Effect of Abiotic Stress on the Production of Carotenoids in *Dunaliella salina* and *Dunaliella bardawil*

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by

DILEEP KUMAR

11MSB0128

School of Bio Sciences and Technology

VIT University, Vellore 632014

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Declaration by the Candidate

I, hereby, declare that the thesis entitled, "**Effect of Abiotic Stress on the Production of Carotenoids in *Dunaliella salina and Dunaliella bardawil***" submitted by **DILEEP KUMAR, Reg. No. 11MSB0128** to VIT University, Vellore in fulfillment of the requirement for the award of the degree of **M.Sc Biotechnology** is a record of bonafide research work carried out by me under the guidance of **Dr. K. M. Gothandam** during the course of the year 2012 – 2013. I further declare that the work reported in this dissertation has not been submitted, and will not be submitted, either in part or in full, for the award of any other Degree or Diploma of this University or of any other Institute or University.

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Signature of the Guide Programme Chair Dean

Internal Examiner External Examiner

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Place : Vellore

Date : 28/05/2013 **Dileep Kumar**

Dedicated to my parents who have been a great source of motivation and inspiration and have supported me all the way

ABSTRACT

The aim of the present study to enhance carotenoid production of *Dunaliella sp*. under stress conditions such as NaCl stress, Nitrogen, sulphur, phosphorous depletion and supplementation of sodium bicarbonate. Pigments were analyzed using spectrophotometer every 7 days interval and amount of carotenoid influenced by these stresses were analyzed by HPLC. *Dunaliella salina* produced optimum carotenoid production were seen in supplementation of sodium bicarbonate 100mM and 150mM concentration (180.6µg/100mg and 180.5µg/100mg) in the medium and for *Dunaliella bardawil* also shows optimum production of carotenoid in 50mM concentration of sodium bicarbonate (8.34µg/100mg). The results indicated that supplementation of sodium bicarbonate in the culture medium could be effectively manipulated to enhance carotenoid production by *D. salina* and *D. bardawil.*

ABBREVIATIONS

D. salina	-	*Dunaliella salina*
D. bardawil	-	*Dunaliella bardawil*
β-carotene	-	Beta-carotene
NaCl	-	Sodium choride
N^-	-	Sulphur depletion
P^-	-	Phosphorous depletion
HPLC	-	High Performance Liquid Chromatography
S.D	-	Standard deviation
UV	-	Ultra-violet

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Chapter 1

Introduction

Algae are relatively simple aquatic organisms that capture light energy through photosynthesis, use it to convert inorganic substances into organic matter. Algae have been regarded as simple plants, but they actually span more than one domain including both Eukaryota, belonging to Chlorophyceae, Rhodophyceae(*Chlorella, Dunaliella* etc) and Prokaryota belonging to Cynophyceae group (Blue green algae eg., *spirulina*) (Gupta *et al.* 1981). Algae ranges from single celled organisms(Micro algae) to multicellular organisms, some with fairy complex differentiated form. The complex form are known as macro algae which includes the marine form such as seaweeds. They are devoid of well differentiated structure such as leaves, roots, flowers and other organ structures that characterize higher plants (Dawson *et al.* 1960; Fretsch *et al.*1977). All algae have photosynthetic machinery basically derived from the cyanobacteria (Dawson *et al.*1960; Fretsch *et al.* 1977), and so produce oxygen as a byproduct of photosynthesis.

These include the production of alternative food sources (Soeder and Binsack, 1978), health-promoting algal preparations (Kawaguchi *et al.*1980) and fine chemicals (Aaronson, *et al.*1980) as well as the use of algae in aquaculture (Persoon and Claus, 1980) and waste-water treatment (Oswald, *et al*. 1965). Two high-value speciality products are currently being produced in significant amounts from microalgae. The first, "linablue", is a phycobiliprotein concentrate produced from Spirulina species, and is used as a colourant in the food industry. The second, B-carotene, is produced from *Dunaliella* sp. and is used as a food colourant, a source of provitamin A and as a therapeutic antioxidant (Borowitzka *et al.*1986; Benemann *et al.*1989; Ben-Amotz and Avron *et al.*1989).

Two *Dunaliella* species were used in this study *D. bardawil* and *D. salina* and species were cultivated on both natural and artificial media under controlled experimental conditions.

1.1. *Dunaliella salina*

It is a unicellular bi-flagellated, naked, halotolerant green alga that is well known for its carotenoid producing capacity and produced carotenoids are mainly stored in a lipid globule form (AviSadka *et al.* 1990). The alga contains one large chloroplast and typical extrachloroplastic organelles, but unlike other green algae, *Dunaliella* lacks a rigid cell wall. Instead, the cell is covered by a plasma-membrane, which allows for rapid volume changes in response to extracellular osmolarity changes (Avron *et al.*1992; Ben-Amotz and Avron *et al.*1992).

Commercially cultivated strain for the production of natural carotenoids are *D. bradawill* and *D. salina.* The classification of *Dunaliella* is given below.

Table 1. Taxonomical classification of *Dunaliella*

Super kingdom	-	Eukaryota
Kingdom	-	Virindiplantse
Subkingdom	-	Phycobionta
Phylum	-	Chorophyta
Class	-	Chlorophyceae
Order	-	Volvocals
Family	-	Dunaliellaceae/Chlorophyceae
Genus	-	*Dunaliella*

(Borowtzka and shiva, *et al.* 2007)

It is a type of green microalgae which produces a large amount of carotenoids which are used as antioxidants and themost important reason to opt this species is that it can survive in high salt concentration and high intensity of light. Stress conditions high light intensity, high salinity, temperature and availability of nutrients acts as inductive features for production of β- carotene, it can proliferate high salinity (Borowitzka, M.A. *et al.*1990). *Dunaliella salina* has shown the higher requirement of inorganic carbon sources as it is grown in high salinity, even though there is lesser solubility of inorganic carbon. So nitrogen is depleted from the media to give stress as nutrients depletion also triggers production of carotenoids (A. Barriga *et al.* Gonzales *et al.*2003).

Intracellular B-carotene hyper-accumulation can be induced by high light intensity (Ben-Amotz *et al.*1986; Ben-Amotz *et al.*1989), nutrient limitation (Ben-Amotz*et al.*1987), or other environmental stresses such as high salt concentrations and high and low temperature (Ben-Amotz and Avron *et al.* 1983). B-carotene accumulation up to 14% dry wt. has been reported (Borowitzka, *et al*. 1986; Ben-Amotz and Avron, 1989).

The accumulated B-carotene is contained within oily globules in the inter thylakoid spaces of the chloroplast and comprises a mixture of the 9-cis and all-trans stereoisomers. Both the total amount of B-carotene accumulated and the all-trans to 9-cis ratio is dependent on the integral amount of light absorbed by the cell during one division cycle (Ben-Amotz, *et al.,* 1982, 1988).

Carotenoid accumulation by microalgae depends on nutritional and it can be stimulated by different NaCl concentration such as: salt stress, supplementation of sodium bicarbonate, nitrogen, sulphur and phosphorus depletion (JayantaTelukdar *et al.* 2012).

1.2. *Dunaliella bradawil*

Dunaliella bardawil, a β-carotene-accumulating halotolerant alga, hasbeen analyzed for the effect of various growth conditions on its pigment content, and compared with *Dunaliella salina*, a β-carotene non accumulating species. In *D. bardawill*, increasing light intensity and light period orinhibiting growth by various stress conditions such as nutrient deficiencyor high salt concentration caused a decrease in the content of chlorophyll per cell and in the increase in the amount of β-carotene per cell.

1.3. Carotenoids

Naturally occurring pigment, responsible for colour of fruits, vegetables and plants. Carotenoids are group of fat soluble pigments present in chloroplasts and chromoplasts of plants &other photosynthetic organisms like algae, some bacteria & fungi. Carotenoids can be of two types: Xanthophyll which containing oxygen in their structure & carotene which solely of hydrocarbon contents and lacks oxygen.

1.4. Biosynthesis and accumulation of carotenoids in *Dunaliella*

Carotenoids are isoprenoids synthesized by the isoprenoid pathway. Isopentanyl pyrophosphate (IPP) is the common precursor of many of the isoprenoid compound. Many of review stated that the initial steps of carotenoid synthesis are common to all carotenogenic organisms (Fraser and Bramley *et al*. 2004). C_{20}-geranylgeranyl pyrophosphate (GGPP) is synthesized through a series of condensation steps.The condensation of two GGPP molecules forms the precursor of most carotenoid, 15-cis-phytoene (C_{40}). This reaction is carried out by phytoene synthase, the first enzyme in the pathway specifically found in carotenogenic organisms (Armstrong *et al*.1990).

Conversion of phytoene to lycopene occurs through two desaturation steps, carried out by two desaturase, phytoene desaturase (PDS) and z-carotene desaturase (ZDS). Lycopene can be cyclized by several different lycopene cyclases to generate carotenes with either β-carotene or

ionone end group (Fig.2). Through two rounds of hydroxylation, β-carotene and α-carotene are converted to the xanthophylls, zeaxanthin or lutein respectively (Moise *et al*.2005). Reports on the enzyme and proteins involved in β-carotene regulation in *Dunaliella* are very scanty. β-carotene is accumulated in intraplastid lipid globules (Gongalez *et al.* 2005), which are stabilized and maintained by a peripherally associated 38 KD protein called the carotene globule protein (CgP). Probably CgP is involved in stabilizing the globules within the chloroplast (Katz *et al.*1995). Rabbani *et al.(*1998) suggested that induction of CgP and deposition of triacylgycerol are in parallel with β-carotene accumulation. In addition, inhibitors of lipid biosynthesis pathway affected the β-carotene formation (Katz *et al.*1995). All these reports imply that the formation and stabilization of globules are the main factors associated with β-carotene accumulation.

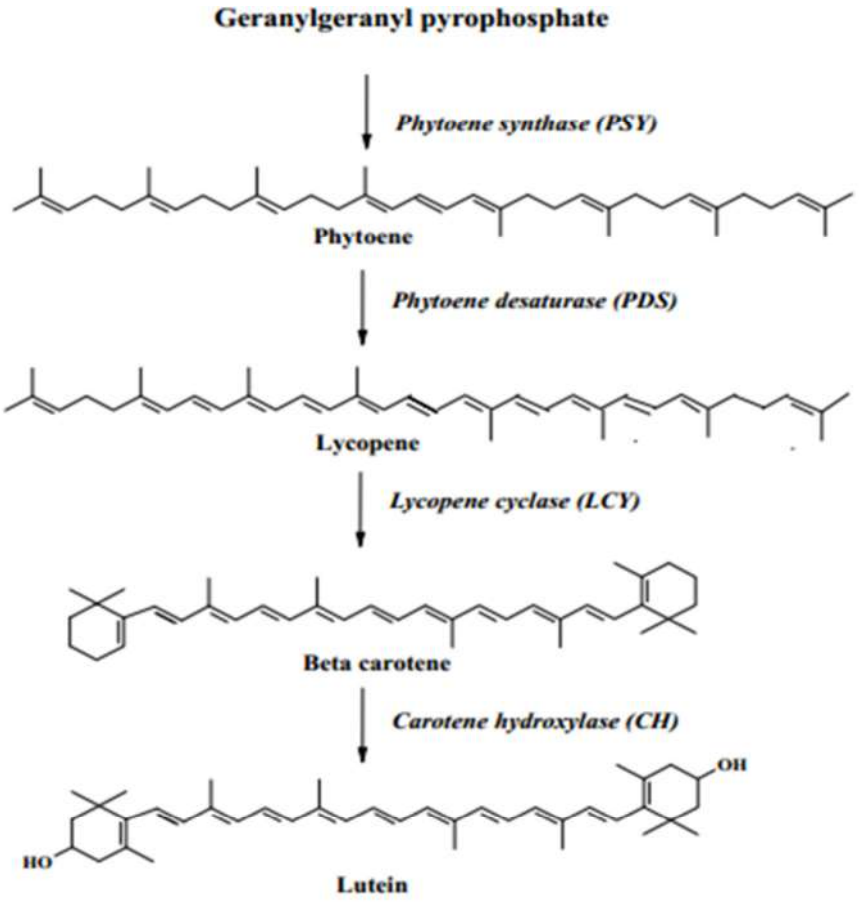

Fig.1 Brief description of carotenoid biosynthetic pathway in *Dunaliella.*

The carotene biosynthesis enzyme phytoene synthase (PSY), phytoenedesaturase (PDS), lycopene cyclase (lcy), and carotenoid hydroxylase (CH) are known to be the key steps in the carotenogenesis.

