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## Microalgal Biotechnology and Bioenergy in *Dunaliella*

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### 1. Introduction

*Dunaliella* is a halotolerant green alga that now belongs to the phylum Chlorophyta and family Polyblepharidaceae (Avron and Ben-Amotz 1992; Garcia et al., 2007). It lacks a rigid cell wall nevertheless it can grow in aquatic environments varied salinities from 0.5 to 5.0 M NaCl (Shariati & Hadi, 2000, Phadwal & Singh, 2003; Jahnke & White, 2003). Under stress conditions, *Dunaliella* species can accumulate significant amounts of valuable chemical matters such as carotenoids (Hosseini Tafreshi & Shariati, 2006; Hadi et al., 2008), glycerol (Hadi et al., 2008), vitamins and proteins (Ghoshal et al., 2002). 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 (Raja et al., 2007). In fact, the accumulation of glycerol in this alga is regulated by external water activity rather than the specific solute effect (Shariati & Lilley, 1994). In this condition, glycerol acts as a 'compatible solute' that protects enzymes against both inactivation and inhibition (Telfer, 2002). 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 (Shariati & Lilley, 1994). Therefore recently, algal biotechnology has made major advances, and microalgae like *Dunaliella* sp. are cultivated for the production of carotenoids and glycerol (Hosseini Tafreshi & Shariati, 2009; Spolaore et al., 2006). The potential ability of carotenoids to act as antioxidants and immunomodulatory agents has led to more active research investigating their application in the prevention of human cancers (Chidambara Murthy et al. 2005). Also, this alga accumulates large amounts of  $\beta$ -carotene (up to 14% of dry weight) in conditions such as high light intensity (Coesel et al., 2008), increased temperature (Ben-Amotz, 1996; Gomez & Gonzalez, 2005), high salinity (Hadi et al., 2008) and nutrient deficiency (Marin et al., 1998) such as sulfate deficiency (Aghaii & Shariati, 2007) and nitrate deficiency (Shariati & Zoofan, 2003). *Dunaliella*  $\beta$ -carotene is used in the food (Dufosse et al., 2005), cosmetic, and pharmaceutical industries as a colorant, antioxidant (Chidambara Murthy et al., 2005), anti-tumor agent, and heart disease preventive (Tornwall et al., 2004), in addition to its characteristic as precursor of vitamin A. Among the parameters that can significantly influence the production of biomass and  $\beta$ -carotene in open ponds is the selection of the area (Hosseini Tafreshi & Shariati, 2006). *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. The climatic conditions in most of regions of world make it into one of the most suitable areas not only for mass culture of *Dunaliella* but also for other algae (Hosseini Tafreshi & Shariati, 2009). On the other hand, microalgae biofuel is necessary for economic sustainability, because the continued use of fossil fuels is not sustainable and resources are finite (Tsukahara & Sawayama, 2005). Also, the oil productivity of many microalgae exceeds the best producing oil crops (Chisti, 2007). In particular, some species have been identified as promising producers of useful lipids for biofuels production with cells containing about 37% oils. *Dunaliella salina* is one such species, which shows lipid accumulation in response to high environmental salinities with content of the cell up to 70% (Takagi et al., 2006). Therefore, it seems that mass culture of *Dunaliella* at a commercial level in world as pilot ponds can be suitable to promote economical productions from this microalga for bioenergy via direct conversion of algal biomass to liquid fuel (Tsukahara & Sawayama, 2005).

## 2. Biology, morphology and taxonomy of *Dunaliella*

At first in 18 centuries, scientists thought that *Dunaliella* is same *Haematococcus*, but after in the first of 19 centuries, they reported that this genus clearly differed from *Haematococcus* and erected the new generic name *Dunaliella* (Avron and Ben-Amotz 1992). So far, twenty-eight species of *Dunaliella* are recognized (fig.1). They comprise five species (shapes of 1-5 in fig.1), all of which occur in freshwater and appear to be very rare whereas 23 species of these (shapes of 6-28 in fig.1) occur in saline environments (Avron & Ben-Amotz, 1992). The cell shape in species of *Dunaliella* varies from ellipsoid, ovoid, cylindrical and pyriform to almost spherical (Borowitzka & Siva, 2007). Cells of a given species may change shape with changing conditions, often becoming spherical under unfavorable conditions (Ben-Amotz et al., 2009). Cell size may also vary to some degree with growth conditions and light intensity (Coesel et al., 2008). The general cell organization has been studied in most detail in *D. salina*, both with the light microscope and the electron microscope. In the following survey, reference is given primarily to *D. salina*, and other species are only mentioned when major differences are evident. A rigid wall is lacking, but there is a distinctive mucilaginous cell coat. The cell coat can be visualized in the light microscope with Indian ink, whereas in thin sections, it is seen as irregular electron-dense material covering the plasmalemma (Avron & Ben-Amotz, 1992). It consists of 25 to 200 nm long fibrils and appears to be largely glycoproteic in nature. The two flagella are apically inserted, equal in length, and usually exhibit a homodynamic pattern of beating (Vismara et al., 2004). The two basal bodies are displaced against each other and carry microtubular flagellar roots. The single chloroplast occupies most of the cell body. It is cup-, dish-, or bell-shaped and has a thickened basal portion containing a pyrenoid. Anteriorly, the chloroplast is sometimes incised into several lobes. The thylakoids of the chloroplast are sometimes arranged in dense stacks of up to 10 units. Stacking of thylakoids was found to be particularly pronounced in cells grown at high light intensity and high salt concentration. Starch grains usually surround the pyrenoid, but may also be found at other places of the chloroplast. In some species (*D. salina*, *D. parva*), the chloroplast may also accumulate large quantities of  $\beta$ -carotene within oily globules in the interthylakoid spaces, so that the cells appear orange-red rather than green (Avron & Ben-Amotz, 1992). The  $\beta$ -carotene globules of *D. salina* were found to be composed of practically only neutral lipids, more than half of which were  $\beta$ -carotene. Most of the reddish forms may lose their red color when grown at low light intensities (Sarmad et al., 2006).

