ANTICANCEROUS AND ANTIOXIDANT ACTIVITY OF BIOACTIVE COMPOUNDS FROM *DUNALIELLA SALINA*

A

DISSERTATION

Submitted in the partial fulfillment of the requirements
For the Award of the Degree of

Masters of Science
(Biotechnology)

Under the Guidance of

Dr. M. Sudhakara Reddy
(Professor, DBT)

Co-Guidance of

Dr. Manoj Baranwal
(Assistant Professor, DBT)

Pritika Singh
301201016

DEPARTMENT OF BIOTECHNOLOGY, THAPAR UNIVERSITY, PATIALA-147001
(July 2014)

DEDICATED TO MY

FAMILY AND FRIENDS

CERTIFICATE

This is to certify that the dissertation entitled "**Anticancerous and Antioxidant activity of Bioactive compounds from *Dunaliella. salina***" submitted by **Pritika Singh** (301201016) in partial fulfillment of the requirement for the award of the Degree of Masters of Science in Biotechnology of Thapar University, Patiala is a record of student's own work carried out by her under my supervision and guidance. The report has not been submitted for the award of any other degree or certificate in this or any other university.

Dr. M.S. Reddy
Supervisor and Professor

Dr. Dinesh Goyal
(Head of Deptt. Of Biotech)

Dr. Manoj Baranwal
Co-Supervisor and Assistant Professor

(Deptt. Of Biotech)

Dr. S.K. Mahopatra
Dean
(Academic Affairs)
Thapar University
Patiala

CANDIDATE'S DECLARATION

I hereby declare that the work presented in the dissertation entitled **"Anticancerous and Antioxidant activity of Bioactive compounds from *Dunaliella. salina*"** in partial fulfillment of the requirement for the award of the Degree of Masters of Science in Biotechnology, is an authentic record of my own work during the academic year 2012-2014, under the supervision of Dr. M Sudhakara Reddy, Professor and Co-supervision of Dr. Manoj Baranwal, Assistant Professor, Department of Biotechnology, Thapar University, Patiala. The report has not been submitted for the award of any other degree or certificate in this or any other university.

Place: Patiala

Date: 18-July-2014

Pritika Singh

301201016

This is to certify that the above statements made by the student are correct and true to the best of our knowledge and belief.

Dr. M.S. Reddy
Supervisor and Professor

Dr. Dinesh Goyal
(Head of Deptt. Of Biotech)

Dr. Manoj Baranwal
Co-Supervisor and Assistant Professor

(Deptt. Of Biotech)

ACKNOWLEGDEMENT

In pursuit of the academic endeavor, I feel that I have been singularly fortunate because inspiration, guidance, love and care -all came in my way and it seems almost an impossible task for me to acknowledge the same in inadequate words.

I consider myself to be very lucky to be associated with my mentor, and Supervisor **Dr. M.S Reddy**, Professor, Department of Biotechnology, Thapar University Patiala for his guidance, invaluable advice and constant support throughout the course of my research work. This thesis never would have been finished without his encouragement and suggestions.

I am grateful **to Dr. Manoj Baranwal**, Co-supervisor, Assistant Professor, Department of Biotechnology, Thapar University Patiala who's ever encouraging and highly positive approach has influenced and benefitted me a lot. I express my deep sense of gratitude for his constant help and guidance accorded during my work.

I am thankful to **Dr. Dinesh Goyal,** Head of the Department of Biotechnology , Thapar University, Patiala, for his kind support during this investigation.

I am sincerely thankful to **Mrs. M Vasundhara**, Assistant Professor, Department of Biotechnology, Thapar University, Patiala who was always more than willing to extend a heling hand during the hour of need.

I am earnestly thankful to **Ms. Neha Lohia**, PhD Scholar, Thapar University for her immense support and friendly approach to all my problems.

Most of the work would have been incomplete without the profound support of Lab assistants. So I owe a word of thanks to **Mr. Babban Yadav**.

Life at Thapar would have been not so relaxing and enjoyable without my friends Apurva, Gurvinder, Ravleen, Poonan, Tajeshwar, thank you for all you support.

The whole credit of my achievement goes to my parents and sister, without the support and faith of whom I would not have been what I am today.

Date: 18-July-2014

Place: Patiala

Pritika Singh

(Pritika Singh)

ABSTRACT

Dunaliella salina is a halotolerant, green microalga which is extensively studied for accumulation of carotenoids under stress conditions and has been consumed as food and natural medicine. Because of its high bioavailability of 9-*cis* form of β-carotene in dry biomass and omega-3 fatty acids, makes it a potent naturally derived antioxidant. The scope of this study was to investigate anticancer, antioxidant and antimicrobial properties of normal and stressed crude extract of *Dunaliella salina.* In the present work various stress parameters such as high salinity, high temperature and nitrogen stress to increase the agglomeration of bioactive compounds were studied. Results indicated that with increase in the stress levels pharmacological activity also increased. On these parameters total cell number, specific growth rate, carotenoid and protein content was analyzed. Non-stressed growth conditions were the optimum growth^{o} conditions for the maximum biomass .i.e., 1.7 M NaCl, 0.75 mM nitrogen^{o} concentration at 28 C, whereas 3.5 M NaCl concentration without nitrogen source at 37 C as were identified as optimum conditions for bioactive metabolites accumulation. Antioxidant activity in non-stressed *Dunaliella* was recorded as 14.96 % by DPPH assay, which significantly increased to 57.3 % under stress conditions. Similarly the cytotoxic effect of microalgae was studied on Human Breast Cancer cell MCF-7. MTT assay showed that 250 µg/ml of nitrogen stressed 0 M and 0.25 M *Dunaliella* cells considerably reduced MCF-7 cell proliferation by 53.7 % and 69.3 %, respectively. The salinity stressed at 3.0 M and 3.5 M NaCl *Dunaliella* extract (250 µg/ml), significantly reduced MCF-7 Cell proliferation by 33.42% and 47.87% respectively. *Dunaliella salina* has potent antibacterial and antifungal activity and these activities were significantly increased under stressed *Dunaliella.* Thus *Dunaliella salina* may be considered as a good candidate to be developed as a wholesome nutrient supplement and can be used to obtain antioxidant and cytotoxic agent.

CONTENTS

LIST OF ABBREVATIONS

M	Molar
D. Salina	*Dunaliella salina*
DMEM	Dulbecco's Modified Eagle's medium
DMSO	Dimethyl sulfoxide
DPPH	1,1-Diphenyl-2-picrylhydrazyl radical, 2,2-Diphenyl-1-(2,4,6-trinitrophenyl)hydrazyl,
ELISA	Enzyme linked immune sorbant assay
MCF-7	Human Breast adenocarcinoma cell line
MHB	Mueller Hinton Broth
MIC	Minimum Inhibitory concentration
MTT	3-(4, 5- dimethylthiazol-2-yl)-2, 5 diphenyltetrazolium bromide
OD	Optical density
PBS	Phosphate buffer saline
PDB	Potato dextrose broth

Chapter 1

INTRODUCTION

INTRODUCTION

Algae are the ancient and the most diverse form of eukaryotic organisms, ranging from multicellular to unicellular forms. Algae are regarded similar to plants due to its photosynthetic capacity, but differ by absence of differentiated systems, such as roots, xylem and phloem (Dawson, 1966). Algal biotechnology has made major advances in the past three decades, as they cover two-thirds of earth biomass and are now being identified for food, pharmaceuticals, biochemicals and alternative medicines. Economically feasible production of added-value compounds with microalgae is possible because microalgae produce biomass and specific biomass ingredients directly from solar irradiation at high photosynthetic efficiencies and high volumetric and areal productivities. As an example, my work focus on discussing the production of bioactive metabolites from *Dunaliella salina*.

Among the diverse algal classification is the genus of unicellular, biflagellate, green microalgae *Dunaliella,* first reported by Michel Fliex Dunal in the year 1838 (Oren, 2005). Among different species of *Dunaliella* such as *Dunaliella teritoletica, Dunaliella salina, Dunaliella viridis, Dunaliella salina* which is halotolerant, unicellular, uninucleate, red-green microalgae lacks a rigid polysaccharide cell wall. It is found in high salinity environments such as salted brines, sea salt fields and oceans.

They have combined properties of higher plants (*viz*. efficient oxygenic photosynthesis and simple nutritional requirements) with biotechnological attributes proper of microorganisms (*viz*. fast growth rates, and ability to accumulate or secrete primary and secondary metabolites, and an inherent self-defense mechanism).

It is becoming commercially and economically important because it can grow and adapt in salinities ranging from 0.05–5.0 M NaCl with constant and low intracellular sodium concentration by regulating the metabolism of osmolyte, glycerol to balance external diverse stress conditions. These stress conditions are not the optimum growth conditions but the adverse conditions like nutrient starvation, osmotic stress, high light, stress temperature conditions. These physiological stress conditions are the key factors which upsurge the bioactive compounds or secondary metabolites in the stationary phase or slow growing vegetative phase i.e. 14% of the dry biomass, which makes *Dunaliella salina* a popular nutrition supplement in China, Japan and Taiwan.

Despite the advancement 90% of the β-carotene is available commercially is produced by synthetic route, which can be harmful if consumed over a longer period of time, and less efficient a pro-vitamin A as compared to naturally derived antioxidants (Goodman *et al., 1999).* It is a beneficial alga as it stores important compounds such as carotenoids (β-carotene), Caulerpenyne, Zeaxanthin, lutein, Cryptoxanthin, violaxanthin, omega-3 fatty acids ,proteins (Cgp) and glycerol, which possess potent antioxidant, anticancer, antimicrobial and immune enhancing properties. Due to its ecological adaption, incredible metabolic versatility, less chance of contamination, easy availability, more therapeutic (14 % of metabolites in dry weight) make *Dunaliella* a promising candidate for commercial exploitation. It is extraordinary because it possess such transient forms of carotene, which are absent in plants, and are ten times stronger than preventing cancer, specially skin and breast cancer (Heiber *et al.*, 2000). It also produces lipids and certain digestible proteins which help treat muscle cramps and enhances immune system.

Hence, the present study is focused on the assessment of bioactive properties of the compounds released under stress conditions by the organism, *Dunaliella salina.*

Chapter 2

REVIEW OF LITERATURE

Review of Literature

2.0 Taxonomy

The scientific acquirement of unicellular red-colored algae inhabitant of hypersaline environment was reported 150 years ago by French engineer Dunal (Oren *et al*., 2005). He saw red-colored, biflagellate cells which he identified as *Haematococcus salinus* because of their analogy with similar algal cells. Later in the year 1905, E.C Teodoresco postulated extensive information on morphology, behavior, cell structure, ecology and formally introduced it as a new genus *Dunaliella* in honor of Dunal (Borowitzka and Borowitzka, 1988).

The taxonomic details were systematically examined by Massyuk in the year 1973. He revised the genus and recognized 29 species. Some of the essential and extensively found species are *Dunaliella bioculate, Dunaliella bardawil, Dunaliella teritolecta, Dunaliella granulata, Dunaliella parva, Dunaliella primolecta* (Borowitzka and Siva 2007). Remuneratively cultivated strains for the production of bioactive compounds are *Dunaliella salina* and *Dunaliella bardawil.*

The classification of *Dunaliella* is given below

Table 1. Taxonomic classification of *Dunaliella*

Super Kingdom	Eukaryota
Kingdom	Viridiplantse
Subkingdom	Phycobionta
Phylum	Chlorophyta
Class	Chlorophyceae
Order	Volvocales
Family	Dunaliellaceae/ Chlorophyceae
Genus	Dunaliella

(Adapted from, Borowitzka and Siva, 2007)

2.1 Organism and life cycle

Dunaliella is a unicellular, photoautotrophic, bi-flagellate, naked green alga. *Dunaliella* is morphologically similar to Chlamydomonas, with the main difference being the absence of a cell wall in *Dunaliella* (Figure 1). It has two flagella of equal length and a single, cup-shaped chloroplast, responsible for photosynthetic activity (Borowitzka and Borowitzka, 1988). *Dunaliella* are generally spherical or pear-shaped with dimensions as 6-15 µm in length and 4-10 µm wide (Amotz *et al*., 1983). Chloroplast contains a large pyrenoid surrounded by a polysaccharide granule (a storage product). One of the most significant character of *Dunaliella*, in contrast to other members of Chlorophyta is that it lacks a rigid polysaccharide cell wall, with the cell protoplast enclosed within an elastic cell membrane. Because of its elasticity it rapidly responds to osmotic changes and proliferates in extremely varied stress conditions (Amotz and Avron , 1981). The major means of osmoregulation is through production of intracellular glycerol at a concentration that is proportionate to the external NaCl concentration (Amotz and Avron *et al,* 1973). Other microorganism such as *E.coli* lack natural carotenoid synthesis capacity. Thus considering its advantages that both production and bioactive accumulation can be enhanced *in-vitro*, which make it a suitable cell factory organism.

Dunaliella is being exploited commercially as a model organism because it can accumulate carotenoids or other bioactive compounds under adverse growth conditions (or cartenogenic conditions). These cartenogenic conditions are not the optimal growth conditions but the adverse growth conditions i.e. high salinity, high light intensity, U.V exposure, stress temperature, privative nutrients (deficiency of nitrogen, phosphorus or sulphur) (Amotz *et al*., 1982 ; Raja *et al*., 2007), which results in agglomeration of carotenoids (or β-carotene) upto 14% of the total dry organic weight. However the percentage falls upto 1.3 % under unsuitable conditions or non-stressed conditions (Jhonson and Schroeder , 1995).

Dunaliella salina can be grown easily ex-situ and in-situ conditions as it has simple growth rewuirements, depending mainly on nitrates as nitrogen source, phosphates as phosphorus source, sodium, magnesium and calcium (Mg^{2+}:Ca^{2+}) has been reported optimal (McLachlan, 1960).

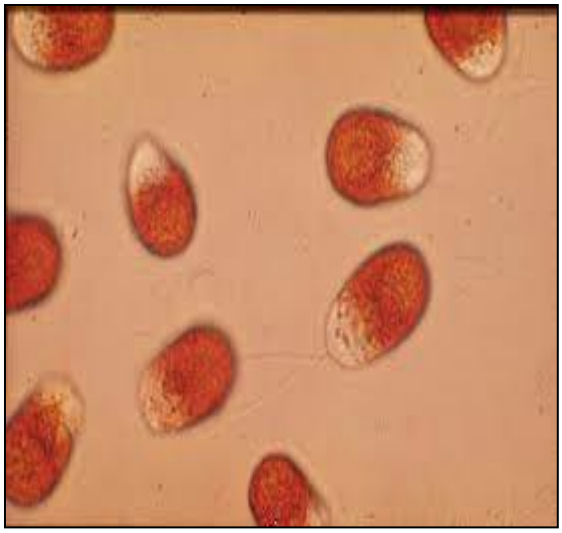


Figure 1- (a.) pear shaped green colored *Dunaliella* cells. (b.) swollen red colored *Dunaliella* cells in stress. (c) Lake Retba famous for its pink color due to deposition of β-carotene (Source Internet).

The genus *Dunaliella* has developed several physiological adaptions including the lack of rigid cell wall, a variable intracellular concentration of glycerol (Kacka and Donmez , 2008), changes in photosynthetic pigments (Estudillo *et al.*,2006) and structural modifications in chloroplast (Bakalova and Panova, 2003).

Electron microscopic imaging showed that bioactive compounds concentrate as lipid globules in the interthylakoid spaces of the chloroplast which give it a bright red coloration, whereas in non-stressed conditions few or no lipid globule is found (Amotz *et al*., 1982).

Under stress conditions the cell membranes shrink and cell swells up. The green cell which is dominated by the chloroplast starts to turn orange although intracellular organelles, including the Nucleus, Mitochondria, Chloroplast, Golgi bodies, and Vacuoles decreases both in volume and surface area (Figure 2).

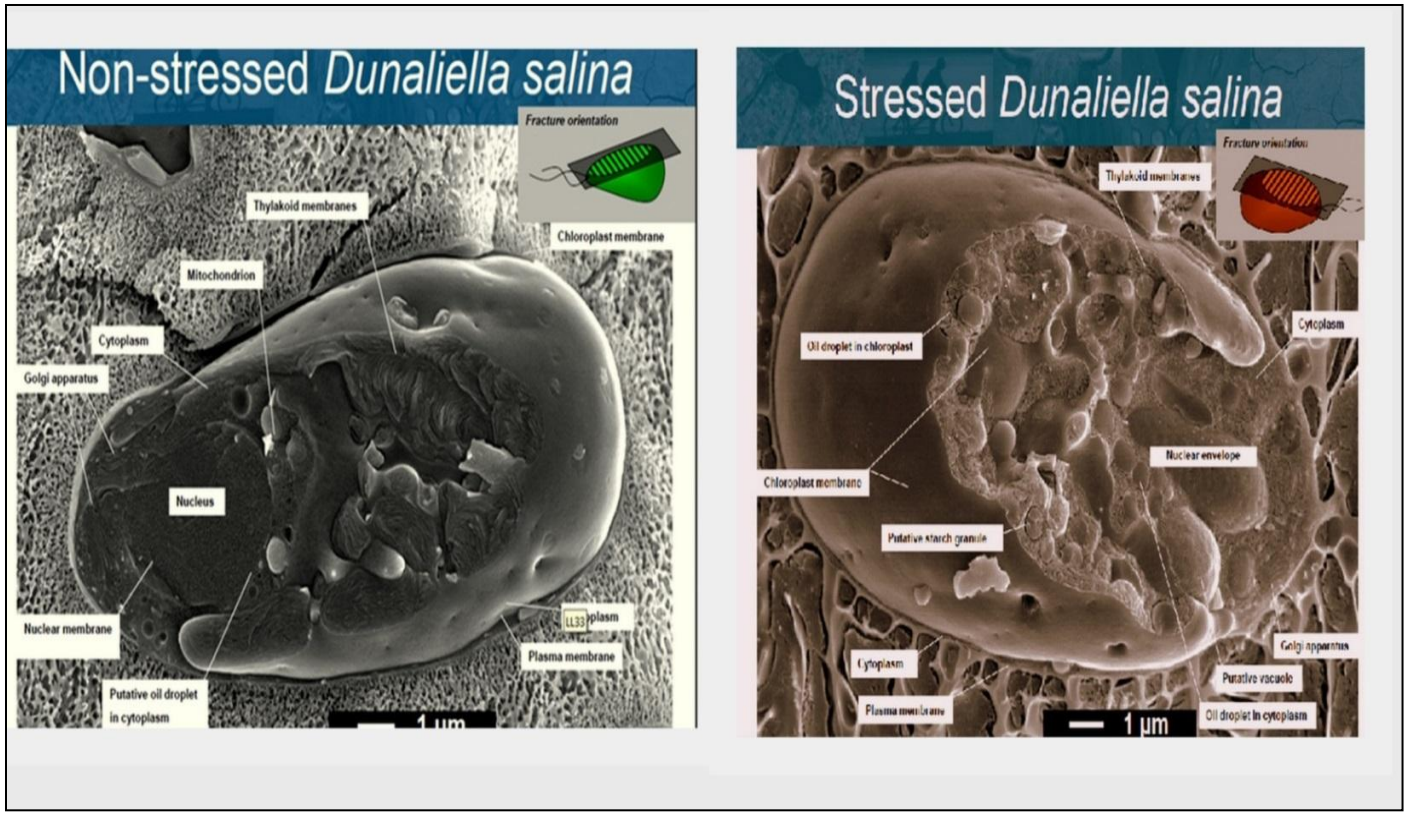


Figure 2. Electron microscopic images showing morphological changes in *Dunaliella salina* cells under stressed and non-stressed conditions (Adapted from Lammer *et al.*, 2011).

It is detected that membrane –free lipid globule exclusively contained β-carotene, neutral lipids (mainly triacylglycerol) and a small amount of protein (Fried *et al*., 1982). This 38 kD Carotene globule protein (Cgp) is induced in parallel with bioactive compounds so as to stabilize the β-carotene contained lipid globule (Katz *et al*., 1995). Bioactive compounds are released as a self-defense mechanism by the algae so as to grow and adapt with the increase in ROS species released by photosynthetic center under stress.

2.2 Carotenoids and other bioactive compounds from *Dunaliella salina*

Natural carotenoids are synthesized universally in photosynthetic organisms, which are diversiform in structure as well as great variation in function. Most of the carotenoids are C_{40} isoprenoids, which are composed of eight isoprene units. Heterogeneity of conjugated double bonds of the polyene chain results in the various characteristic absorption spectra and photochemical properties of carotenoids. These carotenoids form the nutraceutical component in *Dunaliella* and make it the richest source of dietary β-carotene (Amotz and Avron, 1983).

In addition to β carotene, this green alga also synthesizes mixture of secondary carotenoids which are equally important and produced after exposure to specific environmental stimulus (via carotenogenesis) such as, α- carotene, violaxanthin, zeaxanthin, antheraxanthin and cryptoxanthin (Vorst *et al.*,1984), out of which zeaxanthin is a very crucial compound which is produced upto 6 mg by *Dunaliella* under low-light stress conditions as compared to plants which produce only 0.2 mg (Jin *et al*.,2003)

Carotenoids perform several functions in algae, they play important role in photosynthesis in terms of energy and oxygen transport as accessory pigments, and in protecting the algae against the injure caused by photooxidation. They quench singlet oxygen (ROS), dissipating excess energy and scavenging free radicals which were generated from metabolism and would damage the metabolizing tissue. This intrinsic antioxidant activity of carotenoids constitutes the basis for their protective action against oxidative stress (Telfer, 2002).

The isomeric composition of β- carotene from Dunaliella contains mixture of all –*trans* (Figure 3a), 9-*cis*(Figure 3b), 13-*cis* and 15-*cis* forms (Wang *et al.,* 1994). Such a mixture can hardly be obtained via chemical synthesis (Demming-Adam *et al.,* 2002). The predominance of 9-*cis* stereoisomer gives *Dunaliella salina* a potent antioxidant and anticancer features (Jhonson *et al.*,1996). Reportedly, chemically synthesized β-carotene is harmful for smokers and ex-smokers when taken for a longer period of time and can cause cancer instead of treating it, because it interferes with the absorption of other nutrients.

