunaliella* meal, besides the lack of the indigestible cell wall and the presence of high levels of β-carotene (vitamin A) as well as its generally recognized as safe status make it as an excellent poultry and aquaculture feed or food. To this end, the use of *Dunaliella* protein as a supplement in bread has been attempted (Finney. *et al.* 1984). Fabregas and Herrero (1985) also suggested *D. tertiolecta* as a source for single cell protein (SCP). In comparison with other sources of SCP such as yeasts and bacteria, the concentration of nucleic acids (DNA and RNA) in *Dunaliella* meal are less (Mokady 1992), which is desirable because they can have toxic effects. Recently, it was also shown that a combined treatment with the drug fibrate plus β-carotene-rich powder from *D. bardawil* could improve the drug's effect on HDL-cholesterol levels in patients (Shaish *et al.* 2006). On the other side, as *Dunaliella* species contain about 0·2% xanthophylls (mostly lutein), meal from this alga can be used for enhancing the colour of egg yolks (Ben-Amotz *et al.* 1986; Moulton and Burford 1990) and the flesh and shell of aquatic animals, such as prawns and ornamental fishes, to make them more attractive in the market (Pulz and Gross 2004). Shrimp can convert β-carotene to astaxanthin, the most effective substance for pigmentation in shrimp compared with β-carotene and canthaxanthin (Boonyaratpalin *et al.* 2001). Shrimp feed containing *Dunaliella* extract showed higher weight gain and survival compared with the control (Supamattaya *et al.* 2005). Cattle feed rich in *Dunaliella* meal also had better health and fertility attributed to the extra β-carotene (Borowitzka and Borowitzka 1989). Furthermore, the results obtained by Abd El-Baky *et al.* (2004) suggested that *D. salina* cells contain high concentrations of total lipid rich in ω_3 polyunsaturated fatty acids (PUFA) and antioxidants in the unsaponifiable lipid fraction, and hence can be used as a supplement or complete food to enhance the performance and state of the human body or improve a specific bodily function.

Production of glycerol

Glycerol is an important commercial organic chemical and osmoregulator, which have been used in the cosmetic, pharmaceutical and food industries. It is also utilized as an antidrying medium (Craigie and McLachlan 1964). Under appropriate condition (mostly high salinity), *Dunaliella* can accumulate glycerol up to 50% of its dry weight (Ben-Amotz and Avron 1980, 1990). In addition to open ponds, another system for glycerol production employed immobilized *D. tertiolecta* cells within calcium alginate beads, cultured in a bioreactor (Grizeau and Navarro 1986). At present time, glycerol is mostly produced from petrochemical sources and its price is directly proportional to the price of crude oil.

Expression of foreign proteins and analyzing gene function

Conventionally, the production of foreign proteins such as vaccines, antibiotics and human growth hormones as well as various enzymes (to obtaining some metabolites)

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has been practiced using bacteria such as *E.coli*, yeast and cultured plant, insect and mammalian cells. Recently, the advantages of *Dunaliella* as a candidate for synthesizing of novel high-value compounds have become apparent. First, this alga can be easily cultivated in a simple medium of high salinity, which provides a substantial degree of selectivity against most contaminating organisms and thus the cost of the final product is significantly lowered. Second, because of the lack of a rigid cell wall, the introduction of vectors into the cells and the purification of the expressed proteins can be easily accomplished for determination of the transformation efficiency.