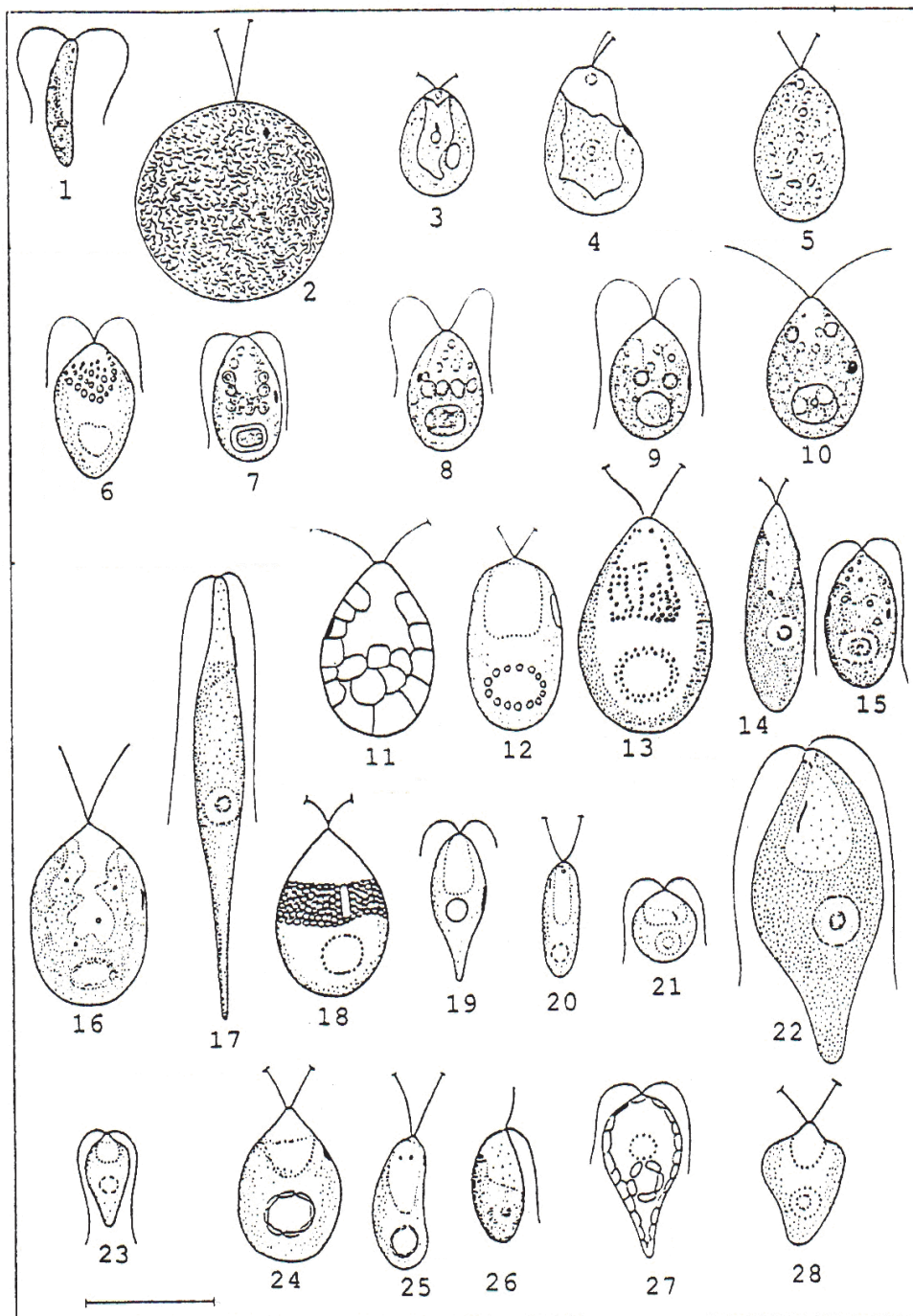


Fig. 1. 1. *D. acidophila*; 2. *D. flagellata*; 3. *D. lateralis*; 4. *D. obliqua*; 5. *D. paupera*, 6. *D. maritima*, 7. *D. polymorpha*, 8. *D. primolecta*, 9. *D. quartolecta*, 10. *D. tertiolecta*, 11. *D. parva*, 12. *D. pseudosalina*, 13. *D. salina*, 14. *D. baas-beckingii*, 15. *D. bioculata*, 16. *D. carpatica*, 17. *D. gracilis*. 18. *D. granulata*, 19. *D. media*, 20. *D. minuta*, 21. *D. minutissima*, 22. *D. ruineniana*, 23. *D. terricola*, 24. *D. viridis*, 25. *D. asymmetrica*, 26. *D. jacobae*, 27. *D. peircei*, 28. *D. turcomanica*. Scale bar: 10  $\mu\text{m}$  (Avron & Ben-Amotz, 1992).

The eyespot (stigma) has an anterior peripheral location in the chloroplast. In some species (especially in *D. salina*), the eyespot may be hardly visible in the light microscope (Avron & Ben-Amotz, 1992). The nucleus is generally obscured in life by a number of granules. It



occupies most of the anterior part of the cell and is often surrounded by anterior lobes of the chloroplast. Ultrastructural studies show that it has a porous envelope and a single prominent nucleolus, which is often surrounded by clumped heterochromatin. Mitochondrial profiles can be seen in various parts of the cell in thin sections. It is showed that the number and size of mitochondria may vary among cells at different stages of growth. Dictyosomes (Golgi bodies) occur in numbers of 2 to 4, each consisting of 10 to 15 cisternae. The endoplasmic reticulum (ER) typically underlies the plasmalemma over most parts of the cell. During hyperosmotic stress periods, there may be marked increases in ER. It appears that ER serves as a temporary reservoir for membrane material in temporary excess during stress periods when major cellular compartments shrink. Vacuoles of different types occur in *Dunaliella*. Vacuoles containing portions of membrane and vesicles as well as granular or thread-like material are often prominent ultrastructural constituents of the cytoplasm in *Dunaliella*. Large lipid globules or vacuoles containing smaller lipid globules similar to those of the chloroplast may also occur at various places in the cell. Asexual cysts may be formed under extreme conditions such as drastic dilution of the medium or drying up of the environment. Sexual reproduction is by isogamy, with gametic fusion proceeding in a manner similar to that in *Chlamydomonas*. The gametes have the same size and the same structural features as growing cells of the same species. Several species of *Dunaliella* appear to be homothallic, whereas *D. salina* has been reported to be heterothallic. The zygote is green or red and is surrounded by a thick, smooth wall. After a resting stage, the zygote nucleus divides forming up to 32 cells, which are liberated through a rupture in the mother cell wall. Meiosis takes place during the germination of the zygote. Cell morphology may be influenced to some degree by environmental growth conditions. Conspicuous changes may occur between logarithmic and stationary growth phases. It has also been clearly shown that salt concentration, light intensity, and temperature may have some effects on, e.g., thylakoid structure, appearance of pyrenoid, and proliferation of the endoplasmic reticulum. Originally, *Dunaliella* and other wall-less green flagellates were all classified in the Polyblepharidaceae within the Volvocales. Subsequently, detailed studies on many of these green flagellates led to the reclassification of certain genera in other taxa. It is placed *Dunaliella* in a separate order of the Chlorophyceae (*Dunaliellales*) (Borowitzka & Siva, 2007). Except for the lack of a rigid wall, *Dunaliella* also has many characteristics in common with members of the Chlamydomonadales (Avron & Ben-Amotz, 1992).

### 3. Osmoregulation and glycerol production

The genus *Dunaliella* contains several species which stand out as being the only eukaryotic and photosynthetic organisms which are able to grow in media containing an extremely wide range of salt concentrations, from 0.05 to 5.5 M NaCl (Avron & Ben-Amotz, 1992), but, optimal growth is different in various species and in order to Hadi et al., (2008) reported that optimal growth of *D. salina* (Iranian strain) was obtained at 2 M NaCl (Fig. 2 and Fig. 6 partition of D). *Dunaliella* was already recognized as a major constituent of saline lakes. It has been shown to be present in all natural hypersaline environments. In contrast to other green algae, cells of the genus *Dunaliella* do not contain a rigid cell wall (Avron & Ben-Amotz, 1992) but have a thin elastic plasma membrane that responds rapidly to changes in osmotic pressure by changes in cell volume (Shariati & Lilley, 1994). Therefore, *Dunaliella* can physically withstand three- to fourfold increases or decreases in osmotic pressure, shrinking or swelling in response, respectively (that know as osmoregulation). However,

much larger changes will lead to cell-bursting during a hypoosmotic stress, and to an irreversible shrinkage under hyperosmotic stress (Avron & Ben-Amotz, 1992).

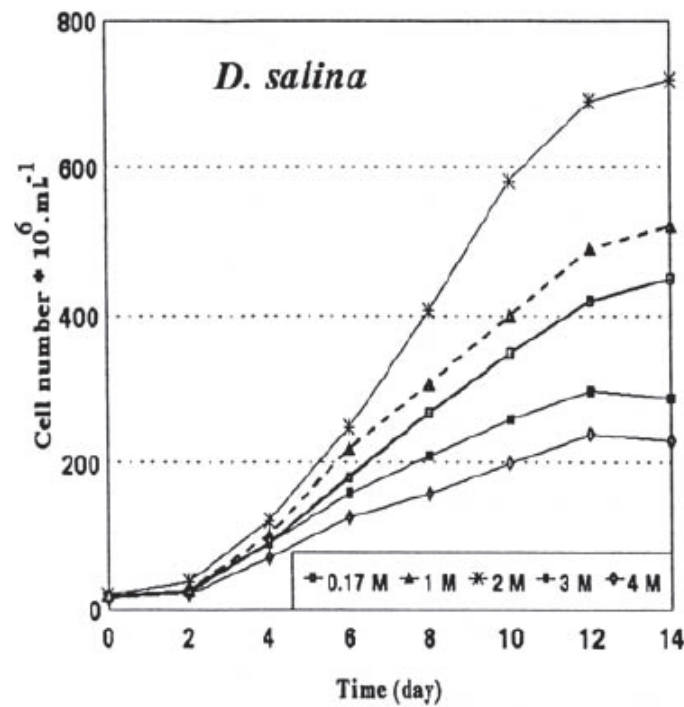


Fig. 2. Growth of *Dunaliella salina* in media containing different NaCl concentrations. The algae can grow in media containing an extremely wide range of salt concentration, from 0.17 M to 4.0 M NaCl (Hadi et al., 2008).

