OnLine Journal of Biological Sciences 2 (4): 220-223, 2002
ISSN 1608-4127

Accumulation of Antioxidant Vitamins in *Dunaliella salina*

Farouk K. El Baz, [1]Ahmed M. Aboul-Enein, [1]Gamal S. El-Baroty
[1]A. M. Youssef and Hanaa H. Abdel-Baky
Botany Department, National Research Center, Cairo, Dokki, Egypt
[1]Biochemistry Department, Faculty of Agriculture, Cairo University, Cairo, Egypt

Abstract: *Dunaliella salina* a β-carotene accumulating halotolerant algae has been analyzed for the effect of various growth conditions on its antioxidant vitamin contents β-carotene, tocopherol and ascorbic acid. Vitamin contents of *Dunaliella salina* grown in culture contained sufficient nitrogen (70 ppm N) and NaCl (10 %) under optimum light intensity 200 W were 1.78, 0.7 and 0.25 %, respectively. Increasing salt concentration (NaCl) to 30 % and high light intensity (400 W) increased vitamins content to 6.43, 0.45 and 0.95 %, respectively. The maximam accumulation of antioxidant vitamins occurred in *Dunaliella salina* was observed when the cells grown under combined stress conditions high NaCl concentration, high light intensity with nitrogen deficiency (5 ppm). β - carotene, vitamin E and vitamin C percentages were 13.14, 1.23 and 2.5 %, respectively. These values represented 738,1751 and 1000 %, respectively when compared with values of *Dunaliella salina* grown under optimum conditions (70 ppm N, 10 % NaCl and light intensity 200 W). The results showed that *Dunaliella salina* could be a potential for mass production of antioxidant vitamins.

Key words: *Dunaliella salina*, β-carotene, tocopherols, ascorbic acid

Introduction

Microalgae is now considered one of the non-conventional source of vitamins, they contain several water and lipid –soluble vitamins. However, a number of vitamins are presented in higher concentrations in microalgae than in conventional foods, which traditional considered rich in vitamins (Fabregas and Herrero, 1990; De Roeck–Holtzhauer *et al.*, 1991, 1993; Brown and Framer, 1994; Venkataraman *et al.*, 1994). Some of the vitamins are of commercial interest such as vitamin E, vitamin C, β-carotene, thiamin, riboflavin, vitamin B and folic acid . Vitamin E, C and pro-vitamin A are produced by some algae in significant quantities and have a great market potential nowadays especially for use as an antioxidant (Harker and Young 1995, Zhang and Lee, 1997; Zhang *et al.*, 1997) . In this respect algal genus *Dunaliella* contains species which possess the unique ability to accumulate largre amounts of β-carotene both in nature or under some laboratory conditions. *Dunaliella berdawil* and *Dunaliella salina* accumulated β-carotene to at least 8–13 % of its dry weight when grown under stress conditions such as high light intensity, high salt concentration, extreme temperatures or nitrate deficiency (Borowitzka and Borowitzka, 1986; Abalde and Fabreges, 1991; Gomez- Pinchetti *et al.*, 1992).

The vitamins content of algae depend on several factors such as the genotype, the stage in the growth cycle, the nutritional status of algae, light intensity (photosynthetic rate) and other factors which affect growth and metabolism (Abalde and Fabregas, 1991). The vitamin content is, therefore, amenable to manipulation by varying the culture conditions as well as by strain selection or genetic engineering (Running *et al.*, 1994; Merchie *et al.*, 1995). Therefore, this experiment was conducted to study the antioxidant vitamins content of *Dunaliella salina* as affected by stress conditions to explore the most suitable conditions that enhance growth and antioxidant vitamins accumulation.

Materials and Methods

Algal source: *Dunaliella salina* was obtained from the Culture Collection of Dr. W. H. Thomas, La Jolla, CA ,U.S.A.