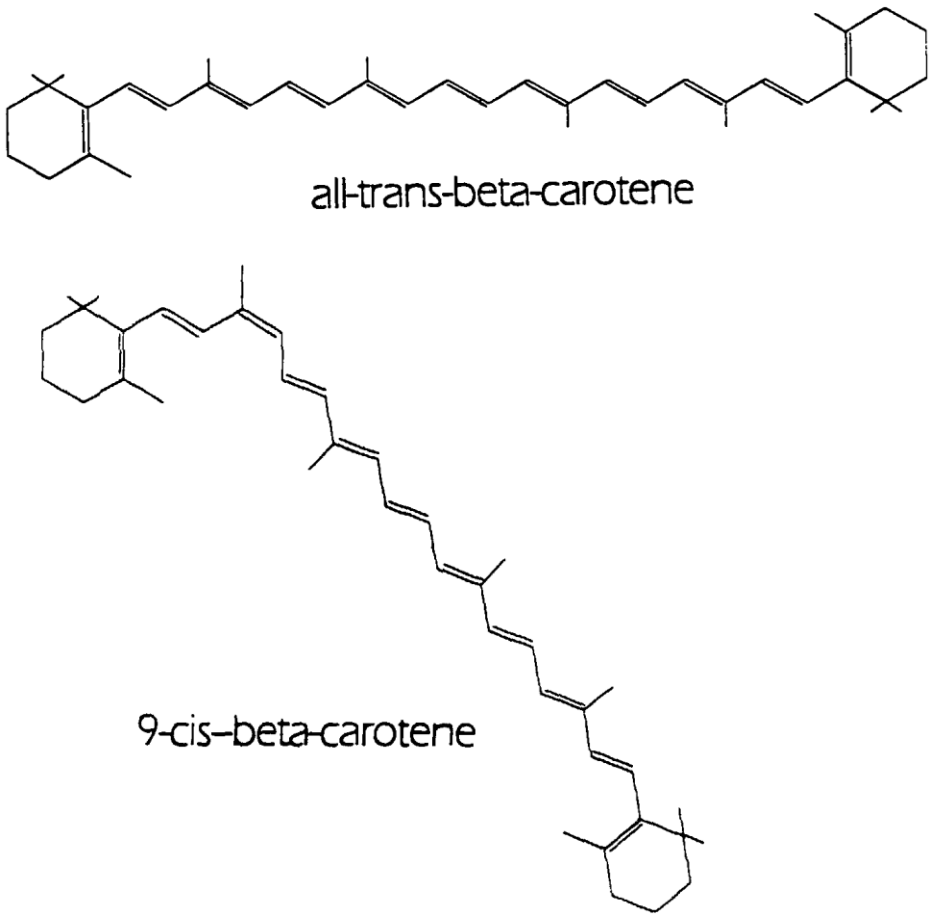


Figure 3. Chemical structures of β-carotene isomers. (a) all-*trans*-β-carotene (b) 9-*cis*-β-carotene

Other than carotenoids it can accumulate other bioactive compounds such as lipids, carbohydrates, vitamins, fatty acids under cartenogenic conditions, which make *Dunaliella* a complete model organism with various cell factory applications.

According to a report by BCC (Business Communications company), the worldwide market value of commercially- used natural carotenoids was US $886.9 million in 2005, and it was expected to break the billion dollar mark by 2015.

The composition of bioactive compounds from *Dunaliella* is given in the following table,

Table 2. General Chemical Composition of *Dunaliella*

BIOACTIVE METABOLITES	COMPOSITION (dry weight)	REFRENCES
β-carotene	2-8%(non-stressed cells) 14% (stressed cells)	Ben Amotz and Avron,1983
Protein	30%(non-stressed cells) 50%-60%(stressed cells)	Borowitzka and Borowitzka, 1980
Lipid	6%(low salinity) 19%(high salinity)	
Carbohydrates	11% (non-stressed cells) 40%(stressed cells)	
Fatty Acids	Palmitic acid (31%), Linoleic acid(20%), Linolenic acid(17%), Oleic acid(13%)	
Thiamine, Pyridoxine, Riboflavin, Nicotinic acid, Biotin and α-tocopherol	Detectable levels	

2.3 Biosynthesis and regulation of carotenoids in *Dunaliella*

Carotenoids are derived from the C_5 isoprenoid precursors, isopentyl diphosphate (IPP) and its isomer dimethyl diphosphate (DMAPP) (Shewmaker *et al.,* 1999). The biosynthesis of isopentenyl diphosphate (IPP), catalyzed by 1-deoxyxylulose-5-phosphate synthase (DXS) coded by the *dxs* gene (Lichtenthaler, 1999), which is then converted to GGPP acts as a substrate for formation of phytol (chlorophyll side chain) and phytoene, catalyzed by *psy gene.*

Thus the cartenogenic pathway is briefly divided into three steps; Geranylgeranyl pyrophosphate (GGPP) biosynthesis, the Lycopene biosynthesis and the generation of carotenoids with cyclohexene rings.

GGPP formation, takes place by non-mevalonate pathway by sequential addition of three IPP molecules to a DMAPP molecule.

Lycopene formation takes place by a metabolic route via the sequential generation of phytoene and other intermediates mentioned in (Figure 4).The process of cyclization is dominant in *Dunaliella salina*, in this process two cyclohexane rings are added to either or both ends of lycopene under the catalysis of lycopene cyclase (LYC) leads to the formation of δ-, α-, γ- and β-carotene.

Two types of LYC have been found ; lycopene β-cyclase (LYC-B) and lycopene ε-cyclase (LYC-E), which helps in synthesis of β carotene and α carotene.

Regulation of carotenogenesis in *Dunaliella salina* is divided into three steps of : (i) sensing of the induction signal, (ii) signal transduction, and (iii) regulation of carotenoid biosynthesis (Figure 5).

The stress conditions such as high salinity, light intensity, temperature act as signal which induce various signal transduction cascades, and results in activation of ROS scavenging enzymes and leads to overproduction of carotenoids. In other words, the slower the growth rate and/or the higher the light intensity, the more β-carotene is produced per cell.

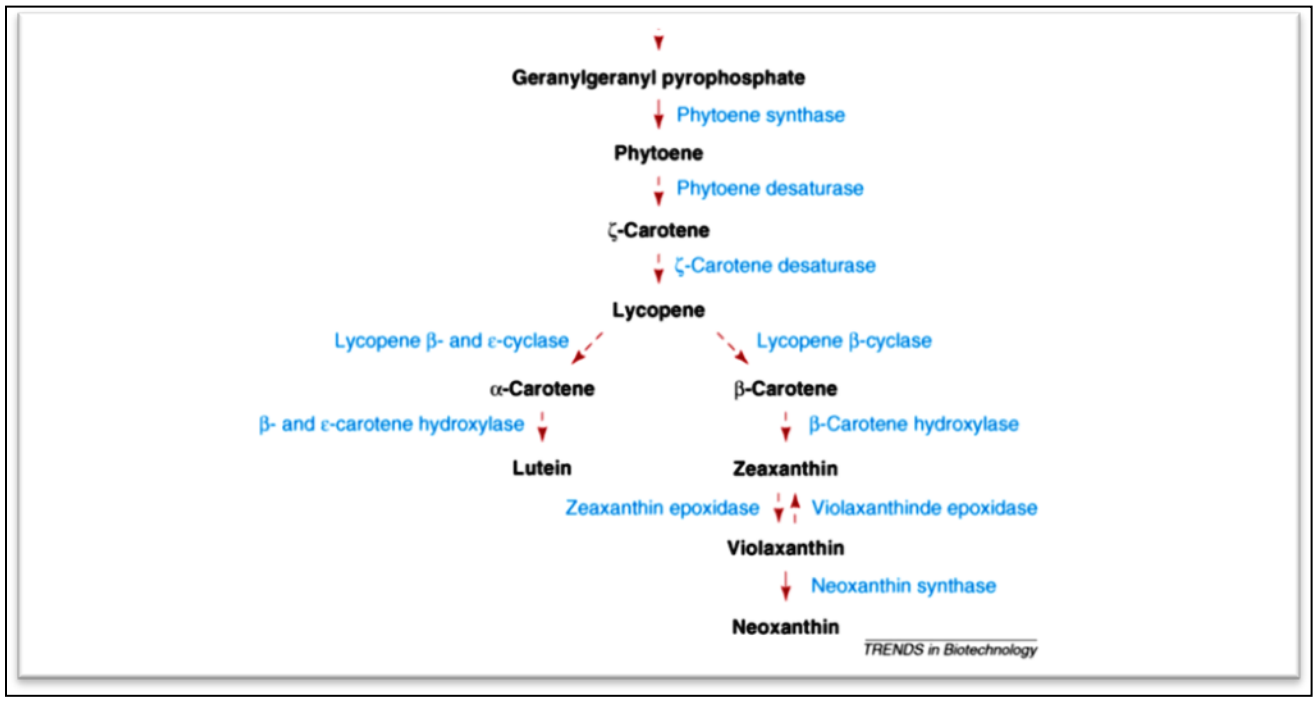


Figure 4. Pathway of carotenoid biosynthesis in microalgae. The first committed step of the carotenoid pathway is the condensation of two geranylgeranylpyrophosphate molecules by phytoene synthase (enzyme names are written in blue) (Adapted from Rabbani. S. *et al.*, 1988)

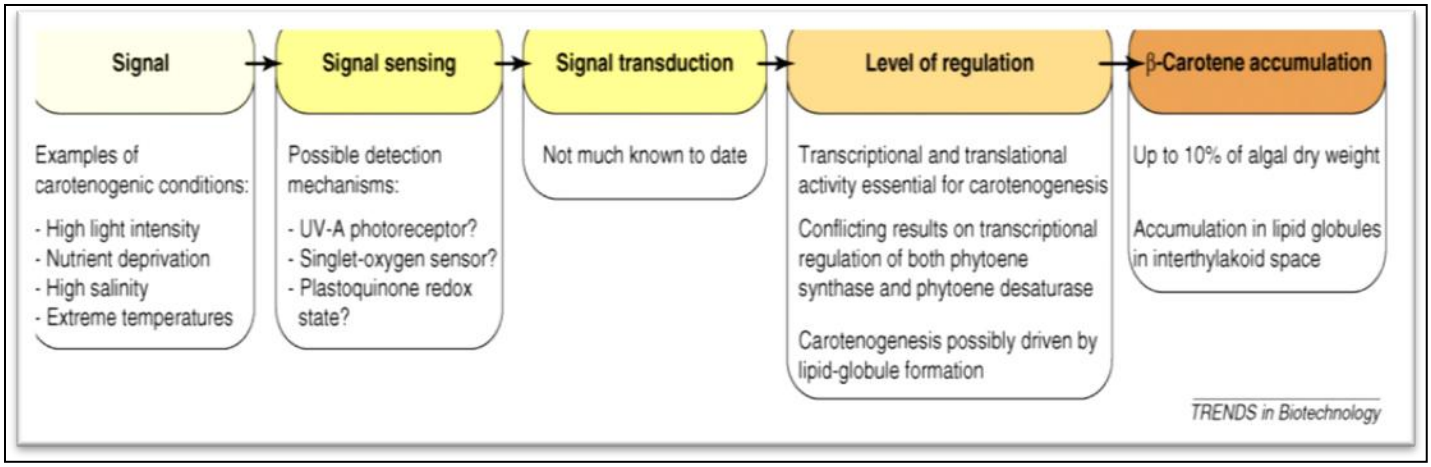


Figure 5. Regulatory mechanism for the induction of β-carotene overproduction in *D. salina*. Adapted from (Lammer *et al.*, 2005).

2.4 Bioavailability of bioactive compounds

Not only carotenoids that are stored under stress by *Dunaliella* but various other metabolites (Table 3) that increase under high light and nitrogen stress conditions.

Carotenoids

The efficiency of absorption of carotenoids from natural sources such as fruits and vegetables is less than 5 %,on the other hand the efficiency of absorption increases in carotenoid containing nutritional supplements to 70 % (Murthy, 2002). Carotenoids have substantial commercial value owing to their color and antioxidant properties. Typical markets of application include food, feed, cosmetics and nutraceuticals. In foods, carotenoids exist as part of the complex matrix, comprised of indigestible polysaccharides and digestible polysaccharides and protein.. Cleavage of digestible β- carotene in enterocyte is reduced to vitamin-A and also produces apocarotenol intermediates like zeaxanthin, α-carotene, astaxanthin (Wang *et al*., 1993) which affect growth regulation and other cellular activities (Krinsky *et al.*, 1994).

Lipids

Marine sources are widely regarded as possessing interesting lipid compositions, which make them attractive as a source for lipid extraction. The main polar lipids found in these substrates include monogalactosyldiacylglycerols (MGDG), digalactosyldiacylglycerols (DGDG), and

phosphatidylglycerols (PG). In *Dunaliella*, main osmolyte 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).

Glycerol accumulates in *Dunaliella* in response to osmotic stress, but the most important physiological role of glycerol is that of a compatible solute, that is, a substance that at high concentrations protects enzyme activity.

Minerals

Whole dried *Dunaliella salina* is exceptionally rich in nutrient and antioxidant minerals, particularly selenium and magnesium. Selenium is a powerful antioxidant that aids in detoxification and immune health. Magnesium is vital for healthy cellular metabolism, energy production and nerve and muscle function.

Amino acids

Dunaliella salina has a high content of useable protein (amino acids). Amino acids are the basic building materials of life, required for the synthesis of muscles, skin and connective tissues, hormones, enzymes and neurotransmitters.

2.5 Bioactive properties from Bioactive metabolites

Carotenoids on biotransformation convert to retinol, vitamin A and other products which provide this microalgae its importance of acting as a potent antioxidant and anticancer supplement.

Pharmacological activity in humans can be classified as;

- Antioxidant
- Immunomodulatory
- Vitamin A(Retinol)
- Antimicrobial
- Feed and food formulation
- Skin protection
- Cardiovascular diseases, prevention of cataracts and muscular degeneration

Antioxidant Activity

Dunaliella salina contains rich mixture of natural carotenoids including beta-carotene, alpha-carotene, lutein, zeaxanthin and cryptoxanthin, which lead to potent antioxidant activity. In human beings, oxidation reactions driven by reactive oxygen species can lead to DNA denaturation and protein damage or mutation; these may in turn lead to several syndromes, *viz*. cardiovascular diseases, some kinds of cancer and degenerative diseases, and ageing at large (Halliwell *et al*., 2007).

As potent biological antioxidants, carotenoids are able to absorb the excitation energy of singlet oxygen radicals into their complex ringed chain thus promoting energy dissipation, and protecting tissues from chemical damage. They can also delay propagation of such chain reactions as those initiated by degradation of polyunsaturated fatty acids which are known to dramatically contribute to the decay of lipid membranes, thus seriously hampering cell integrity (Guerin *et al*., 2003). Study showed significant prevention of cognitive impairment in Alzheimer's disease by using extracts from *Dunaliella*, (Nakashima *et al.,* 2001*).* Recent studies have shown decrease in DNA strand breakage, increase erythtrocytes, copper, zinic dismutase activity, decreased serum lipid peroxide level (Murthy *et al*.,2003).

Immunomodulatory Activity,

Dunaliella has the ability to stimulate the immune-system, thus being potentially involved in more than 60 life-threatening diseases including various form of cancer, coronary heart diseases, premature ageing and arthritis. It has been demonstrated in male nonsmokers supplemented with 15 mg day^{-1} of naturally synthesized beta-carotene significantly increase the percentage of monocytes expressing MHC Class II molecule HLA-DR (Stahl *et al*., 1998).

Also few studies have shown antibody proliferative response of spleen cells from BALB/c mice. Some secondary metabolites also enhance the release of Interlukein-1α from peritoneal adherent cells which might contribute to the enhancement of antibody production in B cells which might pay help in some serious subjects like AIDS (Okai *et al.,*1996). *Dunaliella salina* has favorable effect on different levels of complement C3, C4 and antioxidant capacity in rainbow trout *(Oncorhynchusmykiss).* These metabolites have shown to enhance natural killer cell activity in elderly men, and also increased lymphocyte response to mitogens in male smokers (Raja *et al*., 2003).

Anticancer activity

Epidemiological studies encompassing β-carotene from *Dunalliela* which contains readily bioavailable 9-*cis* and all-*trans* stereoisomers (40 % and 50 %, respectively), have indeed provided evidence of a lower incidence of several types of cancer (Amotz *et al*., 1999). It has been postulated that mechanism behind Anticancerous activity of *Dunaliella* may be that Secondary carotenoids gets metabolically converted to retinoids which modulate gene expression of factors linked to differentiation and cell proliferation, and prevent cancer. It also modulates the gene expression of connexin-43 resulting in the induction of gap junctions which inhibits neoplastic transformation (Jiang *et al.,* 2005). The magical substance which make it a renowned anticancer agent is that is possess a dominant 9- *cis* stereoisomer which is 50 times more powerful than any other naturally obtained carotenoid. *D.teritiolecta* has strong antiproliferative activity of violaxanthin on human mammary cancer cell line, MCF-7 and LNCaP cells (Pasquet *et al.,* 2011). *D.Salina* was also found to induce apoptosis and cell cycle arrest in the G0/G1 phase of A549 lung cancer cells (Sheu *et al.,* 2008). It inhibits the proliferation of breast cancer, colon cancer, lung cancer and liver cancer (Raja *et al*., 2005).

Antimicrobial activity

Little is known about the antimicrobial activity of *Dunaliella*, but it may possess antifungal activity. The presence of an indolic derivative was detected in the extract which has never been reported in *Dunaliella salina*, together with polyunsaturated fatty acids and compounds related to carotene metabolism, such as, β-ionone, neophytadiene, contribute to its antimicrobial activity (Leon *et al.,* 2008).

Other activity

Bioactive compounds like proteins and carotenoids from *Dunaliella* are known to influence the intracellular communication (Stahl *et al*., 2002). Lycopene is known to increase the Cyclin D levels which decreases the phosphorylation of retinoblastoma protein and resulting in suppression of growth (Nahum *et al*., 2001). It also cures symptoms of vitamin A deficiencies.

Other activity also includes antiageing, hepatoprotection, hyperlipidemic and hypercholesterolemic protection (Murthy *et al*., 2003).

Some of the bioactive compounds with their bioactive properties are summarized in (Table 3)

The recognized therapeutic value of bioactive metabolites in prevention and treatment of degenerative diseases has indeed opened new avenues for development of mass production systems. Advances in knowledge of the underlying physiology, biochemistry and molecular genetics of specially carotenoid-producing microalgae are now urged which would have a major impact upon development and optimization of this (and alternative) microalga-based technologies.

Table 3. Bioactive compounds with bioactive properties in *Dunaliella salina.*

• β Carotene	Pro vitamin A, Anticancer, Antioxidant, cardiovascular protection, Immunomodulatory. (Olson and Krinsky(1995), Stahl *et al*., (2002))
• Caulerpenyne	Anti-proliferative activity in human neuroblastoma cell line, cytotoxic effect on HeLA, Inhibitory action on Urinary Bladder Cancer cells. (Chidambara *et al*.,(2005)
• Violaxanthin	Anti-proliferative activity on MCF-7, A431and inducing apoptosis (Pasquet V *et al*., (2011))
• Zeaxanthin, lutein, Cryptoxanthin, Cantaxanthin	Antioxidant activity (Raja.R *et al*.,(2007), Used in cosmetic preparations and cancer treatment (Kumaresan *et al*., 2008), Prevention of age-related macular degeneration and cosmetic preparations (Seddon *et al*., 1994), Used in animal feed (Krupa *et al*., 2010).
• Sterols and phytosterols, Threitol, Galactosylglycerol, Glycerol-3-phosphate • 3, 3, 5-Trimethylheptane,n-Hexadecane	Important role in lowering LDL cholesterol, anti-inflammatory and anti-atherogenic activity (Francavilla *et al*. (2010) Neuromodulatory Activity (Francavilla *et al*. (2012) Antimicrobial activity, pharmacological activity(Krishnakumar *et al*.,(2013)
• Proteins(Adenine, Adenosine-5-monophosphate, glyciene, β-Alanine,guanine,)	Osmoregulation under stress conditions, Various metabolic functions (Lammers, 2011)

OBJECTIVES OF STUDY

1. Optimization of media, culture conditions, nutrient and environmental factors for the growth and production of bioactive compounds.
2. To study the antioxidant activities and antimicrobial activities of crude extract of *Dunaliella salina* grown under optimal and stress conditions.
3. In-vitro study of anticancer activity of crude extract of *Dunaliella salina* grown under optimal and stress condition on MCF-7 breast cancer cells

Chapter 3

***M**aterials &**M**ethods*

MATERIALS AND METHODS

3.0 Procurement of Microalgal Culture

Algal genotype used in present study:

- *Dunaliella salina*, culture used was obtained from Birla Institute of Scientific Research, Jaipur, India
- *Dunaliella salina*, culture was procured from CAS in Botany, University of Madras.

3.1 Maintenance and Production of *Dunaliella*

3.1.0 Culture conditions

The microalgae were harvested in two different mediums. *Dunaliella salina* isolated from a saline source was grown in AS-100 medium (Vonshak, 1986). The chemical composition of the medium is mentioned in Table 4. *Dunaliella salina* isolated from marine source was grown in De-Walens medium (Orset and Young,1999). The chemical composition is mentioned in Table 5. The cultures were maintained in 250ml of Erlenmeyer flask containing 100 ml of medium at 28^{o}C temperature. Cultures were manually shaken twice in a day and a continuous photoperiod with 50 µ mol $m^{-2}s^{-1}$ illumination was provided by white florescent lamps (Philips, India) for 20 days. Sub culturing was done after every 20th day.

Table 4. Chemical Composition of AS-100 medium

CHEMICALS	**CONCENTRATION (MOLES)**
NaCl	AS REQUIRED
$MgCl_2.6H_20$	0.73M
KNO_3	0.75mM
$MgSO_4$	5mM
KH_2PO_4	0.2mM
$CaCl_2$	0.3mM
$NaHCO_3$	50mM
Tris HCl	40mM
Chelated Iron solution $FeCl_3$	2µM
Na-EDTA	5 µM
TRACE ELELMENTS $MnCl_2$	1 µM
$CuCl_2$	1 µM
$CoCl_2$	1 µM
$ZnCl_2$	1 µM
$(NH_4)_6MO_7O_2.4H_2O$	1 µM

Media was autoclaved at 121^oC at 20 psi for 15 min. To avoid precipitation KH_2PO_4 and $FeCl_3$ were autoclaved separately and $NaHCO_3$ was filter sterilized before being added to medium. Final pH was adjusted to 7.5

Table 5. Chemical composition of De-Walens medium

CHEMICALS	**CONCENTRATIO(gL^{-1})**	**CONCENTRATION(MOLES)**
NaCl	58.44g	2.14M
$FeCl_3.6H_2O$	77.9µg	4.81µM
$MnCl_2.4H_2O$	360 µg	0.54mm
H_3BO_3	33mg	0.54mm
Na_2EDTA	44mg	0.12mm
$NaH_2PO_4.2H_2O$	20mg	0.13mm
$NaNO_3$	100mg	1.18mm
$ZnCl_2.4H_2O$	20mg	10 µM
$(NH_4)_6MO_7O_2..4H_2O$	19 µg	0.28 µM
$CoCl_2.6H_2O$	9 µg	0.08 µM
$CuSO_{4.5}H_2O$	20 µg	0.08 µM
*Seawater	1L	

All stocks were autoclaved separately and mixed together under laminar so as to avoid precipitation. Final pH of the medium was adjusted to 8.0.

*Seawater was formulated artificially by following composition:-

CHEMICALS	**CONCENTRATION(g/L)**
Solution A	
NaCl	41.6
KCl	1.172
$NaHCO_3$	0.34
H_3BO_3	0.45
Solution B	
$MgCl_2$	18.4
$CaCl_2$	2.6

*Solution A and solution B was prepared separately and mixed together to make final volume of one liter.

3.2. Maintenance of the Germplasm

The axenic cultures of *Dunaliella salina* were preserved at ambient temperatures on 1.5% agar slants of AS-100 Medium (Figure 6). The cultures were kept at 28^0C under 12/12 (light and dark cycle) illuminated with fluorescent white lamps with average light intensity.

3.3. Modification of Culture Conditions for Production of Bioactive Metabolites.

Three stress parameters, salinity, temperature and nitrogen-concentration were considered to find out capacity to accumulate carotenoids or bioactive compounds of *Dunaliella* cells.

Effect of Salinity

To study the effect of growth and accruement of bioactive metabolites the *Dunaliella* cultures were subjected to grow in AS-100 medium comprising of different salinities such as 1.0 M, 3.0 M and 3.5 M.

Effect of nutrient stress

In order to know the effect of nutrients on growth and production of carotenoids, algal cells were cultivated under nutrient stress associated with a macronutrient i.e. Nitrogen limiting and Nitrogen excess conditions. The cells were harvested in AS-100 medium containing different nitrogen concentrations such as 0M and 0.25M.