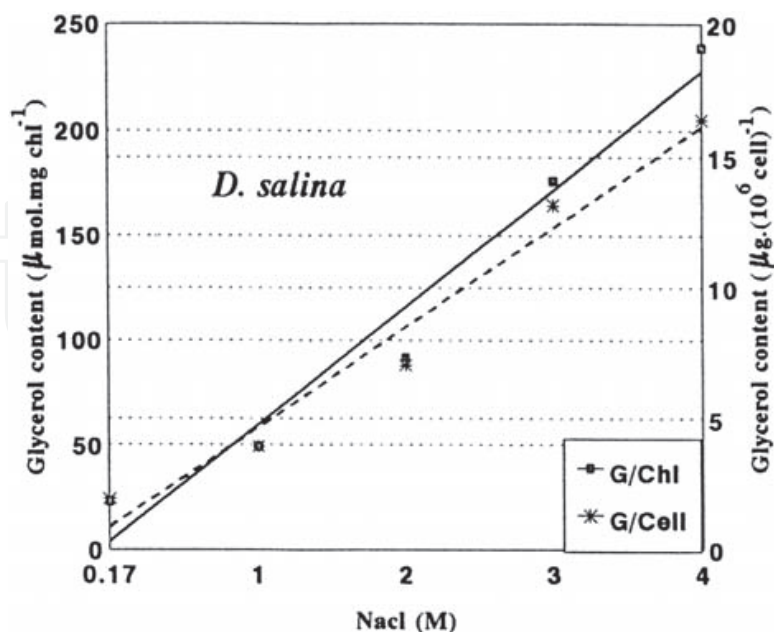


Fig. 3. Intracellular glycerol content as a function of the extracellular NaCl concentration in *Dunaliella salina*. Interacellular glycerol and chlorophyll content was determined in cells grown in media containing the indicated salt concentration (Hadi et al., 2008).

It seems that in *Dunaliella*, osmolyte of main for balancing the extracellular osmotic stress is glycerol, because its intracellular concentration intensively accumulates in response to any osmotic stress such as high medium salinity (Shariati, 2003; Hadi et al., 2008). Also, it shown that its intracellular concentration was linearly related to the medium osmotic pressure (Fig. 3). When cells of *Dunaliella* grown at high salinity, the intracellular glycerol concentration exceeds 50% and is sufficient to account for essentially all of the osmotic pressure required to balance the extracellular osmolarity (Avron & Ben-Amotz, 1992). Glycerol production in *D. salina* grown in different salt concentrations is shown in Fig. 3. Also, glycerol production in *D. salina* grown under saltshock conditions (by transfer of algae from medium containing 1 to 3 M NaCl) is shown in Fig. 4. However glycerol accumulates in *Dunaliella* in response to osmotic stress but it seems that physiological role and function of glycerol may be different in each algae. Another possible physiological role of glycerol is that of a compatible solute, that is, a substance that at high concentrations protects enzyme activity.

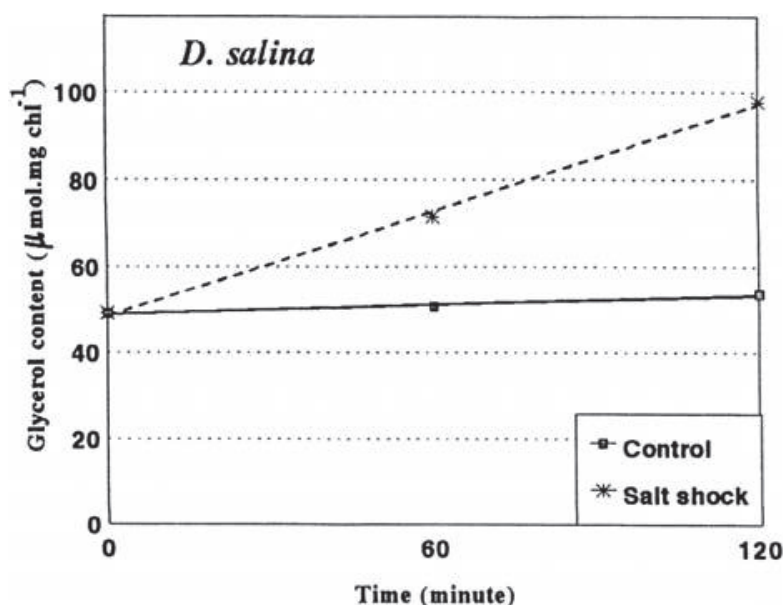


Fig. 4. The response in glycerol production of *Dunaliella salina* grown in 1.0 M NaCl and subjected to dense stress by transfer to 3.0 M NaCl. Time was measured from the moment of transfer to the higher salt concentration (Hadi et al., 2008)

Glycerol in *Dunaliella parva* can accumulate to the maximum content under saturated NaCl conditions, which is about 8 M or 55% of cell weight (Alkayal et al., 2010). When cells of *Dunaliella* expose an osmotic shock, the initial reaction is physical in nature. Within seconds water enters or leaves the cell osmotically, causing marked volume changes which bring the cells back into osmotic equilibrium with the medium. Thereafter, depending on the direction and extent of the osmotic shock and on the metabolic conditions of the cells, glycerol is synthesized or eliminated via temperature-dependent enzymatic pathways, accompanied by water reentry or efflux, respectively, so that the cells regain approximately their original volume (Avron & Ben-Amotz, 1992). Glycerol is produced in *Dunaliella* either by photosynthetic  $\text{CO}_2$  fixation or by starch degradation. The contribution of these metabolic pathways to glycerol synthesis depends on the availability of light, the starch reserve pool, and the size of the salt stress. In the dark, *Dunaliella* produces glycerol exclusively by degradation of starch, and the capacity of the cells to recover from hyperosmotic shock

depends on their starch reserve pools. Hyperosmotic shock in the light greatly stimulates the rate of glycerol production and in parallel enhances starch degradation, indicating that starch degradation also has a significant contribution to glycerol production in the light. This complex interplay between photosynthesis and starch degradation probably results from the excessive demand for glycerol synthesis on one hand, and from the inhibition of photosynthesis by hyperosmotic shock on the other hand. Starch synthesis is also strongly inhibited following hyperosmotic shock. Hypoosmotic shock induces in *Dunaliella* a decrease in glycerol content and a parallel increase in starch content, indicating metabolic conversion of glycerol to starch. Hypoosmotic shocks also induce a transient inhibition of photosynthesis and a substantial, but not complete, inhibition of glycerol synthesis. These and other observations suggest that *Dunaliella* utilizes a dynamic interconversion between glycerol and starch, the two major carbon pools, as well as photosynthesis, to meet the osmotic requirements which are set by the external salt concentration (Avron & Ben-Amotz, 1992). Also, Chen & Jiang (2009) reported that changes of shape and volume of *D. salina* cell cultured chronically at various salinities were minor, but when the salinity was changed rapidly, the variations of cell shape and cell volume of *D. salina* were significant, which were recovered basically after 2 h except treating by high salinity. In addition, they indicated that, it was found some lipid globules in the surface of *D. salina* cells when the salinity increased from 2.0 to 4.0–5.0 M NaCl rapidly. In this researchs when *D. salina* was cultured chronically at various salinities, the accumulation of single cell glycerol increased with increased salinity, and *D. salina* also could rapidly decrease or increase single cell glycerol contents to adapt to hypoosmotic or hyperosmotic shock (Chen et al., 2011). Biosynthesis of glycerol in *Dunaliella* is carried out mostly within the chloroplast and partly in the cytosol and may be broadly divided into the production of dihydroxyacetone phosphate and its conversion to glycerol, as is summarized in Fig. 5. Four enzymes catalyze the interconversions between dihydroxyacetone phosphate and glycerol in *Dunaliella*: two reversible steps, namely a glycerophosphate dehydrogenase and an NADPH-specific dihydroxyacetone reductase, and two irreversible steps, namely a glycerol phosphate phosphatase (GPase) and a dihydroxyacetone kinase. Dihydroxyacetone reductase which catalyzes the interconversion of glycerol to dihydroxyacetone, is NADP<sup>+</sup>-specific, has an exceptionally low affinity for glycerol, and an almost absolute specificity towards both glycerol and dihydroxyacetone phosphate. Dihydroxyacetone kinase, mediating the phosphorylation of dihydroxyacetone by ATP, has an absolute specificity towards dihydroxyacetone. The chloroplastic glycerophosphate dehydrogenase, catalyzing conversion of dihydroxyacetone phosphate to glycerol-3-phosphate with either NADH or NADPH, has unusual catalytic and regulatory properties and is distinct from the cytosolic isoenzyme in its stability towards detergents and high salt. These enzymes compose the so-called glycerol cycle (Avron & Ben-Amotz, 1992).