Growth conditions: Algae was cultivated in a growth medium containing 5mM $MgSO_4$, 0.3 mM $CaCl_2$, 0.2 mM KH_2PO_4, 1.5 μ M $FeCl_3$, 6 μM Na-EDTA, 50 mM $NaHCO_3$, 0.75 mM KNO_3, 7 μM H_3BO_3, 0.8 μM $MnCl_2$, 0.02 μM $ZnCl_2$ and 20 μM Tris –HCl, pH 8.0 (Ben-Amotz and Avron, 1988). KNO_3 was used as a nitrogen source with two different concentrations 70 and 5 ppm N. Also NaCl was used at different concentrations 1.7, 3.4 and 5.1M. Algae was cultivated in 2 L flasks . The cultures were gassed with 1.5 % volume CO_2 in air and algae were cultivated at 25 ± 3 °C, pH 8.5. The cultivated flasks were illuminated by continuous cool white fluorescent lamps at two different light intensity levels 200 and 400 W.

Growth measurements: The growth of *Dunaliella salina* was measured by dry weight methods and Optical Density (O.D) as described by Payer (1971).

Harvesting: Stationary-phase cells were harvested at 4°C by centrifugation at 6000 rpm for 15 min.

Extraction and identification of carotenoids: Carotenoids were extracted with petroleum ether according to Bjornland *et al.*, (1984). The β-carotene was identified by Reverced Phase –Thin Layer chromatography (Sherma and Fried, 1990) octadecyl–boned silica (RP-18, F254) plate 0.25 mm thickness was used with the solvent system, petroleum ether (40-60°C): acetonitrile: methanol (25 : 25 : 50, v/v/v). β-carotene was identified as a one spot at Rf 0.16.

Determination of β-carotene: β-carotene was determined by spectrophotometric method at 450 nm as described by Semeneko and Abdullaev (1980).

Extraction and determination of tocopherols: Tocopherols were extracted from algal cells with acetone: ethanol (7: 3, v/v) according to AOAC (1995) and were determined by spectrophotometric method at 534 nm using bathophenthroline as coloring reagent (AOAC., 1995).

Extraction and determination of ascorbic acid: Vitamin C was extracted by meta-phosphoric acid (2 % w/v) according to Augustin *et al.* (1985). The vitamin was determined by spectrophotometric method using 2, 6 di-chlorophenol indophenol dye as described by Augustin *et al.* (1985).

Results

β- carotene contents: β-carotene content (%) of *Dunaliella salina* grown on culture contained sufficient nitrogen source (70 ppm N) and NaCl (10%) under normal and high light intensity were 1.78 and 2.5% respectively (Table 1, Fig. 1a). Increased concentration of NaCl in growth medium to 20 and 30 % , increased β-carotene contents. They reached to 3.4, 4.9 % and 5.3, 6.43 % under the two mentioned light intensities which represented 190, 275 and 297, 361 % increase respectively compared to control treatment. β-carotene content (%) of *Dunaliella salina* grown in limit nitrogen (5 ppm) and high salt concentration (30 %) under low and high light intensity were 8.59 and 13.14 %, respectively. Therefore, β-carotene accumulation depended on both salt concentration and nitrogen content in medium as well as light intensity.

The maximal accumulation of β-carotene was obtained in *Dunaliella salina* cells when grown under combined stress conditions (nitrogen deficient (5ppm N), high NaCl concentration (30 %) and high light intensity (400 W). β-carotene values reached 13.14%

with yield 129.2 mg/l. This values represented more than 738 % compared to *Dunaliella salina* grown on normal culture conditions (Table 1).

Tocopherol contents: Nitrogen deficiency (5 ppm) increased the tocopherols content of *Dunaliella* cells from 0.07 to 0.25% more than 3571% increase compared with nitrogen rich media (Table 2, Fig. 1b). In addition, tocopherols content (%), was increased sharply with increase NaCl concentration in the nutrient solution. Also, the tocopherol content of *Dunaliella salina* show an inverse dependence on light intensity, being lowest at the low light intensity. For example, the tocopherol (%) was reached the maximum 1.23 % when *Dunaliella salina* grown on culture contained 5 ppm nitrogen, high NaCl concentration (30 %) under high light intensity. While at low light intensity, tocopherol (%) was reached only 0.41 %. These values, represented 1757 and 586 % compared to *Dunaliella salina* grown on optimum conditions.