Effect of Temperature

To further characterize the growth and accumulation of bioactive compounds *Dunaliella* cells were cultivated under different temperature regimes. Two temperature regimes were assayed, (a) The cultures were kept at 37^oC for 20 days, (b) The cultures were oscillating between 15^oC-25^oC. All the stress conditions were grown in triplicates with a non- stressed culture which acted as control.

3.4 Measurement of Growth and Productivity

3.4.0 Measurement by morphology study

Morphology of stressed as well as non-stressed *Dunaliella* cell was studied under inverted microscope (Nikon Eclipse E200 Biological microscope) after every 10^{th} day and size measurement was studied. Cell growth was also measured by visualizing the change in color from green (non-stressed) to yellow (stressed), (Figure 7).

3.4.1. Measurement by Cell Count

Growth of *Dunaliella* was measured in terms of cell number and counted 10 times in 20 days using Neubauer Haemocytometer. Since the algae is motile, cells were fixed by adding a drop of Lugol's Iodine solution (Mohan *et al.*, 2012). The cell count was expressed as cells ml^{-1}.

3.4.2. Measurement by Optical Density

In order to know the growth optical density (OD) was also measured at 560 nm. Specific growth rate μ_{max} (division day^{-1}) was estimated using standard formula (Gracia *et al.*, 1984).

$$\mu_{max} = \frac{3.3\ (\log N - \log N_o)}{t}$$

where N is the optical density of final reading; N_o is the optical density of Initial reading; t is the time interval (in days)

3.5. Pigment Estimation

3.5.0 Chlorophyll estimation

Log phase culture of *Dunaliella* cells was taken and centrifuged at 8000 rpm for 15 mins. Supernatant was discarded and algal pellet was diluted with 80% acetone and water. This was subjected to vortexing for few minutes and then kept in dark conditions for 24hr. Cells were again centrifuged at 3000 rpm for 10 mins. The absorbance of the supernatant was measured

spectrophotometrically at 645 nm and 661.5 nm respectively. Concentration of chlorophyll a ,b and total content was assayed according to Lichtenhaler equation (Gracia *et al.*, 2005).

Chl a =11.24 x $O.D_{661.5}$ - 2.04 x $O.D_{645.0}$

Chl b =20.13 x $O.D_{645.0}$ – 4.19 x $O.D_{661.5}$

Total chlorophyll – chl a+ chl b

= 7.05 x $O.D_{645}$ + 18.08 x $O.D_{645.0}$

3.5.1. Carotenoid estimation

Carotenoids were extracted in acetone in the same manner as mentioned above and analyzed spectrophotometrically by measuring absorbance at 645nm. The amount of carotenoids were evaluated with help of Davis formula (Gracia *et al.*, 2005), using extinction coefficient as 2500. Concentration of carotenoids was expressed in µg/ml.

Carotenoids content (mg/vol) = ($O.D_{450.0}$ x volume of sample taken) / 2500

3.6 Extraction and Downstream Processing of *Dunaliella* Cells and Bioactive Compounds.

In the non-stressed *Dunaliella* cells extraction was done when cells reached a maximum specific growth rate i.e. on 18th day. Whereas in the stressed conditions the growth rate is slow so the extraction is done after 20th day when the cells are in stationary phase as they start accumulating secondary metabolites under stationary growth cycle.

3.6.0. Extraction of algal cells

A homogeneous suspension of *Dunaliella* from each triplicate flask was taken in graduated falcons and centrifuged at 5000 rpm for 15min. Supernatant was discarded and pellet was washed with distilled water by centrifuge at 5000 rpm for 10 mins to remove extra salts (Lee *et*

al., 2003). Microalgal pellet is dried for 48h at 37^{o}C. The dried algal pellet is resuspended in 100% ethanol or acetone with strong vortexing for 15sec. Cell wall was disrupted using ultrasonication for 10mins. Cells were homogenized with solvents for 4 days and then filtered using 0.2µm filter units to get crude ethanolic extract. Supernatant was then rotary evaporated (Figure 7) under reduced pressure at 30^{o}C to remove all the ethanol (Pronyuk *et al.,* 2005). Final dried biomass was weighed and mixed with acetone or ethanol reconstituted extracts in DMSO and stored at -20^{o}C (Raja *et al*., 2007; Ming *et al.,* 2008).

3.6.1. Analysis of bioactive compounds

Carotenoids determination: Sample of the above storage conditions were analyzed for the content of carotenoids spectrophotometrically same as mentioned in (3.5).

Total protein determination: Protein content was determined using the method of Lowry *et al.,* (1951), using Bovine Serum Albumin as standard.

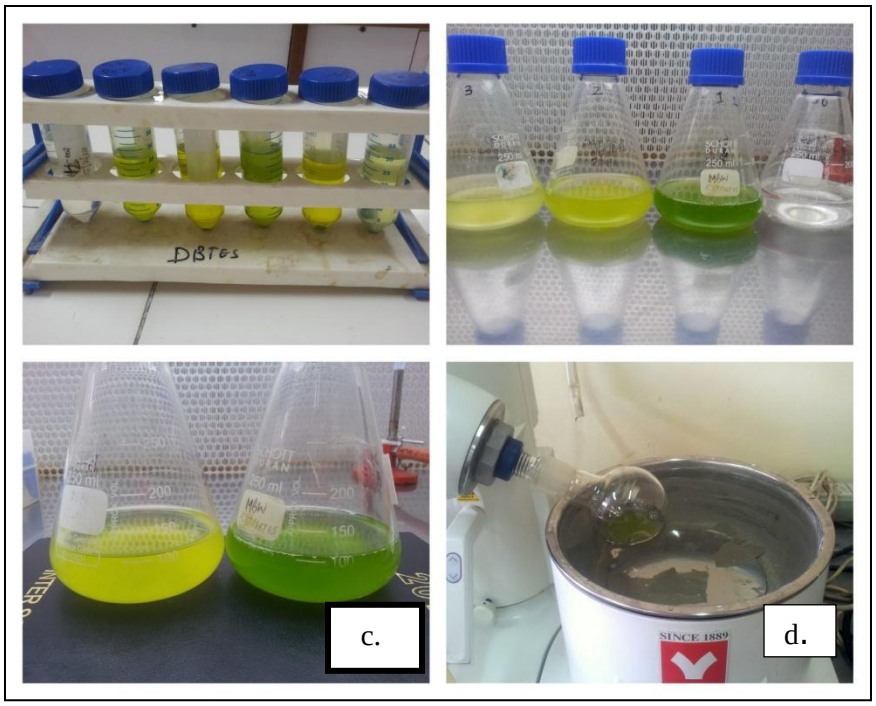
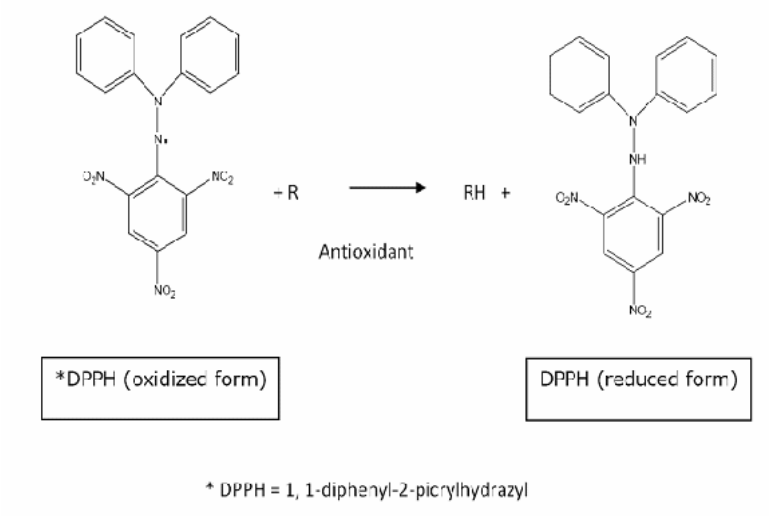


Figure 7. (a.) Harvesting of algal cells in acetone (b.) *Dunaliella* cells in stressed conditions depicting the color change as the number of days increases (c.) *Dunaliella* cells showing accumulation of carotenoids with change in color from green to yellow.(d.) Drying of the extracts by Rota evaporation.

3.7 Biological Activity of *Dunaliella* and Its Bioactive Compounds

3.7.0. In-vitro antioxidant activity of crude extract of Dunaliella salina.

In order to know the antioxidant capacity of crude extract of both non-stressed and stressed *Dunaliella* cells were subjected to well-known antioxidant assay namely, DPPH (2,2-diphenyl-1-picrylhydrazyl) assay and total reducing assay (Singh *et al.*, 2002). Extracts were subjected to following protocol using Ascorbic acid as a standard antioxidant.



Antioxidant and free radical scavenging activity by DPPH assay.

When DPPH is mixed with a substance that can donate a hydrogen atom, (free radical) then this gives rise to the reduced form (Blois, M.S, 1958) and violet color is reduced to pale yellow giving rise to nonradical form, 2,2-diphenyl-1-picrylhydrazyl is reduced to 2,2-diphenyl-1-picrylhydrazine (yellow, non-radical).

100mg/ml of the crude extract was diluted appropriately in methanol to make a final concentration of 250µg/ml and was mixed with 170 µL of DPPH in methanol (100µM), added in wells of a 96-well microtitre plate. Appropriate blanks 10% DMSO diluted in methanol and methanol (only media) and standard of 100µg/ml Ascorbic Acid were run simultaneously. Plate was incubated in dark for 45 mins, after which the absorbance of the solution was measured at 517 nm in a ELISA microtitre plate-reader. Changes in the absorbance were noted (Blios, 1958)

Scavenging activity was expressed as the inhibition percentage calculated using following formula,

$$\%\text{Radical scavenging activity} = \frac{\text{Control}_{abs} - \text{Sample}_{abs}}{\text{Control}_{abs}}$$

Reducing power assay

Total reducing power was determined by Duh and Yen (1997). Substances which have reduction potential reduce potassium Ferricyanide (Fe^{3+}) to Potassium Ferrocyanide(Fe^{2+}) which then reacts with ferric chloride to form ferric ferrous complex that has an absorption maxi mum at 700 nm.

Potassium ferricyanide + Ferric chloride ⟶ Potassium ferrocyanide + Ferrous chloride

Different concentration of crude extract (50 µg/ml, 100 µg/ml, 120 µgml, 150 µg/ml) of *Dunaliella* cells were taken and mixed with 2.5 ml of phosphate buffer (0.2 M, pH 6.6) and 2.5 ml of potassium ferric cyanide (1%). Reaction mixture was incubated in a water bath at 50ºC for 30 min. after incubation 2.5 ml of Trichloroacetic acid (10% of TCA) was added and centrifuged at 6500 rpm for 10 min. From the upper layer, 2.5 ml solution was mixed with 2.5 ml distilled water and 0.5 ml of $FeCl_3$ (0.1%). Absorbance of all the solution was measured at 700 nm. Ascorbic acid is used as a positive control. Increase in absorbance of the samples indicated the reducing potential of the samples.

3.7.2 In-vitro antimicrobial activity of Dunaliella salina

Antibacterial activity

In-vitro antibacterial susceptibility of *Dunaliella* extract was screened against two Gram-negative bacteria (*Escherichia coli and Pseudomonas aeruginosa*) and two Gram- positive bacteria (*Bacillus megaterium and Staphylococcus aureus*) using Microplate- Broth dilution assay. The Antimicrobial activity was determined using MH broth (HiMedia) and using a color indicator, (MTT), 3-(4, 5- dimethylthiazol-2-yl)-2, 5 diphenyltetrazolium bromide which was reduced to formazan (purple color) by living cells. Antimicrobial activity does not exhibit reduction of MTT into formazan. In the screening of antimicrobial compounds, the microplate method provides a potentially useful technique for determining antimicrobial activity of large numbers of test samples, requiring small amounts of substances; this can be particularly important if the antimicrobial is scarce as is the case for many natural products. This method can also be used for a wide variety of microorganisms, is not expensive and presents reproducible results.

Preparation of inoculum

Microbial cultures to be assayed are prepared 24hrs before in MHB broth at 37^0C. Antibiotic concentration of 50 µg/ml is prepared in MHB media.

Standard for Inoculum preparation

To standardize the inoculum density for a susceptibility test, a Barium sulphate ($BaSO_4$) turbidity standard, equivalent to a 0.5 McFarland standard or its optical equivalent (e.g., latex particle suspension), was used. A $BaSO_4$ 0.5 McFarland standard was prepared as 0.5 mL aliquot of 0.048 mol/L $BaCl_2$ (1.175% w/v $BaCl_2.2H_2O$) added to 99.5 ml of 0.18 mol/L H_2SO_4 (1% v/v) with constant stirring to maintain a suspension. The correct density of the turbidity standard was verified by using a spectrophotometer with a 1 cm light path and matched cuvette to determine the absorbance. The absorbance at 600 nm was between 0.144 to 0.146 for the 0.5 McFarland standard. The Barium Sulfate suspension was transferred in 4 to 6 mL aliquots into screw-cap tubes of the same size as those used for growing or diluting the bacterial inoculum (NCCLS, 1997).

Table 6. McFarland Standard

McFarland Standard No.	0.5	1	2	3	4
1.0% Barium chloride (mL)	0.05	0.1	0.2	0.3	0.4
1.0% Sulfuric acid (mL)	9.95	9.9	9.8	9.7	9.6
Approx. cell density ($1X10^8$ CFU/mL)	1.5	3.0	6.0	9.0	12.0
Absorbance*	0.146	0.210	0.449	0.661	0.850

Preparation of plate

Plates were prepared under aseptic conditions. *Dunaliella* extracts were diluted in MHB media to make a final stock concentration of 10 mg/ml containing 10%DMSO, so that final

concentration of extract in the microwells is less than 1%per well. All wells except the first three rows were filled with test sample (50µl) diluted with media to make final well volume of 200 µl per well. First well was filled with media blank and second well was filled with growth control blank containing broth, DMSO, except the antimicrobials. Each test and growth control well was inoculated with 10 µl of 0.5 McFarland adjusted overnight grown cultures (18-24 hours old culture) of culture, to each well.. All experiments were performed in triplicate and the microtitre trays were incubated at 37ºc for 24h. Bacterial growth was detected formerly by Optical density (ELISA reader) and later, by addition of 20 µl of MTT solution. The trays were again incubated at 37ºC for 4h, and in those wells, where bacterial growth occurred, MTT changed from yellow to purple. Then the antimicrobial activity was measured using micro-plate ELISA reader (Multiskan Spectra Readers, Thermo) at 620 nm wavelength. Percentage inhibition was calculated using the following formula:

$$\%\text{Inhibition} = \frac{\text{Control abs} - \text{Sample abs}}{\text{Control}_{abs}}$$

The above experiments were performed in triplicates and for three times each for a particular microorganism.

Antifungal activity

In-vitro antifungal activity of crude *Dunaliella* extract was vetted against *Candida. albicans*. Minimum inhibitory concentration of the extract was investigated by using 96-well plate method as mentioned above. *Candida* was grown in PDB medium and trays were incubated for 48h instead of 24h, positive control tested was Amphotericin drug (50 µg/ml).

3.8 Cytotoxic Effect of Crude Extract of *Dunaliella* and Its Bioactive Compounds on Human Breast Cancer Cell Line

Anticancer activity of crude extract of microalgae was evaluated using MTT assay in the MCF-7 estrogen- receptor positive breast cancer cell line. The extract was prepared from a stock of 100 mg/ml, filtered using 0.2 µm filtration assembly and diluted to 10 mg/ml in DMEM media containing 10% DMSO.

3.8.0 CELL CULTURE

3.8.1 Preparation of complete DMEM liquid medium

9.6 g of powder DMEM media was suspended in 900 ml tissue culture grade water (or Mili Q water) and constantly, stirred gently until the powder was completely dissolved and autoclaved for 15 minutes at 121°C and 15 lbs pressure in an autoclave. After autoclaving allow it to cool to room temperature and then add 49.3 ml of 7.5% sodium bicarbonate solution and 20 ml of 200 mM L-glutamine solution to 1 liter of medium and stirred until dissolved. pH was adjusted to 4.0 using 1N HCl or 1N NaOH. pH of the medium was adjusted ±0.2 below the desired pH since the pH tends to rise during filtration. The final volume was made up to 1000 ml with tissue culture grade water. The medium was immediately sterilized by filtering through a sterile membrane filter with porosity of 0.22 micron, using positive pressure rather than vacuum to minimize the loss of carbon dioxide. Liquid medium was stored at 2-8°C and in dark till use.

DMEM Media is supplemented with 10% heat inactivated Fetal Bovine Serum and Streptomycin (100 µg/ml), Penicillin (100 IU/ml), Amphotericin (2 µg /ml) and Tylosin (1 ml/l). This is called complete DMEM.

3.8.2 Preparation of Phosphate Buffer saline

One litre of 1X PBS was prepared by adding 8 g of NaCl, 0.2 g of KCl, 1.44 g of Na_2HPO_4, 0.24 g of KH_2PO_4 was added in 800 ml of double distilled water pH was adjusted to 7.4 using HCl or NaOH. Volume was made up to 1 litre by double distilled water. PBS was autoclaved for 20 minutes at 121°C. After autoclaving PBS was filter sterilized and stored at 4°C temperature.

3.8.3 Maintenance of MCF-7 Cell Lines

Cell lines were cultured in complete DMEM medium and maintained in a humidified incubator with 5% CO_2 for 37°C in T75 or T25 culture flasks. Vertical laminar was used for working with MCF-7 cells and inverted microscope was used for routine examination of cells (Figure 8). Incubator and lab was regularly maintained by fumigation and by changing of water in incubator to maintain 5 % CO_2 level.

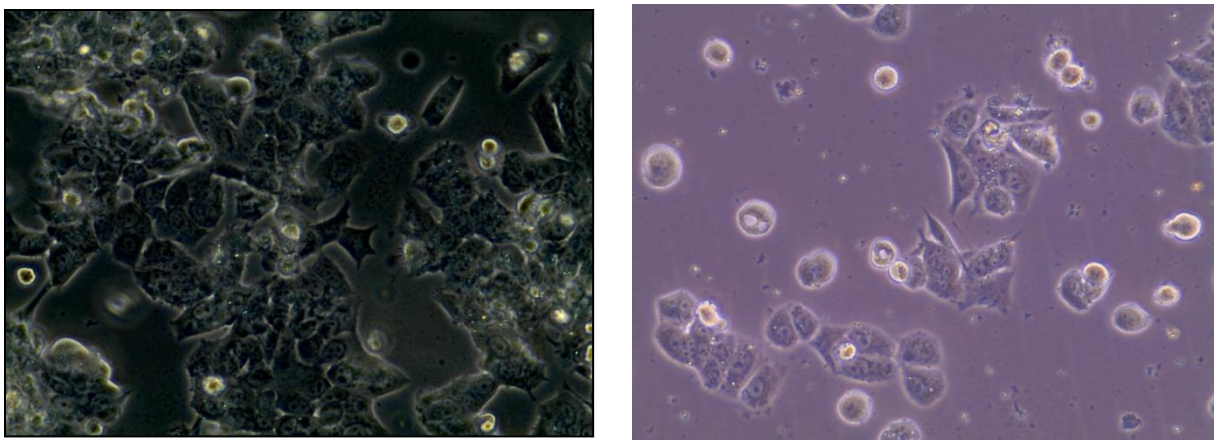


Figure 8. Epithelial MCF-7 cells, under 40-X magnification

a) Revival of MCF-7 cells:

1. Removed the vial containing cells from storage and thawed quickly in a 37°C water bath.

2. Immediately added 2 volumes of complete growth medium to the vial containing frozen and mixed very gently.

3. Centrifuged the cells at 1000 rpm for 10 minute at room temperature.

4. Discarded the supernatant.

5. Gently resuspended the cells incomplete growth medium.

6. Plated the cells in tissue culture T-flask and incubated at 37°C and 5% CO_2.

b. Sub culturing of cells:

1. Used complete DMEM to grow MCF-7 cells in T-flask and maintained the temperature at 37°C in humidified, concentrated CO_2 (5%) atmosphere.

2. Once MCF-7 cells reached approximately 80% confluence in plates, removed media from flask and rinsed with 1X PBS.

3. Added 2-3 ml of warm (37°C) 0.25% Trypsin solution to cells to detach the cell layer. Observed under an inverted microscope (Detachment should happen between 5 and 15 minutes). If cells are not detaching properly, place the flask back in 37°C incubation chamber. Do not agitate the cells during dispersal, either by hitting or shaking the flask. This may cause clumping as the cells detach.

4. Once MCF-7 cell layer is dispersed, Trypsin was deactivated by adding same volume of complete growth medium in flask and then, transferring them to tubes or eppendorfs. Aspirate cells by gently retropipetting.

5. Centrifuged cells in growth medium for 10 minutes at 1000 rpm.

6. Removed trypsin growth medium suspension from tube.

7. Resuspended the pellet (MCF-7 cells) in 2ml PBS and centrifuged at 1000 rpm for 10 minutes.

8. Resuspended the cells in 1 ml of complete medium.

9. Counted the cells using Hemocytometer (10 µl cells + 10 µl of trypan blue+ 80 µl complete DMEM).

10. Resuspended the cells in complete DMEM media (5 ml for T25 flask and 15 ml for T75 flask).

11. Observed culture daily by eye and under an inverted microscope to ensure culture is free of contamination and culture has not reached confluence.

In most cases, cultures at a high cell density exhaust the medium faster than those at low cell density as is evident from the change in pH. A drop in pH is usually accompanied by an increase

in cell density, which is an indicator to subculture the cells. Cells may stop growing when the pH is between 6-7 and loose viability between 6-6.5. Media in flask turns pale red if there is drop in pH whereas it turns pinkish if pH becomes high. In routine culture medium is changed after every two days.

3.8.4 Cell counting and Viability Testing

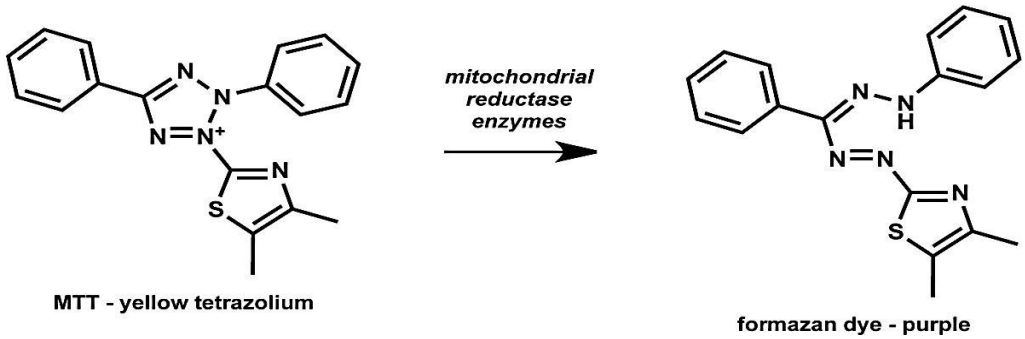
Cell counting was done with the help of Hemocytometer using Trypan blue as a stain. Trypan blue is a stain that penetrates through the cell wall of dead cells and stains them in blue color while live cells remain unstained. 10µl of cell suspension, 80µl of media and 10µl of 0.4% Trypan blue solution (made in 1X PBS) were mixed. Now cell suspension was diluted 10 times to the original cell suspension, and this diluted suspension with Trypan blue was loaded on Hemocytometer. Hemocytometer was focused on using the 10X objective of the microscope and cells were counted in all 4 sets of squares of hemocytometer using 40X objective of the microscope.