#### 4. $\beta$ -carotene

$\beta$ -carotenes are aliphatic-alicyclic components that composed of five carbon isoprene groups and belong to carotenoid components. Those containing only hydrogen and carbon.  $\beta$ -carotenes are the pigments included in many plants and algae. Some of plants and red-orange foods owe their typical color principally to  $\beta$ -carotenes.  $\beta$ -carotenes are very widely distributed in nature; their various roles include provitamin A activity, absorption of light energy, triplet chlorophyll and singlet oxygen quenching, antioxidation activity, oxygen





interthylakoid spaces of the chloroplast. The  $\beta$ -carotene-rich *Dunaliella* strains are widely distributed in salt water bodies which contain more than 10% salt and most dominantly in high salt habitats approaching NaCl saturation (Shariati & Maddadkar Haghjoo, 1998). The orange-reddish color of many salt-lanes in high light intensity ecological niches is usually due to the color of the  $\beta$ -carotene rich *Dunaliella* (Fig. 6 partition B). Under non-inducing, non-accumulating conditions (optimum conditions, 1-2 M NaCl), *D. salina* is green (Table 1 and Fig. 6 partition of D) and contain only about 0.3 %  $\beta$ -carotene, similar to the content in plant leaves and other algae. Following induction and growth under appropriate cultivation conditions, the  $\beta$ -carotene is accumulated within oily globules in the interthylakoid space of the chloroplast to more than 10% of the algal dry weight, which is the highest content of  $\beta$ -carotene of any known alga, plant, or other microorganism. The extent of  $\beta$ -carotene accumulation and the rate of synthesis depend on certain physiological growth parameters, namely light intensity, salt concentration, temperature (Madadkar Haghjou & Shariati, 2007; Madadkar Haghjou et al., 2009), and nutrient deficiency. 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  $\beta$ -carotene per cell. However, these conditions at the same time decrease the cell number per unit culture volume by affecting cell viability. Therefore, it is recommended by one group of authors that adjusting light and salinity likely is one of the best strategies to achieve optimal b-carotene production in mass cultures of *D. salina* (Marin et al. 1998). The Iranian strain of *D. salina* grown in medium containing 1-2 M NaCl appeared green in color (Table 1 and Fig. 6 partition of D), while at 4 M NaCl an orangered color was observed (Table 1 and Fig. 6 partition of A and C). The high accumulation of carotenoids is the main reason for the change to this orange-red color in *D. salina* at 4 M NaCl.

Alga Color	Car./Chl. Ratio	Total Chl. ( $\mu\text{g ml}^{-1}$ )	Total Car. ( $\mu\text{g ml}^{-1}$ )	NaCl (M)
Green	0.39	3.25	1.25	0.17
Green	0.61	4.17	2.57	1
Green	0.82	6.01	4.57	2
Orange	1.53	2.14	3.32	3
Orange-red	2.58	1.10	2.84	4

Table 1. Total carotenoid (Car.), total chlorophyll (Chl.) contents, carotenoid/chlorophyll ratio, and alga color of *Dunaliella salina* grown at the indicated concentrations of NaCl (M) at one week following inoculation (Hadi et al., 2008).

The amount of carotenoid accumulated in *D. salina* after 7 days of growth are depicted in Table 1. *D. salina* had the highest carotenoid/chlorophyll ratio (2.58) at 4 M NaCl (Table 1). Prieto et al., (2011) reported that highest carotenoid production was achieved with this culture system operated following the two-stage strategy. Also they indicated that closed tubular photo-bioreactor provided the highest carotenoid contents (10% of dry weight) in

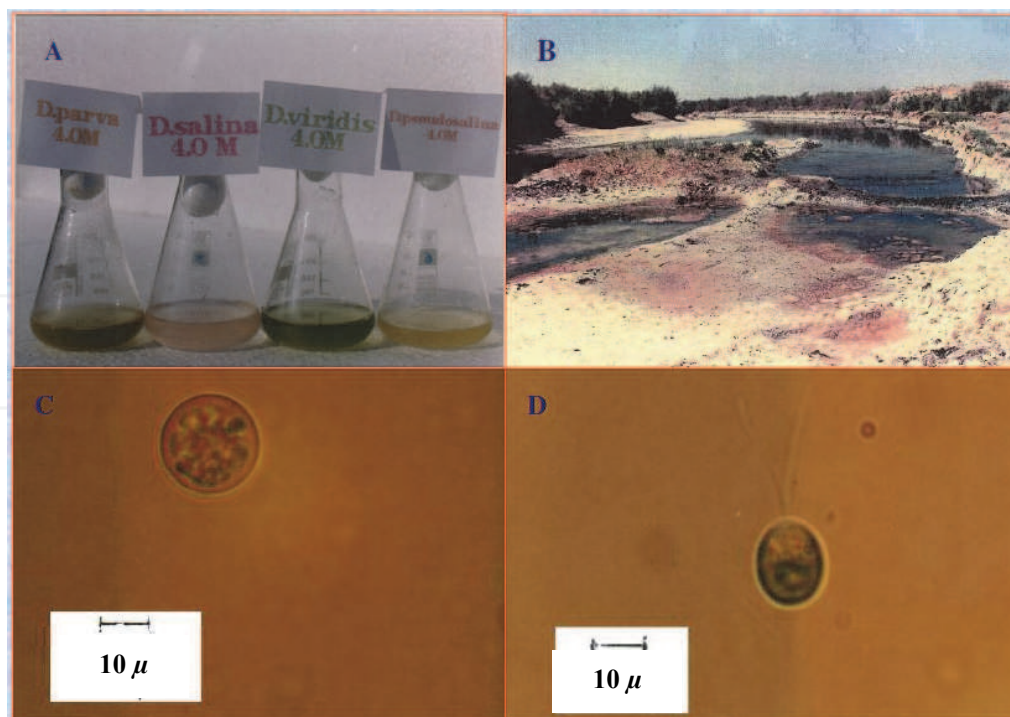


Fig. 6. Four species of *D. Parva*, *D. salina*, *D. viridis* and *D. pseudosalina* grown in 4.0 M NaCl, the liquid medium of *D. salina*, *D. Parva* and *D. pseudosalina* cause cells have rich  $\beta$ -carotene so, those are about orange colour (A), the pools of salt evaporation in Gave-Khooni Salt Marsh, Iran that cause present *D. salina* containing  $\beta$ -carotene have orange colour (B), shape of *D. salina* grown in 4 M NaCl (stress conditions) that cell have rich  $\beta$ -carotene so, it is orange colour (C) and shape of *D. salina* grown in 2 M NaCl at optimum growth that cell have rich chlorophyll so, it is green colour (D) (Hadi, 1996).