1.5. Carotenoids as nutrient

Antioxidants are a group of compounds that are produced by the body or that occur naturally in many foods. They may work together in the body to maintain our health by protecting against damages caused by free radicals, which can injure healthy cells and tissues, causing them to lose their structure and function. Oxygen is a highly reactive atom that can becomepart of potentially damaging molecules called free radicals. Free radicals are unstable and react rapidly and destructively with biomolecules such as protein, lipid, DNA and RNA in the body. Generation of free radicals is associated with peroxidation of lipids and proteins. This peroxidation results in cell structural damage and tissue injury, and finally leads to various health disorders such as Alzheimer's disease, cancer, atherosclerosis, diabetes, mellitus, hypertention and ageing. Antioxidant functions are associated with reduced DNA damage and lipid peroxidation and maintained immune function that are thought to prevent the development of some diseases (Packer and Colman, 2000; Valko *et al.* 2007).

Objective of the study

Chapter 2

Objective of the study

To evaluate the production of carotenoid under different stress conditions in *Dunaliella salina and Dunaliella bardawil.*

- Optimization of media, culture conditions, nutrient and environmental factors for the growth and production of β-carotene.
- Salt stress – 1M, 2M, 3M, 4M and 5M.
- Nitrogen depletion – complete removal of nitrogen source.
- Sulphur depletion – complete removal of sulphur source.
- Phosphate depletion – complete removal of phosphate source.
- Supplementation of sodium bicarbonate**.**
- Separation and chromatographic analysis of carotenoids present in stress condition by HPLC.

Review of literature

Chapter 3

Review of literature

Microalgae *Dunaliella salina* and *bardawil* include a very diverse group of eukaryotic and photosynthetic organisms that play important ecologicalroles and synthesize a vast array of natural products. Microalgal species have been widely used as simple model systems for research on higher plants due to shared physiological and biochemical reactions (Harris 2001, Hicks *et al.* 2001). With fast growth rates and low production costs relative to other transgenic expression systems, microalgae provide useful cell factories for the production of valuable chemical compounds and recombinant products (e.g., biofuels, novel carotenoids, vaccines and antibodies) (Fletcher et al. 2007, Mayfield *et al.* 2007, Greenwell et al. 2010).Various aspects of alga culture and the use of microalgae as bioreactors have been reviewed extensively (Chapin 1991, Dunahay *et al.* 1996, Geng *et al.* 2003, Rosenberg *et al.* 2008). Among eukaryotic microalgae, the relatively unique ability to accumulate glycerol and β-carotene in response to osmotic stress has made the halotolerant, unicellular, green alga *Dunaliella salina* an ideal model organism for dissecting the molecular mechanism of osmotic stress responses (Cowan *et al.* 1992, Pick 1998). However, as *D. salina* is one of a few microalgae currently cultivated on an industrial scale, this organism is rapidly becoming the focus of intense fundamental and applied research. The purpose of this review is to survey recent developments into cellular, biochemical, molecular biological, and biotechnological studies of *D. salina* with a focus on isoprenoid biosynthesis.

2.1. Carotenoid in Microalgae

Carotenoids produced by microalgae generally found in within oily globules in theinterthylakoid spaces of the chloroplast. Carotenoids can be of two types: Xanthophyll which containing oxygen in their structure & carotene which only of hydrocarbon contents and lacks oxygen (Grung*et al.* 1992), which are also essential nutrients for aquatic animals and humans.

These carotenoids are a group of fat soluble pigment (Ikan, 1991; Mastuno and Hiaro, *et al.* 1989) which are isoprenoid polyenes. There are over 600 known carotenoids, which are splits into two classes, xanthophylls and carotenes (Cunningham and Gantt, *et al.*1998). Carotenes are made up of carbon and hydrogen, without the oxygen group. Carotenoids with molecules containing oxygen, such as lutein and zeaxanthin, are known as xanthophylls (Cunningham,; Hirschberg, *et al*. 2001). Carotenoid form an important group of colorant too. Algae belonging to Chlorophyceae contain α-carotene, β-carotene, lutein, violaxanthin and neoxanthin with some species also accumulating astaxanthin (Johnson and Schroeder 1995; Grung *et al.* 1992). The

pigment are usually associated with in lipid globules located in the inter thylakoid space of the chloroplast within plastid (Ben-Amotz and Avron, *et al.* 1983)but occur as extra plastidic carotenoid in green algae *Haematococcus* (Lang,1968) *Dunaliella* produces produces carotenoid during all stages of growth, while *Haematococcus* synthesizes carotenoids during the formation of aplanospores after cessation of growth.

2.2. Methods of Carotenoid Induction

The ability of microalgae to survive in diverse and extreme conditions is reflected in the tremendous diversity and sometimes unusual pattern of carotenoids obtained from these microalgae. Moreover, some of these microalgae can also modify carotenoid metabolism efficiently in response to changes in environmental conditions (Walker *et al*.2005).Under optimal growth conditions, large amounts of algal biomass are produced but with relatively low carotenoid contents (Figure 1), which constitute about 5–20% of their dry cell weight. Essentially, microalgae biomass and carotene compete for photosynthetic assimilate and a reprogramming of physiological pathways is required to stimulate carotenoid biosynthesis. Under unfavorable environmental or stress conditions many microalgae alter their carotenoid biosynthetic pathways towards the formation and accumulation of neutral carotene (20–50%).

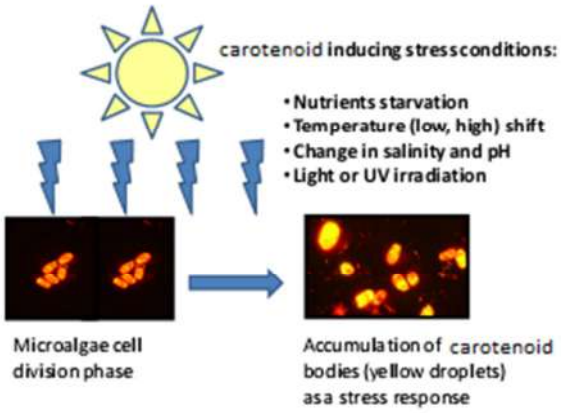

Figure 2. Carotenoid induction in microalgae under stress condition.

There has been a wide range of studies carried out on carotenoid induction techniques in microalgae such as the use of nutrients stress, including nitrogen and/or phosphorus starvation, light irradiation, pH, temperature, heavy metals and other chemicals. The following paragraphs

review the different carotenoids induction techniques and discuss their potential in different microalgae species.

2.3. Nutrient starvation

The supply of the limiting nutrients is eliminated under the state of nutrient starvation, where the growth of microalgae is affected, and the cells modify its physiological functions with accumulation of certain molecules to withstand in stress conditions. Nitrate and sulphate starvations are shown to cause the accumulation of β-carotene in the wild type (Becker*et al.* 2004). Nitrogen and phosphorus limitations affect the photosynthetic apparatus of *D. tertiolecta*; the optimum concentration of phosphorus for the growth of *D.salina* and *D. viridisranges* from 0.002 g/l to 0.025 g/l of KH_2PO_4, and high concentrations (>5 g/l) inhibit growth (Milko *et al.* 1962). The ratio of accessory photo protective pigments (α-carotene and β-carotene) to chlorophyll a increased under nitrogen (Geider *et al.* 1998) and phosphorus limited conditions (Krom and Brenner *et al.* 1991). Thecarotene/chlorophyll ratio increased by 33 times under N_2-starvation conditions in aqueous two phase systems (León *et al.* 2003). On the other hand, starvation had no observable effect on the levels of glycerol and α-carotene accumulation (Orset and Young *et al.*2000).

In the absence of chloride and sulphate, the growth of *D. salina*is greatly inhibited (Massyuk 1965). The optimum value of Cl^{-1}:SO_4^{-2}ratio for growth of *D. salina* is 3.2,whereas a high ratio of 8.6 would be required forβ-carotene accumulation. Interactions of anions and cations have specific effect on the alga. For example, MgSO4 supports growth of some *Dunaliella* sp. but $MgCl_2$ does not (Fujii *et al.* 1983). *D. salina* was grown separately in 11 different nutrients present in De Walne's medium at different concentrations to optimize the chlorophylls and β-carotene as well as the growth rate (Raja *et al*. 2004). Although different elements, namely, Zn, Co, Cu and Mo, are included in the growth medium of *Dunaliella*, there are no reports to show an absolute requirement for most of these elements (Borowitzka and Borowitzka*et al.*1988).

2.4. Salinity-Induced carotenoid production

The physiological mechanisms by which the euryhaline microalga *Dunaliella* tolerates rapid changes in the water potentials of the external medium are well documented. In response to high salinity and high irradiances, the micro-organisms employ different strategies to maintain the osmotic balance. Many halophilic bacteria involve accumulation of K^+ and Cl^-ions (Oren *et al*. 2002), but the intracellular salt concentration of *Dunaliella* is low. Such a low concentration of Na^+ levels are achieved by the activity of a Na^+pump in the cytoplasmic membrane (Katz and Pick*et al.*2001) and by Na^+ extraction coupled with direct electron transport (Ehrenfeld and Cousin *et al*. 1984). Size alteration takes place in lipid and plastoglobuli, chloroplast, nucleus and other cell organelles (Berube*et al.*1990). The low intracellular ionic concentrations and the need forosmotic equilibrium are met by the accumulation of glycerol as a compatible solute (Chitlaru and Pick *et al*. 1991). The accumulation of glycerol is regulated by the external water activity rather than the specific solute effect.

Electron spin resonance studies on *Dunaliella* suggested that physical changes brought by shrinking and swelling actas a sensor of changing environmental salt concentrations Curtain *et al.* 1983). The changes in lipid motion (membrane fluidity) can affect the activity of membrane-associated enzymes, which could initiate glycerol synthesis or dissimilation (McMurchie and Raison *et al.* 1979). Oren-Shamir *et al.* (1989) suggested that the activity of plasma membrane is essential for the recovery of *Dunaliell*a from a hypertonic shock. The flux of carbon between starch production in chloroplast, synthesis of glycerol in the cytoplasm and accumulation of β-carotene are some of the important physiological responses produced under stress conditions. In *D. salina,* the optimum salinity for growth lies between 18 and 22% NaCl whereas; the optimum salinity for carotenoid production is > 27% NaCl (Borowitzka *et al.* 1984). Maximum yield of β-carotene occurs at an intermediate salinity; however, the actual salinity has to be higher than the optimum level to avoid the problems of predatory protozoa and the non-carotenogenic algal competitor, *D. viridis* (Borowitzka and Borowitzka*et al.*1988).