Until recently, most eukaryotic algae were refractory to any type of genetic manipulation, mainly because of the unavailability of the correct promoters necessary for the efficient expression of foreign genes. Therefore, there was no reliable approach for genetic transformation and metabolic engineering of *Dunaliella*. Geng *et al.* (2003) introduced the first report of expression of a foreign gene in *D. salina*. They observed the stable transformation of this alga with hepatitis B surface antigen under the control of the ubiquitin-Ω promoter by electroporation. Walker *et al.* (2005) also reported both transient and stable transformation of *D. tertiolecta* by electroporation with the *ble* antibiotic resistance gene under the control of the native *RbcS1* promoter and terminator regions. The transformation efficiency of this later work was low and the foreign DNA was quickly degraded. In another report, Sun *et al.* (2005) transformed *D. salina* by an electroporation procedure to obtain individual clones that retained resistance to 10 mg l^{-1} Zeocin for at least 6 months, while about 90% of the cells lost their resistance in the presence of 5 mg l^{-1} Zeocin during subculturing. More recently, a successful novel transformation of *D. salina* using glass beads has been reported (Feng *et al.* 2007).

In spite of their benefits, using constitutive strong promoters sometimes triggers gene silencing, which in turn affects cell growth (Geng *et al.* 2003; Jiang *et al.* 2005; Li *et al.* 2007). To overcome this problem, Li *et al.* (2008) reported that the upstream region of the nitrate reductase gene may be a useful promoter for inducible expression of foreign genes in *D. salina*. This expression system can be easily induced by nitrate and repressed by ammonium. They developed a vector, which was able to switch on or off the expression of heterologous genes and proteins in transformants.

Another major problem in genetic manipulation of higher eukaryotes is that expression levels of the introduced genes are low, mainly because of the effect of chromatin structure or regulatory elements locating at the near of transgenes randomly integrated on chromosomes, a phenomenon called ‘ position effects’ (Peach and Velten 1991). To overcome this problem, one approach has been developed in which new chromatin elements are used to neutralize the negative effects of elements causing low expression level of transgene (Kim *et al.* 2004). Recently, to increase expression level of transgenes in *Dunaliella*, Wang *et al.* (2007a) isolated a DNA fragment named DSM 2 from *D. salina*. They finally reported that this sequence could improve the expression level of chloramphenicol acetyltransferase transgene by more than 4·5 fold in this alga. It is clear that further investigations are required to develop methods to improve the frequency and stability of transformation and to find new promoters and regulatory sequences to promote the expression of heterologous genes in *Dunaliella*.

Molecular biology is being used to reveal the roles of the endogenous genes in *Dunaliella* and to analyse the tolerance of abiotic stresses at the molecular level. *Dunaliella salina* is a useful model to study the mechanisms of photoadaption and light-harvesting under stress conditions. Because the liquid culture medium of this alga can be easily manipulated, the micro-environmental parameters can be simply tested. As an example, the carotene biosynthesis-related (*Cbr*) gene which encodes one of the light stress proteins, is cloned from *D. bardawil* (Lers *et al.* 1991) and *D. salina* (Levy *et al.* 1992). Recently, using realtime PCR, Chen *et al.* (2007a) showed that the mRNA levels of this gene increased rapidly after exposure to high light intensity and salt concentration. Their results demonstrated that the CBRs have some roles in adaptive responses to photodamage results from photoinhibition. Wei *et al.* (2007) studied the roles of light harvesting complex II (LHCII) protein at the molecular level under extreme conditions. They isolated an additional *LhcII* cDNA sequence from *D. salina* and amplified the corresponding gene named *DsLhcII-3*. They found that when *D. salina* was shifted from low light to high light or from low light to dark, a rapid reduction in mRNA level of *DsLhcII-3* occurred within 2 and 4 h, respectively. These data suggested that the control mechanisms acting in the dark and in the light may be different. In another approach, Park *et al.* (2006) showed that about 1% of the ESTs obtained from the cDNA library of *D. salina* created from irradiance-stress could be assigned to the genes encoding proteins that are known to be increased in response to high light intensities. These were included in antioxidant genes, such as *Fe-SOD* and *APX*, *Cbr* mRNA, a LHC protein homologue and the ATP-dependent *Clp* protease gene.

