Extracellular polymeric substances (EPSs) from *Dunaliella salina* having antioxidant and immunostimulating activity

A Dissertation report

Submitted in the fulfillment of the requirement

For the award of the degree of

Master of Technology

(Biotechnology)

By

Sakshi Sharma
601404017

Under the guidance of

Dr. M. Baranwal
(Assistant professor)

Dr. M.S Reddy
(Professor)

DEPARTMENT OF BIOTECHNOLOGY

THAPAR UNIVERSITY, PATIALA -147001

CERTIFICATE

This is to be certified that thesis entitled "Extracellular polymeric substances from *Dunaliella salina* having antioxidant and immunostimulating activity" submitted by Sakshi Sharma, 601404017 in partial fulfillment of the requirement of the award of degree for Master of Technology in Biotechnology of Thapar University, Patiala is the 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. Manoj Baranwal

Supervisor and Assistant Professor

Dr. Dinesh Goyal

(Head of Deptt. of Biotech)

Dr. M S Reddy

Co-supervisor and Professor

(Deptt. of Biotechnology)

Dr. S. S Bhatia

Dean of academic affairs

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CANDIDATE'S DECLARATION

I hereby declare that work presented in the dissertation entitled "Extracellular polymeric substances from *Dunaliella salina* having antioxidant and immunostimulating activity" in the partial fulfillment of the requirement of the award of the degree Master of Technology in biotechnology is an authentic record of my own work during academic year 2015-16 under the supervision of Dr. M Baranwal (Assistant Professor) and Dr. M.S Reddy (Professor) Department of Biotechnology ,Thapar University, Patiala. The report has not been submitted for the award of any other degree or the certificate in this or any other university.

Date: 15/07/16

Place: Patiala

Sakshi Sharma

601404017

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Sakshi Sharma

601404017

CONTENTS

ABSTRACT

D. salina is a unique unicellular, biflagellate species of genus *Dunaliella* which contain no cell wall in saline environments. *Dunaliella* is known for its growth in wide range of salt concentration where range varies from 0.05 M to 5.0 M and other stress conditions like temperature stress, light intensity stress, nutrient stress etc. Extracellular polymeric substances are complex high molecular weight biopolymers containing main components like carbohydrates, lipids, proteins, nucleic acids etc. In present work, extracellular polymeric substances EPSs have been extracted from D. *salina* and its antioxidant and immunostimulating activity were studied.

Further, stress conditions of phosphate (2.8mM and 7mM) and nitrate (9.8mM and 24.5mM) were given to *D. salina* to study its growth, EPSs production and bioactive properties. Normal growth condition of these two parameters was 1.4mM KH_2PO_4 and 4.9mM KNO_3.

Cell growth and count was found to be increased at 9.8 mM KNO_3as compared to normal conditions. On the other hand phosphate (KH_2PO_4) stress resulted in decrease in cell growth and count.

EPSs produced under normal conditions were checked for protein and glucose contents in it and the result confirm the presence of these two components in it. Under stress conditions, protein and carbohydrate concentrations were increased. Antioxidant effect was assessed of EPSs produced under normal conditions and it was found that these effects become more pronounced with concentrations.

1 mg/ml of EPSs (normal condition) has shown the increase in proliferation of Concanavalin A stimulated peripheral blood mononuclear cells (PBMCs). Further, 1 mg/ml of EPSs extracted in all stress conditions has shown proliferative response of PBMCs indicating the immunostimulating properties of EPSs.

Present results suggest that EPSs produced under normal and stress condition possess bioactive properties which may be used for therapeutic purpose.

LIST OF ABBREVIATIONS

BSA	Bovine serum albumin
ConA	Concanavalin
EPSs	Extracellular polymeric substances
D.salina	*Dunaliella salina*
DPPH	1, 1-Diphenyl-2-picrylhydrazyl radical, 2-Diphenyl-1-(2, 4, 6-trinitrophenyl) Hydrazyl
DMSO	Dimethyl sulfoxide
PBS	Phosphate buffer saline
O.D	Optical Density
M	Molar
MTT	3-(4, 5-dimethylthiazol-2-yl) -2, 5-diphenyltetrazolium bromide

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Introduction

Cultivation of microalgae nowadays has received much attention globally. Microalgae are cultivated for highly valuable biomolecules like lipids, pigments, proteins, polysaccharides, nucleic acids etc. Those are used in various industrial applications. Microalgae are mostly used in nutrient supplements, natural food colorants, animal feed etc. Cultivation of microalgae has attracted many researchers as microalgae have many advantages over higher plants. Microalgae have higher productivity than plants; and cultivation of microalgae can be done easily without the need of fresh water or arable land. Most of the species of microalgae can be cultivated on natural seawater or on the free land that is already not in the requirement of any other agricultural use.