2.5. Temperature Stress

Ben-Amotz (1996) observed that decreasing the temperature from 30 to 10°C caused a 4-fold increase in the 9-cis/all-trans-β-carotene ratio. In contrast to the above report, Gomez and Gonzalez (2005) have stated that the temperature decreases from 26 to 15 °C did not affect these ratios. The accumulation ofβ-carotene at low temperatures was stimulated under high irradiances (1,000μmol/m2/s) rather than at low irradiances as observed by Ben-Amotz (1996).

2.6. Light Irradiation Stress

Irradiance stress is created whenever the incident light intensity is greater than that needed to saturate photosynthesis. *Dunaliella* produces β-carotene in excess to over-come the irradiance stress and inhibits high photo inhibitory activity when blue light is used, intermediate with white and non-existent with red light (Ben-Amotz *et al.* 1989). When exposed to high-intensity blue light, the following sequence of events occur: photo inhibition of O_2 evolution, photo destruction of carotenoids in the order 9-cis-β-carotene, all-trans-β-carotene, chlorophyll and finally the destruction of the cell (Ben-Amotz *et al.*1989).

The accumulation ofβ-carotene (9-cis/all-trans isomers ratio) depends on the integral light intensity to which *Dunaliella is* exposed during a division cycle (Lers *et al.*1990). This ratio is promoted at low (20 to 50 μmol / photons/m^2/s^{-1}) rather than high irradiances (200 to 1250 μmol / photons/m^2/s^{-1}; Orset and Young *et al.* 2000). It has also been observed that the accumulation of β-carotene and its isomeric ratio are strongly dependent on the light intensity and the quality of light used (Senger *et al*. 1993).

In addition to the visible wavelength, ultraviolet (UV) radiation is also found to affect β-carotene production. Visible radiation is composed of 9% of near UV radiation (UV-A and UV-B: 290–400 nm) at the surface of earth, of which UV-B is absorbed by the stratospheric ozone leaving

the UV-A to reach the earth. Inhibition of phytoplankton photosynthesis by natural UV radiations in particular UV-A has been reported for several phytoplankton species (Herrmann *et al.* 1997). When the UV-A is supplemented along with high PPFDs, there is an increase of 80–310% inthe carotenoid and chlorophyll ratio per unit protein (Jahnke*et al*. 1999). Similar studies conducted by Salguero *et al.* (2005) proved that an 84h exposure to UV-A conditions stimulated an increase in the total carotene content with 3–5-fold increase in lutein and zeaxanthin contents. UV-Aexposure has the advantage of easy application andremoval, but under open-door cultivations, its applicationand control becomes a difficult task (Salguero *et al*.2005).

2.6. Regulation of β-carotene production

A review of the literature indicates that only a few reports are available on the enzymes and proteins involved in β-carotene regulation. β-carotene is accumulated into extra plastid lipid globules (Garcia-Gonzalez *et al*. 2005), which are stabilized and maintained by a peripherally associated 38 KD protein called the carotene globule protein (CgP). Probably, CgP is involved in stabilizing the globules within the chloroplast (Katz *et al.* 1995). Induction of CgP and deposition of triacylglycerol are in parallel with β-carotene accumulation. In addition, inhibitors of lipid biosynthetic pathway affected the β-carotene formation (Katz *et al.*1995). All these reports imply that the formation and stabilization of globules are the main factors associated withβ-carotene accumulation. Furthermore, an increased production of abscissic acid (ABA) and enhanced conversion of ABA to phaseic acid into the culture medium were found to regulate the β-carotene metabolism. The subsequent release of these compounds shifted the chemical equilibrium to favour carbon flux through the isoprenoid pathways with enhanced carotenoid biosynthesis (Cowan and Rose *et al.* 1991).

2.7. Characterization

2.7.1. Measurement of total carotenoid concentration by spectrophotometer

Spectrophotometer is a quantitative measurement method which uses the reflection or transmission properties of a material as a function of wavelength. Spectrophotometer is much less expensive and much faster than HPLC, however it is not able to estimate the individual content of carotenoids (Henriques *et al.* 2007). Carotenoids in solution follow the Beer-Lambert law in which absorbance (A) is equal to concentration multiplied by extinction coefficient ($A^{1\%}$). The extinction coefficient ($A^{1\%)}$ is the absorbance of a 1 % (10 g/liter) solution of carotenoid, in a defined solvent, in a 1-cm path-length cuvette, at a specific wavelength (λ). This equation can be used to determine the concentration of carotenoid in a standard sample or in a mixture or extract of carotenoids. The extinction coefficient can also be expressed in molarity (Wrolstad *et al.* 2005).

2.7.2. High-Performance Liquid Chromatography (HPLC)

High-performance liquid chromatography (or high-pressure liquid chromatography, HPLC) is a chromatographic technique to separate a mixture of compounds and is used in biochemistry and analytical chemistry to identify and quantify the individual components of the mixture. HPLC is used for analysis of carotenoids levels due to its ability to distinguish between similar conformational structures of carotenoids. The rapidity, non-destructiveness and ease in automating as well as small amount of required sample make the new HPLC methods a suitable technique for analysis of carotenoids (Oliver and Palou *et al.* 2000).

Materials and Methods

Chapter 4

Materials and Methods

3.1. Culture collection

Dunaliella salina and bardawill culture was obtained from CAS botany, Madras University and cultivated for stress analysis.

3.2. Chemicals used for experiments

Acetone (70%) for determination chlorophyll content and total carotenoids by spectrophotometrically, Standard β-carotene, solvents used for study were of analytical and HPLC grade. Experiments on cultivation and chemical analysis was done both in *D.salina* as well as *D.bradawill*.

3.3. Cultivation of *D. salina*

The cultures were maintained in Dewalne's medium using 10 ml strain and 90 ml medium.For media preparation marine water was used and cultivation of stressed culture done within 30 days in Dewalne's medium at 24±1temperature with manual shaking once or twice a day and kept for 12 hours light and 12 hours dark.The chemical compositions of the medium are given in table:

Table No. 2 : Compositions of Dewalne's medium

Chemicals	Quantity(L^{-1})	Quantity(Moles)
NaCl	125.06g	2.14M
$FeCl_3$	779.22mg	4.18mM
$MnCl_2.4H_2O$	360mg	1.82mM
H_3BO_3	*33.3mg*	*0.54mM*
$C_{10}H_{14}N_2Na_2O_8.2H_2O$	44.652mg	0.12mM
NaH_2PO_4	20.28mg	0.13mM
$NaNO_3$	100.3mg	1.18mM
$ZnCl_2$	20.829mg	0.10mM
$(NH_4)_6Mo_7O_{24}.4H_2O$	9mg	7.28nM
$CoCl_2.6H_2O$	19.04mg	0.08mM
$CuSO_4.5H_2O$	20mg	0.08mM

The media were dispensed into autoclaved 500ml flasks and sterilized at 121^0C at 15psi for 20 minutes. After media autoclaving 10% culture was inoculated in all flasks and incubated at 25^0C in 1000 lux light intensity. The cultures were observed for growth, chlorophyll content and carotenoid contents specially β - carotene in every 7 days intervals. And these parameters were compared with control culture.

3.4. Stress Study

During media preparation we used sodium chloride (Nacl) stress, Phosphate, Sulphur, Nitrogen depletion, and Supplementation of sodium bicarbonate. For Nacl stress 1M, 2M, 3M, 4M and 5M concentration was used. Control was made with standard Dewalne's medium.

3.5. Stress conditions

High light intensity, high salinity, temperature and availability of nutrients acts as inductive features for production of β- carotene, it can proliferate in salinity as high as 1M to 5M NaCl. Salinity effect on lag phase growth and lag phase length. *Dunaliella salina* and *Dunaliella bardawill* has shown the higher requirement of inorganic carbon sources as it is grown in high salinity, even though there is lesser solubility of inorganic carbon. So nitrogen, sulphur, phosphate, control and sodium bicarbonate is depleted from the media to give stress as nutrients depletion also triggers production of carotenoids.

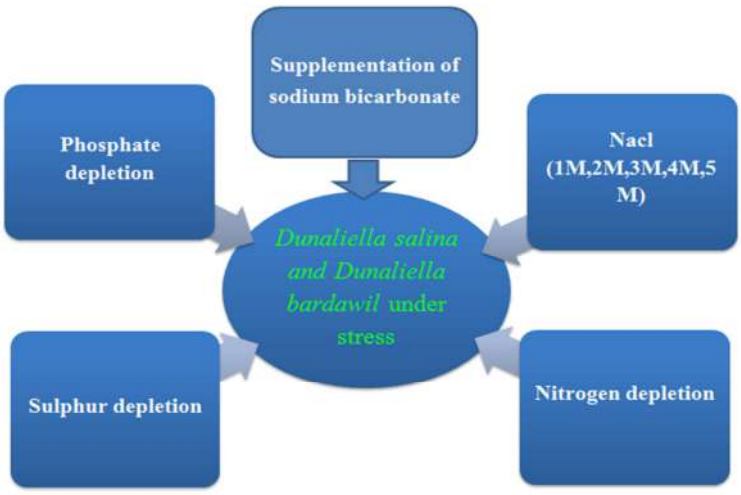

Fig 3. Stress study on *Dunaliella* species

3.6. Modification of culture conditions for carotenogenesis

In order to induce accumulation of carotenoids, algal cells were subjected to various stress conditions like light, salt and nutrients (Nitrogen, Phosphate, Sulphur) and addition of sodium bicarbonate as a carbon source.

3.7. Salinity stress

Dunaliella bardawil and *Dunaliella salina* were inoculated in De walne's media containing different salt concentration (1M, 2M, 3M & 4M) and incubated in automated chamber at 25±1°C with manual shaking of once or twice with 16 hours light and 8 hours dark.

3.8. Nutrient Stress

3.8.1. Nitrogen Starvation

To study the effect of nitrogen starvation in *Dunaliella salina* and *Dunaliella bardawil*. The algal culture were inoculated in nitrogen free De walne's media, the medium was deprived of nitrogen sources sodium nitrate ($NaNO_3$) and ammonium molybdate hydrate ($(NH_4)_6Mo_7O_{24}.4H_2O$).The algal culture were incubated in automated chamber at 25±1°C with manual shaking of once or twice a day with 16 hours light and 8 hours dark.

3.8.2. Sulphur Starvation

To study the effect of sulphur starvation in *Dunaliella salina* and *Dunaliella bardawil*.The algal culture were inoculated in sulphur free De walne's media, the medium was deprived of sulphur sources Copper (II) sulfate ($CuSO_4.5H_2O$) and and replaced with copper (II) chloride .The algal culture were incubated in automated chamber at 25±1°C with manual shaking of once or twice a day with 16 hours light and 8 hours dark.

3.8.3. Phosphorus Starvation

To study the effect of Phosphorus starvation in *Dunaliella salina* and *Dunaliella bardawil*. The algal culture were inoculated in phosphorus free De walne's media, the medium was deprived of phosphorus sources, sodium dihydrogen phosphate monohydrate (NaH_2PO_4).The algal culture were incubated in automated chamber at 25±1°C with manual shaking of once or twice a day with 16 hours light and 8 hours dark.

3.9. Carbon Supplementation

Dunaliella bardawil and *Dunaliella salina* were inoculated in De walne's media containing sodium bicarbonate as alternative carbon source in varied concentration (e.g. 50mM, 100mM, 150mM, & 200mM) to study the effect of alternative carbon source in lipid productivity and

incubated in automated chamber at 25±1°C with manual shaking of once or twice with 16 hours light and 8 hours dark.