Table 1: Effect of nitrogen and NaCl concentration and light intensity on ascorbic acid content of *Dunaliella salina*

	Light intensity					
	Low light intensity (200W)			High light intensity (400W)		
Treatments	Yield (mg/l)	(%)	100%	Yield (mg/l)	(%)	100%
70ppm nitrogen & 10% NaCl (control)	26.42	1.78	100	33.27	2.5	112
70ppm nitrogen & 10% NaCl (control)	38.45	3.4	190	64.7	5.3	297
70ppm nitrogen & 30% NaCl	45.62	4.9	275	71.3	6.43	361
5ppm nitrogen & 10% NaCl (control)	29.3	3.15	177	51.8	4.27	240
5ppm nitrogen & 10% NaCl	64.1	7.71	433	101.3	9.92	557
5ppm nitrogen & 30% NaCl	68.8	8.59	483	129.2	13.14	738

100%: Calculated % compared to algae grown under optimum conditions.

Ascorbic acid contents: Vitamin C was gradually increased as a result of increased NaCl concentration from 0.25 to 0.41 % at 30 % NaCl (Table 3, Fig. 1c). The maximum vitamin C (%) value was obtained when NaCl and nitrogen concentration were 30% and 5 ppm. The maximum accumulation of vitamin C (2.5 %) and its yield 24.62 mg/l was observed in medium containing high salt concentration and nitrogen deficient and exposed to high light intensity. This value represented 1000 % compared to *Dunaliella salina* grown on optimum conditions Generally, *Dunaliella salina* accumulated 13.1 % β-carotene, 1.2 % tocopherols and 2.5 % ascorbic acid (which is over the conventional source), when grown under stress growth conditions (high salt concentration, nitrogen deficient and high light intensity).

Table 2: Effect of nitrogen NaCl concentration and light intensity on tocopherols content of *Dunaliella salina*.

	Light intensity					
	Low light intensity (200W)			High light intensity (400W)		
Treatments	Yield	% (mg/l)	100%	Yield	% (mg/l)	100%
70ppm nitrogen & 10% NaCl (control)	0.9	0.07	100	2.5	0.19	271
70ppm nitrogen & 10% NaCl (control)	1.5	0.13	186	2.8	0.23	329
70ppm nitrogen & 30% NaCl	1.8	0.2	286	4.9	0.45	643
5ppm nitrogen & 10% NaCl (control)	2.3	0.25	357	7.4	0.61	871
5ppm nitrogen & 10% NaCl	3.1	0.37	529	9.4	0.92	1314
5ppm nitrogen & 30% NaCl	5.3	0.41	586	12.1	1.23	1757

100%: Calculated % compared to algae grown under optimum conditions.

Table 3: Effect of nitrogen NaCl concentration and light intensity on ascorbic acid content of *Dunaliella salina*

	Light intensity					
	Low light intensity (200W)			High light intensity (400W)		
Treatments	Yield	% (mg/l)	100%	Yield	% (mg/l)	100%
70ppm nitrogen & 10% NaCl (control)	3.2	0.25	100	3.0	0.23	132
70ppm nitrogen & 10% NaCl (control)	3.8	0.29	116	8.9	0.73	292
70ppm nitrogen & 30% NaCl	4.6	0.41	164	10.5	0.95	380
5ppm nitrogen & 10% NaCl (control)	4.9	0.47	188	14.5	1.2	480
5ppm nitrogen & 10% NaCl	9.4	1.33	532	20.5	2.01	804
5ppm nitrogen & 30% NaCl	12	1.5	600	24.62	2.5	1000

100%: Calculated % compared to algae grown under optimum conditions.

Discussion

The present study indicates that *Dunaliella salina* accumulates a significant quantity of β-carotene, vitamin C and vitamin E (reached to 129.0, 24.62 and 12.1 mg / l respectively). The increase of these antioxidant vitamins seems to occur as a response to nitrogen deficiency and high NaCl concentration in nutrient solution and exposed to high light intensity. Similar observation was given by Bortowizke and Bortowizke (1986); Ben Amotz and Avron (1988) and (1990) and Gomez- Pinchetti *et al.* (1992) who showed that *Dunaliella salina* has ability to accumulate large amounts of β - carotene (up to 10 – 14 % dry weight) when grown under extreme environment conditions (high salt concentration, high light intensity and nitrogen deficient). The extent of carotenoids accumulation depend on the high salinity, high light intensity and also, increased under nitrogen starvation (Semenenko and Abdullayev, 1980; Ben Amotz and Avron, 1988; 1990).