Dunaliella salina (D.salina), an unicellular, biflagellate green microalga is known to grow in hypersaline environment worldwide which is kind of extremophile. *D. salina* has become a model representative organism for the study of the salt stress conditions and its adaptation in such conditions. *D. salina* first, has been reported by Michel Fliex Dunal in 1838. D. salina lacks rigid polysaccharide cell wall. This microalga reproduces by longitudinal division of motile cell or by the fusion of two motile cells for the formation of zygote. (Borowitzka M.A., 2007).

Extremophiles are those organisms which are not only tolerate extreme conditions/ harsh environment, but also need such environmental extremes for their survival and growth. The term "*Extremophiles*" was first coined by Mac Elroy in 1974. Their classification usually based on the environment in which they survive or grow. These Extremophiles are responsible for the production of high-value products of biological importance as a result of their adaptation to such extreme conditions. Extremophiles are known producers of Extracellular polymeric substances (EPSs) under such conditions. Recently EPS from *Dunaliella* has been reported and it was found that under salt stress D.salina make emulsions of polysaccharides with stability and is comparable to bacterial polysaccharides (Kazak Hande *et al.,* 2010).

EPSs are important class of polymeric substances which includes polysaccharide, lipids, proteins and nucleic acids. These are metabolites release from microalgal cell and accumulate generally on the cell surface. These biometabolites provide protection to the cells from harsh environment and play role in membrane stability. It has been found that EPSs isolated from species like *Vibrio sp*. and *Pseudomonas sp.* have, antitumor, antiviral and immunostimulatory effects (Okutani, 1984). Demand of natural polymers in industries has also increased in recent years which have led interest to isolate these from new sources like microalgae etc (Mishra *et.al,* 2009). These exoploymers previously were extracted in bacteria and cyanobacteria only. Now a day, researchers are trying to extract such exoploymers from algal species like *Chromonas sp*, *Spirullina sp*. and *Dunaliella sp*.

Our present investigation is focused on the extraction of such extracellular polymeric substances (EPSs) from *Dunaliella salina* under normal and stress conditions and assessment of their like antioxidant and immunomodulation activity.

Chapter-2

Review of literature

REVIEW OF LITERATURE

Dunaliella salina is considered as the best model organism for the production of various important biometabolites under variable environmental conditions. Researchers have been studied the physiology of green unicellular alga *Dunaliella* for several decades.

2.1 Taxonomy:

Dunaliella salina is the halophilic representative of *Dunaliella* genus. This unicellular microalga is responsible for most of the initial production in hypersaline environments (Oren A., 2014). For the very first time description of this unicellular microalga was given by Dunal as a red colored biflagellate alga. He named this microalga as *Haematococcus salinus* and *Protococcus*. Dunal had found it in the Mediterranean coast of France. Teodoresco in 1905 named it as *Dunaliella salina* in the honor of M.F Dunal. (Borowitzka M.A., 2007). Massyuk has examined taxonomical details systematically of total number of 29 species. Various other varieties and forms were also examined. Some of the intraspecific taxa have been described later. Taxonomy of genus *Dunaliella* is very difficult and challenging as this organism can survive or grow under wide range of environmental conditions. Every species of this genus has wide morphological variability. (Borowitzka M.A., 2007).

Table1: Taxonomical classification of *Dunaliella salina:*

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

(Adapted from, Borowitzka and Siva, 2007)

2.2 Morphology:

Shape of the cells in the different species of *Dunaliella* varies from ellipsoid, ovoid, cylindrical to almost spherical, pyriform or fusiform. The symmetry of cell can be radial, bilateral or somewhat symmetrical (flattened, curved or dorsoventrically curved) (Amotz and Avron, 1990). The changing shape of the cells depends on the environment change. Most often cells become spherical before returning to original shape under stress conditions like osmotic shock. Temperature stress like low temperature is responsible for the formation of amoeboid cells or irregular shaped cells. This effect is caused due to cytoskeleton microtubule break down under low temperature (Borowitzka and Siva, 2007).