3.10. Analytical methods

3.10.1. Estimation of pigments

3.10.1.1. Chlorophyll content a & b

Spectrophotometric determination of chlorophyll 'a' chlorophyll 'b' from the algal samples was carried out. From all the flasks 1ml*Dunaliella* cells culture were taken aseptically into a graduated tubes and centrifuged at 8000 rpm for 10 minutes. Then the fresh weighted samples were re-suspended in 80% acetone and centrifuged at 8000rpm for 10minutes. The pellet was extracted in acetone by homogenizing and extract was kept in dark for 1 hour. The absorbance of supernatant was measured spectrophotometrically at 645 nm and 662 nm against acetone blank. Concentration of chlorophyll a, b were calculated by the equation of Lichtenhaler (1987) and expressed as (µg/mL).

Chlorophyll a = $(11.24 \times O.D._{661}) - (2.04 \times O.D._{645})$ µg/ml

Chlorophyll b = $(20.13 \times O.D._{645}) - (4.19 \times O.D._{661})$ µg/ml

3.10.1.2. Carotenoids content

Carotenoids were extracted in acetone as mentioned above and analyzed spectrophotometrically by measuring the absorbance at 450 nm. Content of carotenoids was calculated according to method of Davis (1976) using extinction co-efficient 2500. Content of carotenoids were also expressed as Pico gram $cell^{-1}$.

Carotenoid content (mg/vol.) = $(OD_{450} \times$ volume of sample taken) / 2500 µg/g cells

3.10.2. Statistical analysis

All the experimental analysis of stressed samples were done in triplicate and average of chlorophyll a, chlorophyll b and β-carotene production for both samples *Dunaliella salina* and *Dunaliella bardawill*. Result value were expressed as mean ±SD in experiment. All the tests were considered to be statistically significant and highly significant respectively.

3.10.3. Algal sample extraction for HPLC

All *Dunaliella salina* and *bardawill* culture NaCl depletion (1M, 2M, 3M, 4M, 5M), N^-, P^-, S^-, sodium bicarbonate as well as control were taken. All culture was taken in 50 ml falcon tubes, centrifuged all one by one at 8000 rpm for 10 minutes. Than after transfer supernatant and kept finally 1 or 2 ml supernatant with remained pellet and vortex for 1-2 minutes properly. Than vortexed samples transfer in 2 ml eppendrof tubes, centrifuged again at 17500 rpm for 10 minutes. Transfer supernatant and dry with tissue paper, and weight were taken as a total biomass, later all pellet samples kept in -80^o C.

Each 2 ml of eppendrof tubes pellet samples kept for lyophilization over night at - 40^o C. After lyophilization each pellet samples Nacl (1M, 2M, 3M, 4M, 5M), N-, S-, control as well as control were weighted and later 5M depletion culture were removed due to no growth was present. 1 ml of acetone were added in all pellet samples and vortexed properly and kept in normal freeze temperature. Than after all samples were centrifuged at 8000 rpm for 10 minutes, transfer supernatant in new 2 ml eppendrof tubes as a HPLC samples and kept for overnight these samples for HPLC work (wright *et al.* (1997).

3.10.4. HPLC analysis of carotenoids

High performance liquid chromatography was employed to separate and quantify the individual carotenoids as detailed previously (A. RangaRao, G.A. Ravishankar *et al.* 2006). The chromatographic separation of extracted carotenoids was performed on a reverse phase C18 column maintained at 25^o in an isocratic elution mode. The mobile phase comprising acetonitrile/methanol/dichloromethane (70:10:20) was maintained at flow rate of 1 mL/min. The carotenoids were monitored using UV detector set at a wavelength of 450 nm. Carotenoids were identified by their characteristic spectral properties, relative mobilities and co-chromatography with authentic standards. Amount of individual carotenoids were determined by integration of the peak area.

Result and Discussion

Chapter 5

Result and Discussion

When *Dunaliella salina* and *Dunaliella bardawil* cells are stressed, they start to produce carotenoids. The green cell which is dominated by the chloroplast starts to turn orange. The chloroplast shrinks, chloroplast membranes decrease in size and carotenoid containing lipid globules are formed.

For the production of carotenoids in both sp. *Dunaliella salina* and *Dunaliella bardawil* different kinds of salinity and nutrients NaCl salt concentration (1M, 2M, 3M, 4M), N^-, S^-, & P^- as well as supplementation of sodium bicarbonate were used which cultivated under normally *in vivo* lab condition. Every one week of time interval stress culture were examined by a taken picture as a 7^{th}, 14^{th}, 21^{st}& 30^{th} days. The following pictures are mentioned here in different time interval:

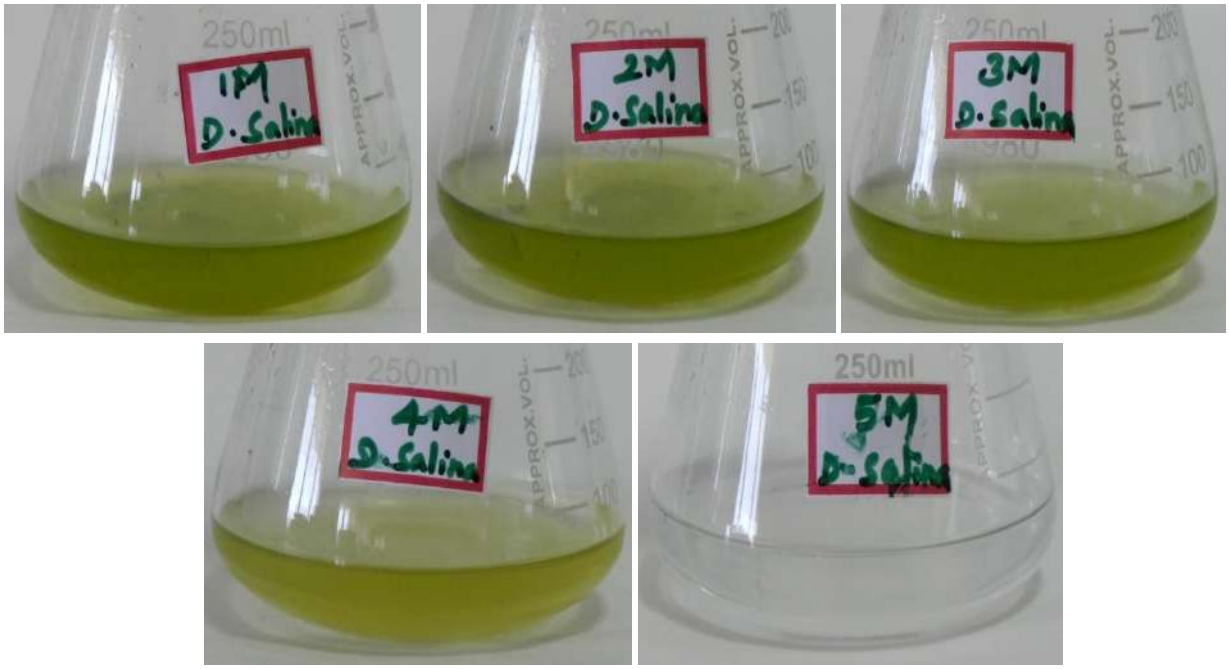

Fig.4. *Dunaliella salina* cultures under different level of salt stress. In which from 1M to 4M NaCl concentration showing slightly carotenoid production and in 5M NaCl concentration no growth were present (7^{th} days).

Fig.4.*Dunaliella salina* cultures under different level of nutrient stress under nitrogen and sulphur depleted medium. In which nitrogen showing high carotenoid production comparison to sulpher and control (7^{th} days).

Fig 6.*Dunaliella bardawil* cultures under different level of salt and nutrients stress. In which from 1M to 4M showing only chlorophyll content and in 5M completely blank due to no growth. In other part nutrients stress control and sulphur depletion culture are showing only chlorophyll content but in nitrogen and phosphorous depleted culture medium showing slightly growth of carotenoid production (7^{th} days).

4.1. Analysis of chlorophyll content a, b & total carotenoid

β -caroteneconcentrations were measured in µg/100 mg cells and chlorophyll a & b concentrations were measured in µg/ml for control, 1M to 4M NaCl stress, Nitrogen, Sulphur, Phosphate, control and Supplementation of sodium bicarbonate depleted culture after every 1 week interval by spectrophotometrically. Experiments were carried out in triplicates and average of chlorophyll a, chlorophyll b and β-carotene production for both samples *Dunaliella salina* and *Dunaliella bardawill* were given in tables and graphs for 7th day, 14th day, 21st day and 30th day.

Table 3.OD analyzed on 7th, 14th, 21st& 30th day of *Dunaliella salina* for salinity and nutrients stress.

7th day

Stress culture	Chlorophyll a (µg/ml)	Chlorophyll b (µg/ml)	Total carotenoid (pg/cell)
1M	1.63±0.04	0.59±0.26	0.14±0.00
2M	1.47±0.01	0.55±0.04	0.13±0.00
3M	1.37±0.04	0.36±0.02	0.12±0.00
4M	0.84±0.03	0.29±0.01	0.05±0.00
N-	0.48±0.02	0.20±0.02	0.09±0.00
S^-	1.52±0.09	0.31±0.07	0.07±0.00
P^-	0.04±0.02	0.91±0.14	0.12±0.00
Control	0.93±0.02	0.16±0.01	0.04±0.00

14th day

Stress culture	Chlorophyll a (µg/ml)	Chlorophyll b (µg/ml)	Total carotenoid (pg/cell)
1M	3.27±0.09	0.95±0.09	0.37±0.01
2M	2.96±0.03	1.11±0.10	0.28±0.00
3M	2.75±0.10	0.74±0.06	0.26±0.00
4M	1.69±0.10	0.59±0.03	0.25±0.01
N^-	0.98±0.06	0.41±0.04	0.11±0.00
S^-	3.05±0.21	0.63±0.17	0.14±0.01
P^-	0.18±0.02	0.05±0.01	0.36±0.12
Control	1.87±0.06	0.34±0.03	0.09±0.00

21st day

Stress culture	Chlorophyll a (µg/ml)	Chlorophyll b (µg/ml)	Total carotenoid (pg/cell)
1M	4.01±0.02	1.02±0.03	0.60±0.00
2M	2.78±0.03	0.64±0.05	0.49±0.00
3M	3.62±0.03	0.93±0.06	0.59±0.00
4M	2.44±0.02	6.22±0.02	0.47±0.00
N^-	1.09±0.04	0.48±0.07	0.81±0.00
S^-	0.10±0.01	0.11±0.00	0.35±0.03
P-	0.22±0.02	0.11± 0.00	0.264±0.03
Control	2.26±0.02	1.19±0.03	0.11±0.00

30th day

Stress culture	Chlorophyll a (µg/ml)	Chlorophyll b (µg/ml)	Total carotenoid (pg/cell)
1M	4.73±0.04	1.04±0.04	0.82±0.00
2M	2.61±0.04	0.23±0.04	0.71±0.00
3M	4.46±0.03	1.12±0.05	0.79±0.18
4M	3.93±0.04	4.43±0.04	0.69±0.00
N^-	1.11±0.10	0.99±0.57	0.29±0.00
S^-	0.24±0.03	0.09±0.02	0.39±0.04
P^-	0.81±0.15	0.50±0.43	0.28±0.01
Control	2.62±0.06	2.05±0.08	0.13±0.00

Table No 4.OD analyzed on 7th, 14th, 21st& 30thdays of *Dunaliella bardawil* for salinity and nutrients stress

7th days

Stress culture	Chlorophyll a (µg/ml)	Chlorophyll b (µg/ml)	Total carotenoid (pg/cell)
1M	1.03±0.00	5.57±0.06	0.11±0.00
2M	1.54±0.04	7.22±0.07	0.13±0.00
3M	2.34±0.03	8.23±0.11	0.17±0.01
4M	0.41±0.03	3.23±0.04	0.23±0.00
5M	0	0	0
N^-	1.11±0.03	5.12±0.03	0.18±0.00
P^-	1.27±0.01	1.08±0.02	0.011±0.00
S^-	3.23±0.48	6.13±0.01	0.17±0.00
C	1.23±0.02	7.1±0.05	0.25±0.00