Cell count was calculated using the formula:

$$\text{Cell count} = \frac{\text{Total number of cells counted}}{\text{Number of chambers counted}} \times \text{Dilution Factor} \times 10^4 \text{ cells/ml}$$

3.8.5 Assessment of effect of extract on MCF-7 cell growth using MTT Assay

Cell proliferation of MCF-7 cells was measured using a 3-(4, 5- dimethylthiazol-2-yl)-2, 5 diphenyltetrazolium bromide (MTT assay). In viable cells, NADPH dependent Oxidoreductases present in the cytosolic compartment of active cell reduces tetrazolium dye MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) to insoluble Formazan. (Mosmann *et al., 2001*).



Freshly trypsinized MCF-7 cells were taken (20,000 cells/well) and plated in 96-well flat bottomed microtiter plate along with 80 µl complete DMEM. This plate was pre-incubated at 37°C and 5% CO_2 incubator for 12-16 hrs for attachment to surface of plate. Different concentrations of each extracts were added to the wells along with their corresponding controls and final volume was maintained as 200µl. Plate was incubated in 5% CO_2 incubator at 37^0C for 72 hours. After 72 hours of culture, 20 µl of MTT reagent (5 mg/ml) was added to each well and incubated for 4 hours to observe reduction of MTT to formazan crystals. After incubation, 170µl of supernatant was removed and formazan crystals were dissolved in 100 µl of DMSO. In order to properly dissolve formazan crystals, 10 minutes incubation was given at 37^0C and O.D. was taken at 570 nm and 630nm as reference O.D. Final O.D. was calculated as difference between both O.D. by micro titer plate reader.

The percentage growth inhibition was calculated using following formula;

$$\%\text{Inhibition} = \frac{\text{Control abs} - \text{Sample abs}}{\text{Control}_{abs}}$$

3.8.6 Cytotoxic effect of extracts on Human PBMC'S from whole Blood

Considering the possible effects of applied antitumor drugs on normal healthy immunocompetent cells, *Dunaliella* crude extracts were evaluated against healthy PBMC's.

Isolation of Peripheral Blood Mononuclear cells:

1. Blood from healthy human volunteers was obtained with heparinized syringes and was placed into sterile polypropylene tubes
2. Blood samples were diluted with PBS and mixed properly using a pipette
3. The blood samples were layered using PBMC Histopaque 1077 density gradient.
4. Centrifuged the tubes at room temperature (18-20°C) for 30 minutes at 400 X g
5. Extra plasma was removed to about 1cm of the white cell interface.

6. White monolayer cell interface was collected carefully without disturbing and transferred to a fresh falcon.

7. Cells were washed twice with PBS and Centrifuged for 10 minutes at room temperature at 380 X g- repeat this procedure twice, until the pellet turns white

8. Resuspended the white cells for counting in 1ml Cell culture medium. (DMEM) and adjust the cell count to $2x10^4$ cells/well.

3.8.7 Assessment of effect of extract on PBMC's cell growth using MTT Assay

Duanliella extracts that showed anticancer activities were further subjected to evaluate their effect on normal Human cells. 4 µl of mitotic agent, Poke weed mitogen was also added and compared with normal cells. Anticancer drug , Paclitaxel was used as a negative control

Cytotoxic activity of the extract was assessed using MTT, as mentioned above.

The percentage growth inhibition was calculated using following formula;

$$\%\text{Inhibition} = \frac{\text{Control abs} - \text{Sample abs}}{\text{Control}_{abs}}$$

2.9 Statistical Analysis

Data were statistically analyzed by an analysis of variance (ANOVA) and when observed differences were significant, the means were compared by Tukey's honestly significant difference test. The Pearson co-efficient was calculated using Graph-Pad Prism software. All the experiments in this study were performed in triplicates. Graph pad prism (5.0) software was used for all analysis.

Chapter 4

Results &Discussion

RESULTS AND DISCUSSION

4.0 Optimization of Cultivation, Growth and Nutritional Compositions of *Dunaliella salina* Biomass

Background

Microalgae are diverse organisms inhabiting in many ecosystems ranging from Salt lakes and desert sands to marine and fresh water environments (Guschina *et al.*,2006).

In present study, *D.salina* isolated from both the environment was analyzed for various bioactive metabolites and their pharmacological activities under laboratory conditions were studied. Different parameters such as light intensity, temperature, macro and micronutrients play a pivotal role in growth. Several studies have been carried out which point out that stress conditions such as high temperature, high salinity, nitrogen imbalance play a significant role in *de novo* carotenogenesis. Hence in this section, experiments were conducted to evaluate the influence of above oxidative stress on growth rate and accumulation of bioactive metabolites in crude extract of *D.salina.*

4.1 Maintenance and Production of *Dunaliella salina*

Initially the suspension culture of *D. salina* was maintained in 250ml Erlenmeyer flask containing 100 ml of AS-100 media and De-Walens media respectively. These mother cultures were perpetuated at 28^{o}C under continuous light provided by white lamps (Philips, India). Cell growth was monitored and after 30 days when the cell count was 75x10^{6} cells/ml, mother cultures were sub cultured and various stress conditions were given with *D.salina* of ~10^{6} cells/ml as inoculum.

Dunaliella. salina cells were also maintained on agar slants by taking the pelletized cells and streaking them on solid medium. The slants were kept at 28^{o}C, under continuous light intensity for 35 days (Figure 9) and later stored at -20^{o}C.

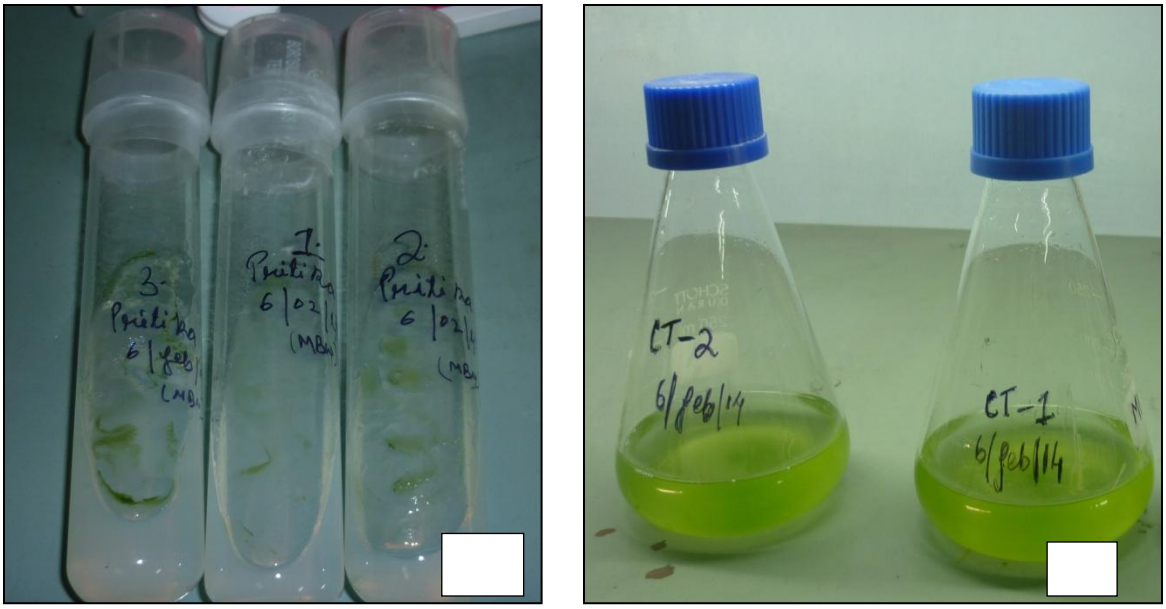


Figure 9. (a) *D.salina* growing on solid agar slant (b) *D. salina* vegetative stage, green culture growing in 250 ml flask under laboratory conditions

4.2 Optimization of Non- Stressed *Dunaliella salina*

The non- stressed *D.salina* isolated after the first subculture was optimized and growth rate, carotenoids and maximum Biomass was measured.

4.2.1 Measurement of growth and productivity

Measurement by morphology study

Non-stressed cultures of *Dunaliella salina* where ovoid or pear shaped, biflagellate cells in vibrating motion under light microscope. The size was measured using Nikon Eclipse E200 inverted microscope, which was approximately 7.6 µm in length and 4 µm in width. Whereas the *D.salina* isolated from marine source had the same morphology but were slightly bigger, 9 µm in length and 6µm in width (Figure 10). Flagella length was equal to size of the cell. The cells appeared to be green in color (Figure 11).

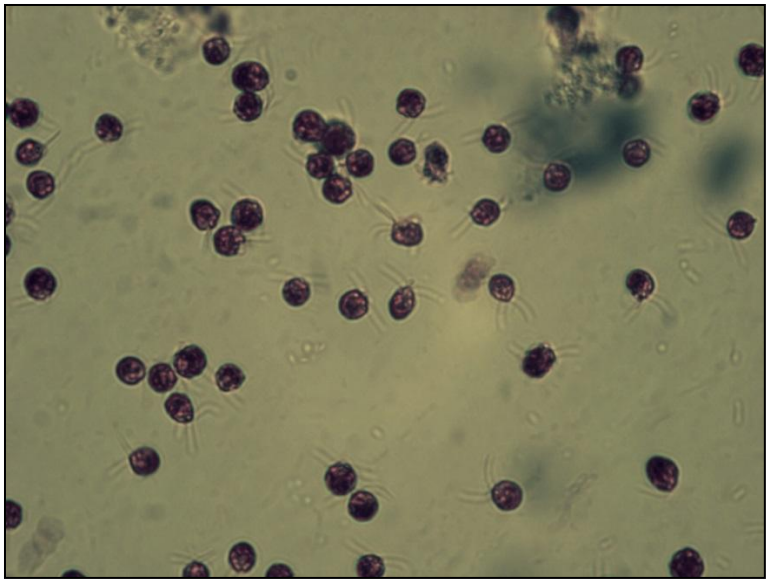


Figure10. Non stressed *Dunaliella* cells from marine source under Inverted microscope. Flagella are clearly visible ,cells are in vegetative phase.

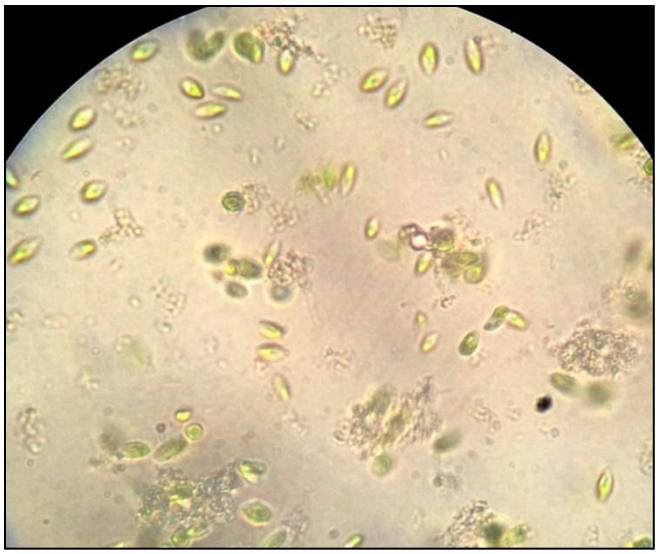


Figure 11. Non stressed, green colored *Dunaliella. salina* cell under light microscope, cells are in vegetative phase.

Measurement by Cell Count

Under the optimum growth conditions i.e. 1.7 M NaCl, the cell concentration of *D.salina* from the saline source increased linearly and attained a log phase on the 6^{th} day. The maximum cell count was seen on the 18^{th} day i.e. 28.8 x 10^6 cells/ml (Figure 12).

D.salina cells from the marine source showed a higher cell count and attained a higher cell concentration on the 12th day i.e. 37.6 x 10^{6} cells/ml as compared to the other strain. The maximum cell count was obtained on the 18th day i.e. ~40 x 10^{6} cells/ml (Table 7).

Table 7. Cell count of *Dunaliella salina* cells per ml.

Days	Saline source	Marine source
2	4.33 ±1.1 ^{f*}	4.0 ± 2.2 ^{d}
4	9.50 ±2.8 ^{e}	11.6 ± 3.7 ^{c}
6	15.0 ±1.0 ^{c}	19.6 ±4.2 ^{bc}
12	22.67 ±3.7 ^{c}	37.6 ± 11.1 ^{a}
18	28.83 ±1.4 ^{b}	39.57 ±5.8 ^{a}
20	27.67 ±5.7 ^{a}	36.67 ±9.7 ^{a}
22	25.67 ±2.3 ^{a}	22.3 ±4.2 ^{b}
24	16.3 ±0.5 ^{ab}	17.7 ±5.2 ^{c}

*Results are expressed as mean value, ± the standard deviation. Means followed by the same letter within the column are not significant at $p<0.05$.

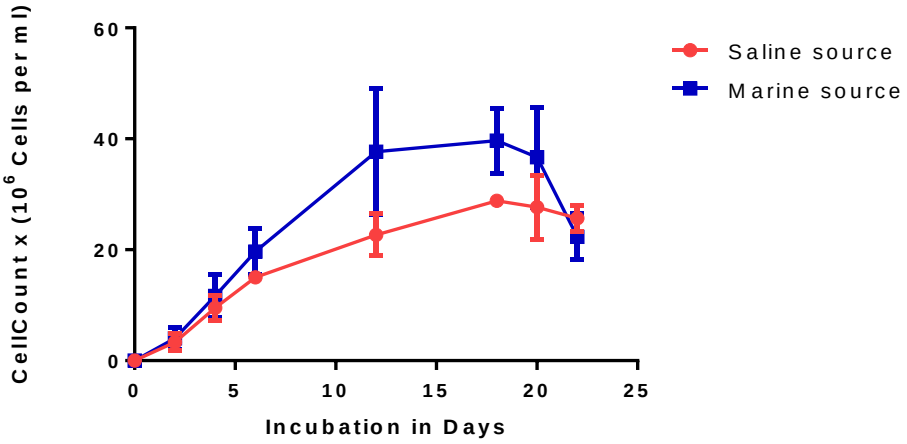


Figure 12. Cell count of *D.salina* under optimum growth conditions (All data presented are the mean of three replicate)

Measurement by optical Density

Under the optimum growth conditions i.e. 1.7 M NaCl at 28^{o}C, the specific growth rate i.e. divisions per day was calculated. Results show that both the strains have a slow growth rate. Specific growth rate decreased on the 1st day and thereafter it increased. The exponential phase was obtained approximately on the 6th day and the growth entered stationary phase on the 17th day. After the 20th day a decline in the growth was seen (Figure 13). Maximum growth rate was seen i.e. 0.869 (Table 8). The Pearson coefficient for specific growth rate was, 0.8921 which states that specific growth is positively correlated with increase in number of days.

Table 8. Specific growth rate of *D. Salina* cells.

Days	Saline source	Marine source
0	0.006	0.002
2	0.0107	0.022
4	0.395	0.422
6	0.869	0.982
12	0.753	0.812
18	0.652	0.711
20	0.562	0.42
22	0.226	0.217

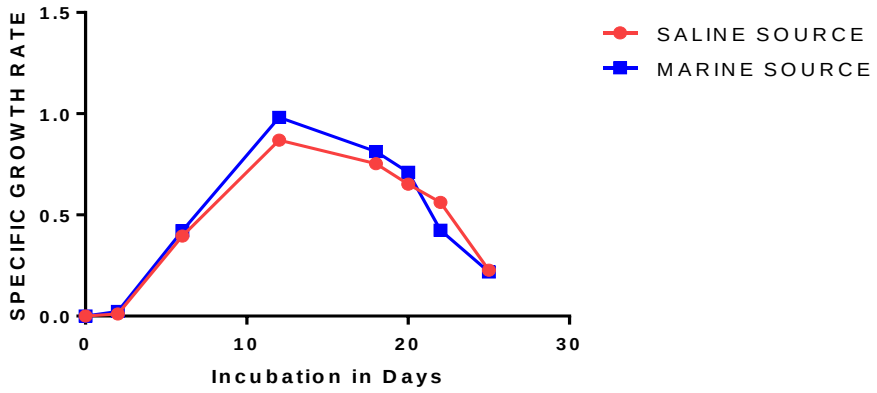


Figure 13. Specific growth rate in non-stressed strains of *D.salina*

4.3 Chlorophyll and Carotenoids in the Vegetative Stage of *Dunaliella salina* Under Laboratory Conditions

Chlorophyll and carotenoid content of *D.salina* from the salt lake was estimated for a period of 20 days under laboratory conditions which was measured for three times during the course. (Figure 14). Chlorophyll a and b content was found to be maximum at 20th day i.e. 1.2 ±0.2 mg/gL^{-1} of the dry weight, which is equivalent to 0.00012µg 100 mg^{-1} of the dry green Biomass. Whereas the carotenoid content was more, 3 mg/gL^{-1} of the dry weight which is equivalent to 0.003µg 100 mg^{-1} of the dry green Biomass, and from marine source was 0.0016µg/ml 100 µg^{-1} of the dry weight. The time course data on accumulation of chlorophyll and carotenoids is represented in following (Table 9)

Table 9. Carotenoids(s) $_{saline}$/chlorophyll(s) $_{saline}$ µg/ml content from saline source carotenoids (m)$_{marine}$/ chlorophyll(m) $_{marine}$ µg/ml content from marine source

Days	Carotenoids$_s$	Total chlorophyll$_s$	Carotenoids$_m$	Total chlorophyll$_m$
3	0.0001±0.03^{a*}	0.36695±0.031^{b}	0.0005±0.056^{bc}	0.4409±2.45^{b}
12	0.00115±0.05^{a}	0.507715±0.1^{a}	0.00095±0.124^{b}	0.34185±1.45^{a}
20	0.0033±3.39^{a}	1.2985±2.31^{a}	0.0016±1.404^{a}	0.39545±0.78^{b}

*Results are expressed as mean value, ± the standard deviation. Means followed by the same letter within the column are not significant at $p<0.05$.

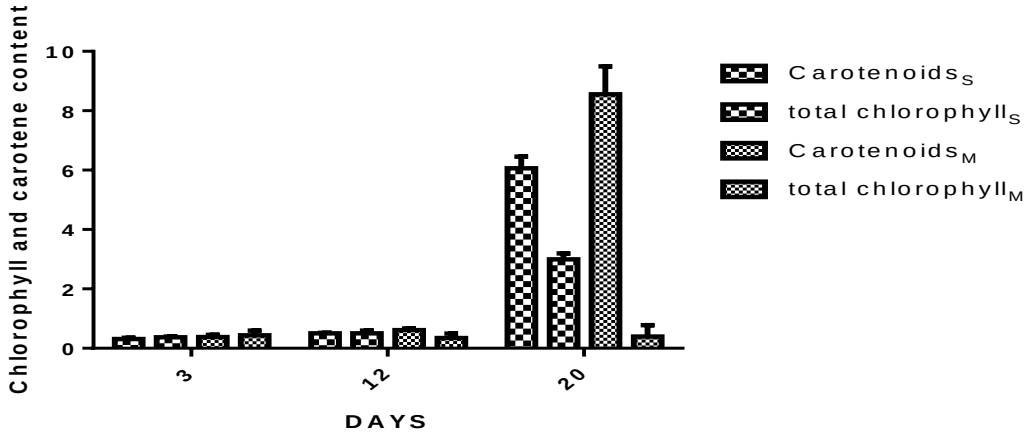


Figure14. Chlorophyll a, b and carotenoid content in *Dunaliella salina* under optimum growth conditions

Dunaliella cells were extracted in acetone and reconstituted in DMSO after establishment of the optimized growth rate and pigment content in the non-stressed green *Dunaliella salina* cells.

4.4 Evaluation of Bioactive Property from Vegetative Crude Extract of Green *Dunaliella* Cells

Preliminary antimicrobial activity was analyzed of the crude ethanolic extract of *Dunaliella* cell with concentration of (10mg/ml in MHB media with 1% DMSO) of both the strains. Antibacterial activity of green *Dunaliella* cells was determined against 4 bacteria and one fungus, based on MTT assay.

Antimicrobials produced by *Dunaliella* salt strain against *B.megaterium* has shown maximum inhibition (Figure 15) which is highest as compared to all the other bacteria. *Dunaliella* marine strain gave maximum suppression with *E.coli*. (Table 10).

Table 10. Percentage Inhibition of *Dunaliella salina* extract with different microorganisms

Microorganisms	**Saline source**		**Marine source**	
	Inhibition(%)	**log values**	**Inhibition(%)**	**log values**
E.coli	26.3 ±0.8 [a*]	0.231	33.1 ±8.4 [a]	0.281
S.aureus	16.4 ±6.5 [b]	0.151	10.9 ±2.1 [bc]	0.103
P.aeruginosa	13.6 ±4. 7 [b]	0.127	20 ±5.2 [a]	0.183
B.megaterium	54.9 ±16.2 [a]	0.437	19.1 ±9.8 [b]	0.174
C.albicans	22.12 ±5.1 [bc]	0.199	18.75 ±4.1 [b]	0.171

***** Results are expressed as mean value, ± the standard deviation. Means followed by the same letter within the column are not significant at $p<0.05$.

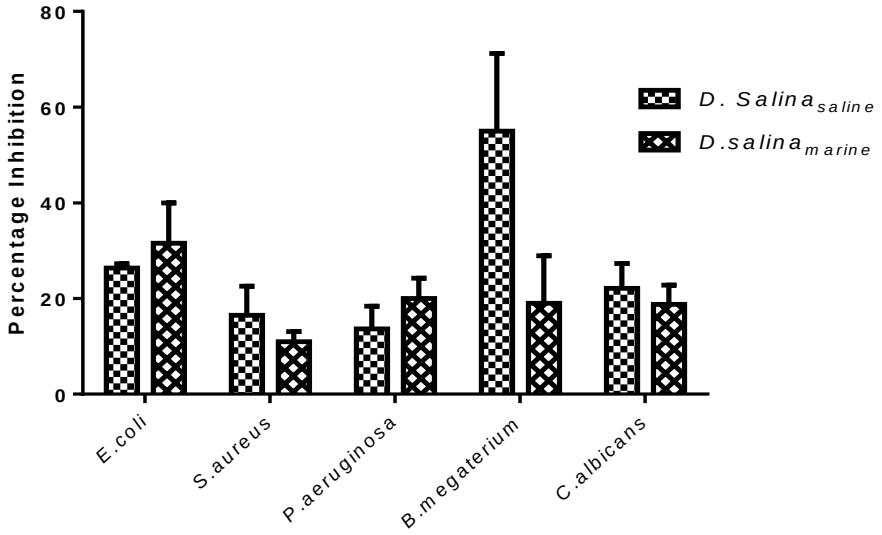


Figure 15. Antimicrobial activity of *D.salina* strains under optimum growth conditions. Bars indicate the standard deviation of three experiments.

Thus the above results depict if a non-stressed *Dunaliella* cells which is growing under optimum conditions of 1.7M salinity, at 28^{o}C under continuous illumination can show a significant level of antimicrobial activity. Then by giving different oxidative stress to the algae cells we can increase the production of bioactive compounds so as to enhance the pharmacological and commercial importance of *Dunaliella salina.*

4.5 Enhancement of Intracellular Bioactive Compounds Under Different Stress Conditions

In this section, influence of different stress conditions; salinity stress, temperature stress and nitrogen stress on growth, bioactive metabolite production and bioactive properties of *Dunaliella* cells were studied.

4.5.0 Measurement of growth and productivity

4.5.1. Effect of salinity

Morphology of salinity stressed on Dunaliella salina cells

Morphological changes were seen in stressed *Dunaliella* cells at stationery phase after 20th Day. Color of the Erlenmeyer flask changed from green to yellow showing the increase of carotenoids (Figure 16). Salinity stress results in change in morphology (Figure 17) . The *D.salina* possessed

flagella of equal length. The cells appeared to be swollen due to accumulation of secondary metabolites in the basal region. The shape was spherical rather than pear shaped. Cells turned Orange/Red due large accumulation of β carotene under high salt conditions (Jayappriyan *et al*., 2010). Size was measured using Nikon Eclipse E200 inverted microscope, which were ~14µm and 6 µm in length.