Dunaliella salina generally lacks a rigid polysaccharide cell wall but contains mucilaginous cell coat. This can be seen under light microscope. It has been seen as electron dense material covered along with plasmalemma structure. Cells of the microalgae are motile; biflagellate with flagella inserted at the anterior cell structure (Amotz and Avron, 1990). *Dunaliella* cell contains single, large chloroplast which takes large space of the cell body. It can be of cup-shaped, disc-shaped or bell-shaped. Thickened basal portion has pyrenoid. Irregularly stacked thylakoids are present in its chloroplast.

Fig1: A *Dunaliella salina* cell. (Adapted from Morais *et al.*, 2015)

2.3 Reproduction:

Vegetative reproduction *D.salina* starts from infurrowing along with nuclear division. It was seen first from anterior flagellar end of the cell followed by all around the cell. It was found that whenever exposed to stress condition like high salinity, its cells form palmella stage. Due to this reason its cells become more rounded in shape during stress conditions (Borowitzka and Siva, 2007). Aplanospores have also been reported in old cultures of *D .salina*.

Sexual reproduction in *D .salina* occurs through isogamy as it is heterothallic also. Reproduction can be stimulated by decrease in the salinity of the medium. Somewhere variations in the sexual reproduction have been seen due to variations in the cell shape of *Dunaliella.*

2.4 Ecology:

Dunaliella salina is worldwide found in salt lakes, saltern ponds and crystallizer pond at high salt concentration i.e. above saturation where other oxygenic phototrophs are not actually able to grow. It is the sole primary producer which is responsible for the fixation of carbon for entire ecosystem. This species mostly has been studied in different saline environments like the Dead Sea, Great Salt Lake, Solar saltern, Death valley etc. Two main species which are found in such conditions are *D.salina* and *D.viridis.* β-carotene rich red-colored species were never observed in Dead sea and Great Salt Lake. According to survey reports on 53-lakes in Antarctica only three lakes contain this genus. β-carotene rich species only present the crystallizer brines (Oren, 2014).

2.5 Regulation of salt stress in D.salina:

Salt stress has been observed in the various species of *Dunaliella* which include (*D. salina, D.bioculata, D.viridis, D.tertilacta and D.parva*). This genus actually uses intracellular glycerol and glycine betaine to save itself from drastic environmental effects like hypersaline conditions (Mishra, 2008). Researchers have explained this adaptation under high salt concentrations by two ways in *Dunaliella*. One is rapid adjustment of intracellular metabolites by osmoregulatory mechanism. Another way is the biosynthesis of lipids and carotene biosynthesis by *de novo* synthesis. When sudden fluctuations occur in osmolarity of the medium it leads to the lipid changes in plasma membrane. Long term exposure to the salinity is responsible for the *de novo* protein biosynthesis. There induces a stress response based transcripts which will be responsible for the assimilation of carbon, iron or carotene production etc. Other stresses like temperature stress generally induces reactive oxygen species (ROS) which further leads to the stress induced transcription and translation .These proteins or biometabolites actually provide protection from harsh environments. Various other secondary metabolites also produced during this are isoprenoids, terpenoids etc (Ramos A. *at el.*, 2011)

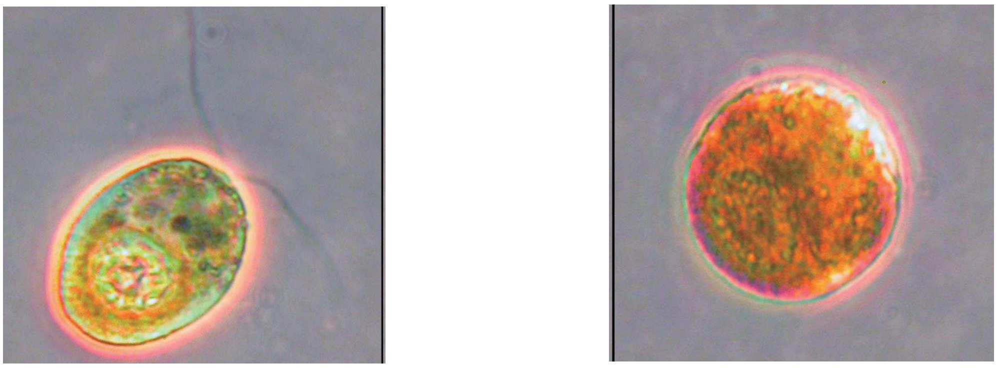


Fig 2: *Dunaliella salina* in different culture conditions a) Green (Normal condition) non-stressed cell b) Under stress conditions cells turned orange due to carotenoid production. (Adapted from Ramos A. *at el.*, 2011)