14th days

Stress culture	Chlorophyll a (µg/ml)	Chlorophyll b (µg/ml)	Total carotenoid (pg/cell)
1M	1.07±0.02	4.08±0.09	0.18±0.00
2M	1.68±0.00	5.73±0.14	0.24±0.00
3M	3.57±0.03	6.35±0.10	0.32±0.01
4M	0.84±0.22	2.72±0.00	0.17±0.00
5M	0	0	0
N^-	0.97±0.05	3.69±0.04	0.32±0.00
P^-	1.03±0.01	0.51±0.00	0.21±0.00
S^-	3.47±0.72	4.65±0.01	0.23±0.00
C	1.59±0.01	5.42±0.03	0.29±0.00

21st days

Stress culture	Chlorophyll a (µg/ml)	Chlorophyll b (µg/ml)	Total carotenoid (pg/cell)
1M	1.08±0.06	2.55±0.08	0.013±0.00
2M	1.67±0.03	3.84±0.03	0.012±0.01
3M	3.37±0.08	5.76±0.08	0.10±0.00
4M	0.79±0.00	1.90±0.07	0.15±0.00
5M	0	0	0
N-	0.90±0.04	2.44±0.01	0.15±0.00
P^-	0.64±0.02	0.33±0.00	0.20±0.00
S-	2.62±0.04	3.21±0.02	0.23±0.00
C	1.61±0.04	4.00±0.10	0.39±0.00

30th days

Stress culture	Chlorophyll a (µg/ml)	Chlorophyll b (µg/ml)	Total carotenoid (pg/cell)
1M	0.59±0.07	1.17±0.14	0.10±0.00
2M	0.79±0.05	1.96±0.11	0.16±0.00
3M	1.67±0.04	3.09±0.05	0.26±0.00
4M	0.18±0.05	1.00±0.07	0.22±0.01
5M	0	0	0
N-	0.37±0.02	1.50±0.09	0.26±0.00
P^-	0.27±0.03	0.23±0.07	0.25±0.00
S^-	1.03±0.04	1.47±0.06	0.34±0.00
C	0.72±0.02	2.25±0.02	0.42±0.00

Table No 5. OD analyzed on 7th, 14th, 21st& 30th days for *Dunaliella salina* for carbon supplementation

7 th days

Stress culture	Chlorophyll a (µg/ml)	Chlorophyll b (µg/ml)	Total carotenoid (pg/cell)
0mM	0.12±0.06	1.36±0.27	0.05±0.00
50mM	1.82±0.03	0.19±0.13	0.09±0.00
100Mm	0.86±0.03	0.36±0.16	0.12±0.00
150Mm	0.93±0.09	0.01±0.05	0.15±0.00
200mM	0.16±0.03	0.18±0.05	0.16±0.00

14st days

Stress culture	Chlorophyll a (µg/ml)	Chlorophyll b (µg/ml)	Total carotenoid (pg/cell)
0mM	3.19±2.56	1.99±0.99	0.08±0.04
50m	3.69±0.01	0.62±0.04	0.19±0.00
100m	1.95±0.01	0.87±0.03	0.24±0.00
150m	1.30±0.03	0.35±0.07	0.30±0.00
200m	0.46±0.02	0.07±0.04	0.32±0.00

21st days

Stress culture	Chlorophyll a (µg/ml)	Chlorophyll b (µg/ml)	Total carotenoid (pg/cell)
0Mm	1.81±1.56	0.58±0.60	0.05±0.03
50m	2.31±0.15	0.25±0.16	0.12±0.00
100m	1.10±0.09	0.50±0.13	0.15±0.00
150m	0.84±0.10	0.15±0.06	0.19±0.01
200m	0.24±0.04	0.12±0.02	0.20±0.01

30th days

Stress culture	Chlorophyll a (µg/ml)	Chlorophyll b (µg/ml)	Total carotenoid (pg/cell)
0mM	9.36±0.15	2.85±0.09	0.22±0.00
50m	7.33±0.03	1.20±0.03	0.39±0.00
100m	3.43±0.03	1.92±0.03	0.49±0.00
150m	2.61±0.04	0.69±0.03	0.61±0.00
200m	0.93±0.01	0.02±0.05	0.64±0.00

Table No 6. OD analyzed on 7th, 14th, 21st& 30th days for *Dunaliella bardawil* for carbon supplementation

7th days

Stress culture	Chlorophyll a (µg/ml)	Chlorophyll b (µg/ml)	Total carotenoid (pg/cell)
0mm	1.78±0.03	0.51±0.01	0.07±0.00
50m	3.78±0.02	0.31±0.00	0.25±0.01
100mm	1.32±0.08	0.61±0.03	0.12±0.00
150mm	0.78±0.17	0.34±0.01	0.13±0.00
200mm	1.08±0.07	0.47±0.04	0.08±0.00

14th days

Stress culture	Chlorophyll a (µg/ml)	Chlorophyll b (µg/ml)	Total carotenoid (pg/cell)
0mm	3.41±0.08	1.12±0.02	0.15±0.00
50mm	7.88±0.04	0.72±0.01	0.17±0.00
100mm	3.71±0.02	1.35±0.03	0.19±0.00
150mm	2.14±0.05	0.89±0.01	0.24±0.00
200mm	2.53±0.03	1.05±0.05	0.21±0.00

21th days

Stress culture	Chlorophyll a (µg/ml)	Chlorophyll b (µg/ml)	Total carotenoid (pg/cell)
0mm	5.48±0.04	1.67±0.03	0.18±0.00
50mm	11.3±0.12	1.24±0.02	0.024±0.00
100mm	5.67±0.03	1.79±0.00	0.26±0.00
150mm	3.02±0.02	0.91±0.04	0.33±0.00
200mm	3.08±0.05	1.32±0.01	0.22±0.00

30th days

Stress culture	Chlorophyll a (µg/ml)	Chlorophyll b (µg/ml)	Total carotenoid (pg/cell)
0mm	7.02±0.09	2.24±0.07	0.27±0.00
50mm	16.1±0.17	1.64±0.08	0.29±0.00
100mm	7.39±0.14	2.86±0.05	0.38±0.00
150mm	4.49±0.06	1.88±0.03	0.43±0.00
200mm	5.13±0.08	2.34±0.04	0.34±0.00

4.2. Separation and chromatographic analysis of carotenoids present in stress condition culture by HPLC

Chromatogram has shown clear separation of chlorophyll and β-carotene as seen in both green and red cell extracts of *Dunaliella.* The β-carotene eluted after 30 min and was identified with standard β-carotene. A clear separation of carotenoids was noticed when methanol was used as the solvent. The chromatogram of *Dunaliella* shows the presence of β-carotene as the major component along with other carotenoids. The identified peaks were confirmed by determination of relative retention time and by spiking with standard β-carotene.

4.2.1. Salinity and nutrients stress: HPLC chromatogram analysis of *Dunaliella salina* and *Dunaliella bardawil*

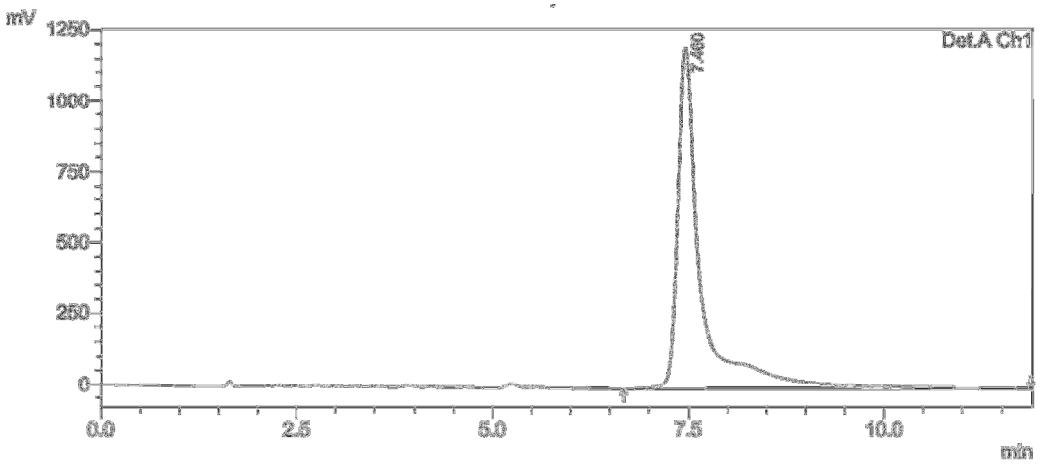

Fig.7. HPLC chromatogram of standard β-carotene with retention time at 7.460 minutes

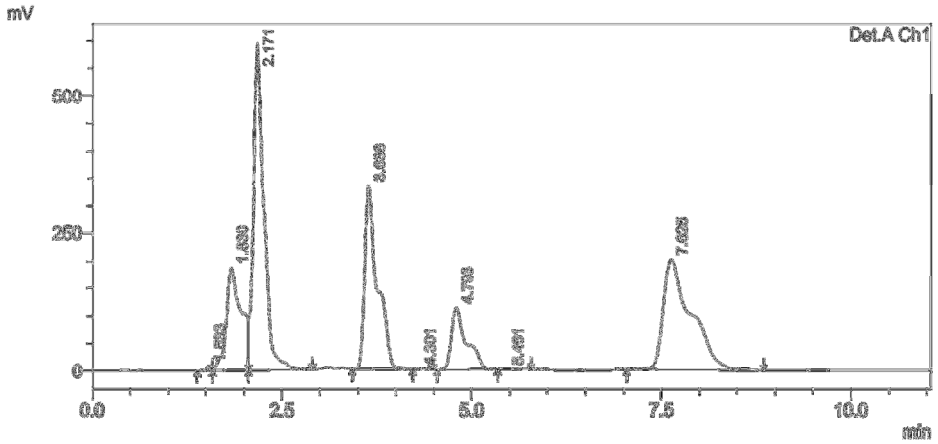

Fig.8. HPLC chromatogram of control *Dunaliella salina* algae with β-carotene at retention time 7.625 minutes.

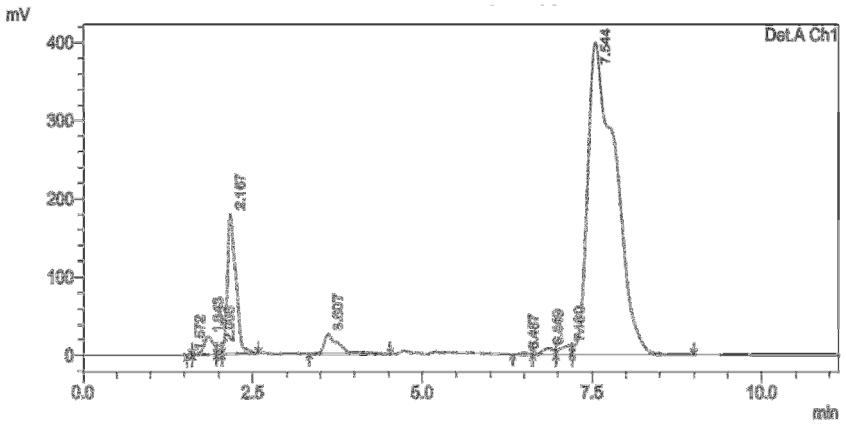

Fig.9.HPLC chromatogram of 1M (Nacl) *Dunaliella salina* algae with β-carotene at retention time 7.544 minutes.