The salinity tolerance of *Dunaliella*, its simple cellular structure and easy cultivation make it an ideal organism to study environmental stress resistance mechanisms at the molecular level. For this purpose, further development of functional genomics methodology applicable to this alga is required to explore gene functions. In recent work, the potential of double-stranded interfering RNA (RNAi)

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in *D. salina* was evaluated (Sun *et al.* 2008). Transient silencing was achieved of phytoene desaturase gene (*pds*), a key gene in carotenoid biosynthesis. This initial work using RNAi technology could be further developed for analyzing any gene of interest in microalgae. Recent research is providing a better understanding about the regulation of carotenogenesis in *Dunaliella* at the molecular level and ways to enhance carotenoid accumulation in the alga. Some reports showed that under the condition of high light intensity (Rabbani *et al.* 1998) and low nitrogen (Sanchez-Estudillo *et al.* 2006), the steady-state mRNA levels of Phytoene desaturase (*Pds*) and levels of Phytoene synthase (Psy) and Pds proteins in *Dunaliella* cells was stable. In contrast, Coesel *et al.* (2008) recently showed that the steady-state levels of these transcripts increased when *D. salina* was shifted to high light or high salt under nutrient-deficient regimes, whereas such response was inhibited under nutrient rich conditions. The same research group in another publication showed that the levels of steady-state lycopene β-cyclase (*Lcy-*β) transcript and of carotenoids accumulation in *D. salina* were up-regulated in all stress conditions tested including salt, light and nutrient depletion, and on this basis they suggest again that nutrient availability is an important factor affecting on carotenogenesis and expression of carotenoid-related genes (Ramos *et al.* 2008).

Recently, the first mitogen-activated protein kinase (*MAPK*) gene, important in stress-signalling transduction, was cloned from *D. salina* by Lei *et al.* (2008). The expression level of this gene decreased when the alga was exposed to hyperosmotic medium and low temperature. The role of this component at the cellular level needs to be further studied. In another study, Wang *et al.* (2007b) obtained a gene encoding the 14-3-3 protein from *D. salina* for the first time. This cell cycle-related protein shared homology with that of other eukaryotes. A better understanding of the regulation of cell division in *Dunaliella* could provide new ways to accelerate growth in culture.

Several important genes from *Dunaliella* have recently been cloned and characterized. Examples include DNA photolyase, which is involved in repairing UV-induced DNA damage (Yi *et al.* 2006), ω3 fatty acid desaturase (Lyukevich *et al.* 2003), a nitrate transporter gene named *DsNRT2·1* (He *et al.* 2004), a 5-enolpyruvylshikimate-3-phosphate synthase (Yi *et al.* 2007), plastidic glycerol 3-phosphate dehydrogenase (He *et al.* 2007) and a sodium-dependent phosphate transporter gene, *DvSPT1*, from *D. viridis* (Li *et al.* 2006).

Wastewater treatment

Typically, there are three levels of wastewater treatment: primary, secondary and tertiary. At the primary level, materials like grit, debris and large particles are removed using filtration or other conventional methods. Secondary treatment usually involves the breakdown of organic matter in a biological treatment utilizing various microbes. Algae may contribute to the secondary process in a symbiotic relationship where the algae consume the inorganic nitrogen, phosphorous and CO_2 products of organic matter decomposition by microbes. In turn, the oxygen, generated by the algae via photosynthesis, supports the microbes. Further removal of inorganics such as heavy metals and nutrients may be accomplished at the tertiary level by biological or chemical treatments. Among the various biological tertiary treatments, exploitation of microalgae has been attractive because of their high capacity to take up inorganic matter (Talbot and de la Noue 1993). Microalgae can be easily mass cultivated outdoor in high-rate algal ponds, where effective oxidation ponds and an algal reactor are combined (Araki *et al.* 2001). Alternatively, algae can be used in immobilized microbe bioreactors. In addition to absorbing some heavy metals, these systems are capable of bio-transforming and bio-depredating petroleum aliphatics and aromatics, chlorinated halocarbons and pesticides (Portier and Miller 1991). Topics focused on the use of immobilized algae for treating wastewater and removal of organics, heavy metals and other pollutants are reviewed elsewhere (Mallick 2002; Moreno-Garrido 2008).