The relationship between the low nitrogen concentration in culture medium and accumulation of mentioned vitamins in *Dunaliella salina* cells are clear in the present studies. To understand the reason of these observations, Semenenko and Abdullaev (1980); Arad *et al.* (1993); Rice *et al.* (1994) and Bar *et al.* (1995) reported that the cell division of microalgae grown under nitrogen starvation conditions are blocked but the preservation of photosynthesis occurred. This situation leads to store specific substances (triglycerides, polysaccharides and carotenoids). The accumulation of these compounds was attributed to the fact that carbohydrates and carotenoids do not require nitrogen for their synthesis. Also, fat, carbohydrate and carotenoids synthesizing enzymes may be less susceptible to disorgaization than in the system responsible for other compounds synthesis (Fogg, 1975). For example *Dunaleilla salina* when grown under nitrogen starvation and hypertonic conditions the accumulated glycerol increased up to 40 % (Borowitzka and Borowitzka, 1986).

The increased synthesis of β-carotene in *Dunaliella salina* when grown in limit nitrogen medium may be function as a ' carbon sink'. Extreme carotenoids formation in this algae occurs when one or more metabolic intermediate pathways are inhibited by lack of substrate; however, photosynthesis still continues, at a reduced rate. In fact, photosynthesis must continue to supply sufficient energy for essential metabolic processes such as the Na^+ -efflux system and glycerol synthesis. One by –product of this photosynthesis is 3- phosphoglyceric acid and this must be further metabolized and the excess either stored or excreted. Furthermore, the stored form of this photosynthetically produced organic carbon must not inhibit cell function. β-carotene is a suitable neutral' compound that could serve this function. Many

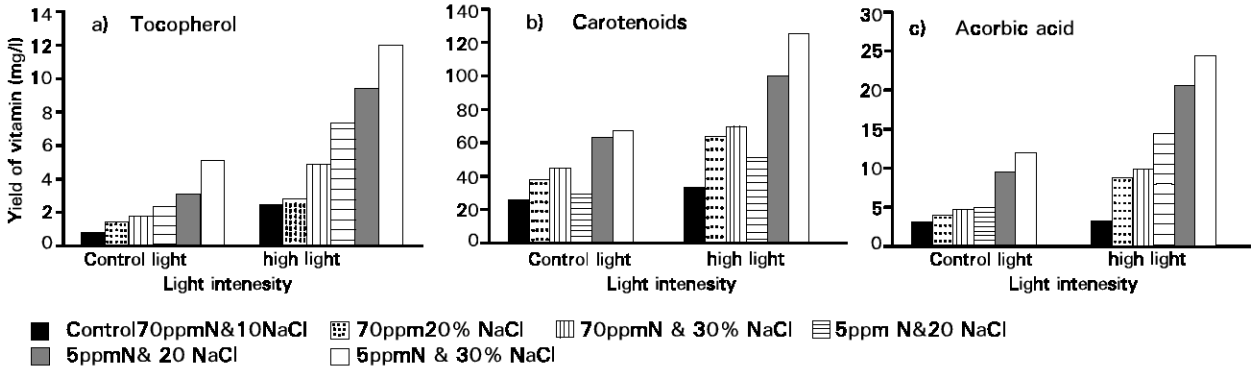

Fig. 1a-c: Tocopherol(a), carotenoids(b) and ascorbic acid (c) content of *Dunaliella salina* grown under indoor conditions as affected by different nitrogen conditions as affected by different nitrogen and NaCl concentrations and light intensity

other algae excrete large quantities of organic compounds once nutrient limitation sets in (Bortowizke and Bortowizke, 1986). This has been suggested to result from an imbalance between production and utilization of photosynthetic products . Thus the β-carotene may serve not only in a photoprotective function but also as a carbon sink. The latter hypothesis accounts for the observed effects of salinity, light and nutrient limitation on carotenogenesis (Bar *et al.*, 1995).