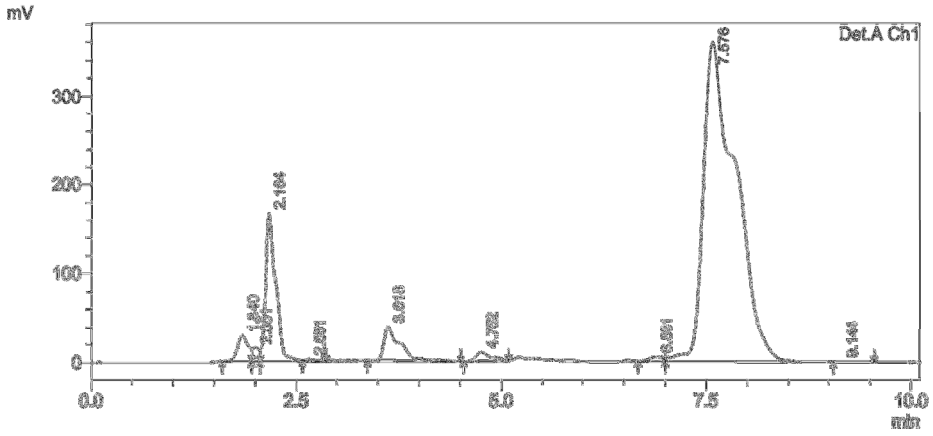

Fig.10. HPLC chromatogram of 2M (Nacl) *Dunaliella salina* algae with β-carotene at retention time 7.576 minutes.

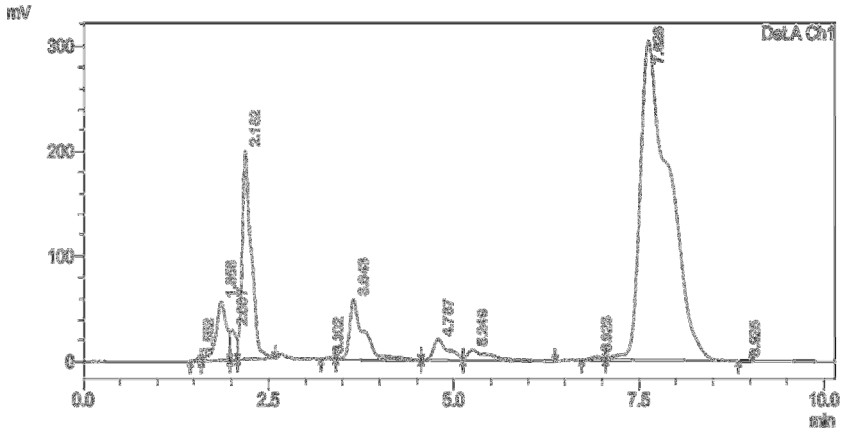

Fig.11. HPLC chromatogram of 3M (Nacl) *Dunaliella salina* algae with β-carotene at retention time 7.626 minutes.

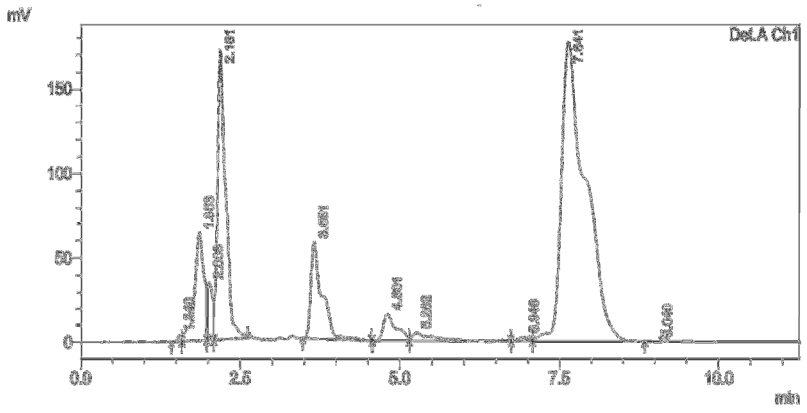

Fig.12. HPLC chromatogram of 4M (Nacl) *Dunaliella salina* algae with β-carotene at retention time 7.641 minutes.

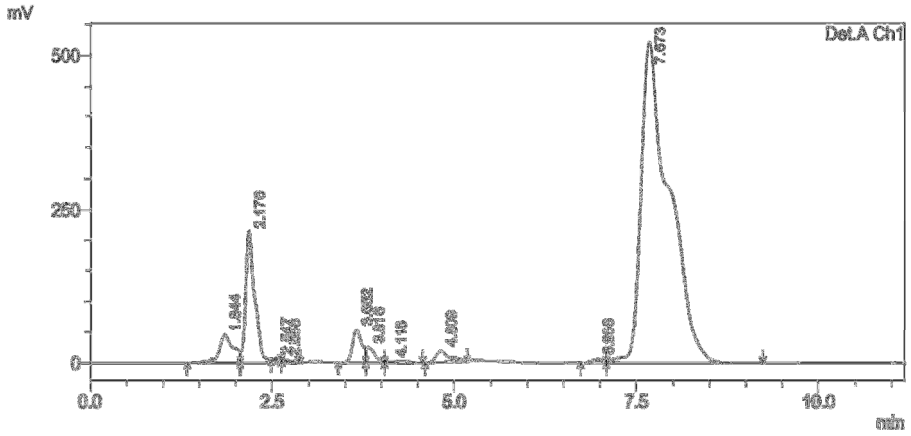

Fig.13. HPLC chromatogram of N-*Dunaliella salina* algae with β-carotene at retention time 7.673 minutes.

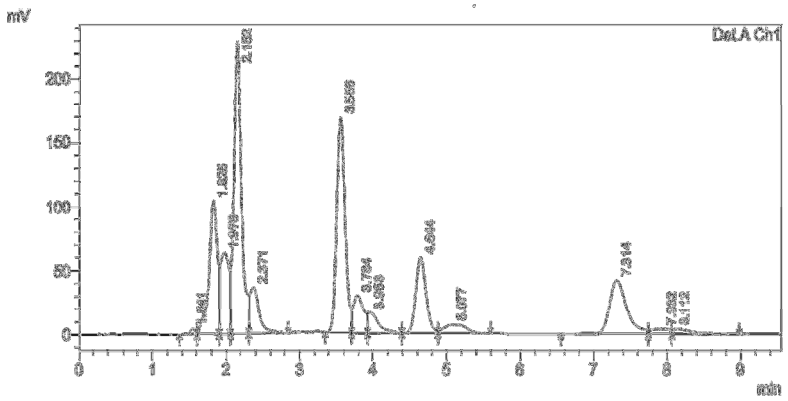

Fig.14. HPLC chromatogram of S-*Dunaliella salina* algae with β-carotene at retention time 7.314 minutes.

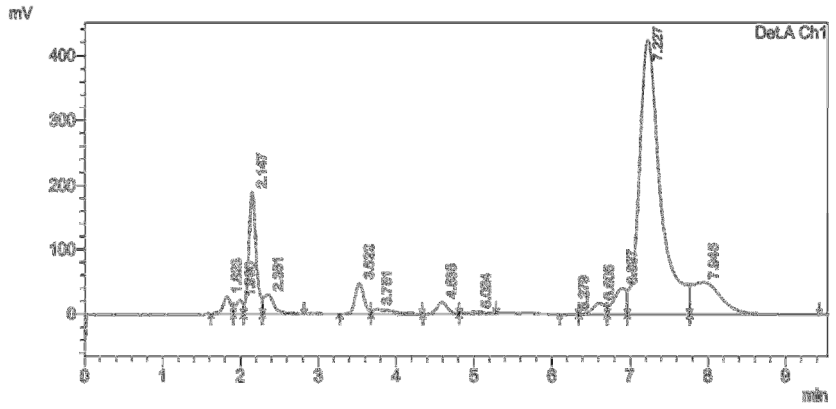

Fig.15. HPLC chromatogram of P*Dunaliella salina* algae with β-carotene at retention time 7.227 minutes.

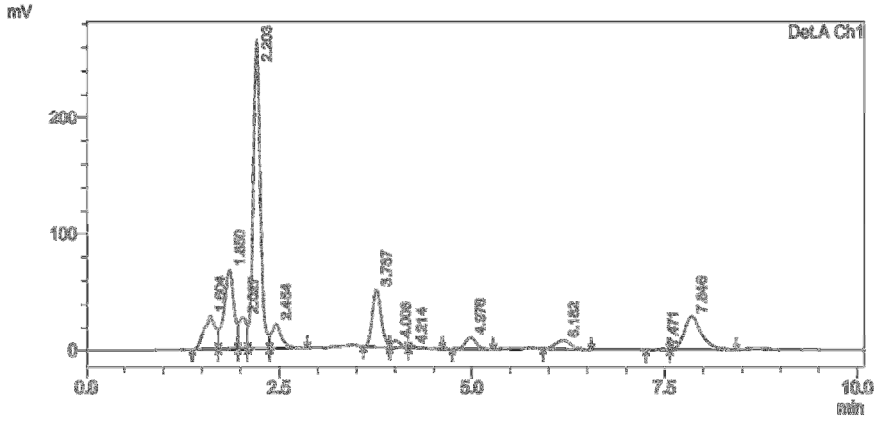

Fig.16. HPLC chromatogram of Control (Nacl) *Dunaliella bardawil* algae with β-carotene at retention time 7.846 minutes.

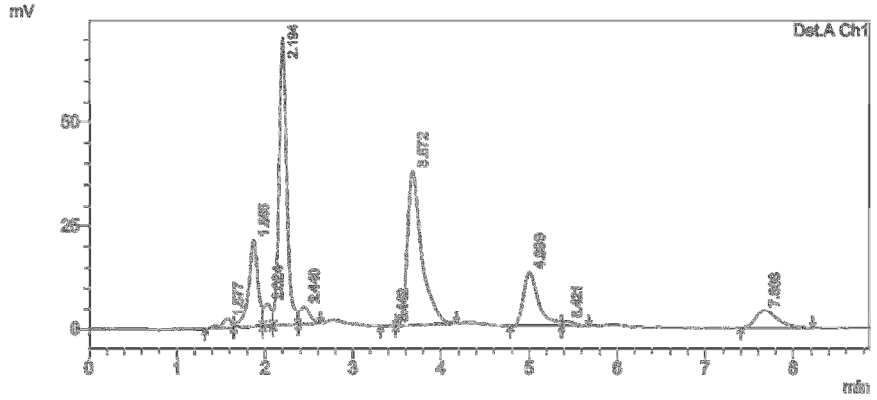

Fig.17. HPLC chromatogram of 1M (Nacl) *Dunaliella bardawil* algae with β-carotene at retention time 7.668 minutes.

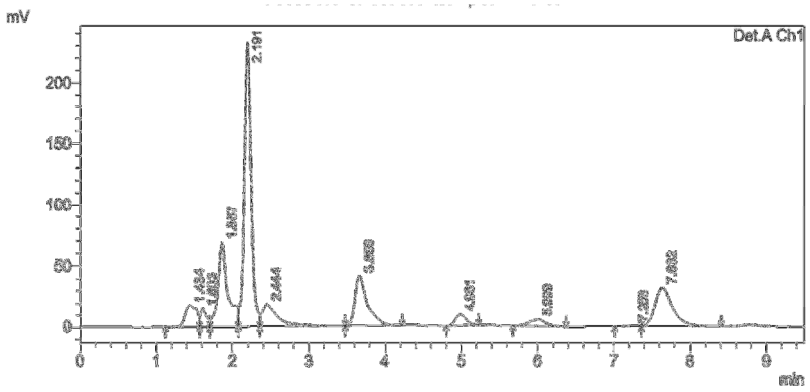

Fig.18. HPLC chromatogram of 2M (Nacl) *Dunaliella bardawil* algae with β-carotene at retention time 7.632 minutes.