Free and immobilized *Dunaliella*, like other microalgae, have potential for use in wastewater management programmes, because of their ability to remove, accumulate compounds such as NH_4^+ and PO_4^{3-} and heavy metals like copper and arsenic from the wastewater and effluents (Takimura *et al.* 1996; Yamaoka *et al.* 1999). It has been shown that *D. tertiolecta* contains the heavy-metal binding peptides, phytochelatins, and can be used in bioremediation for removing heavy metals from the environment (Tsuji *et al.* 2002). *Dunaliella tertiolecta* is also proposed as an ideal candidate for tertiary treatment of saline wastewaters with a high level of nitrogen-ammonia (Belle 2007). The same alga could take up inorganic arsenic from the marine environment and metabolize it to simple methylated species and arsenoriboses (Foster *et al.* 2008). Thakur and Kumar (1999) demonstrated a high N and P removal efficiency of the *D. salina* immobilized in Ca-alginate beads. Alternatively, dead or nongrowing living algal biomass can be exposed to the waste as an absorbent agent for metal adsorption or absorption. This later method could be selective, efficient and easy to operate as well as cost-effective for treating a large amount of wastewater. By this method, absorption and removal of heavy metals like chromium(VI) ions from saline waters have found to be considerable (Donmez and Aksu 2002).

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Bioindicators

Algae like *D. tertiolecta* are attractive as bioindicators for the evaluation of ecotoxicity of anthropogenic compounds on environments. The integration of such bioassays into environmental monitoring for effluents and wastes can finally lead to pollution prevention and environment protection. Compared with other test organisms like animals, algae have greater sensitivity to various effluents from municipalities and industries (Lewis *et al.* 1998). The ability to grow in severe conditions and lack of a rigid cell wall (eliminating a potential barrier to the permeation of substances to be assayed) make *Dunaliella* one the best options for use in testing the environmental toxicity among the microalgae. For instance, *D. tertiolecta* was selected as an environmental biosensor and indicator, meeting most of the criteria for a bioassay organism (Reish and Lemay 1988). It was also proposed as a standard organism for testing seawater toxicity (APHA, AWWA, WEF 1998). Sacan and Balcioglu (2006) studied the responses of this alga to effluent from an aluminium planting plant and to the seawater from a pharmaceutical plant. The toxicity of Irgarol alone and in binary mixtures with other pesticides like the fungicide chlorothalonil, and the herbicides atrazine and 2, 4-D has also been examined (DeLorenzo and Serrano 2006). The authors noted that overlap of certain pesticide applications in the coastal zone may increase the toxicological risk to resident phytoplankton populations. In a more recent study, Santin-Montanya *et al.* (2007) tested seven microalgae genera to develop an assay for herbicides in marine environments. Their results showed that *D. primolecta* had the best potential among the other microalgae in growing in a defined and optimized medium and in using as a biodetector of four representative herbicides including alloxydim, sethoxydim, metamitron and clopyralid. In another study, the toxicity of typical mutagenic phenols assayed using *D. salina* (Chen *et al.* 2007b).