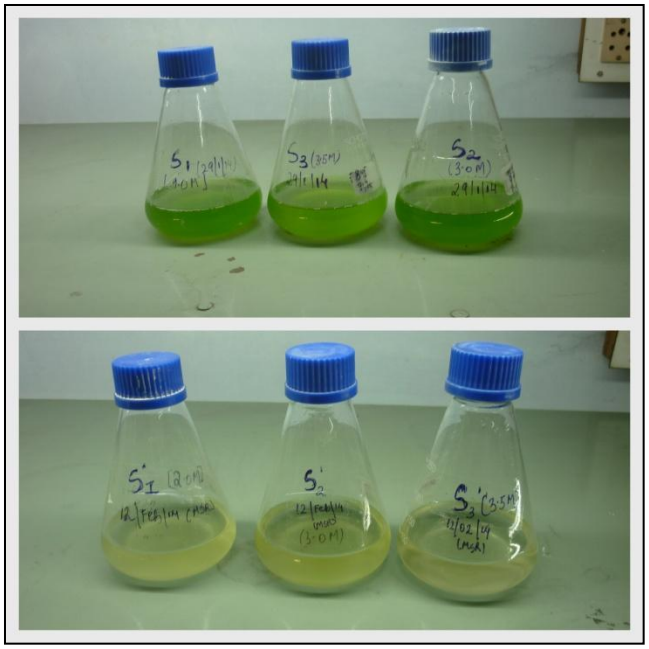


Figure 16. Yellow colored culture of *Dunaliella* representing metabolite production at different NaCl concentrations (stress condition) after 20th day, whereas green colored culture of *Dunaliella* on the 6th day at log phase (growth condition).

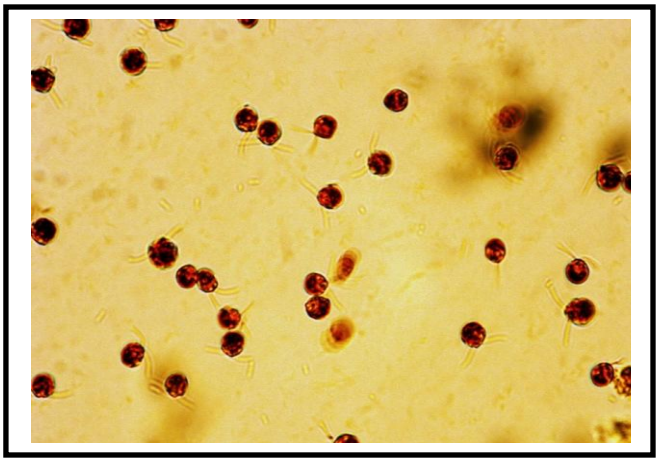


Figure 17. Salinity stressed *Dunaliella* cells from under Inverted microscope. Cells are red in color and in stationary phase.

Growth Measurement

D.salina cells were able to grow in all the concentrations of NaCl tested. A maximum of cell count was recorded at 1.0 M NaCl concentration of $34.16x10^6$ cells/ml. While cultures containing 3.5M NaCl concentration showed a less cell count i.e.$13.5\ x10^6$ cells/ml (Figure 18). Log phase culture was obtained up to 12-18 days in different salt concentrations after that cells entered a stationary growth phase and later the cell concentration decreased (Table 11). 1.0 M showed a highest specific growth rate (μ) of 0.7631 divisions per day. Specific growth rate of 3.0M and 3.5M NaCl concentration was in negative during the initial days and thereafter it increased slowly (Figure 19).

Table 11. Effect of Salinity on cell concentration and cell viability (μ)

Days	**1.0M**		**3.0M**		**3.5M**	
	Cell conc.	**μ**	**Cell conc.**	**μ**	**Cell conc.**	**μ**
0.0	0.007±0.1 [d]	0.005	0.005 ±2.5 [e]	0.001	0.003 ±2.7 [cd]	0.004
2.0	4.16 ±1.5 [d]	0.061	2.56 ±0.5 [d]	0.015	2.467 ±4.2 [d]	-0.045
6.0	10.50 ±0.7 [c]	0.234	10.13 ±5.7 [dc]	0.271	4.50 ±0.8 [c]	0.123
12.0	17.86 ±1.7 [bc]	0.763	22.63±3.3 [b]	0.785	9.10±0.2 [b]	0.559
18	34.18 ±1.5 [a]	0.611	25.6 ±1.7 [a]	0.662	13.5 ±3.3 [a]	0.288
20	27.66 ±6.5 [b]	0.404	26.33±2.6 [a]	0.427	17.6 ±1.2 [a]	0.336
22	14.30 ±0.5 [b]	0.231	21.53 ±1.0 [c]	0.359	9.6±2.8 [b]	0.017

***** Results are expressed as mean value, ± the standard deviation. Means followed by the same letter within the column are not significant at $p<0.05$.

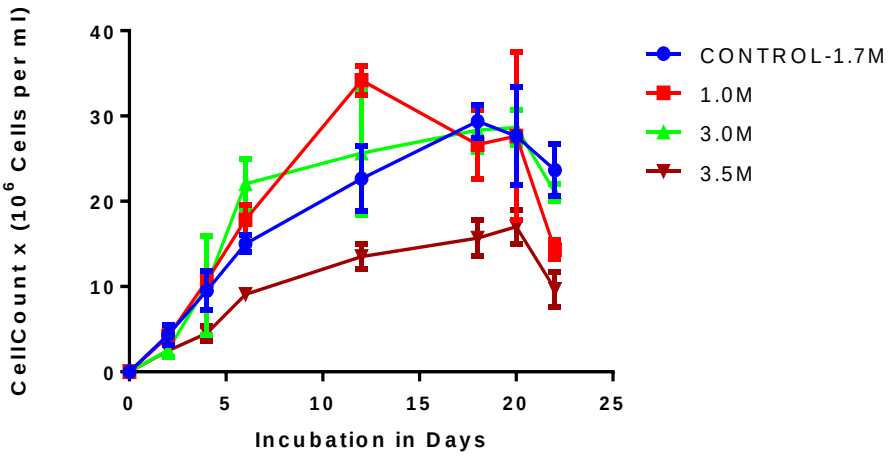


Figure 18. Effect of salinity on cell count of *D.salina* (All data presented are the mean of three replicate)

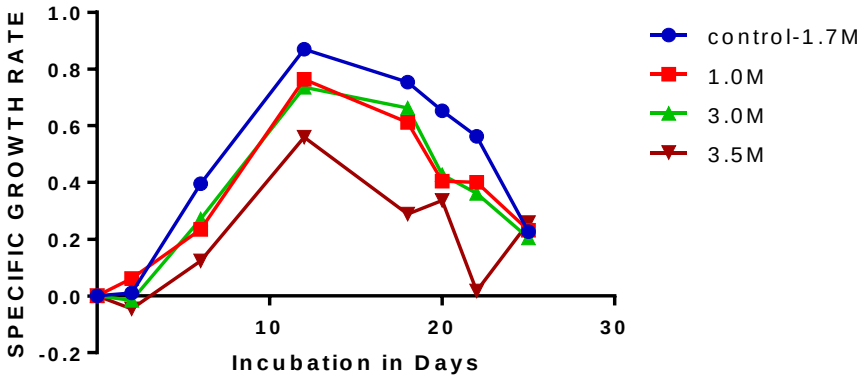


Figure 19. Effect of salinity on cell growth by specific growth rate

Measurement of Carotenoids and Proteins.

Salinity has a lot of effect on accumulation of carotene and proteins . With increase in stress to 3.5 M NaCl the amount of carotene accumulated is 10mg/ gL^{-1} which is equivalent to 0.010µg/ml (Table 12) of the dry weight which was estimated on the 20^{th} day. Total Chlorophyll was highest in 1.0M Thus, as the NaCl concentration increases growth slows down as seen in 3.5M has a longer log phase but has the highest accumulation of carotenoids.

Table12. Effect of NaCl concentration on Biomass and carotene content

Treatments	Carotenoids(mg/ml)	Chlorophyll(mg/ml)	Biomass(mg/ml)
Control 1.7M	0.0033 ± 3.39^{c}	0.0145 ± 0.012^{b}	714
1.0M	0.00415 ± 0.54^{bc}	0.0125 ± 1.123^{a}	414
3.0M	0.00535 ± 2.03^{b}	0.00985 ± 1.112^{a}	213
3.5M	0.00951 ± 3.02^{a}	0.00615 ± 2.198^{b}	175

*****Results are expressed as mean value, ± the standard deviation. Means followed by the same letter within the column are not significant at $p<0.05$.

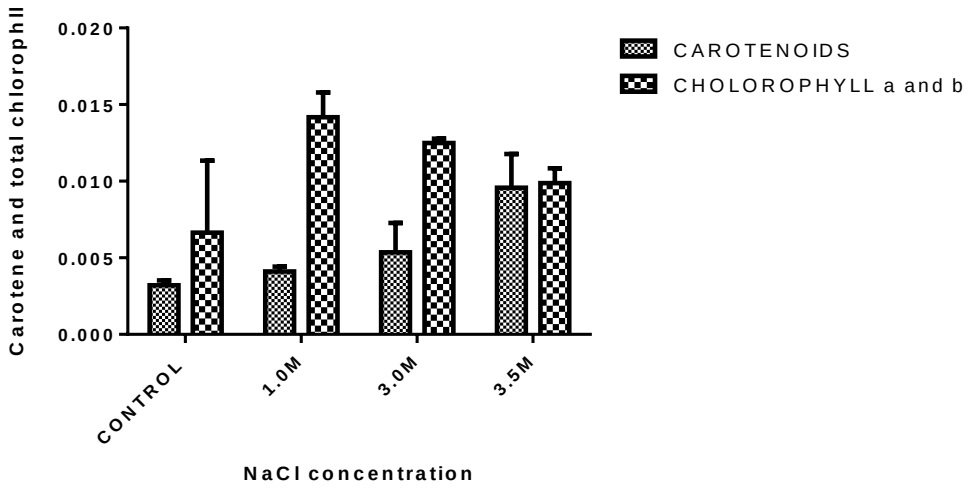


Figure 20. Effect of salinity on carotene and chlorophyll production µg/ml 100µg^{-1} of the dry biomass (All data presented are the mean of three triplicates)

Effect of salinity on the protein content was determined (Table 13). Maximum protein content was measured in pg/ml which was highest in 3.5M i.e. 185.32pg/ml. Although in non-stressed *Dunaliella* cell, amount of protein was 114 pg/ml.

Table 13. Effect of NaCl Concentration on protein content.

Treatments	Protein content(pg/ml)
Control	114.0 ±2.33^{bc*}
1.0M	104.97 ±4.5^{c}
3.0M	139.61 ±1.8^{b}
3.5M	185.32 ±5.3^{a}

*Results are expressed as mean value, ± the standard deviation. Means followed by the same letter within the column are not significant at $p<0.05$

4.5.2 Effect of Nitrogen

Morphology of Nitrogen stressed Dunaliella cells

Morphology of *Dunaliella cells* in different nitrogen concentration was studied. Initially the color change was strikingly visible, as the Erlenmeyer flask containing 0.25M nitrogen showed a dark green color as compare to without nitrogen, with a very slow growth and color change (Figure 21). Shape and size was similar to that of the salinity stressed cells, only a slight variation was seen in size, i.e. 7µm in width. Cells showed a bright red appearance (Figure 22)

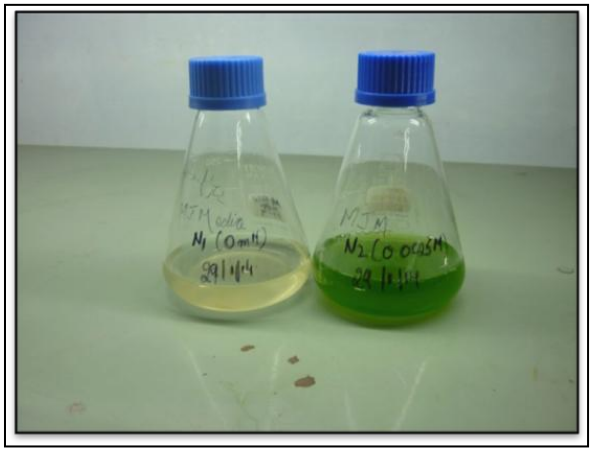


Figure 21. Nitrogen stressed *Dunaliella*, Green color depicts the cells are in the exponential growth phase, whereas the 0M nitrogen takes longer time and starts *de novo* synthesis of bioactive metabolites to adapt the stress (Amotz *et al.*, 1982)

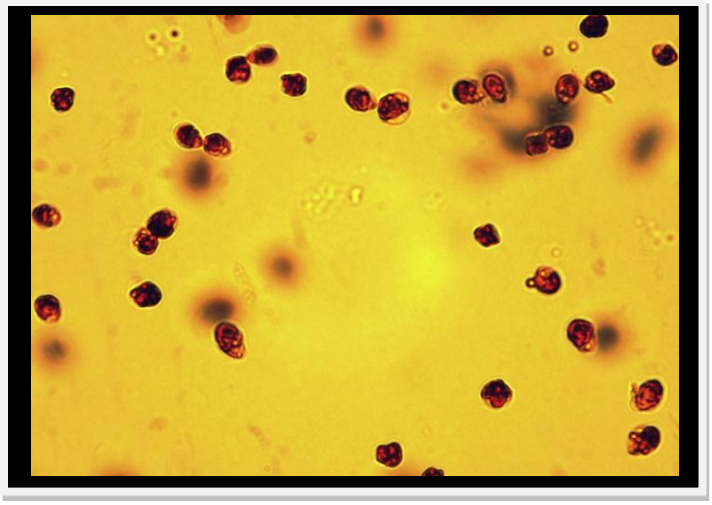


Figure 22. Nitrogen stressed *Dunaliella* cells from under Inverted microscope. Cells are red in color and in stationary phase.

Growth Measurement

D.salina cells were able to grow in nitrogen depleted (0M) and nitrogen excess conditions (0.25M). Growth was monitored in terms of cell count/ml. As concentration of nitrogen decreased, cell number and specific growth rate decreased and carotenoids and chlorophyll increased. In medium without nitrogen source, cell number (12.9x 10^6cells/ml) was less than half at 0.25 M concentration. Maximum cell concentration at 0.25M was seen on the 18^{th} day of 36.6x10^6cells/ml (Figure 23). Specific growth rate was half in 0M as compared to 0.25M (Table 14), the maximum specific growth rate in 0m was 0.207 divisions per day (Figure 24)

The Pearson coefficient (0.813) was interdependent and showed a positive correlation with increase in days and specific growth rate, and gave a less p value<0.0002.

Table 14. Effect of nitrogen on cell concentration and cell viability

Days	0 M Cell count/ml	μ	2.5 M Cell count/ml	μ
0	0.001 ±0.4^{f}	0.005	0.00 ±0.8^{d}	0.001
2	1.50 ±0.5^{e}	0.017	3.00 ±0.5^{d}	0.038
6	5.50 ±2.7^{d}	0.0598	10.16 ±1.7 ^{c}	0.253
12	12.66 ±2.5^{c}	0.136	24.63 ±3.3^{b}	0.585
18	16.98 ±1.5^{ab}	0.207	36.03 ±1.7 ^{a}	0.315
20	20.33 ±1.5^{a}	0.195	32.6 ±2.6^{b}	0.247
22	15.50±0.5^{b}	0.179	24.53±3.8^{b}	0.159

*Results are expressed as mean value, ± the standard deviation. Means followed by the same letter within the column are not significant at $p<0.05$.

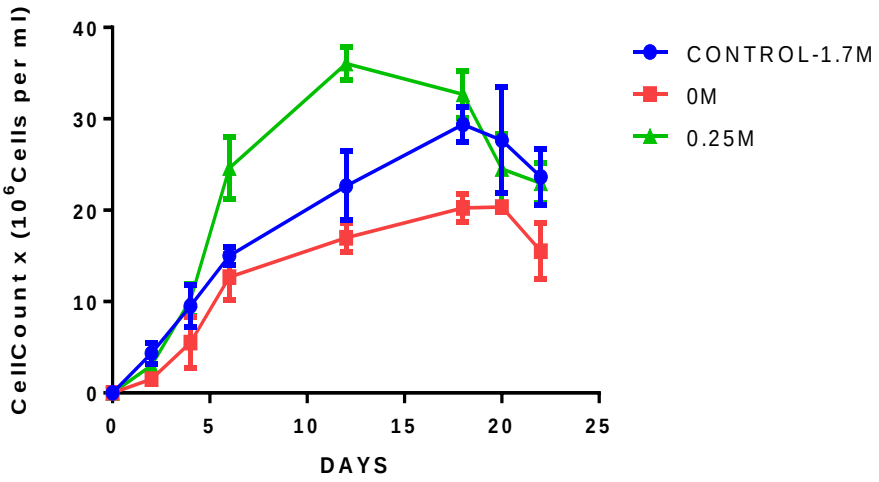


Figure 23. Effect of Nitrogen stress on cell growth of *D.salina*

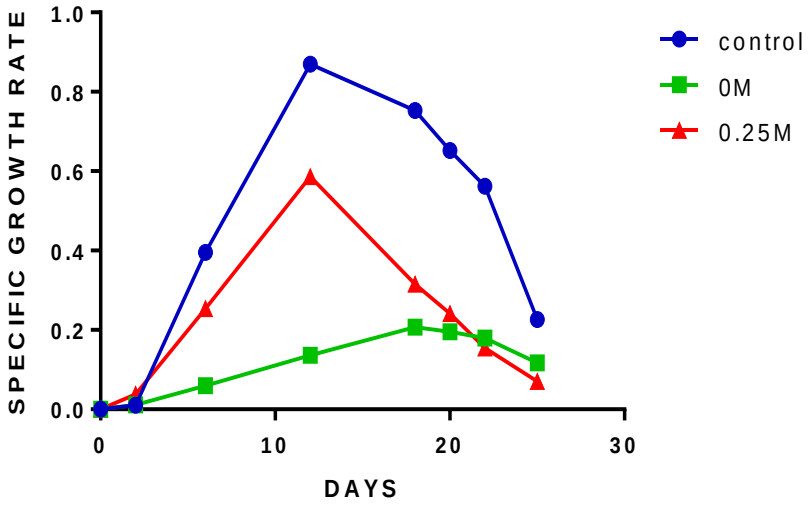


Figure 24. Effect of Nitrogen stress on cell viability of *D.salina*

Measurement of bioactive compound (Carotenoid and Proteins)

The amount of intracellular carotene was more in nitrogen starved condition i.e. 8.7 mg/ gL^{-1} which is equivalent to 0.087µg/ml 100 μg^{-1} of the dry weight, estimated on the 22nd day (Figure 25). Total Chlorophyll was highest in 0.25M which is 0.04 µg/ml, although the amount of biomass produced is more in 0.25 M which is an exception. (Table 15).

Table 15. Effect of nitrogen stress on Biomass and carotene content.

Treatments	Carotenoids(mg/ml)	Chlorophyll(mg/ml)	Biomass(mg/ml)
Control 0.75mM	$0.0033\pm3.39^{b*}$	0.007 ± 0.012^{c}	714
0.0M	0.00115 ± 1.54^{c}	0.028 ± 0.13^{b}	200
0.25M	0.0075 ± 2.03^{b}	0.040 ± 3.2^{a}	817

*Results are expressed as mean value, ± the standard deviation. Means followed by the same letter within the column are not significant at $p<0.05$.

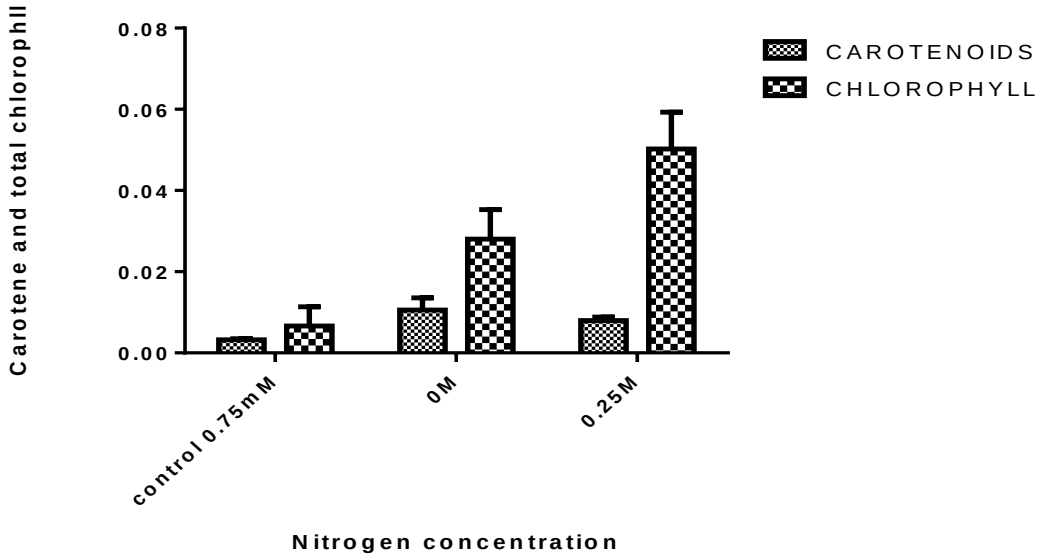


Figure 25. Effect of different Nitrogen concentration on carotene and chlorophyll production µg/ml (All data presented are the mean of three triplicates)

Effect of Nitrogen concentration on the protein content was determined (Table 16). Nitrogen starvation causes a significant reduction in the intracellular concentration of proteins (Lammer, 2010) as seen in the study *D.salina* without nitrogen source had the lowest protein content.

Table 16. Effect of Nitrogen Concentration on Protein content.

Treatments	**Protein content(pg/ml)**
Control	114.0 ±2.33^{b*}
0.0M	98 ±6.7^{a}
0.25M	109.61 ±0.8^{b}

*Results are expressed as mean value, ± the standard deviation. Means followed by the same letter within the column are not significant at $p<0.05$.

4.5.3 Effect of Temperature stress

Morphology of temperature stressed Dunaliella salina cells

Morphology of *Dunaliella cells* in different Temperature conditions of 15-25 ^{0} C and 37 ^{0} C was studied under continuous white lamp with 12/12 light and dark cycle. Stressed *Dunaliella* cells showed spherical shape under inverted microscope. Cells growing at high temperature were more swollen due to accumulation of metabolites and had a size of 13.76 µm while cells growing at 25^{0} C was comparatively less shortened, length of 7.6 µm similar to that of non-stressed *D.salina* cells. The change in yellow/red color was more lucid at 37^{0} C (Figure 26).

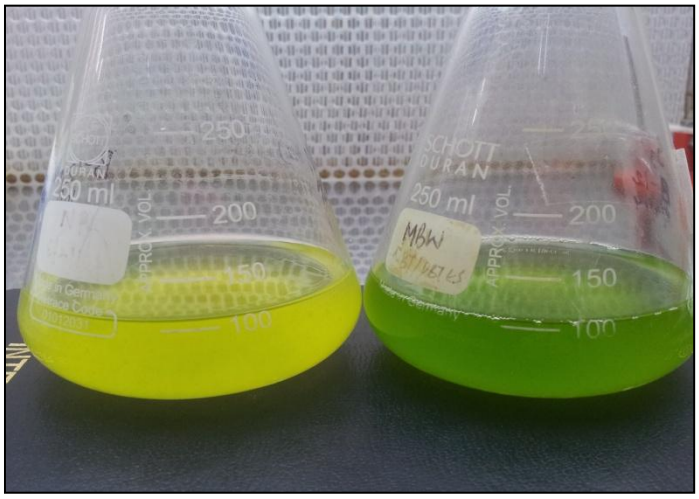


Figure 26. *D.salina* cells on 18th day, under temperature stress conditions

Growth Measurement

D.salina cells were able to grow in temperature stress conditions. Growth was monitored in terms of cell count/ml and cell viability was analyzed spectrophotometrically. At 25^0C maximum cell number (32.34 x 10^6cells/ml) and growth rate (0.8952 divisions per day) were observed.(Table 17). At 37^0C maximum cell number was 23.53x 10^6 cells/ml (Figure 27) and specific growth rate was 0.395 (Figure 28).