*Dunaliella* biomass and  $\beta$ -carotene abundance (90% of total carotenoids) as well as the highest 9-cis to all-trans  $\beta$ -carotene isomer ratio (Prieto et al., 2011). In addition, Hosseini Tafreshi & Shariati (2006) reported that Iranian strain G of *Dunaliella salina* had the highest potential for  $\beta$ -carotene accumulation and suitable for outdoor cultivation (Fig. 7). The higher the light intensity and the slower the growth rate of the alga, the higher the cellular  $\beta$ -carotene content. Highest  $\beta$ -carotene content per cell can be obtained by exposing the nitrogen-deficient cells to high light intensity in a short period of 1 to 2 d. The effect of light quality on carotenogenesis indicates that  $\beta$ -carotene biosynthesis and accumulation in *D. bardawil* is independent of light quality within the photo-synthetically active radiation region. The commercial production of large quantities of the  $\beta$ -carotene-rich *Dunaliella* provides natural  $\beta$ -carotene for different nutritional, dietary, and clinical studies for evaluation in comparison with the synthetic  $\beta$ -carotene. A few possible reasons for the increased synthesis of  $\beta$ -carotene and its possible function in *Dunaliella* have been suggested and investigated (Avron & Ben-Amotz, 1992):

1. Carbon storage:  $\beta$ -carotene is accumulated and stored as an extra-photosynthetic product for later use under limited growth rate. This hypothesis was studied by a "carbon sink" utilization which revealed that the *Dunaliella* cells do not consume the accumulated  $\beta$ -carotene on transfer to darkness or to a  $\text{CO}_2$ -free medium in the light.
2. Singlet oxygen quencher:  $\beta$ -carotene protects against chlorophyll catalyzed singlet oxygen and possibly other excited chlorophyll damaging agents. This hypothesis was analyzed by

electron micrographs of the  $\beta$ -carotene-rich *Dunaliella*. Due to the large distance between the  $\beta$ -carotene globules and the thylakoid located chlorophyll, and due to the short lifetime of these damaging compounds, the massively accumulated  $\beta$ -carotene cannot be effective through this mechanism.

3. Absorption effect:  $\beta$ -carotene protects the cell against injury by high intensity radiation under limited growth conditions by acting as a screen to absorb excess radiation. This hypothesis is well accepted through the observation that the  $\beta$ -carotene-rich *Dunaliella* shows maximal photoprotection against high irradiation with blue light, and minimal photoprotection against light irradiation with red light. Strains unable to accumulate  $\beta$ -carotene and the  $\beta$ -carotene-poor *D. bardawil* show low photo-protection and die when exposed to the extreme irradiation while the  $\beta$ -carotene-rich *D. bardawil* survives and flourishes. Thus, the function of  $\beta$ -carotene in *Dunaliella* seems to be photo-protection through its absorption properties. The oily nature of the globules at the periphery of the cup shaped chloroplast functions structurally most efficiently for this purpose.

In mammals, the structure of  $\beta$ -carotene possessing two  $\beta$ -ionone rings, one at either end, and a long one plane polyene chain, provides the optimal structure for enzymatic cleavage to two molecules of vitamin A and thus, the highest efficiency as a vitamin A precursor. Many factors affect the biological activity of  $\beta$ -carotene metabolism and its potency as a vitamin A precursor, namely, physical form, oily solubility, isomerization, dietary fat level, state of oxidation, presence of dietary antioxidants, the diet composition, animal studied, age, sex, and many more. Acetyl-CoA, and in a few organisms valine and leucine, are considered the starting compounds in the biosynthesis of  $\beta$ -carotenes. These early steps of the pathway, leading to C5 isoprenoid units and the subsequent prenyl diphosphate intermediates, are common to all classes of terpenoids. Only the later stages after geranylgeranyl, diphosphate (GGDP) and after phytoene, which is a product of condensation of two molecules of GGDP, are unique to the formation of  $\beta$ -carotenes. In most order, the general biosynthesis of  $\beta$ -carotene can be divided into four stages (Fig. 8): (1) formation of GGDP from mevalonic acid; (2) condensation to form phytoene; (3) desaturation of phytoene to lycopene; and (4) cyclization of lycopene to form  $\beta$ -carotene (Ye et al., 2008). The ability of  $\beta$ -carotene in *Dunaliella* to protect the algae from death by high blue irradiation was utilized to select mutants of *D. bardawil* which accumulate a higher content of  $\beta$ -carotene. Both the production rate of phytoene and the conversion rate of phytoene to lycopene and  $\beta$ -carotene are accelerated in the isolated mutants. By the analysis of the effect of protein synthesis inhibitors, it was suggested that the mutants are affected in the mechanism which regulates the activation of the carotene biosynthetic pathway, most probably at the metabolic steps which precede GGPP and which allow enhanced production of both  $\beta$ -carotene and chlorophyll, and possibly at a later site after phytofluene. Recent epidemiological and oncological studies suggest that normal to high levels of  $\beta$ -carotene in the body may protect it against cancer. Humans and animals fed a diet high in carotenoid-rich vegetables and fruits and who maintain higher than average levels of serum  $\beta$ -carotene have a lower incidence of several types of cancer. The interest in a natural source of  $\beta$ -carotene is increasing with the buildup of information relating carotenoids to preventive medicine. Of special interest in this regard is the observation that natural  $\beta$ -carotene, as found in *Dunaliella* and in most fruits and vegetables, contains a mixture of all-trans  $\beta$ -carotene and 9-cis  $\beta$ -carotene together with a few other stereoisomers, as discussed above. The requirement for better absorbed  $\beta$ -carotene for disease prevention purposes has created a new market of high commercial potential for the *Dunaliella*  $\beta$ -carotene stereoisomeric



mixture. Large scale cultivation of *Dunaliella* for  $\beta$ -carotene production is based on autotrophic growth in media containing inorganic nutrients with carbon dioxide as a carbon source. Commercial attempts are being made to apply the basic biological information of  $\beta$ -carotene optimization to mass production of  $\beta$ -carotene in outdoor ponds of *Dunaliella* in areas located where the solar light output is maximal and the high salt concentration can eliminate foreign grazers. Two modes of cultivation are being used in large-scale bioreactors of *Dunaliella*. In the more common, the intensive mode, attempts are made to control all factors affecting cell growth and chemistry. The growth limitation is usually provided by controlling the availability of the nitrogen supply on continuously grown induced cells of *Dunaliella*. In the other mode, the extensive growth, *Dunaliella* grows very slowly in nearly saturated brine where the high salt concentration is used to control consistent production of  $\beta$ -carotene. In addition, since commercial activity in the microalgae extractable chemical sector is currently limited to two main products, *Dunaliella*-derived carotenoid pigments as a human nutritional supplement and genetic modification of *Dunaliella* strains, transgenic strains should also be explored (Jin & Melis, 2003). It seems that  $\beta$ -carotene accumulation protects cells against the deleterious effects of high intensity irradiation by absorbing light in the blue region of the spectrum (Ben-Amotz, 1993).

Ben-Amotz (1995) cultivated *D. salina* by a new two-phase growth strategy for  $\beta$ -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 (Hosseini Tafreshi and Shariati 2006). 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  $\beta$ -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  $0.9 \times 10^6$  cell  $\text{ml}^{-1}$ . 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  $\beta$ -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  $\beta$ -carotene accumulation is also controversial. Garcia-Gonzalez et al. (2005) also found that a suitable approach for the production of high quality  $\beta$ -carotene with high 9-cis isomer content is the cultivation of *Dunaliella* in closed tubular photobioreactors, which have low mutual shading. 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* (Hosseini Tafreshi & Shariati, 2009). 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  $\beta$ -carotene contents per unit time and per unit volume (Hosseini Tafreshi & Shariati, 2009). The optimization procedure should be done by testing the best strains and the most effective strategies under optimal conditions. Recently, Mojaat et al. (2008) studied the effects of  $\text{Fe}^{2+}$  ions and organic carbon source on growth and

carotenogenesis of *Dunaliella salina*. In their study, a significant increase in  $\beta$ -carotene contents per cell was observed, with a maximum value of 70 pg cell<sup>-1</sup> when the culture was supplemented with acetate and FeSO<sub>4</sub>. The approach might be a good alternative method for production of carotenoids by alga in photobioreactors after optimization (Hosseini Tafreshi & Shariati, 2006).