Production of bioactive compounds and biofuels

In addition to those mentioned before, *Dunaliella* species produce several other bioactive compounds like valuable enzymes, vitamins and pharmaceuticals. For example, one unique enzyme (dihydroxy acetone reductase) has been obtained from *D. salina* and commercially sold (Ben-Amotz and Avron 1990). Several promising pharmaceutical activities have been reported for *Dunaliella*. For example, it is demonstrated that *D. tertiolecta* had antihypertensive, bronchodilator, antiseritonin, polysynoptic block, analgesic, muscle relaxant and antioedema activities (Villar *et al.* 1992; Borowitzka 1995). Chang *et al.* (1993) also indicated that algal cells of *D. primolecta* contained a number of different antibiotic substances. They found that a crude extract from this alga strongly inhibited the growth of *Staphylococcus aureus*, *Bacillus cereus*, *Bacillus substilis* and *Enterobacter acrogenes*. In another study with the petroleum ether, hexane, ethanolic extracts of *D. salina* also showed good antimicrobial activity against several micro-organisms of importance for the food industry including *Escherichia coli*, *Staphylococcus aureus*, *Candida albicans* and *Aspergillus niger* (Herrero *et al.* 2006a). Their results from gas chromatography–mass spectrometry showed 15 different volatile compounds and several fatty acids including palmitic, alpha-linolenic and oleic acids that could have been responsible for the antimicrobial activity.

In recent years, an ingredient from *D. salina* with strong ability to stimulate cell proliferation and turnover and to improve the energy metabolism of skin has been launched by Pentapharm (Basel, Switzerland) (Stolz and Obermayer 2005).

Dunaliella has also been used in biotransformation process to produce a specific set of metabolites. In one study, Ca-alginate immobilized *D. parva* used by Hatanaka *et al.* (1999) to produce (R) 1,2-propanediol from hydroxyacetone. Propanediol is a valuable and expensive glycol.

Recently, microalgae have been considered for producing biofuels, especially biodiesel (Huntley and Redalje 2007). Properties like rapid growth and high accumulation of oils (exceeding 80% by weight of dry biomass) make microalgae an attractive potential source for biodiesel, a replacement for fossil diesel (Chisti 2007). Some species of *Dunaliella*, like *D. tertiolecta*, may share this potential, with cells containing about 37% oils. *Dunaliella tertiolecta* is a fast-growing species which means it has a high rate of absorption of CO_2. The pyrolysis characteristics of *D. tertiolecta* were studied by thermogravimetric analysis (Zou *et al.* 2007). In addition, Park *et al.* (1998) proved that the hydrocarbon productivity of *D. salina* 1650 was similar to that from *Botryococcus braunii*, which was known to economically produce liquid fuels. The effect of salt concentration on lipid and triacylglyceride contents of *Dunaliella* cells was also tested (Takagi *et al.* 2006). It was found from this study that addition of 0·5 or 1·0 mol l^{-1} NaCl at mid-log phase or the end of log phase during *Dunaliella* cultivation with initial NaCl concentration of 1·0 mol l^{-1} further increased the lipid content of the cell up to 70%.

Conclusion

New findings concerning the carcinogenic effects of synthetic β-carotene and particularly the beneficial properties of *Dunaliella* natural β-carotene for improving human body function will promote demand for the natural

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product. This will lead to further development by the traditional commercial manufacturers and is likely to attract new producers into the mass culture of *Dunaliella* for β-carotene production and other biotechnological purposes. New pilot plants are under development in India, Chile, Mexico, Cuba, Iran, Taiwan, Kuwait and Japan. The ability to induce, modify and scale up *Dunaliella* to produce a series of uncommon carotenoids of high nutritional and medical value, like phytoene and phytofluene, also opens a new field in the area of *Dunaliella* biotechnology. The cultivation of *Dunaliella* in photobioreactors in diverse autotrophic, heterotrophic and mixotrophic culture modes as well as in two-phase systems are other promising approaches requiring further development to make them more economically competitive in future.

Exploitation of reliable approaches for genetic transformation and metabolic engineering of *Dunaliella* combined with its use as a biological source for mass-producing high-value proteins such as vaccines, antibiotics and enzymes, seriously under consideration by several research groups, could open an interesting new facet of microalgal biotechnology in future. Finally, it seems that the enormous potentialities of different species of this fantastic alga for exploitation in various biotechnological areas such as wastewater management programmes, designing of biosensors, production of new antibiotic substances and production of biofuels will make *Dunaliella* a main topic for many future microalgal investigations.

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