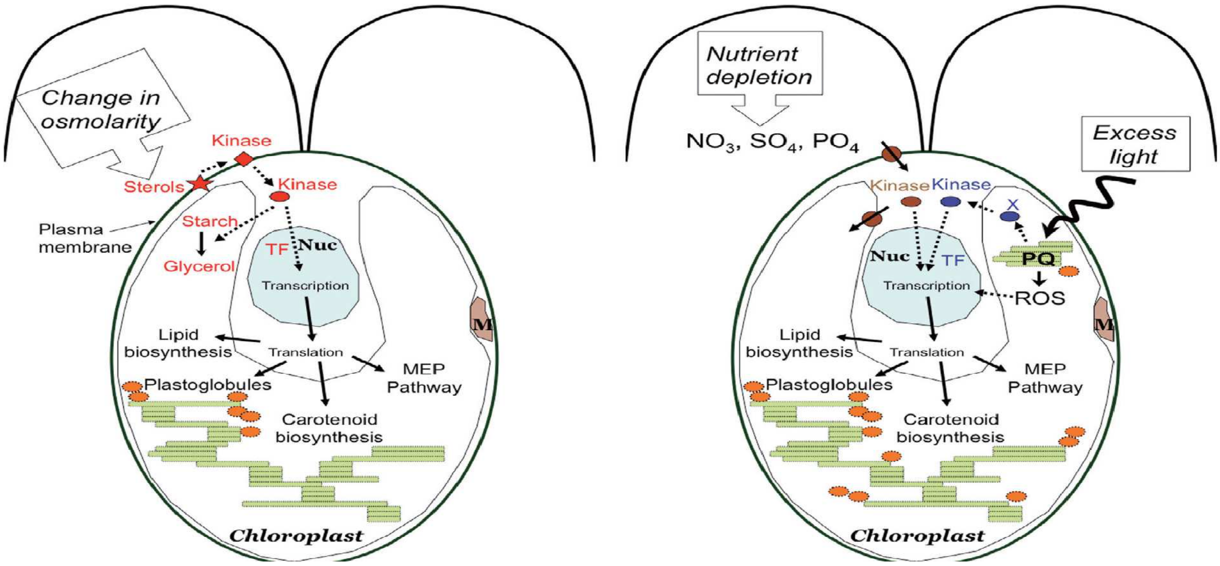


Fig3: Various metabolic pathways in *D.salina* in stress response. (Adapted from Ramos A., 2011)

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.

2.5 Osmotic response in *Dunaliella*:

Osmotic response to osmotic stress involves three main stages which includes metabolic changes and structural changes**. First** stage is immediate response where change in cell shape, size occurs within 5min after providing osmotic stress. At least two-fold change can occur in the cell volume within seconds. Expansion of plasma membrane also resulted from its fusion with various vesicles throughout cytoplasm. This gives a clue that *Dunaliella* has special membrane space which helps in rapid decrease and increases in cell volume and prevents the cell from apoptosis**. Second** response is short term response which involves the balancing of osmotic stress with the help of glycerol concentration regulation. When subjected to a hyperosmotic shock (salt stress) the cells of the *Dunaliella* which contains no cell wall rapidly shrink and synthesis of glycerol occur and this results in the increase of the internal osmolarity until the cells again come to their original volume. The same process occurs in the hypoosmotic (dilution) shock where rapid swelling takes place followed by a decrease in internal glycerol concentration. **Third** and last term stage is the longest one in which gene expression induced due to stress and this will leads to the synthesis of salt induced protein (Chen *et al.,* 2009).