Table 17. Effect of Temperature on cell concentration and cell viability.

Days	**25^0C**		**37^0C**	
	Cell count	**μ**	**Cell count**	**μ**
0.0	0.001^{f} ±0.3	0.0100	0.00^{b} ±0.6	0.012
2.0	5.03 ±0.64^{e}	0.0107	3.06 ±0.5^{b}	0.027
6.0	19.66 ±4.7^{c}	0.813	5.46 ±3.7^{b}	0.395
12.0	32.56 ±3.9^{a}	0.515	11.03 ±3.3^{b}	0.285
18	29.62 ±6.5^{a}	0.376	23.53 ±1.7^{a}	0.198
20	22.83 ±1.5^{b}	0.351	13.6 ±2.6^{c}	0.174
22	25.50 ±2.5^{b}	0.217	10.53 ±3.8^{c}	0.389

*Results are expressed as mean value, ± the standard deviation. Means followed by the same letter within the column are not significant at $p<0.05$.

The Pearson coefficient, 0.8110 was interdependent and showed a positive correlation with increase in days and specific growth rate.

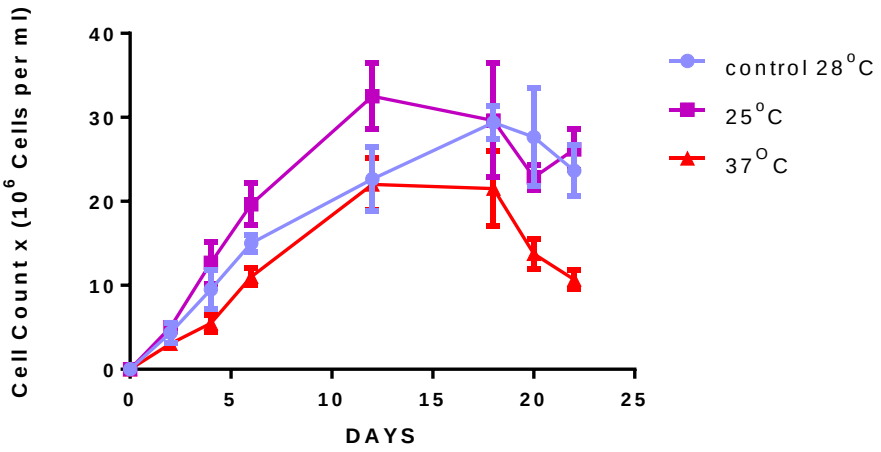


Figure 27. Effect of Temperature on cell count of *D.salina* (All data presented are the mean of three replicate)

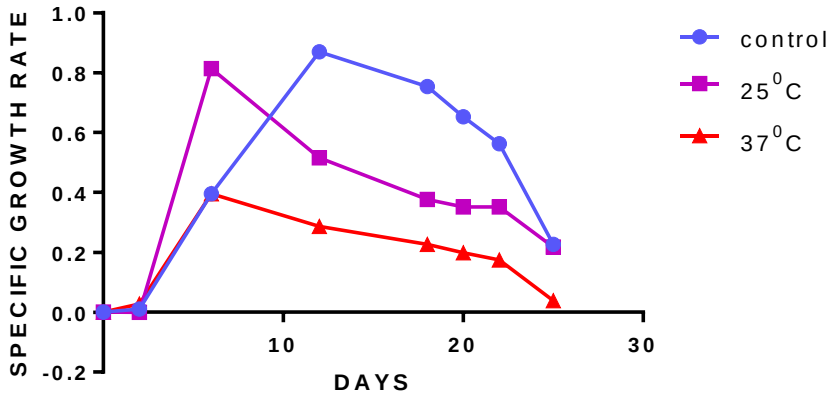


Figure 28. Effect of Temperature on cell viability of *D.salina*

Measurement of bioactive compound (Carotenoid and Proteins)

Higher growth rate, cell number and chlorophyll content in *Dunaliella* were observed at 26 ^{0}C than 37 ^{0}C, whereas high carotenoids and proteins were observed at 37 ^{0}C i.e. 13.1 mg/ gL^{-1} which is equivalent to 0.013µg/ml of the dry weight estimated on the 20th day (Table 18).

Total Chlorophyll was highest at 37 ^{0}C which is 0.045µg/ml (Figure 29).

Table 18 Effect of Temperature stress on Biomass and carotene content.

Treatments	Carotenoids(mg/ml)	Chlorophyll(mg/ml)	Biomass(mg/ml)
Control 0.75mM	$0.0033 \pm 3.9^{b*}$	0.007 ± 0.1^{a}	714
0.0M	0.005 ± 0.5^{a}	0.013 ± 1.1^{b}	542
0.25M	0.013 ± 1.7^{a}	0.0574 ± 6.2^{a}	425

*Results are expressed as mean value, ± the standard deviation. Means followed by the same letter within the column are not significant at $p<0.05$.

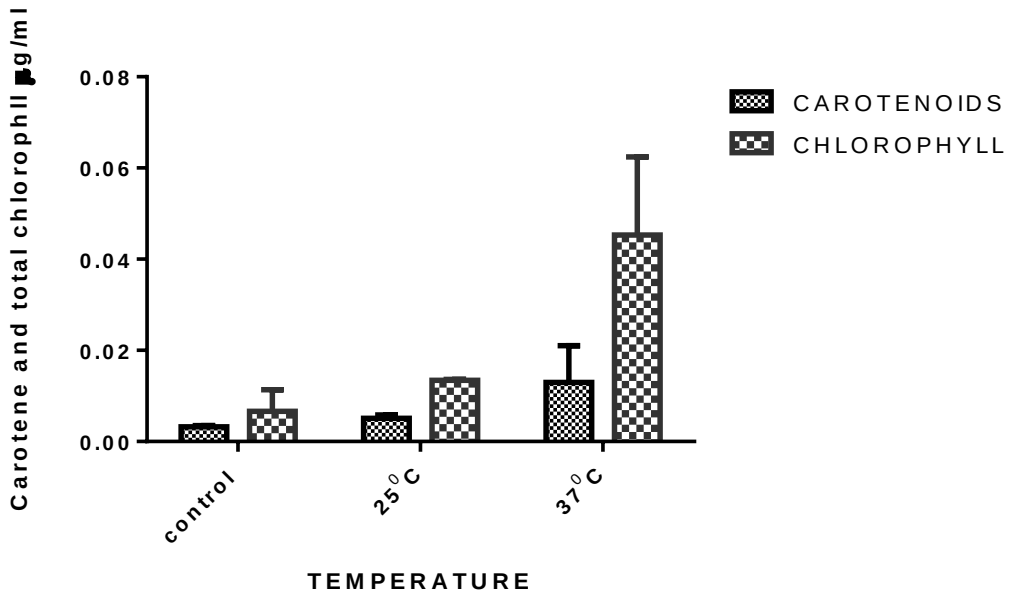


Figure 29. Effect of different Temperature stress on carotene and chlorophyll production µg/ml $100\mu g^{-1}$ of the dry biomass (all data presented are the mean of three triplicates)

Effect of Temperature stress on the protein content was determined (Table 19). Maximum protein concentration was seen in 37^0C i.e. 155.17pg/ml

Table 19. Effect of Temperature on Protein content

Treatments	Protein content(pg/ml)
Control	114.0 ±2.3[a*]
25^{0}C	120 ±0.7[a]
37^{0}C	155.17 ±0.2[a]

*Results are expressed as mean value, ± the standard deviation. Means followed by the same letter within the column are not significant at $p<0.05$

4.6.Biological Activity of Crude Extract of *Dunaliella Salina*

Background

This section deals with the evaluation of *in-vitro* biological activity of algal biomass as well the bioactive compounds with emphasis on antioxidant , free radical, antimicrobial and anticancer activity. In order to evaluate these properties, biomass was successively extracted using ethanol or acetone and reconstituted with DMSO.

4.6.1 Antioxidant Activity of Dunaliella extract.

Antioxidant activity of both the vegetative (non-stressed) and stressed *Dunaliella* cells were studied using DPPH assay and reducing power assay. Both the vegetative non-stressed cells from both the sources marine and saline showed antioxidant activity (Figure30). Marine algae showed a higher antioxidant activity as compared to the saline source i.e. 0.382 log value of % antioxidant activity. All the activity is summarized in Table 20.

4.6.2 Antioxidant activity of the stressed Dunaliella cells

Antioxidant activity i.e. free radical scavenging activity of the *Dunaliella* cells under stress conditions was demonstrated using DPPH assay. Algal extracts of 10mg/ml diluted in methanol were able to reduce 2,2-diphenyl-1-picrylhydrazyl (violet) to 2,2-diphenyl-1-picrylhydrazine (yellow). Results show that 10% DMSO diluted with methanol has no effect on free radical scavenging activity. The results were more significant in the stress conditions. The log values are summarized in Table 20.

Effect of salinity

Salinity stressed *Dunaliella* crude extracts have shown good free radical scavenging as compared to ascorbic acid. In case of 3.5M, free radical scavenging activity of 57.3% . As the concentration of stress increases the antioxidant activity increases as seen in (Figure 31).

Effect of nitrogen

Under nitrogen stress antioxidant activity also increased as compared to standard of ascorbic acid. In case of nitrogen nutrient starved i.e. 0M nitrogen concentration showed more free radical scavenging activity i.e. 0.354 which is equivalent to 42.58 % which had showed more carotenoid accumulation as compared to 0.25M nitrogen (Figure 32).

Effect of Temperature

More Free radical scavenging, is seen more under high temperature of 37^0C i.e. 33.3% (percentage of log value). Whereas algae growing under 25^0C has shown a relatively low value of 4.91%. Thus as the concentration of stress increases the antioxidant activity increases (Figure 33).

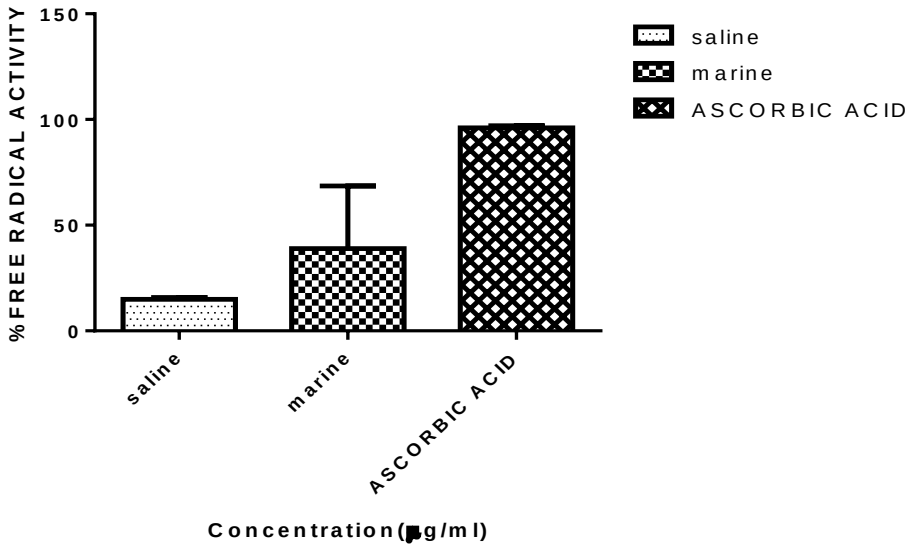


Figure 30. Antioxidant activity of two strains of non-stressed *Dunaliella* cells (All data presented are the mean of three triplicates)

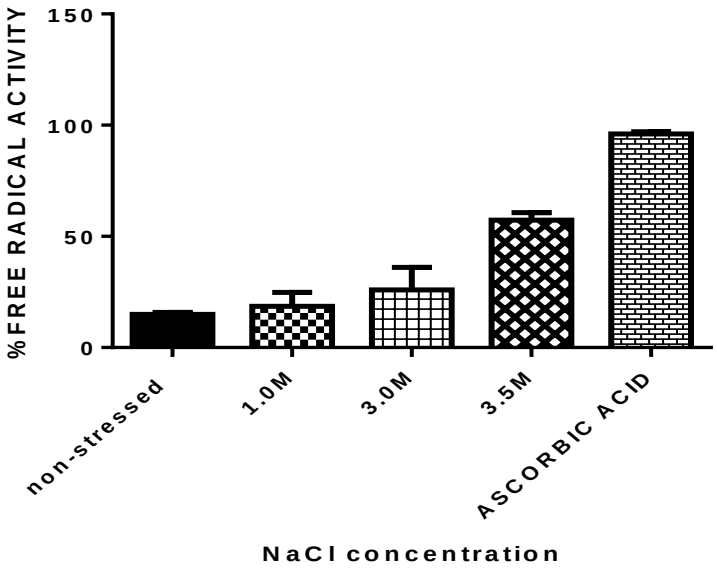


Figure 31. Percentage of Log values of Salinity stressed *Dunaliella* cells (The results are mean value of triplicates)

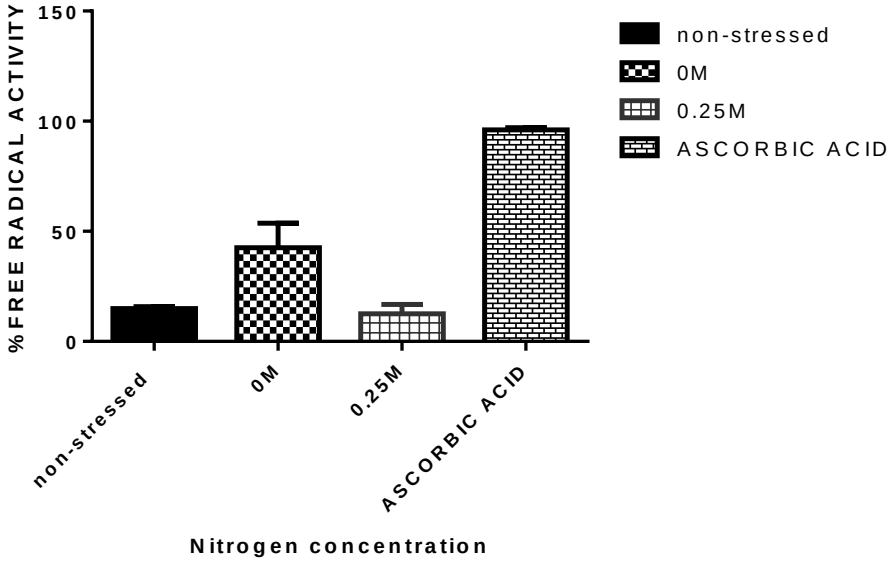


Figure 32. Percentage of Log values of Nitrogen stressed *Dunaliella* cells (The results are mean value of triplicates)

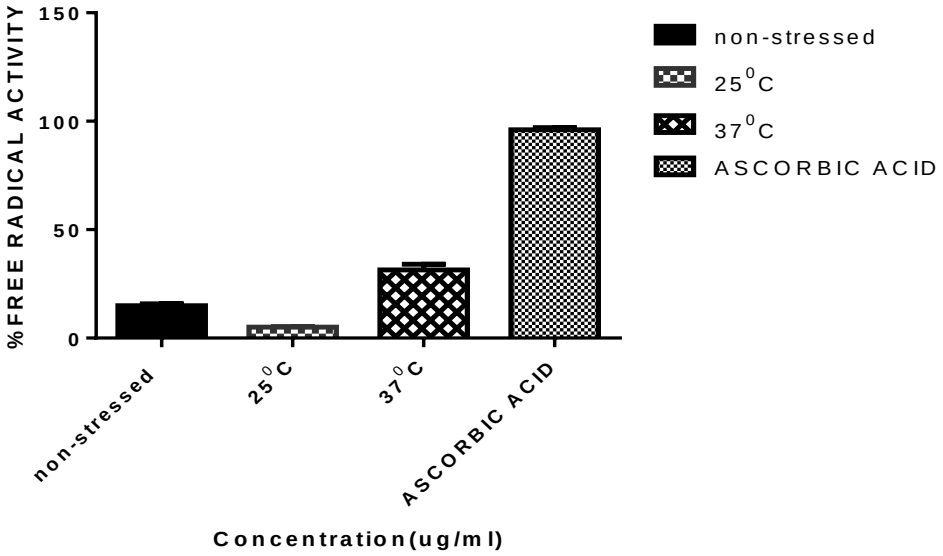


Figure 33. Percentage of Log values of Temperature stressed *Dunaliella* cells (The results are mean value of triplicates)

Table 20. Summarized Antioxidant values of all the stress treatments.

Stress treatments	**Antioxidant Activity (%)**	**Log Values**
Non-stressed	14.96%±0.7	0.138^{bc*}
1M NaCl	21.5%±6.3	0.194^{b}
3.0M NaCl	25.97%±10.2	0.230^{b}
3.5M NaCl	57.3%±3.3	0.452^{a}
0M N_2	42.52%11.1	0.354^{a}
0.25M N_2	12.60%±4.3	0.118^{c}
25^0C	14.96%±0.2	0.047^{d}
37^0C	33.3%±2.3	0.287^{ab}

*Results are expressed as mean value, ± the standard deviation. Means followed by the same letter within the column are not significant at $p<0.05$.

4.6.2.1 Total Reducing power assay

Reducing power is associated with antioxidant activity. Compounds with reducing power including antioxidants and lipids act as electron donor and reduce the oxidized intermediates lipid peroxidation processes, so that they can act as primary or secondary antioxidants. During this assay yellow color of test solution changed to various shades of blue depending on the concentration of reducing power of stressed algal extract (Figure 34).

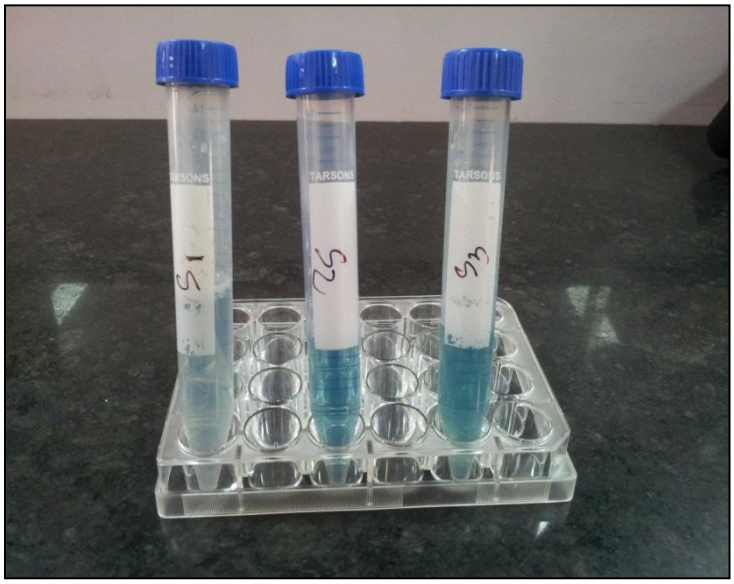


Figure 34. Change in color from (yellow to dark blue) with the increase in stress conditions.

Effect of salinity

All the extracts of *D.salina* showed increase reducing power with increase in the concentration of extract from (50 µg/ml to 200 µg/ml). As the stress increased, 3.5 M NaCl depicted a significant reducing potential. At 200 µg/ml the change in color from yellow to blue was seen in minutes, giving a value of 0.7885 at 700 nm as compared to ascorbic acid (Table 21).

Parallel to this non-stressed *D.salina* showed less than half value at same concentration.

Thus with increase in stress other non-polar compounds with carotenoids also accumulate and produce a potent reduction ability (Figure35).

Figure 35. Reducing power of crude extract of Salinity stressed *D.salina*

Table 21. Effect of salinity stress on total reducing power

Concentration(µg/ml)	Non-stressed	1.0M	3.0M	3.5M	A.A
50	0.10±0.4 ^{Bb}	0.041±0.2 ^{aB}	0.12±0.2 ^{aB}	0.217±0.4 ^{dA}	0.249±1.4 ^{cA}
100	0.193±0.1^{abB}	0.089±1.1 ^{aB}	0.246±0.4^{bAB}	0.434±1.2 ^{cA}	0.518±0.4 ^{baA}
150	0.075±2.1^{abB}	0.130±0.6 ^{aB}	0.308±0.2 ^{bB}	0.581±0.5 ^{bA}	0.642±0.3 ^{bA}
200	0.091±0.4 ^{aB}	0.198±0.3 ^{aC}	0.449±0.1 ^{cB}	0.788±0.4 ^{aA}	0.969±1.6 ^{aA}

*Results are expressed as mean value, ± the standard deviation. Means followed by the same letter within the column are not significant at $p<0.05$

Effect of Nitrogen

As compared to ascorbic acid, nitrogen stressed *D.salina* showed a potent reducing potential. But in comparison to DPPH assay, 0.25 M nitrogen equally possessed antioxidant potential, and gave a reducing power of 0.7235 at 200 µg/ml, exhibiting that there might be other non-polar components or lipids which gave this activity (Table 22). However reducing power was still higher in 0 M (Figure 36) and increased linearly with increase in concentration of crude extract.

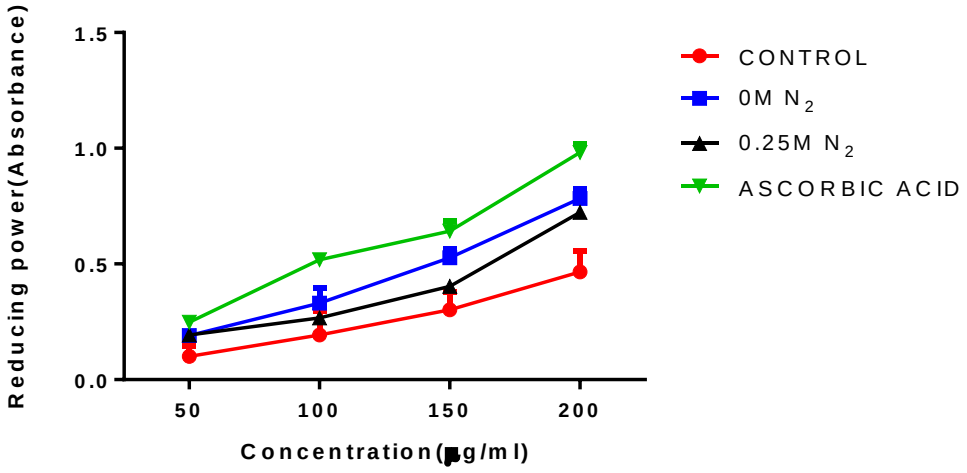


Figure 36. Reducing power of crude extract of Nitrogen stressed *D.salina*

Table 22. Effect of salinity stress of *D.salina* on Total reducing power

Concentration(µg/ml)	Non-stressed	0M	0.25M	A.A
50	0.10±0.04 ^{Bb}	0.191±0.04^{c}	0.193±0.4 ^{c}	0.249±1.4 ^{cA}
100	0.193±0.1 ^{abB}	0.331±3.4^{c}	0.267±0.1 ^{c}	0.518±0.4 ^{bA}
150	0.075±2.1^{abB}	0.525±1.0 ^{b}	0.403±2.3 ^{cC}	0.642±0.3 ^{bA}
200	0.091±0.4 ^{aC}	0.783±4.2^{aB}	0.723±3.4 ^{aAB}	0.969±1.6 ^{aA}

*Results are expressed as mean value, ± the standard deviation. Means followed by the same letter within the column are not significant at $p<0.05$

Temperature stress

With increase in the temperature reducing power also increased, as seen in 37^0C gave the highest value compared to all other conditions at 200 µg/ml (Table 23). Results showed with increase in concentration of the extract reducing potential also increased linearly (Figure37).