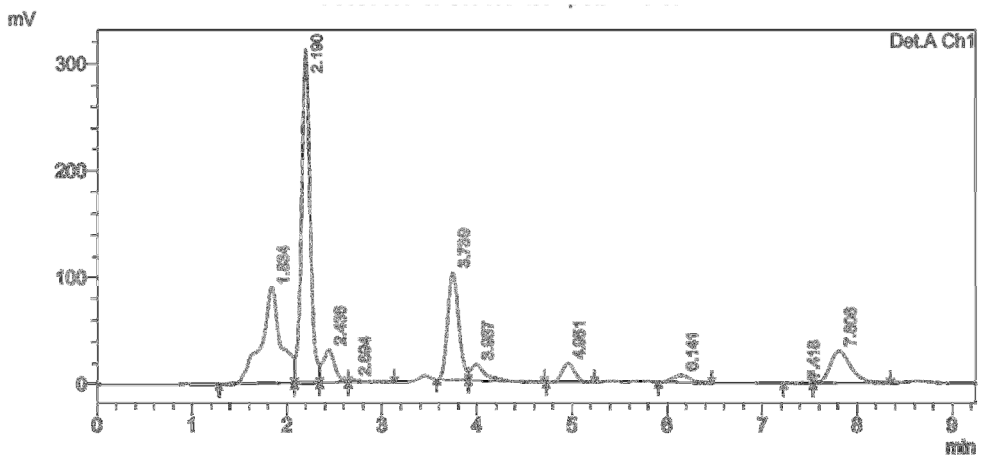

Fig.19. HPLC chromatogram of 3M (Nacl) *Dunaliella bardawil* algae with β-carotene at retention time 7.806 minutes.

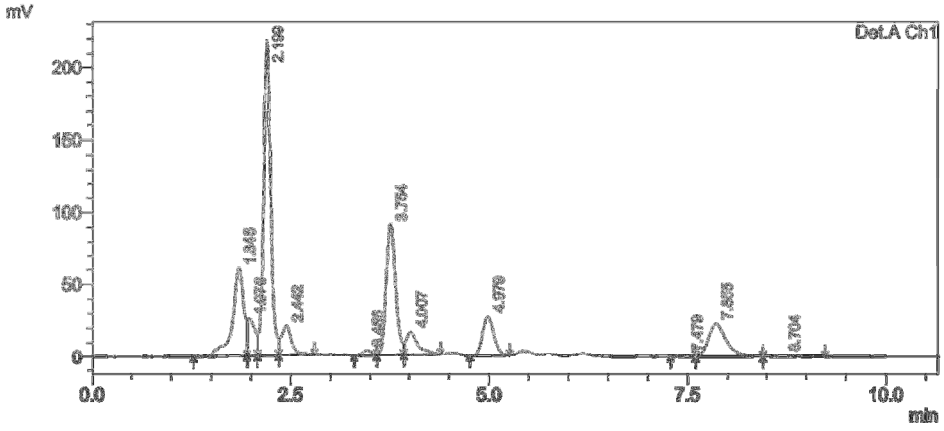

Fig.20. HPLC chromatogram of 4M (Nacl) *Dunaliella bardawil* algae with β-carotene at retention time 7.855 minutes.

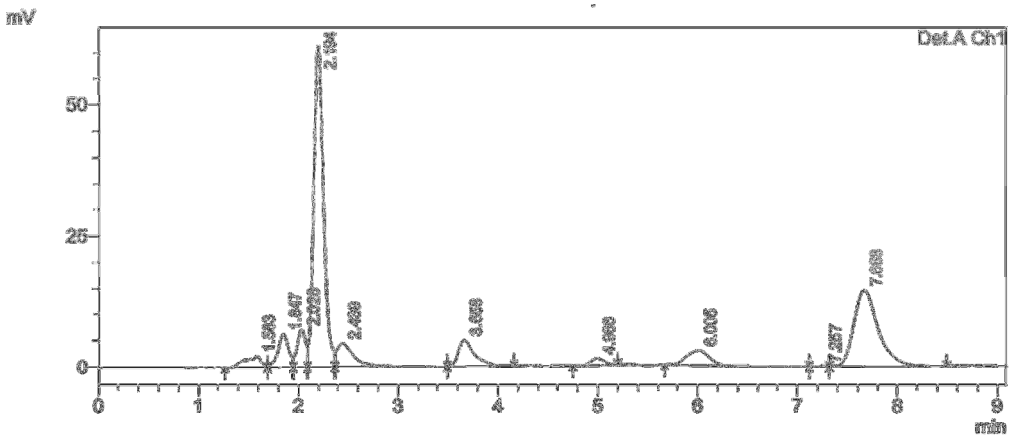

Fig.21. HPLC chromatogram of N-*Dunaliella bardawil* algae with β-carotene at retention time 7.663 minutes.

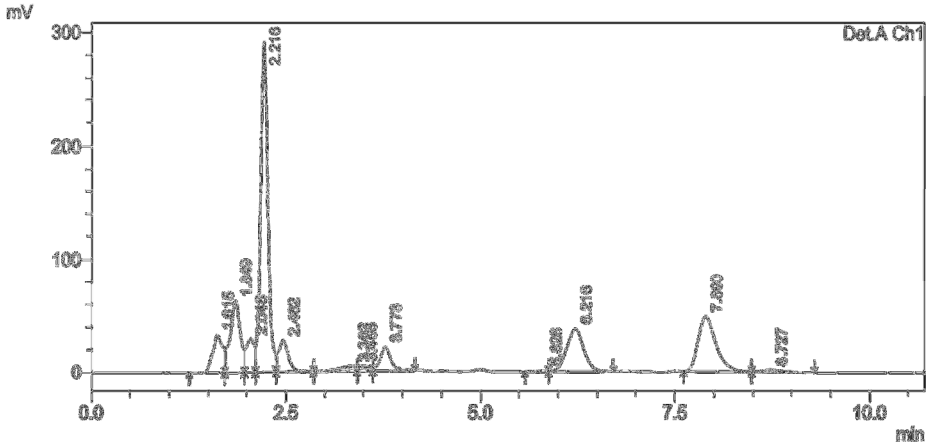

Fig.22. HPLC chromatogram of S-*Dunaliella bardawil* algae with β-carotene at retention time 7.890 minutes.

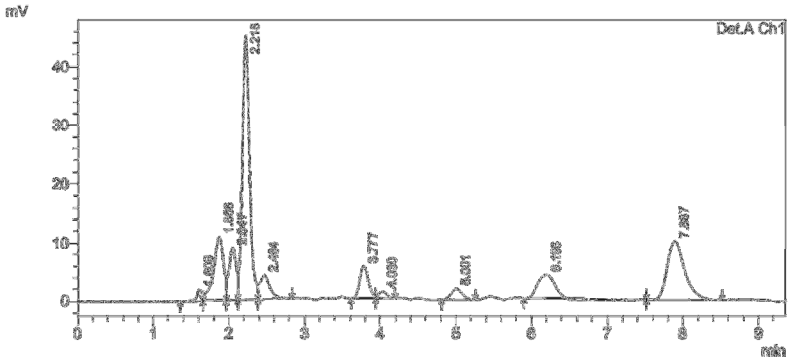

Fig.23. HPLC chromatogram of P-*Dunaliella bardawil* algae with β-carotene at retention time 7.887 minutes.

4.2.2. Carbon supplementation: HPLC chromatogram analysis.

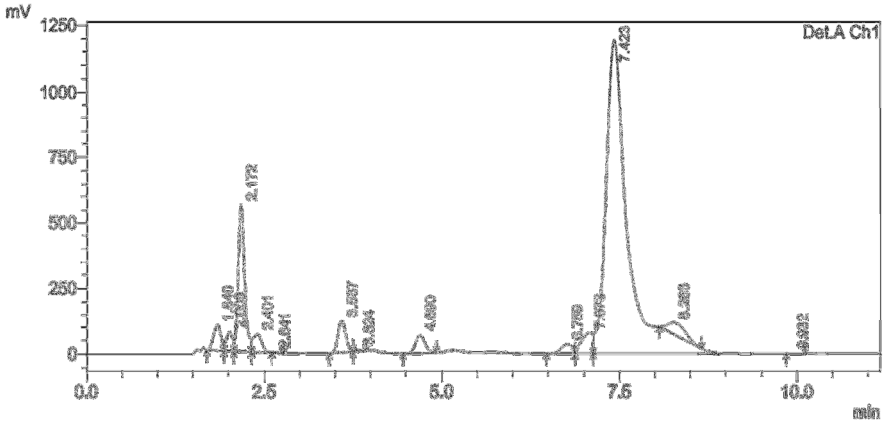

Fig.24. HPLC chromatogram of 0 mM *Dunaliella salina* algae with β-carotene at retention time 7.423 minutes.

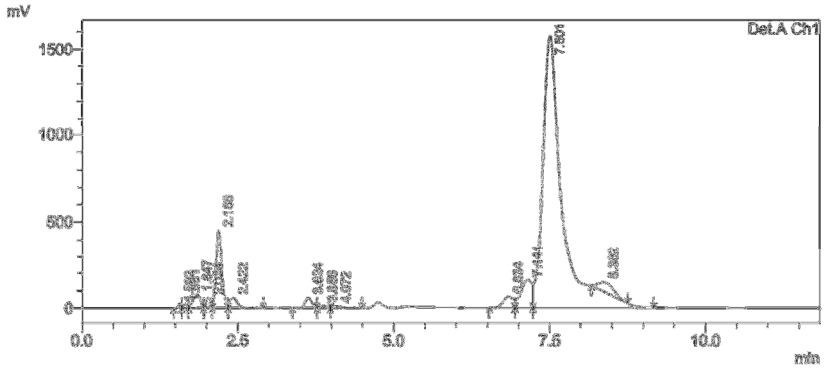

Fig.25. HPLC chromatogram of 50 mM*Dunaliella salina* algae with β-carotene at retention time 7.501 minutes.

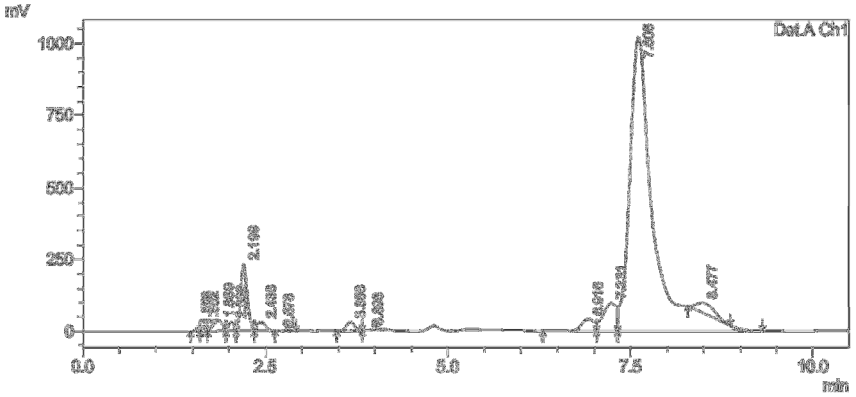

Fig.26. HPLC chromatogram of 100 mM*Dunaliella salina* algae with β-carotene at retention time 7.605 minutes.