Algae has different enzyme systems for oxidizing the glycerol to dihydroxy acetone and the phosphorylation of the latter to dihyroxy acetone –phosphate is thought to be activated. Then dihyroxy acetone –phosphate is converted to glucose by a process which called gluconeogenesis and stored as polysaccharide (Ben Amotz and Avron, 1981). The glucose can be converted to L-gluconate by lactonation prossess produce the L-gluconolactone. Ascorbic acid is synthesized by dehydrogenation of L-gluconolactone to 2- ketogluconolactone, then the ascorbic acid formed by enolation of 2- ketogluconolactone.

The metabolic pathway for accumulation of fatty material when algae cells grown under nitrogen deficiency leads to catabolism of their compound throughout the β-oxidation to produce the excess of acetyl CoA. Thus the accumulation of carotenoids and tocopherol in algae cells cultivated under nitrogen starvation were increased, hence acetyl CoA serves as a precursor for synthesis of tocopherol and carotenoids. In other words, the accumulation of carotenoids and tocopherol in algal cells grown under nitrogen starvation may be due to the stimulation of lipolysis lead to production of acetyl CoA precursor. Tocopherol is synthesized by condensing the phytyl pyrophosphate with homogentistic acid to methyl phytyl quinones and subsequently methylation (Jsniszowska, 1987; Ogbonna *et al.*, 1998). However, tocopherol and vitamin C were accumulated in *Dunaliella salina* grown under stress conditions to protect the photosynthetic system from oxygen radical generated from high light intensity. They might act as antioxidants to quench the singlet oxygen radical and break down the chain reaction of lipid peroxidation (Malanga and Puntarule,1995; Malanga *et al.*, 1997).

The cellular content of antioxidant vitamins of *Dunaliella salina* increased with the increase of NaCl concentration levels β-carotene (4.9 %), vitamin C (0.41%) and vitamin E (0.2 %) (when cells grown under nitrogen sufficient medium, high salt concentration (30 %) and optimum light intensity) . Similar trend was observed for increasing the accumulation of antioxidant vitamin as result of increasing the light intensity. However, variation in β - carotene., vitamin C and vitamin E content of *Dunaliella salina* have been shown to result from light intensity ,salts concentration and nitrogen deficiency in culture medium. *Dunaliella salina* grown at a range of light intensities showed a linear increase in total carotenoids with increasing light intensity. The main reason for accumulation of carotenoids in intracellular of *Dunaliella salina* when exposed to high light intensity was attributed to the photoprotective function of these compounds which that chlorophyll a can be protected from bleaching in high light by β-carotene which quenches the singlet oxygen generated during photoxidation (Bortowizke and Bortowizke (1986); Foote *et al.* (1970) and Bar *et al.* (1995). The accumulation of carotenoids in some microalgae grown under high light intensity was also, found by Vectel *et al.* (1992) and Rise *et al.* (1994). Under high irradiation the photosynthetic apparatus does not sufficient to utilize light energy and the excess energy leads to the formation of highly active oxygen molecules. In this case, the primary carotenoids can not scavenge the radicals sufficiently. Additional mechanisms, therefore, are required for eliminating radicals or for reducing the illumination reaching the cell components under such conditions. Green microalgae such as *Chlorella* and *Haematococcus*, accumulate secondary carotenoids in response to stress conditions and may serve as protective agents against the effects of photo oxidation. Also, the carotenoids were accumulated in lipid layer of thylakoid membranes of alagl cells and act as a light filter to reduce irradiation of cell components, to prevent photo oxidative damage and to reduce water losses.

As a general mechanism the present results showed that the *Dunaliella salina* grown under combined stress conditions: (high NaCl concentration, high light intensity and nitrogen deficiency) correlate with accumulation of more efficient antioxidant vitamins. The negative effect of the various environmental stresses is at least partially due to the generation of active oxygen species (AOS). The AOS are produced during normal aerobic metabolism by the interaction between O_2 and electrons leaks from electron transport chains in the chloroplasts and mitochondria (Halliwell and Gutteridge, 1990). The AOS molecules (OH (hydroxyl), H_2O_2 (hydrogen peroxide), O_2 (superoxide) and O_2^1 (singlet oxygen) are not controlled by protective systems and may destroy proteins, lipid and pigments such as chlorophyll under stress conditions. The algae increased the production of antioxidant or elevated activities of protective enzymes to detoxify and eliminate the highly reactive oxygen species. The antioxidant defense system includes hydrophobic molecules such as carotenoids and α tocopherol to remove the singlet oxygen. While the hydrophilic antioxidant ascorbate and glutathion are effective chemical scavengers of oxygen radicals (Polle and Rennenberg, 1994; Shalate and Tal, 1998; Tausz *et al.*, 1998).