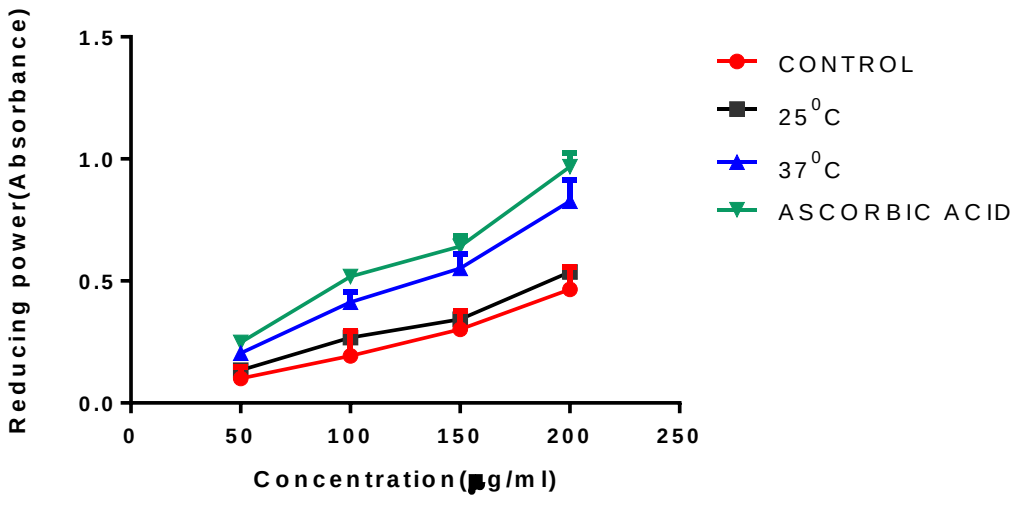


Figure 37. Reducing power of crude extract of Temperature stressed *D.salina*

Table 23 Effect of temperature stress of *D.salina* on total reducing power.

Concentration(µg/ml)	Non-stressed	25^0C	37^0C	A.A
50	0.10±0.04 **bB**	0.131±0.1 **dA**	0.206±0.3 **aB**	0.249±1.4 **cA**
100	0.193±0.1**abB**	0.265±0.3 cA	0.413±2.1 **bA**	0.518±0.4 **bA**
150	0.075±2.1**abB**	0.344±0.2 **bB**	0.551±4.4 **bA**	0.642±0.3 **bA**
200	0.091±0.4 **aB**	0.536±1.1 **aBC**	0.827±0.1 **aAB**	0.969±1.6 **aA**

*Results are expressed as mean value, ± the standard deviation. Means followed by the same letter within the column are not significant at $p<0.05$

4.5.3 Antimicrobial Activity of the Stressed *Dunaliella salina*

As discussed earlier non-stressed *Dunaliella* showed antimicrobial activity ,this forms the basis that under oxidative stress the algae should possess the same. The results of the antimicrobial testing of 10 mg/mL algal extract revealed that *Dunaliella salina* have potential antimicrobial activity when compared to Ampicillin (50µg/ml)

Antibacterial activity

The ethanolic crude algal extract of the eight stress conditions showed different activities against an array of Gram positive, Gram negative bacteria. Salinity stress with 3.0M and 3.5M NaCl concentration were active against *Staphylococcus aureus* and gave values 0.426 and 0.592 (log values of percentage inhibition), (Figure38). An oxidative stress of high temperature showed significant antimicrobial activity with all microorganisms (Table 24).

Table 24. Summarized Antibacterial values of all the stress treatments

Treatments	*E.coli*		*S. aureus*		*P. aeruginosa*		*B. megaterium*	
	%inhibition	*Log values*	*%inhibition*	*Log*	*%inhibition*	*Log values*	*%inhibition*	*log values*
Non stress	26.3 ±0.8 c	0.231	16.4±6.0d	0.151	13.6±4.7e	0.127	54.9 ±11ab	0.437

1M NaCl	22.6 ±3.7 [c]	0.220	40.6±9.2[bc]	0.340	32.8 ±2.5[cde]	0.283	32.6 ±7.2[b]	0.282
3.0M NaCl	52.3±9.3 [ab]	0.420	53.2±2.1[b]	0.426	47.9 ±2.6[bcd]	0.40	64.0 ±5.3[a]	0.661
3.5M NaCl	26.6±6.9 [c]	0.235	80.8±8.8[a]	0.592	63.4 ±8.5[abc]	0.30	73.9 ±2.7[b]	0.36
0M N_2	67.5±1.7 [a]	0.516	23.7±4.3[c]	0.228	87.3 ±4.1[a]	0.62	59 ±12.3[ab]	0.46
0.25M N_2	63.4±3.7 [a]	0.492	54.6±2.7[b]	0.436	73.7 ±6.1[ab]	0.54	89.0%3.9[a]	0.670
25^{0}C	40.5±10.2 [bc]	0.340	21.6±1.1[bc]	0.196	11.7 ±0.7[e]	0.110	26±7.9[b]	0.231
37^{0}C	48.3±0.3 [ab]	0.396	87.1±2.1[a]	0.626	88.1 ±2.2[a]	0.631	56.0±12.3[ab]	0.44

*Results are expressed as mean value, ± the standard deviation. Means followed by the same letter within the column are not significant at $p<0.05$

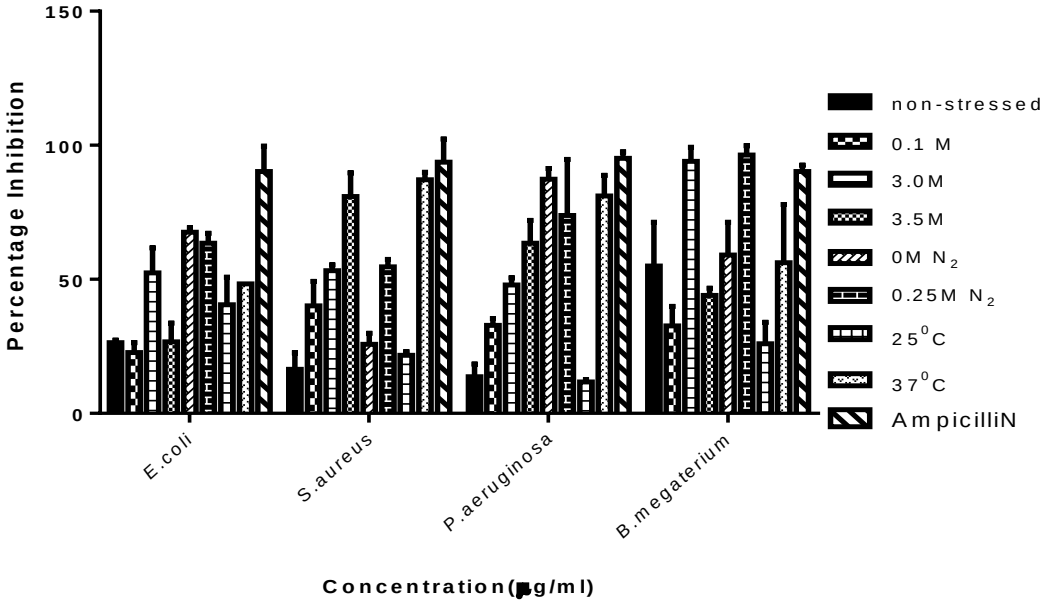


Figure 38. Antibacterial activity of stressed *Dunaliella* extract, as compared to positive control(The results are mean value of triplicates).

Antifungal Activity

Stressed *Dunaliella* cells showed substantial antifungal activity against C*andida albicans*, which is very interesting and is reported here for the first time (Table 25).

Table 25. Summarized Antifungal values of all the stress treatments.

Treatments	***C.albicans* %inhibition**	***Log values***
Non-stressed	22.12 ±5.7 [d]	0.199
1M NaCl	66.8 ±3.1 [abc]	0.511
3.0M NaCl	68.4 ±2.9 [a]	0.521
3.5M NaCl	76.9 ±2.0 [a]	0.570
0M N_2	45.8 ±7.5 [b]	0.377
0.25M N_2	79.9 ±7.3 [a]	0.587
25^0C	69.13 ±9.9 [ab]	0.525
37^0C	80.2 ±0.7 [a]	0.588

*Results are expressed as mean value, ± the standard deviation. Means followed by the same letter within the column are not significant at $p<0.05$

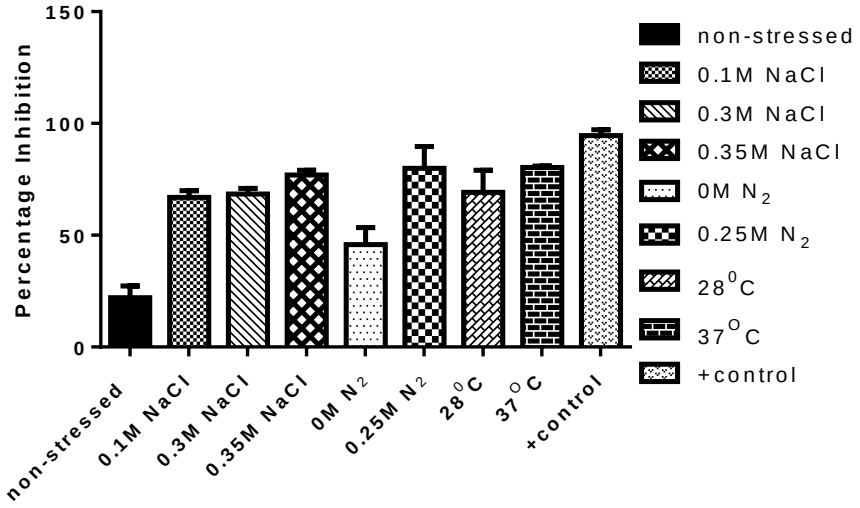


Figure 39. Antifungal activity of stressed *Dunaliella* extract, Amphotericin (50µg/ml) as positive control

4.6 Anticancer Activity of *Dunaliella. Salina* Cells on Human Breast Cancer

Background

In-vitro screening of the compounds hold advantages over *In-vivo* work such as the ability to control the experimental environment, cost and time efficiency (Keloff *et al.*, 1953).

MCF-7 Estrogen-receptor positive Breast Cancer Line was used in screening of the cytotoxic ability of stressed as well as non-stressed *Dunaliella* cells, diluted in DMEM with 10% DMSO.

4.6.0 Cytotoxic assay Non-stressed *Dunaliella salina*

Cytotoxic activity of crude extract non-stressed *Dunaliella* was screened by MTT –viability assay against MCF-7 cells. Paclitaxel was used as a standard to compare with the extract tested against MCF-7 cells. Both the strains of *Dunaliella* extracts indicate anticancer activity as compared to anticancer drug (Table 26). Although the cytotoxic activity is higher in Alga from the marine source (Figure 40).

Table 26. Summarized anticancer values of non-stressed treatments

Treatments	%Suppression	Log value
Saline source	16.19 ±0.3 [a]	0.121
Marine source	30.8 ±15.9 [a]	0.269

*Results are expressed as mean value, ± the standard deviation. Means followed by the same letter within the column are not significant at $p<0.05$

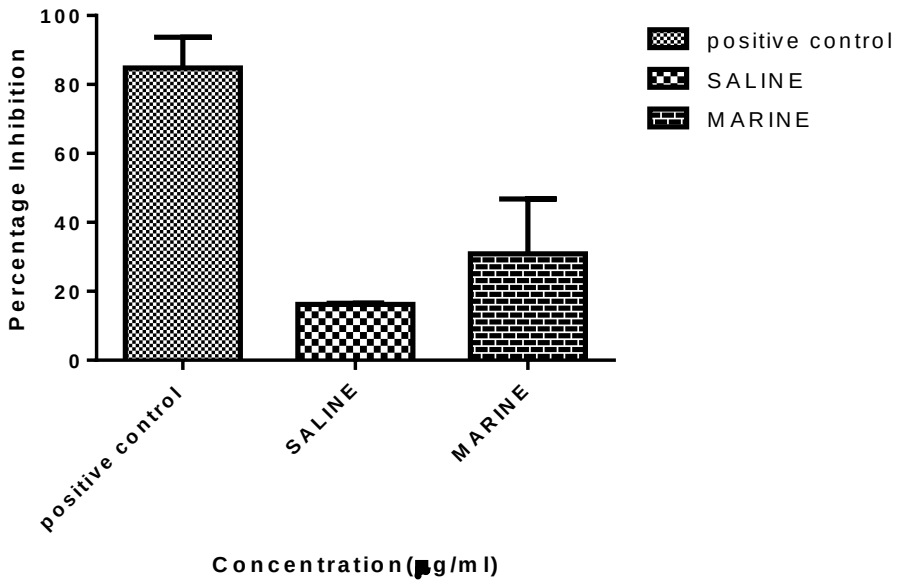


Figure 40. Anticancer activity of two strains of non-stressed *Dunaliella* crude extract, Paclitaxel used as a positive control

4.6.1 EFFECT OF OXIDATIVE STRESS ON ANTICANCER ACTIVITY

With increase in the stress, cytotoxic activity also increased, viable cells show formation of purple color (formazan), (Figure 41). All the extracts were diluted with DMEM to make a final concentration of 10mg/ml, containing 10% DMSO. Anticancer drug, Paclitaxel was used to compare the cytotoxic activity of crude extracts of *Dunaliella.*

Results are values of triplicate wells and the average values of three experiments repeated alternatively.

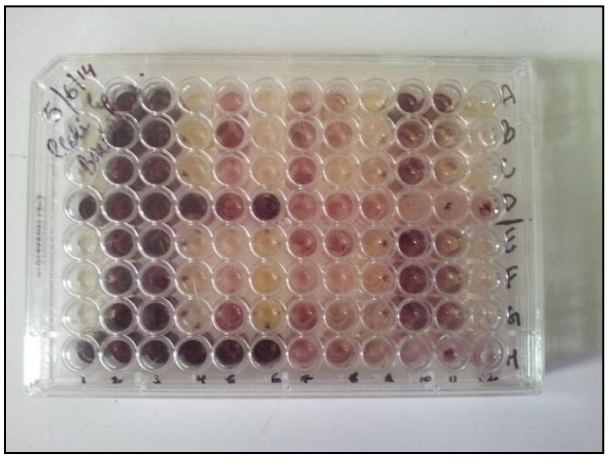


Figure 41. Change in color by addition of MTT, Purple color shows the viable cell

Effect of salinity

Bioactivity of different NaCl stress concentrations (1.0M-3.5M) was determined, final extract was in 10% DMSO, The cytotoxic activity of each crude extract increased as the stress concentration increased. i.e. 3.5M NaCl gave a suppression value of 47.8% (Figure 42) and based on the concentration that induced 50% inhibition on the growth of the MCF-7 cells as compared to the controls in triplicate (Table 27).

Table 27. Effect of salinity stress on anticancer activity .

Treatments	Inhibition(%)	Log values
Non-stressed	12.9±0.3 [b]	0.121
1.0M	13.8±25.0 [c]	0.149
3.0M	33.42±1.17 [ab]	0.288
3.5M	47.8±10.2 [a]	0.391
Paclitaxel	81.44 ±8.2 [a]	0.7991

*Results are expressed as mean value, ± the standard deviation. Means followed by the same letter within the column are not significant at $p<0.05$

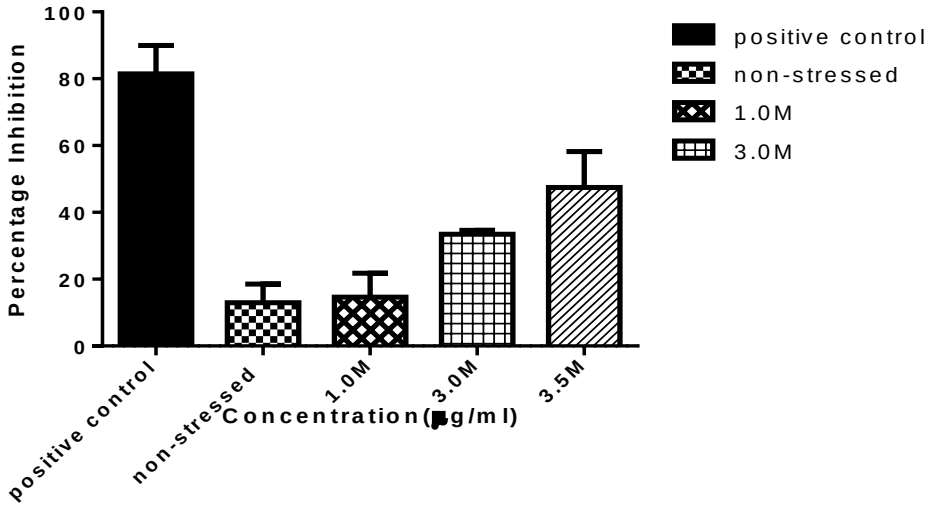


Figure 42. Effect of salinity stress on cytotoxic activity

Effect of Nitrogen

The effect of Nitrogen stressed *Dunaliella* cells leads to more accumulation of bioactive compounds. More activity was seen in 0.25 M nitrogen concentration as compared to Paclitaxel (Figure 43), which is highest among all the stress conditions (Table 28). Although 0 M showed a higher antioxidant which should show a higher anticancer property. This means that some nitrogenous metabolites are produced by algae that increase the cytotoxicity of crude extract

Table 28. Effect of salinity stress on anticancer activity

Treatments	Inhibition(%)	Log values
Non-stressed	12.9 ±0.3 [b]	0.121
0M	53.7 ±8.2 [a]	0.431
0.25M	69.3 ±15.6 [a]	0.538
Paclitaxel	81.44 ±8.2 [a]	0.7991

*Results are expressed as mean value, ± the standard deviation. Means followed by the same letter within the column are not significant at $p<0.05$

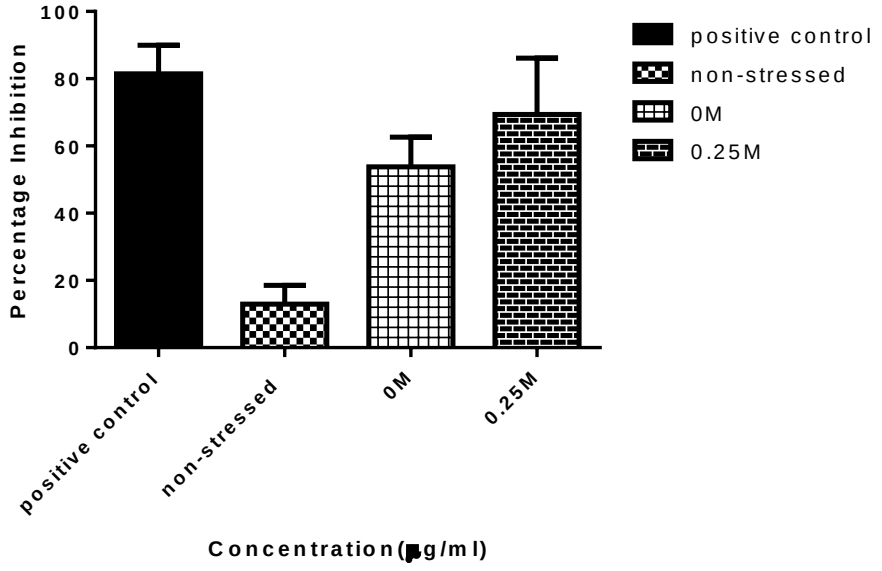


Figure 43. Effect of Nitrogen stress on cytotoxic activity

Effect of Temperature

Temperature stress also showed a significant cytotoxic activity (Figure 44). As the temperature increased , accumulation of bioactive compounds increased linearly so as the anticancer property, when compared to paclitaxel (Table 29)

Table 29. Effect of Temperature stress on anticancer activity.

Treatments	Inhibition(%)	Log values
Non-stressed	12.9 ±0.3 [ab]	0.121
25^{0}C	26.7 ±8.2 [b]	0.231
37^{0}C	50.3 ±15.6 [a]	0.40

*Results are expressed as mean value, ± the standard deviation. Means followed by the same letter within the column are not significant at $p<0.05$

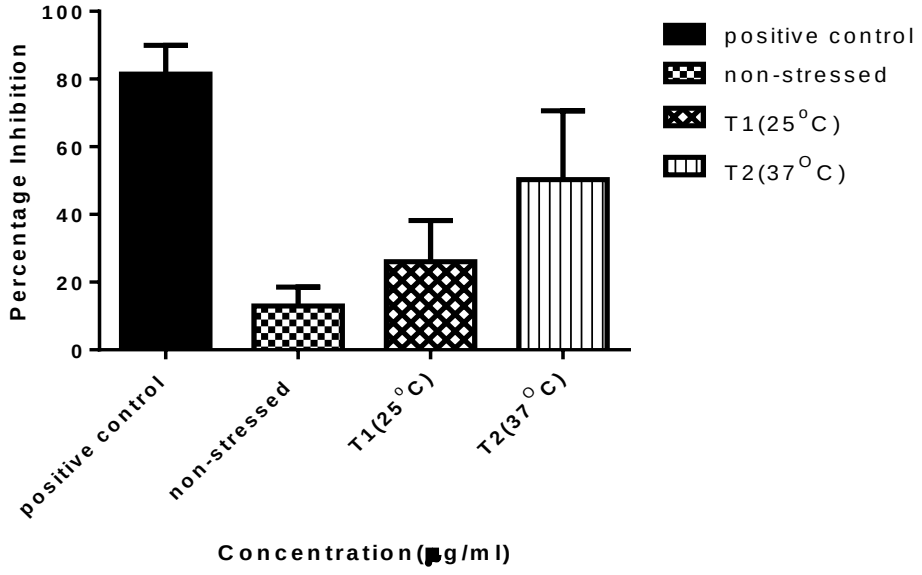


Figure 44. Increase in anticancer activity with increase in temperature stress

4.6.2 Effect of *Dunaliella salina* extracts on growth of immune cells

After evaluating the anticancer property of *Dunaliella*, the cytotoxic effect of crude extract of *Dunaliella* was investigated against normal human immunocompetent peripheral blood mononuclear cells, PBMC'S ($1x10^6$ cells/ml) Stimulated PBMCs (pokeweed mitogen treated) and unstimulated cells were used in culture for the effect of extracts of *D.salina* grown in normal and nitrogen stress condition. Paclitaxel an anticancer agent used as a control. As reported anticancer drugs are immunosuppressive, which was seen in the investigation, Paclitaxalol showed a 60% suppression of immunocompetent PBMC'S unstimulated, As compared to anticancer drug, *Dunaliella* extracts have shown cytotoxic effect on PBMCs (Figure 45,46).