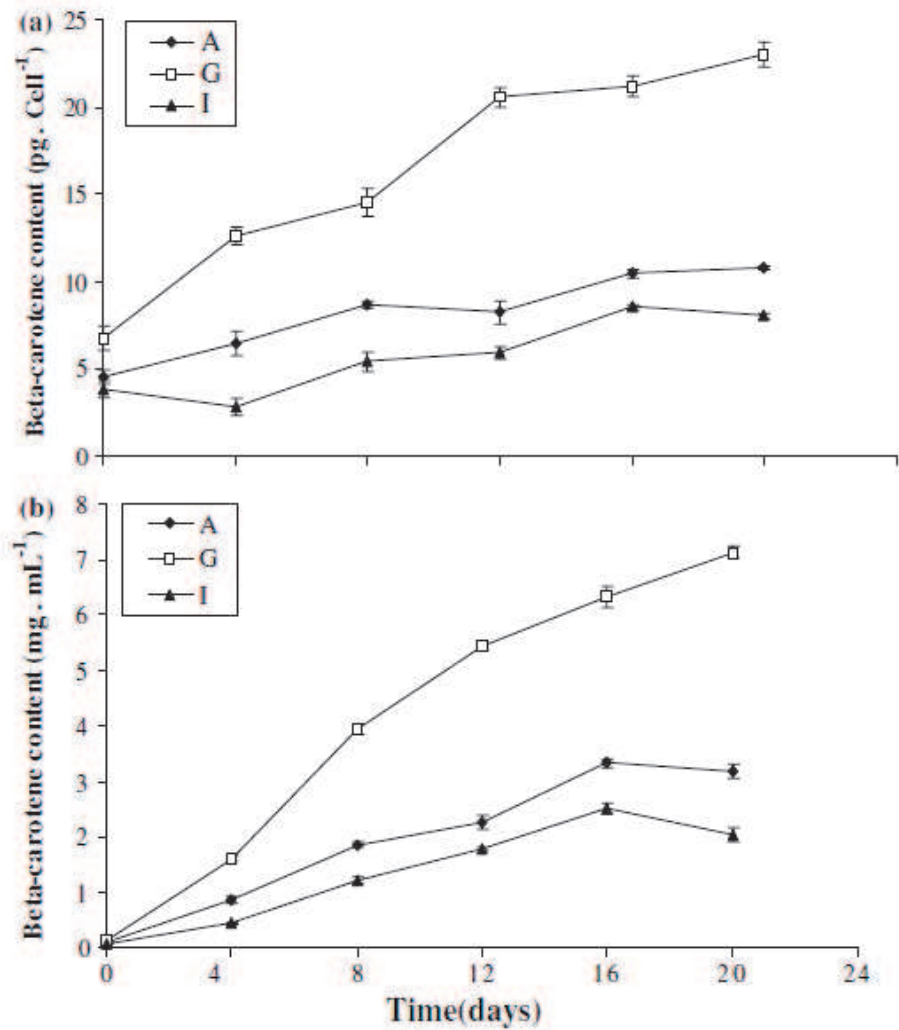


Fig. 7. Quantitative changes in  $\beta$ -carotene as a function of time per cell (a) and per unit volume (b) of three strains of *Dunaliella salina* (A, G and I) in open ponds during the stage 2 (nutrient-poor medium, containing 2.5 M NaCl). The values are means of three replicates  $\pm$  SD (Hosseini Tafreshi and Shariati 2006).

### 5. Production of biofuel from *Dunaliella*

The global economy literally runs on energy. An economic growth combined with a rising population has led to a steady increase in the global energy demands. If the governments around the world stick to current policies, the world will need almost 60% more energy in 2030 than today (IEA, 2007; Vishwanath et al., 2008). The oil productivity of many microalgae exceeds the best producing oil crops (Vishwanath et al., 2008). Past research in the use of hydrothermal technology for direct liquefaction of biomass was very active. Only

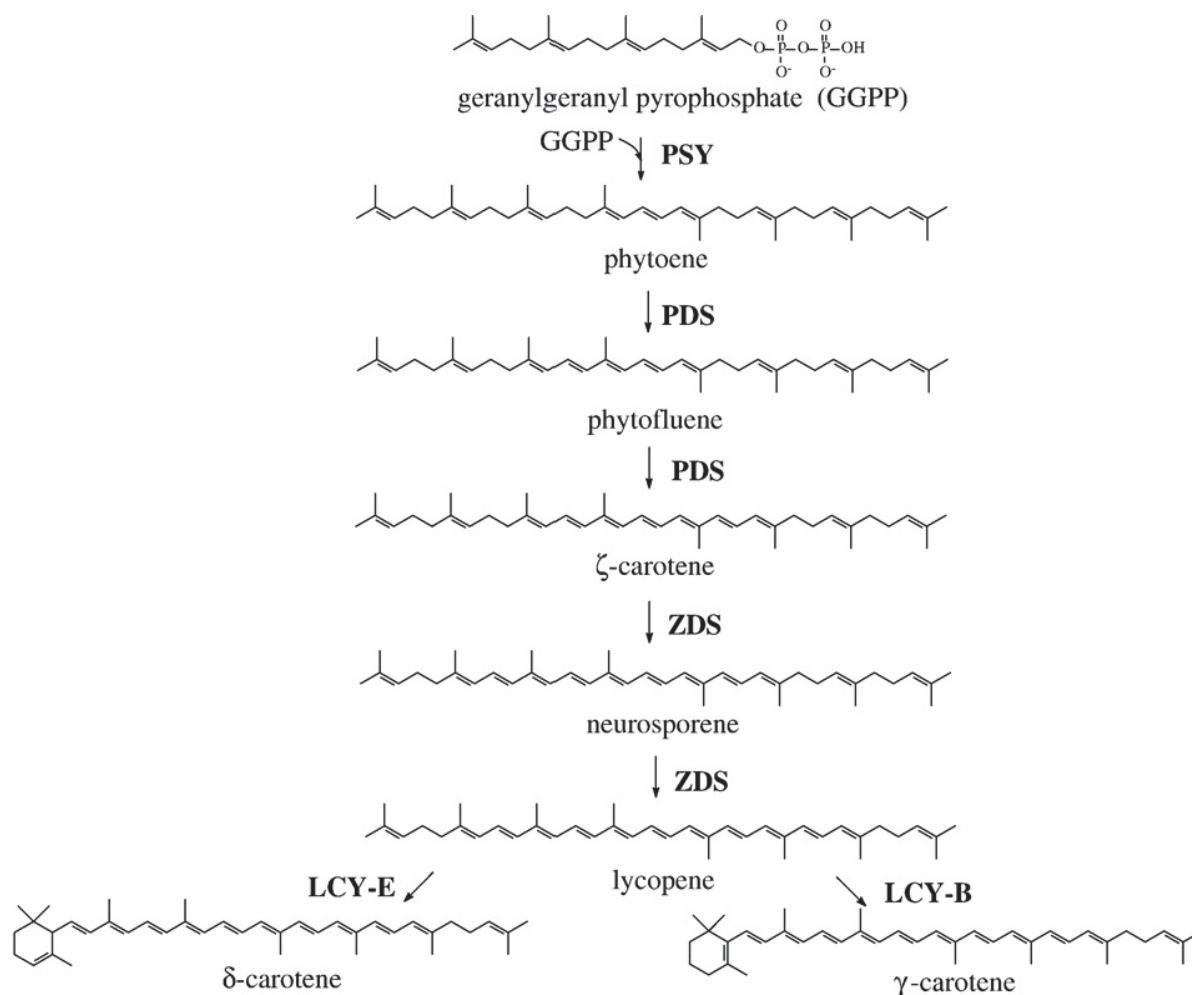


Fig. 8. A hypothetical pathway for carotenogenesis in *Dunaliella*. Enzymes for the relative conversions are in bold. PSY: phytoene synthase; PDS: phytoene desaturase; ZDS:  $\zeta$ -carotene desaturase; LCY-E: lycopene  $\epsilon$ -cyclase; LCY-B: lycopene  $\beta$ -cyclase (Ye et al., 2008).

a few of them, however, used algal biomass as feedstock for the technology. Minowa et al., (1995) report an oil yield of about 37% (organic basis) by direct hydrothermal liquefaction at around 300°C and 10 MPa from *Dunaliella tertiolecta* with a moisture content of 78.4 wt%. The oil obtained at a reaction temperature of 340°C and holding time of 60 min had a viscosity of 150–330 mPas and a calorific value of 36 kJ g<sup>-1</sup>, comparable to those of fuel oil. The liquefaction technique was concluded to be a net energy producer from the energy balance (Vishwanath et al., 2008).