Finally *Dunaliella salina* cells are able to tolerate a very wide rang of salt concentration , high light intensity and limited nitrogen source making it a suitable candidate for outdoor cultivation in region where salt concentration, irradiant and temperature are high. Therefor, these data suggested that *Dunaliella salina* could be a potential candidate for mass production of antioxidant vitamins (provitamin A, vitamin E and vitamin C) in outdoor or indoor culture system.

References

Abalde, J. and J. Fabregas, 1991. β-carotene ,vitamin C and vitamin E content of the marine microalga *Dunaliella tertiolecta* culture with different nitrogen sources. Bioresources Techology, 38: 121-125 .

AOAC, 1995. (Official Methods of Analysis). Association of Official Analytical Chemists , 16 th .edit., K. Hlrich. Arlington Vargenia.

Arad, S., E. Cohen and A. Ben Amotz, 1993 . Accumulation of canthaxanthin in *Chorella emersonii*. Physiologia Plantarum., 87:232- 236

Augustin, J., P. B. Klein, D. Becker and B. P. Venugopal, 1985. Vitamin C. In: Methods of vitamin assay. Marcel dekker, inc. Now York, 323.

Bar, E., M. Rise, M. Vishkautsan and A. Shoshana, 1995. Pigment and structural changes in *Chlorella zofingiensis* upon light and nitrogen stress. J. Plantphysiol., 146: 527 –534.

Ben-Amotz, A. and M. Avron, 1988. The wavelength dependence of massive of carotene synthesis in *Dunaliella bardawil*. J. Phycol., 25: 178- 183.

Ben-Amotz, A. and M. Avron, 1990. The biotechnology of cultivating the halotolerant algae *Dunaliella*. Trends Biotechnol., 8: 121–126.

Ben-Amotz, A., Z. A. Kay and M. Avron, 1981. Glycerol β-carotene metabolism in the halotolerant alga *Dunaliella*: a model system for biosolar energy conversion, TIBS, 297–299.

Ben-Amotz, A., A. Shaish and M. Avron, 1991. The biotechnology of cultivating *Dunaliella* for production of β- carotene rich algae. Bioresource Technology, 38: 233–335.

Bjornland, T., G. Borch and S. Liaaen–Jenen, 1984. Configurational studies on red algae carotenoids. Phytochem., 23: 1711.

Borowitzka, A. M. and J. L. Borowitzka, 1986. *Dunaliella* In: Micro-algal biochology, Ed. Borowitzka, A.M. and Borowitzka J. L., Cambridge New York, 28-58.

Brown, R. M. and L. C. Farmer, 1994. Riboflavin content of six species of micro–algae used in mariculture. J. Appl. Phycol., 6: 61- 65.

De-Roeck-Holtzhauer, Y., C. Claire, F. Bresdin, L. Amicel and A. Derrien, 1991. Vitamin free amino acid and fatty acid compositions of some marine planktonic microalgae used in a quaculture. Botanica Marina, 36: 32S-34S.

De-Roeck-Holtzhauer, Y., I. Quere and C. Claire, 1993. Vitamin analysis of five planktonic micro-algae and one macro-algae. J. Appl. Phycol., 3; 259–264.

Fabregas, J. and C. Herrero, 1990. Vitamin content of four marine microalgae. Potential use as source of vitamin in nutrition. J. Indust. Microbiol., 5: 259-264.

Fogg, G. E., 1975. Metabolic pattern and growth in alga cultures and phytoplankton ecology. 2nd Ed., Univ. Wisconsin Press, pp: 52–61.