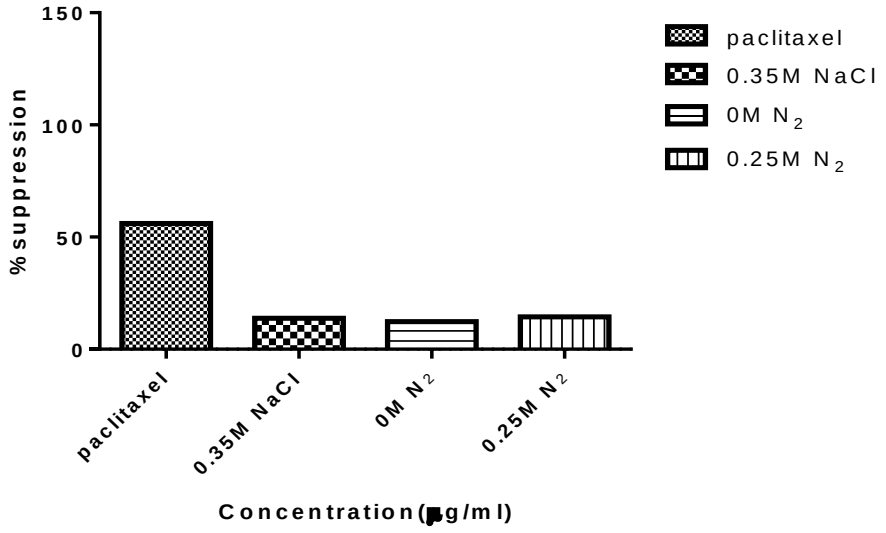


Figure 45. Percentage inhibition of unstimulated PBMC'S by stressed *Dunaliella* cells

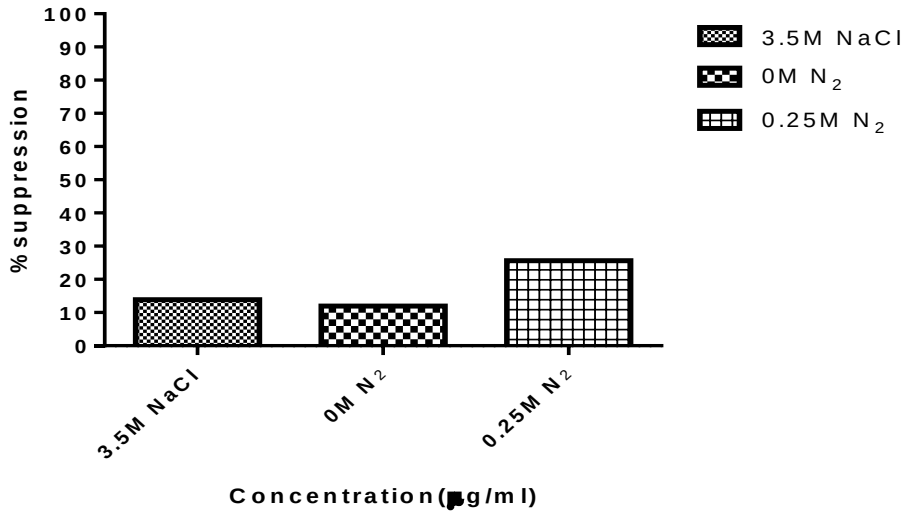


Figure 46. Percentage inhibition of stimulated PBMC'S by stressed *Dunaliella* cells

4.7 Discussion

Dunaliella is a flexible organism that can "not" only grow but also amalgamate various metabolites that have high therapeutic potential (Ben-Amotz, 1982). *D.salina* was able to grow at different physiological stress conditions and produced active compounds that showed anticancer, antioxidant and antimicrobial activity from the crude extract. In this research, efforts were made to optimize the media and growth variables under semi (controlled) conditions for production of dry biomass. It has been reported that with increase in stress conditions like, high salt, high temperature and nutrient starvation results in enhancement of primary and secondary metabolites with an inverse relationship with cell growth as seen in (Figure 18, 23 and 27). Thus 3.5 M salinity without or excess nitrogen and high temperature showed less cell growth but maximum metabolites. Proteins concentration is highest in 3.5 M under high temperature, as reported glycine and guanine and some myo-inositol accumulates under nitrogen excess and high salinity

stress (Lammers, 2012 ; Murthy *et al*., 2005). Studies on antioxidant activity by DPPH (Figure 35, 36 and 37) have shown significant antioxidant activity which is mainly due to carotenoids. Further , Total reducing power (Figure 35, 36 and 37) shows that apart from carotenoids there are other active metabolites which are responsible for antioxidant activity or anticancer activity. *Dunaliella salina* showed convincing antimicrobial activity which might be due to some volatile compounds as well as several fatty acids (mainly palmitic, alpha-linolenic and oleic acids). Based on MTT assay, crude extracts of *D.salina* can be considered as potential cytotoxic agent because it showed substantial inhibition of MCF-7 cell, with no significant effect on PBMC'S. This results confirmed that earlier study that reported anticancer effect of stressed and non-stressed *D.salina* on skin carcinoma cell line A431. Thus this study concluded that as a self-defense mechanism *D.salina* starts accumulation of bioactive compounds under stress conditions which has an intrinsic ability to scavenge free radicals (ROS) which are reported as one of the main cause of cancer (Alejandaro, 2003) and hence holds a robust potential to be used as a wholesome nutrient with therapeutic, health- enhancing and chemopreventive agent.

Thus the present study widens the scope of research that *Dunaliella* is not only tagged with β-carotene but it acquires many important unknown metabolites. It is such an organism which equally matches science and economics.

4.8 Salient features

- Optimization of *D.salina* under stress and non-stressed conditions with respect to cell growth and biomass productivity.
- Under stress conditions the cell growth and cell density decreases with increase in amount of intracellular carotenoids and proteins.
- Biomass productivity is less under stress condition as compared to non-stressed *D.salina.*
- *D.salina* possess antimicrobials which are active against different microorganism, *E.coli, S.aureus, P. aeruginosa, C. albicans.*
- *D.salina* has potent free radical scavenging activity.
- *D.salina* has shown significant inhibition on MCF-7 cells.

Chapter 5

References

REFERENCES

A. Telfer, (2002) What is β-carotene doing in the photo system II reaction center. Philos. Trans. R. Soc. Lond. *B Biol. Sci*., 357: 1431 1439.

Alejandaro, M. S., Mayer, Kirk, R., and Gustafson., (2003). Marine pharmacology in 2000: Antitumor and cytotoxic compounds. *Int J Cancer.* 105, 291-299.

Alley e. Watada, Karl H,. Norris, John T., Worthington ,Devis.,(1971). Estimation of chlorophyll and carotenoid contents of whole tomato by light absorbance technique. *J Food Sci.* 41: 339-342.

Avron M, Edelstein S, Ben-Amotz A., (1987). Feed supplement. UK Patent Application Number 2189675A.

Aebi, H., (1984). Catalase *In vitro* methods of analysis. *Methods in Enzymol* .105 :121–125.

Ben-Amotz A., (1981). Glycerol production in the alga *Dunaliella*. In San Pietro A (ed.) Biochemical and photosynthetic aspects of energy production. Academic Press, New York, 191–208.

Ben-Amotz A., (1987). Effects of irradiance and nutrient deficiency on the chemical composition of *D. bardawil* Ben-Amotz and Avron (Volvocales, Chlorophyta). *J. Plant Physiol*. 131: 479–487.

Ben-Amotz A., Avron M., (1982). The potential use of *Dunaliella* for the production of glycerol, β-carotene and high-protein feed. In San Pietro A (ed.), Biosaline research: A look to the future. Plenum Pub. Corp., New York, 207–214.

Ben-Amotz A, Avron M. (1983). On those factors which determine the massive β-carotene accumulation in the halotolerant alga *D. baradwil*. *Plant Physiol*. 72: 593–597.

Ben-Amotz A., Katz A., Avron M., (1982a). Accumulation of β-carotene in halotolerant algae: purification and characterization of β-carotene-rich globules from *D. bardawil* (Cholorophyceae). *J. Phycol*. 18: 529–537.

Ben-Amotz A., Sussman I., Avron M., (1982b). Glycerol production by *Dunaliella*. *Experiential* 38: 49–52.

Ben-Amotz A., Lers A., Avron M., (1988). Stereoisomers of β-carotene and phytoene in the alga *D. salina*. *Plant Physiol*. 86: 1286–1291.

Ben-Amotz A., Shaish A., Avron M., (1989). Mode of action of the massively accumulated B-carotene of *Dunaliella* in protecting the alga against damage by excess irradiation. *Plant Physiol*. 91: 1040–1043.

Bloch MR., Sasson J., Ginzburg ME., Goldman Z., Ginzburg BZ., Garti N., Perath A., (1982). Oil products from algae. US Patent Number 4341038.

Borowitzka LJ., (1992). Commercial *Dunaliella* production: History of development. In: Villa TG, Abalde J (eds) Profiles on biotechnology:servicio de publicaciones, Universidad Santiago de Compostela, A Coruna :235–245.

Borowitzka LJ., (1981a). The microflora. Adaptations to life in extremely saline lakes. *Hydrobiologia* 8: 33–46.

Blios, M.S.,(1958).Antioxidant Determinations by the Use of a Stable Free Radical. Nature. 181: 1199-1200.

Borowitzka LJ., Borowitzka MA., (1989). β-carotene (Provitamin A) production with algae in Vandamme EJ (ed.). Biotechnology of Vitamins, Pigments and Growth Factors. Elsevier Appl Sci. London: 15–26.

Borowitzka MA, Post F, Borowitzka LJ. (1982). The life cycle of *D. salina. Australasian Soc. for Phycology and Aquatic Botany, Annual Meeting.* 1982: 18.

Brown AD, Goyal A, Larsen H, Lilley RM. (1987). A salt-sensitive mutant of *D. tertiolect*a. A role of carbonic anhydrase. *Arch. Microbiol.* 147: 309–314.

Burton GW, Ingold K.U., (1984). β-carotene: An unusual type of lipid antioxidant. *Science* 224: 569–573.

B. Halliwell, J.M.C. Gutteridge. (1989).Free Radicals in Biology and Medicine Clarendon Press, Oxford: .188–276.

Chidambara, K. N., Vanitha, A., Rajesha, J., Mahadeva, M., Sowmya, P. R., and Ravishankar, A., (2005). In vivo antioxidant activity of carotenoids from *Dunaliella salina* a green microalga. *Life Sci.*76: 1381-1390.

Curtain CC., Looney FD., Regan Dl., Ivancic NM., (1983). Changes in the ordering of lipids in the membrane of *Dunaliella* in response to osmotic-pressure changes. *Biochem J*. 213:131–136.

Christine K. Shewmaker, Julie A. Sheehy, M. Daley1, Susan C, Dang Yang K.,(2002). Seed-specific overexpression of phytoene synthase: increase in carotenoids and other metabolic effects. *Plant J.* 20 :401-404.

Cleidson V., Simone Machado de Souza., Elza F A Smânia., Artur S.,(2007). Screening methods to determine antibacterial activity of natural products. *Brazilian Journal of Microbiology*. 38:369-380

Duh, P.D., Yen, G.H.,(1997). Antioxidant activity of three herbal water extracts. *Food. Chem.* 60 :639-945

Dawson YE.,(1966). An introduction to marine botony Holt, Rinehart and Winston, *Inc*, 35-62.

Demming-Adems, B., G,limore, A.M, Adams, W.W.,(1996). funtions of carotenoids in higher plants. *In Vivo* 10 :403-412.

Emtyazjoo Mo, Z. Moghadasi .,M. Rabbani.; Emtyazjoo Maa, Samadi S., Mossaffa N., (2011). Anticancer effect of *Dunaliella salina* under stress and normal conditions against skin carcinoma cell line A431 in vitro**.** *Iranian Journal of Fisheries Sciences*, 11 283-293.

Emadi, H., P. Amaninejad, M. Emtiazjoo, H. H. Sahhafi.,(2010). Effects of Dunaliella microalgae *(Dunaliellasalina)* on different levels of complement C3, C4 and antioxidant capacity in rainbow trout *(Oncorhynchusmykiss). Adv. Environ. Biol.*, 4: 456-463.

Estudillo, Yolanda F.P ,Renata R.M, Daniel Robledo.J,´ Alberto N.Z., (2006).Regulation of two photosynthetic pigment-related genes during stress-induced pigment formation in the green alga, *Dunaliella salina .Biotehnol let.* 28:787-91

Farouk, K. E.I. Baz., Ahmad , M.Abul-Enein., Gamal, S. E.I Baroty.,Youssef , A.M., andHanaa, H. A. Baky., (2002). Accumulation of antioxidant vitamins in *Dunaliella salina*. *J Biol Sci.,* 2:220-223.

F. M. I. Natrah, F. M. Yusoff, M. Shariff, F. Abas, and N. S.Mariana., (2007). Screening of Malaysian indigenous microalgae for antioxidant properties and nutritional value. *J Appl Phycol*, 6: 711–718.

Francavilla A., Colaianna, M Zotti, M .G .Morgese, P. Trotta, P Tucci, S Schiavone, V Cuomo, L Trabace., (2012). Extraction, characterization and in vivo neuromodulatory activity of phytosterols from microalga *Dunaliella tertiolecta. Curr Med Chem.*9:3058-3067

Fisher M., Pick.U. and Zami.A.,(1994). A salt-induced 60-kilodalton plasma membrane protein plays a potential role in the extreme halotolerance of the alga *Dunaliella. J Biol Chem*. 271 :1359-1367.

Fried, A. et al. (1982) Lipid composition of the halotolerant alga *Dunaliella bardawil*. *Biochem. Biophys. Acta* 713: 419–426

Garcia-Gonzalez M, Moreno J, Manzano JC, Florencio FJ, Guerrero MG., (2005). Production of *Dunaliella salina* biomass rich in 9-*cis*-β-carotene and lutein in a closed tubular photo bioreactor. *J Biotech* 115:81–90.

Goodman G.E, Metch B.J, Omenn., (1984). The effect of long-term beta-carotene and vitamin A administration on serum concentrations of alpha-tocopherol. *Cancer Epidemiol Biomarkers Prev*: 429-432.

Guerin M, Huntley ME, Olaizola M.(2003). Haematococcus astaxanthin: applications for human health and nutrition. *Trends Biotechnol*.21:210–215.

Hejazi M. A., Lamarliere.C. , Rocha J. M. S., Vermu. M., Tramper and R. H. Wijffels., (2002).Selective extraction of carotenoids from the microalga *Dunaliella salina* with retention of viability. *Biotech Bioeng*. 79: 31-36

Heiber AD, King T.J, Morika S, Fukshima LH, Bertam JS, (2000). Comparative effect of all trans 9-cis β-carotene on carcinogen induced neoplastic transformation and connexin 43 expression in murine 10T1/2 cells and on the differentiation of human keritinocytes. *Nutr.Cancer*. 37: 234-344.

Hadi, M. R., Shariati M., Afsharzadeh S. (2008). Microalgal biotechnology: carotenoid and glycerol production by *Dunaliella sp*. algae isolated from the Gave khooni salt marsh, Iran. *Biotech. Bioproc. Eng*., 13: 540-544

J. C. Lee, H. R. Kim, J. Kim, and Y. S. Jang, (2002). "Antioxidant property of an ethanol extract of the stem of Opuntia cus-indica var.saboten*," J. Agric. Food. Chem*., 50, 6490–6496.

Jayappriyan, K.R., R. Rajkumar, L. Sheeja, S. Nagraj, S. Divya and R. Rangasamy (2010). Discrimination between the morphological and molecular identification in the genus Dunaliella. *Int.J.Curr. Res.*, 8: 73-78.

Johnson E.A. and Schroeder W.A. (1995). *Adv. Biochem. Eng./Biotechnol.* 53: 119–178.

Kacka A, Donmez G., (2008). Isolation of *Dunaliella spp*. from a hypersaline lake and their ability to accumulate glycerol. *Bioresource Technol.* 99:8348-8352.

Katz A, Avron M (1985). Determination of intracellular osmotic volume and sodium concentration *in Dunaliella*. *Plant Physiol* 78:817-820.

Keloff, G.J.; Crowell, J.A.; Steele, V.E.; Lubet, R.A.; Boone, C.W. and Malone, W.A. (1999). Progress in cancer chemoprevention. *Annals of Phyochem.*889: 1-3.

Krupa, D.; Nakkeeran, E.; Kumarsen, N.; Vijayalakshmi, G. and Subraminian, R. (2010). Extraction, purification and concentration of partially saturated canthaxanthin from Aspergillus carbonarius. *Bioresource. Technol*., 101: 7598-7604.

Krishna Mohan, S. L. Kothari, P.Sharma ,V. Agarwal ,S. Kachhwaha., (2012). Isolation and Characterization of Dunaliella Species from Sambhar Lake (India) and its Phylogenetic Positionin the Genus Dunaliella Using 18S rDNA .*Natl.Acad.Sci.Lett* 35, 207–213.

Krinsky, N.I., Johnson, E.J.(2005). Carotenoid actions and their relation to health and disease. *Mol. Aspects Med*, 26, 459–516.

Lamers PP, Janssen M, De Vos RCH, Bino RJ, Wijffels RH. (2008). Exploring and exploiting carotenoid accumulation in *Dunaliella salina* for cell-factory applications. *Trends Biotechnol.* 26 :631-638.

Levin G, Yeshurun M and Mokady S., (1997). In vivo peroxidative effect of 9-*cis*-β-carotene compared with that of the all-trans isomer. *Nutr Cancer* 27, 293-297.

Lichtenthaler H.K, Wellburn A.R., (1983). Determinations of total carotenoids and chlorophylls a and b of leaf extracts in different solvents. *Biochem Soc Trans* 11, 591–592.

Lichtenthaler HK., (1999). The 1-deoxy-D-xylulose-5-phosphate pathway of isoprenoid biosynthesis in plants. *Plant Physiol* . 50:47–65.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, J.L., (1951). Protein measurement with Folin-phenol reagent. *J Biol Chem.* 193, 265–275.

Okai Y et al.(1996). Suppressive effects of chlorophyllin on mutagen-induced umu C gene expression in *Salmonella typhimurium* (TA 1535/pSK 1002) and tumor promoter dependent ornithine decarboxylase induction in BALB/c 3T3 fibroblast cells. *Mutat Res*. 370 :11-7.

Oren ,A.,(2005). A hundred years of *Dunaliella* research 1905-2005 saline system,1, 1-14 (http://w.w.w.saline systems.org/contents)

Orset S, Young AJ (2000). Exposure to low irradiances favours the synthesis of 9-cis-β, β-carotene in *Dunaliella salina*. *Plant Physiol* 122:609–617.

Mclachlan J. (1960). The culture of D. tertiolecta Butcher - a euryhaline organism. *J. Microbiol*. 6: 367–375.

Mendiola León, Jose Antonio S., Susana C., Alejandro R., G.I, Elena,(2008). Antimicrobial activity of *Dunaliella salina* sub and supercritical CO_2 extracts. *J Food Protect;* 7:115-118

Mossman T,(1983). Rapid colorimetric assay for cellular growth and survival. Application to proliferation and cytotoxicity assays. *J Immunol Methods* 65, 55-63.

Murthy KNC, Vanitha A, Rajesha J, Swamy MM, Sowmya PR, Ravishanka GA. (2005). In vivo antioxidant activity of carotenoids from *Dunaliella salina*—a green microalga. *Life Sci*. 76:1381–1390.

Ming. J, S., Guan. J, H., Chieh. H, W., Jwo. S, C., Heng. Y, H., Shu. J and Jiang. G, C., (2008). Ethanol extract of *Dunaliella salina* induces cell cycle arreat and apoptosis in A549 Human Non-small cell lung cancer. *In vivo*, 5: 22369-378.

Nakashima, Y., Ohsawa, I., Konishi, F., Hasegawa, T.; Kumamoto, S., Suzuki, Y., Ohta, S.(2009). Preventive effects of *Chlorella* on cognitive decline in age-dependent dementia model mice. *Neur. Lett*., 464: 193–198.

National Committee for Clinical Laboratory Standards (NCCLS), Performance Standards for Antimicrobial Susceptibility Testing; Fourteenth Informational Supplement NCCLS documents M100-S14.NCCLS, Wayne, PA, 2004

Pronyk, C., and G. Mazza. (2009). Design and scale-up of pressurized fluid extractors for food and bioproducts. Review. *J Food Eng.* 95: 215–226.

Rabbani, S. *et al*.,(1998) Induced β -carotene synthesis driven by triacylglycerol deposition in the unicellular alga *Dunaliella bardawil. Plant Physiol*. 116: 1239–1248.

Raja R, Hemaiswarya S, Balasubramanyam B, Rengasamy. R (2006) Protective effect of *Dunaliella salina* (Volvocales, Chlorophyta) against experimentally induced fibrosarcoma on wistar rats. *Microbiol Res*162:177–184

R. Raja & S., Hemaiswarya & R., Rengasamy., (2007) Exploitation of *Dunaliella* for β-carotene production. *Appl. Microbiol. Biotechnol*74:517–52.

Raja R (2003) Studies on *Dunaliella salina* (Dunal) Teod. with special reference to its anticancer properties. Ph.D. Thesis, University of Madras, Chennai, India

Raja R., Anbazhagan C, Lakshmi D, Rengasamy R (2004) Nutritional studies on *Dunaliella salina* (Volvocales, Chlorophyta) under laboratory conditions. *Seaweed Res Util* 26:127–146.

Ramezan A.A, Ali M. Tavana, S. Mohammad, J. Hosseini, K. Moridi , M. Ghorbananli.,(2012). A Method for Antibiotic Susceptibility Testing: Applicable and Accurate. *J Microbiol*. 5 :341-345.

Seddon, J.M.; Ajani, U.A.; Sperduto, R.D.; Hiller, R.; Blair, N.; Burton, T.C.; Farber, M.D.; Gragoudas, E.S. and Haller, .J (1994). Vitamins A, C and E and advanced age related macular degeneration. *J. Am. Medical Asso*., 272: 1413-1420.

Shariati, M. (2003). Characterization of three species of *Dunaliella salina, Dunaliella parva* isolated from salt marsh of Gave Khoni of Isfahan Iran. *Iranian J*. Sci. Technol., 27: 185-190.

Sheu MJ, Huang GJ, Wu CH, Chen JS, Chang HY, Chang SJ, Chung JG.(2008).Ethanol extract of *Dunaliella salina* induces cell cycle arrest and apoptosis in A549 human non-small cell lung cancer cells. *In-Vivo*, 22 :369-78

Stoynova Bakalova, E., Toncheva Panova, T., (2003). Subcellular adaptation to salinity and irradiance in *Dunaliella salina*. *Biologia Plantarum*. 47: 233-236, 2003-

Stahl, W., Sies, H., (1992). Uptake of lycopene and its geometrical isomer is greater from heat-processed tomato juice in humans. *J .Nutr.* 122, 2131–2166.

Stahl,W., Sies,H., Nicoloai, S.(1998). Bioactivity and protective effects of natural carotenoids. Elsevier,1740:101-107.

Vonshak, A., (1986). *Laboratory techniques for cultivation of microalgae.* In: CRC, Handbook of Micro Algal Mass Culture. CRC press, USA, 345-349.

Vorst P, Baard RL.,Mur LR., Korthal HJ, Van D. (1994) Effect of growth arrest on carotene accumulation photosynthesis in *Dunaliella*. *Microbiol.*140: 1411-1477.

Pasquet V, *et al*., (2011). Antiproliferative Activity of Violaxanthin Isolated from Bioguided Fractionation of *Dunaliella tertiolecta* Extracts. *Mar.Drugs.* 9 :819-831.

Wang, X.D., Tang, D.W, Krisnsky, N.I., Russell, R.M. (1991). Enzymatic conversion of beta-carotene in to beta apocaretnols and retinoids in humans, monkey, rat, ferret. *Arch Biochem Biophs*. 285: 8-16.

Yashudhie Tie, Hideyatsu. M., Takashi.W.,Hiroaki.K.,Makoto.T.,Katsusuke. N., (2002.) *In-Vitro* antitumour activity of Bropimine against urinary bladder cancer. *Anticancer Res*:1667-1672.