The key for large scale production of biofuels is to grow suitable biomass species in an integrated biomass production conversion system (IBPCS) at costs that enable the overall system to be operated at a profit. The illustration in Figure 9 is a conceptual model for integrated biomass production (Klass, 1997) that can be adopted for microalgal biodiesel production.

The production of microalgal biodiesel requires large quantities of algal biomass. Most of algal species are obligate phototrophs and thus require light for their growth. Several cultivation technologies that are used for production microalgal biomass have been developed by researchers and commercial producers. The phototropic microalgae are most

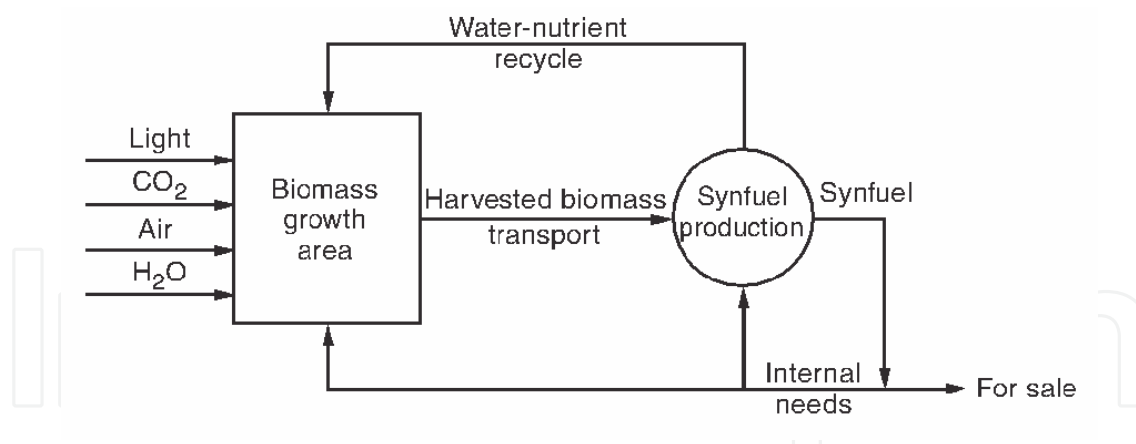


Fig. 9. A conceptual model for integrated biomass production and conversion integration system (Vishwanath et al., 2008).

commonly grown in open ponds and photobioreactors (Patil et al., 2005). The open pond cultures are economically more favorable, but raise the issues of land use cost, water availability, and appropriate climatic conditions. Further, there is the problem of contamination by fungi, bacteria and protozoa and competition by other microalgae. Photobioreactors offer a closed culture environment, which is protected from direct fallout, relatively safe from invading microorganisms, where temperatures are controlled with an enhanced  $\text{CO}_2$  fixation that is bubbled through culture medium. This technology is relatively expensive compared to the open ponds because of the infrastructure costs. An ideal biomass production system should use the freely available sunlight. It is reported the best annual averaged productivity of open ponds was about  $24 \text{ g}^{-1} \text{ dry weight m}^{-2} \text{ d}^{-1}$  (Weisz, 2004). A productivity of  $100 \text{ g}^{-1} \text{ dry weight m}^{-2} \text{ d}^{-1}$  was achieved in simple 300 l culture systems (Patil et al., 2005). This level has been viewed as deriving from the light saturation effect. The light requirement coupled with high extinction coefficient of chlorophyll in algae has necessitated the design and development of novel system for large scale growth. Experiments have also elucidated that algal biomass production can be boosted by the flashing light effect (Matthijs et al., 1996), namely by better matching photon input rate to the limiting steps of photosynthesis. Indeed, the best annual averaged productivity has been achieved in closed bioreactors. Tridici (2004) has reviewed mass production in photobioreactors. Many different designs of photobioreactor have been developed, but a tubular photobioreactor seems to be most satisfactory for producing algal biomass on the scale needed for biofuel production. Closed, controlled, indoor algal photobioreactors driven by artificial light are already economical for special high-value products such as pharmaceuticals, which can be combined with production of biodiesel to reduce the cost (Vishwanath et al., 2008).

## 6. *Dunaliella*-based bioenergy options

In recent years, biofuel production from algae has attracted the most attention among other possible products. This can be explained by the global concerns over depleting fossil fuel reserves and climate change. Furthermore, increasing energy access and energy security are seen as key actions for reducing poverty thus contributing to the Millennium Development Goals. Access to modern energy services such as electricity or liquid fuels is a basic requirement to improve living standards. One of the steps taken to increase access and



reduce fossil fuel dependency is the production of biofuels, especially because they are currently the only short-term alternative to fossil fuels for transportation, and so until the advent of electromobility. The so-called first generation biofuels are produced from agricultural feedstocks that can also be used as food or feed purposes. The possible competition between food and fuel makes it impossible to produce enough first generation biofuel to offset a large percentage of the total fuel consumption for transportation. As opposed to land-based biofuels produced from agricultural feedstocks, cultivation of algae for biofuel does not necessarily use agricultural land and requires only negligible amounts of freshwater (if any), and therefore competes less with agriculture than first generation biofuels. Combined with the promise of high productivity, direct combustion gas utilization, potential wastewater treatment, year-round production, biochemical content of algae and chemical conditions of their oil content can be influenced by changing cultivation conditions. Since they do not need herbicides and pesticides, algae appear to be a high potential feedstock for biofuel production that could potentially avoid the aforementioned problems. On the other hand, microalgae, as opposed to most plants, lack heavy supporting structures and anchorage organs which pose some technical limitations to their harvesting. The real advantage of microalgae over plants lies in their metabolic flexibility, which offers the possibility of modification of their biochemical pathways (e.g. towards protein, carbohydrate or oil synthesis) and cellular composition. Algae-based biofuels have an enormous market potential, can displace imports of fossil fuels from other countries (hence reduce a country's dependence), and is one of the new, sustainable technologies which can count on ever-increasing political and consumer support. The reasons for investigating algae as a biofuel feedstock are strong but these reasons also apply to other products that can be produced from algae. There are many products in the agricultural, chemical or food industry that could be produced using more sustainable inputs and which can be produced locally with a lower impact on natural resources. Co-producing some of these products together with biofuels, can make the process economically viable, less dependent from imports and fossil fuels, locally self sufficient and expected to generate new jobs, with a positive effect on the overall sustainability (Mata et al. 2010). A wave of renewed interest in algae cultivation has developed over the last few years' scientific research, commercialization initiatives and media coverage have exploded since 2007. In most cases, the main driver of the interest in algae is its high potential as a renewable energy source, mainly algae-based biofuels (ABB) for the transport sector. In 2009 FAO published a report detailing various options for algae cultivation, multiple biofuels that can be produced and the environmental benefits and potential threats associated with ABB production. One of the main conclusions of this report is that the economic feasibility of producing a (single) low-price commodity like biofuels from algae is not realistic, at least in the short term. This chapter summarizes some of the technology key findings of the aforementioned report and gives a brief overview of how algae can be cultivated and which biofuels can be produced. The following chapter investigates which other products can be produced from algae, and tries to assess the viability of co-production with bioenergy. Ethanol is commonly produced from starch-containing feedstocks; some algae have been reported to contain over 50% of starch. Algal cell walls consist of polysaccharides which can be used as a feedstock in a process similar to cellulosic ethanol production, with the added advantage that algae rarely contain lignin and their polysaccharides, are generally more easily broken down than woody biomass. Coproducts can potentially be derived from the non-carbohydrate part of the algal biomass. There are a variety of ways to produce biofuel with algae. Figure 10

provides an overview of the options, which are explained in detail in FAO (2009). In this section only the requirements of the algal biomass needed to produce various biofuels are briefly discussed in order to facilitate the selection of different coproduction options further in the report.