Foote, C. S., Y. S. Chang and R. W. Denny , 1970. Chemistry of single oxygen X. carotenoids quenching parallels biological protection. J. Am. Chem. Soc., 92 : 5216–18.

Gomez –Pinchetti, L. J., Z. Ramazahov, A. Fontes and G. Gareia Reina, 1992. Photosynthetic characteristis of *Dunaliella salina* (Chlorophyceae, Dunaliellales) in relation to β-carotene content. J. Appl. Phycol., 4: 11-15.

Harker, M. and J. A. Young, 1995. Inhibition of astaxanthin synthesis in the green algae *Haematococcus pluvialis*. Eur. J. Phycol., 30: 179–187.

Jsniszowska, W., 1987. Intracellular location of tocopherol biosynthesis in *Calendula officinalis* . Phytochem., 26 : 1403 – 1407.

Malanga, G. and S. Puntarulo, 1995. Oxidative stress and antioxidant content in Chlorella vulgaris after exposure to ultra-violet and β-radiation . Physiologia Plantarum, 94: 672 – 679.

Malanga, G., G. Calmanovici and S. Puntarulo, 1997. Oxidative damage to chloroplasts from *Chlorella vulgaris* exposed to Ultraviolet β- radiation. Physiol. Plant, 101: 455–462.

Merchie, G., P. Lavens, P. H. Dhert, M. Dehasque, H. Nelis, A. De-Leenheer and P. sorgeloos, 1995. Variation of ascorbic acid content in different live food organisms. Aquacul., 134: 325-337.

Ogbonna, J., T. Shota and H. Tanaka, 1998. Heterotrophic cultivation of *Euglena gracilis Z* for efficient production of α-tocopherol. J. Appl. Phycol., 10: 67–74.

Payer, H. D., 1971. First report upon the organization and experimental work of the Thailand German project on the production and utilization of single cell green algae as a protein source for human nutrition. Inst. Food Res. Product Development Kasetsar Univ., Bangkok, Thailand.

Poll, A. and H. Rennenberg, 1994. Photo oxidative stress in trees–In causes of photo oxidative stress and amelioration of defense systems in plants. Foyer C.H. and P. M. Mullineaux, eds.199-218. CRC Press Boca Raton.

Rise, M. E., M. Cohen, M. Vishkautsan, H. E. Cojocau, E. Gotrlieb and S. Arad, 1994. Accumulation of secondary carotenoids in *Chlorella zofingiensis*. J. Plantphysiol., 144: 287-292.

Running, A. J., J. R. Huss and T. Ph. Olson, 1994. Heterotrophic production of ascorbic acid by micro-algae. J. Appl. Phycol., 6: 99-104.

Semenenko, E. V. and A. A. Abdullaev, 1980. Parametric control of β-carotene biosynthesis in *Dunaliella salina* cells under conditions of intensive cultivation . Fizioloiya, Rastenii., 27 : 31-41.

Shalate, A. and M. Tal, 1998. The effect of salt stress on lipid peroxidation and antioxidant in the leaf of cultivated tomato and its wild salt-tolerant *Lycopersicon pennellii*. Physiol. Plant, 104: 169–174.

Sherma, J. and B. Fried, 1990. Handbook of thin-layer chromatography. Marcel Dekker , INC . New York, Basel, Hong Kong, 625–662.

Tausz, M., M. Soledad and D. Grill, 1998. Antioxidative defence and photoprotection in poine needles under field conditions. A multivariate approach to evaluate patterns of physiological responses at natural sites. Physiol. Plant, 104: 760–764.

Venkataraman, L. V., T. Somasekaran and E. W. Becker, 1994. Replacement value of blue–green algae (*Spirulina plateensis*) for fish meal and a vitamin, mineral premix for broiler chicks. Britsh poultry Science, 35: 373–381.

Zhang, D. H. and Y. K. Lee, 1997. Enhanced accumulation of secondary carotenoids in a mutant of green algae *Chlorococcum* sp. J. Appl. Phycol., 9: 459- 463.

Zhang, D. H., Y. K. Lee, M. L. Ng and S. M. Phang, 1997. Composition and accumulation of secondary carotenoids in *Chlorococcumm* sp. J. Appl. Phycol., 9: 147- 155.