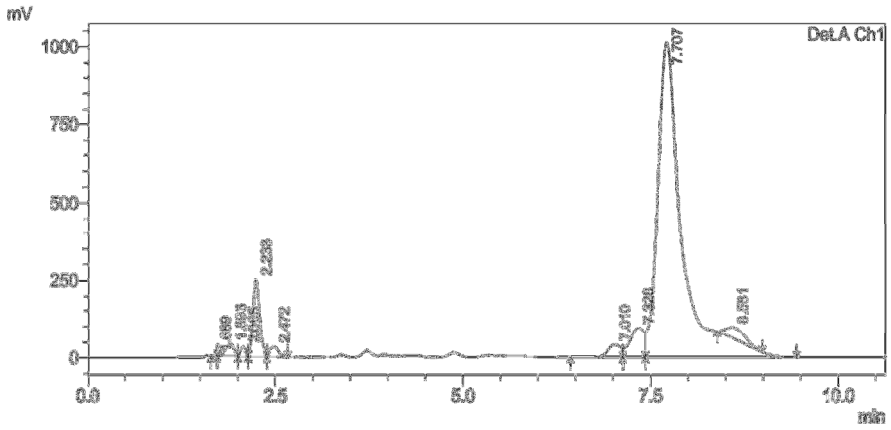

Fig.27. HPLC chromatogram of 150 mM *Dunaliella salina* algae with β-carotene at retention time 7.707 minutes.

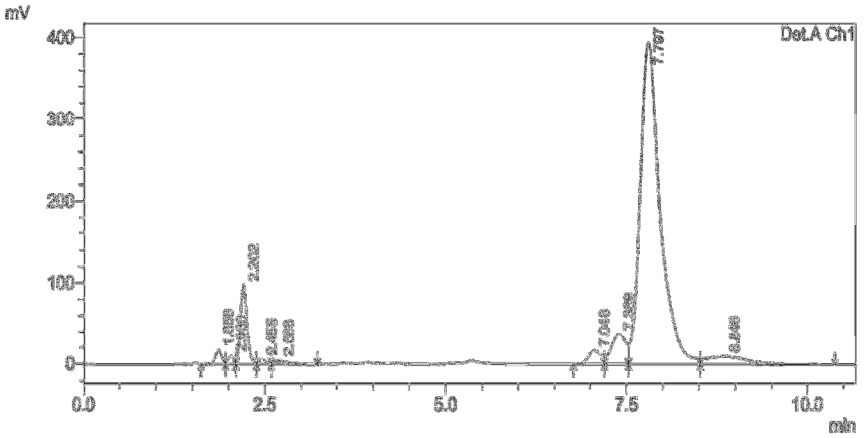

Fig.28. HPLC chromatogram of 200 mM *Dunaliella salina* algae with β-carotene at retention time 7.797 minutes.

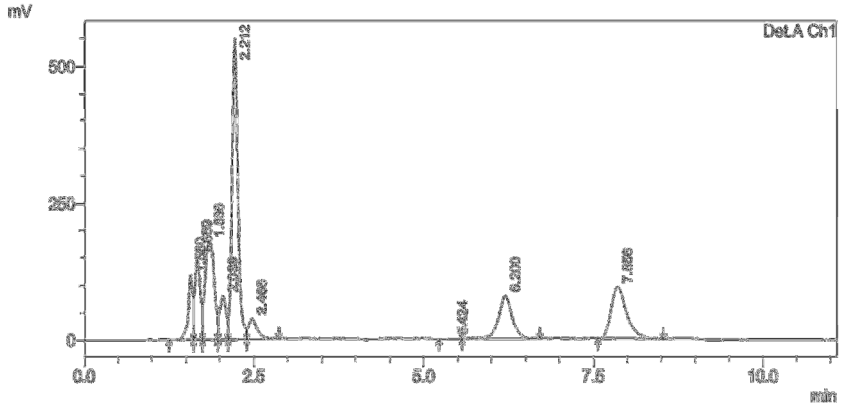

Fig.29. HPLC chromatogram of 0 mM *Dunaliella bardawil* algae with β-carotene at retention time 7.856 minutes.

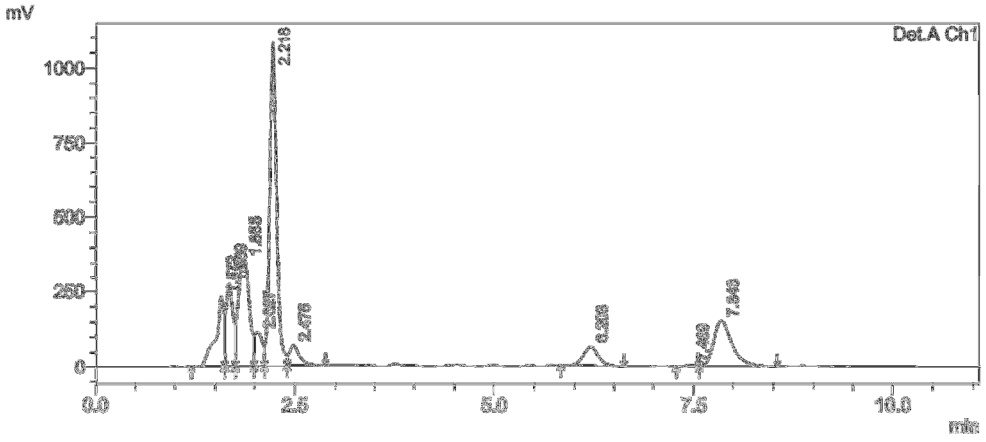

Fig.30. HPLC chromatogram of 50 mM *Dunaliella bardawil* algae with β-carotene at retention time 7.843 minutes.

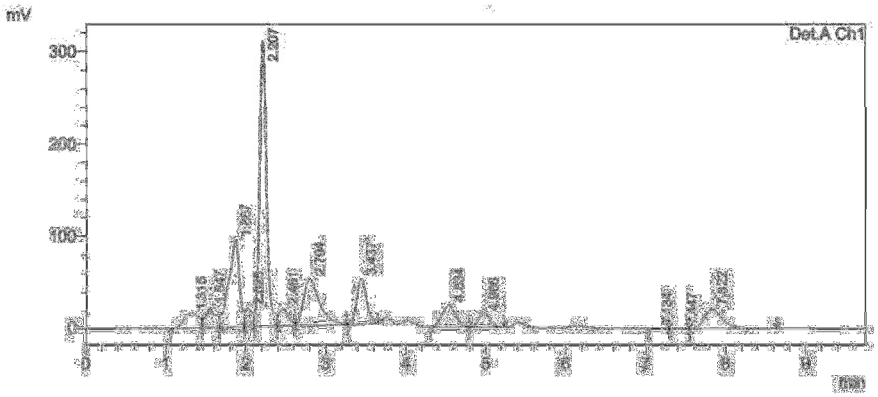

Fig.31. HPLC chromatogram of 100 mM*Dunaliella bardawil* algae with β-carotene at retention time 7.822 minutes.

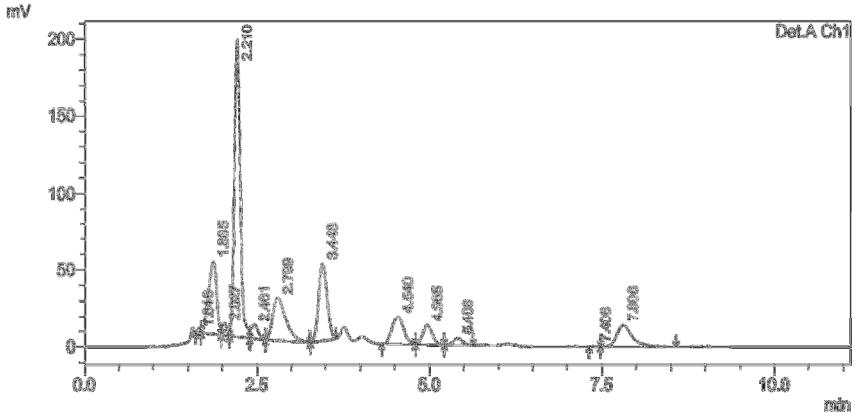

Fig.32. HPLC chromatogram of 150 mM*Dunaliella bardawil* algae with β-carotene at retention time 7.806 minutes.

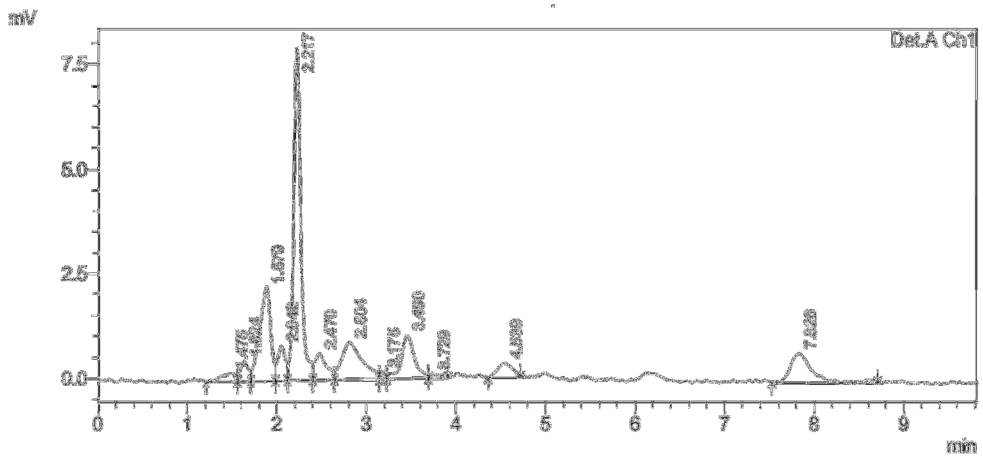

Fig.33. HPLC chromatogram of 200 mM *Dunaliella bardawil* algae with β-carotene at retention time 7.826 minutes.

Table No7 : Carotenoid contents (µg/ 100 mg) in the algae *Dunaliella salina* and *Dunaliella bardawil* at different salinity and nutrients.

Stresses	***Dunaliella salina***	***Dunaliella bardawil***
1M (Nacl)	78.52µg/ 100 mg	0.64µg/ 100 mg
2M (Nacl)	83.64µg/ 100 mg	2.34µg/ 100 mg
3M(Nacl)	56.17µg/ 100 mg	2.11µg/ 100 mg
4M(Nacl)	22.04µg/ 100 mg	1.62µg/ 100 mg
N^-	110.8µg/100 mg	2.32µg/ 100 mg
S^-	5.36µg/100 mg	1.71µg/ 100 mg
P^-	67.21µg/100 mg	1.31µg/ 100 mg
C	50.9µg/100 mg	2.25µg/ 100 mg

Table No8 : Carotenoid contents (µg/ 100 mg) in the algae *Dunaliella salina* and *Dunaliella bardawil* on the supplementation of sodium bicarbonate.

Stresses	***Dunaliella salina***	***Dunaliella bardawil***
0 mm	76.2µg/ 100 mg	4.66µg/ 100 mg
50 mm	134.2µg/ 100 mg	8.34µg/ 100 mg
100 mm	180.6µg/ 100 mg	1.81µg/ 100 mg
150 mm	180.5 µg/ 100 mg	0.89µg/ 100 mg
200 mm	37.20µg/ 100 mg	0.05µg/ 100 mg

Conclusion

Chapter 6

Conclusion

Our finding results suggest that supplementation of sodium bicarbonate can significantly increase the nutrient utilization, photosynthetic efficiency and enhancement of cellular compounds including pigments in microalgae. Thus, concluding that combination of sodium bicarbonate with other stress condition it may leads to better enhancement of carotenoid and also increase cell biomass. These findings indicates using sodium bicarbonate was cost effective and also increase yield of production compare to other sources such as CO_2 gas system, although it depends on species and genus specific. In other stresses like NaCl, N^-, S^- and P^- shows low level in carotenoid production its mainly due to increase in incubation it leads to decrease in biomass so it results in decrease in the level of carotenoid but in case of supplementation of sodium bicarbonate it increases both carotenoid as well as biomass.

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