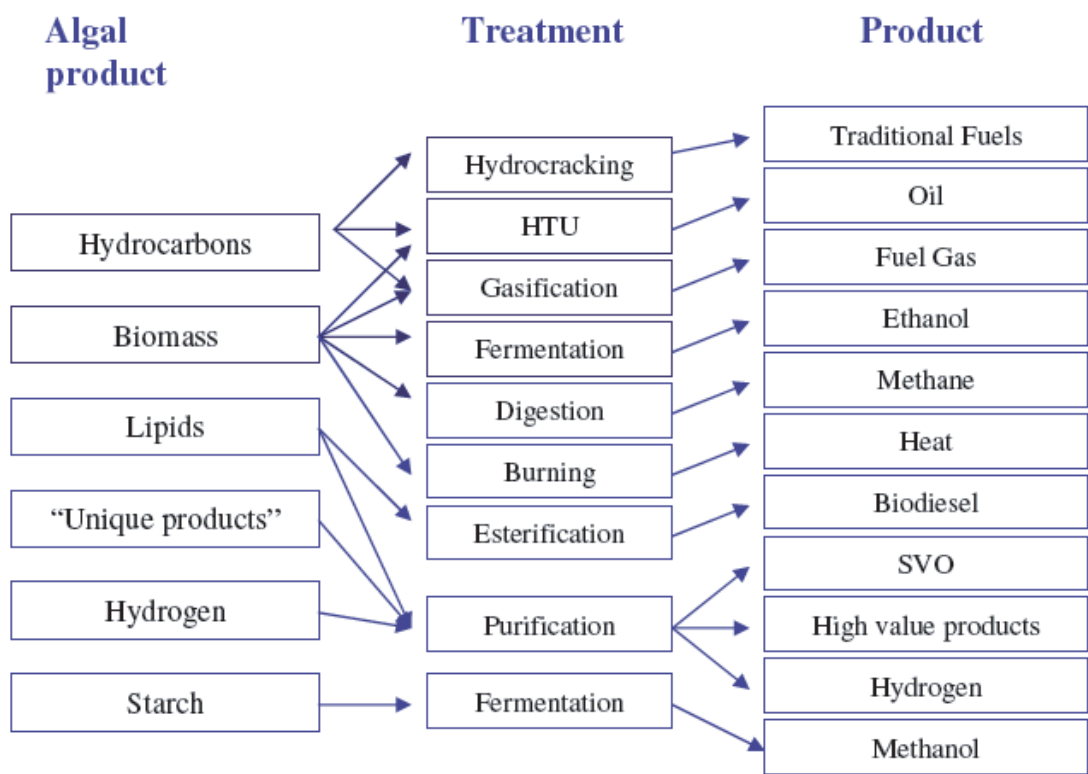


Fig. 10. Overview of algae-to-energy options (FAO, 2009).

Biodiesel production from algal oils has received most attention since algae can contain potentially over 80% total lipids, (while rapeseed plants, for instance, contain about 6% lipids). Under normal growth conditions the lipid concentration is lower (<40%) and high oil content is always associated with very low yields. The various lipids production can be stimulated under stress conditions, e.g. insufficient nitrogen availability. Under such conditions, biomass production is not optimal though, reducing the non-lipid part of the biomass that can be further used as a source for co-products. Biomass has attracted more and more interests as an alternative energy source, since it is a renewable and environmentally friendly source and it fixes CO<sub>2</sub> in the atmosphere through photosynthesis (Yang et al., 2011). Biomass resources mainly include agricultural crops and their waste byproducts, forestry products, marine products and wastes. Among these biomass resources, microalgae are seen as being a future source of third generation bio-fuels and chemicals, due to their fast growth rate in an aquatic medium and high lipid content. Furthermore, microalgae are not lignocellulosic in composition but are comprised of proteins, lipids, non-cellulosic carbohydrates, and nucleic acids, which can be decomposed and hydrolyzed more easily than lignocellulosic biomass. Liquefaction of biomass had received extensive research. Microalgae as feedstock were seldom employed to liquefy. Yang et al., (2004) had investigated the liquefaction of *Microcystis viridis* using the same liquid alkali catalyst (sodium carbonate), and obtained the maximum oil yields of 33–40

wt%. Zou et al., (2009) described the liquefaction of microalgae in the presence of liquid acid catalyst. Ross et al. studied the hydrothermal processing of microalgae using alkali and organic acids, and the yields of biocrude on an organic basis were higher in the presence of organic acids compared with alkali catalysts (Ross et al., 2010). There was a high demand on the liquefaction equipment to avoid the corrosion effects of liquid alkali and acid catalysts. In order to solve these problems, conventional homogeneous catalysts were expected to be replaced in the near future by environmentally friendly heterogeneous catalysts (Perego and Bianchi, 2010). Many efforts had been devoted to the search of solid acid and alkali catalysts in the transesterification of vegetable oils to bio-diesel (Singh and Fernando, 2008), and the upgrading of bio-oils (Peng et al., 2009). The solid heterogeneous catalysts were almost fully recovered from reaction products, were normally easy and safe to dispose of, more selective (Yang et al., 2011). In the industry, microalgae have been used as source for a wide variety of practical and potential metabolic products, such as food supplements, pharmacological substances, lipids, enzymes, biomass, polymers, toxins, pigments, tertiary wastewater treatment, and “green energy”. Microalgae are also important in aquiculture as they are a source of nutrients and have great importance in production of oxygen, in consumption of carbon dioxide, and in consumption of nitrogen-based compounds such as ammonium. The most common procedure for cultivation of microalgae is autotrophic growth. Because all microalgae are photosynthetic, and many microalgae are especially efficient solar energy convertors, microalgae are cultivated in illuminated environments naturally or artificially. Under autotrophic cultivation, the cells harvest light energy and use CO<sub>2</sub> as a carbon source. The main driving force to grow microalgae commercially is harvesting metabolic products, feed for marine and terrestrial organisms, food supplements for humans, or to use the microalgae for environmental processes, such as wastewater treatment, fertilization of soils, biofuels, and phytoremediation of toxic wastes (Perez-Garcia et al., 2011). Microalgae have been recognized as a promising alternative source for oil production. Several species of microalgae can be induced to overproduce specific lipids and fatty acids through relative simple manipulations of the physical and chemical properties of their culture medium. By manipulating fatty acid content, microalgae represent a significant source of unusual and valuable lipids and fatty acids for numerous industrial applications. Microalgae can accumulate substantial amounts of lipids – up to 50% of dry cell weight in certain species. Many microalgae species can grow in brackish water or seawater, thereby avoiding demand for fresh water, a limited resource in many parts of the world. Several species grow very fast; doubling their mass in 24 h. Bioprospection of strains is important to select the best strains that can produce higher amounts of desired metabolic products. Several studies have evaluated the use of several microalgae (Mutanda et al., 2011) but more work still need to be done given the number of existing microalgae. In microalgae, lipids have as a basic function the synthesis of lipoproteic membranes and are important in floating and as an energetic reserve. Accumulation of lipids can be attributed to consumption of sugars at a rate higher than the rate of cell generation, which would promote conversion of excess sugar into lipids. The lipids extracted from microalgae may be used in human nutrition as source of Omega-3 (El-Baky et al., 2004). Accumulation of lipids in the microalgae cells, as well as for other oleaginous microorganisms (high oil producers), depends on diverse factors, such as growth temperature, pH, availability of micronutrients, salinity and other factors. Production of bioactive compounds and bio fuels 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. 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<sub>2</sub>. The pyrolysis characteristics of *D. tertiolecta* were studied by thermogravimetric analysis. 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).

## 7. Conclusion

The alga *Dunaliella* includes many important scientific aspects and application on the physiology (osmoregulation, function of H<sup>+</sup>-ATPase in *D. acidophila*), biotechnology ( $\beta$ -carotene and glycerol production) and bioenergy (bioreactor and biofuel production). Carcinogenic effects of  $\beta$ -carotene and particularly the beneficial properties of *Dunaliella* natural  $\beta$ -carotene will promote demand for the natural 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  $\beta$ -carotene production and other biotechnological purposes. 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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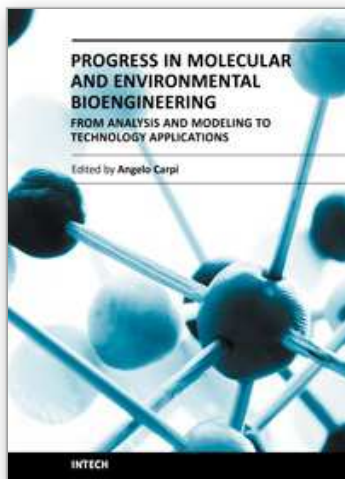
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