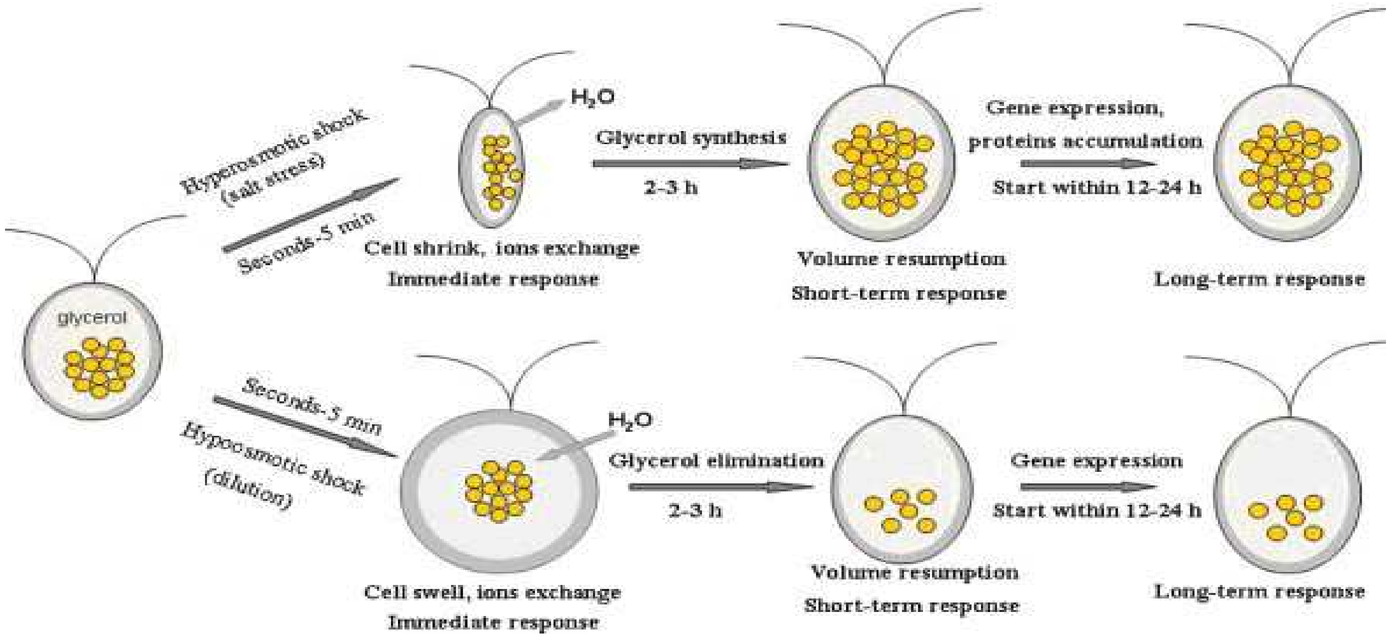


Fig4: Osmotic response in *Dunaliella* under osmotic stress. (Adapted from Chen *et al.,* 2009).

2.6 Bioactive compounds/metabolites from *D.salina* under stress:

Polysaccharides: Polysaccharides produced by this strain is generally increases with increase in the concentration of salt in the environment. On NMR, FT-IR and HPLC analysis xylose, glucose, fructose and galactose were detected as main monosaccharide subunits of polysaccharides. Some of the sulfated-polysaccharides were also recognized on performing reverse-phase HPLC analysis. Complex polysaccharides like nucleic acid-conjugated polysaccharides were also observed on analysis. It is confirmed that *D.salina* contains sulfated polysaccharides with molecular weight of hundreds or thousands of KDa. These polysaccharides are thermostable up to 250-300º C. Pseudoplastic rheological properties were confirmed in such polysaccharides released by *Dunaliella* (Olivier, 2013).

Proteins: Some of the important and specific protein like membrane bound proteins (carbonic anhydrase-60KDa and transferrin-like protein 150 KDa), and some of the enzymes for example: dihydroxy acetone reductase can be induced by change in salinities of growth media/environment of *Dunaliella* which further leads to change in the osmolarity of the medium.

Amino acids: In *Dunaliella*, proline content has been reported under high salinity conditions. Proline provides protection against stress i.e. from degradation and maintains carbon, nitrogen and other energy sources after stress. Proline also protects cells from dehydration. It improves the growth of such salt stressed cultures. It plays an important role as an osmoprotectant for membranes in microalgae from salt inhibition (Mishra *et al.*, 2008). *Dunaliella salina* is rich in useable protein (amino acids). Amino acids act as basic building block of life, required for the synthesis of muscles, neurotransmitters, tissues, hormones, enzymes etc.

Minerals: Minerals were basically detected by ICP (Inductive coupled plasma) method by many researchers. Dried form of *Dunaliella salina* is exceptionally rich in nutrients like selenium, magnesium and other antioxidants. Selenium is a powerful antioxidant that is very helpful in detoxification and immune health. Magnesium is important for healthy cellular metabolism, muscle function, nerve function and energy production. Basically, k^+, $Mg^{2+,}$ Na^+ are the main minerals which were detected under different saline conditions. (Mishra *et al.*, 2008).

Lipids: Marine sources like algae/microalgae are widely known to possess interesting lipid composition. The main polar lipids found in these substrates include monogalactosyldiacylglycerols (MGDG), digalactosyldiacylglycerols (DGDG), and phosphatidylglycerols (PG). *Dunaliella* has glycerol as main osmolyte for balancing the extracellular osmotic stress. Its intracellular concentration intensively increased with response to any osmotic stress such as elevated salinity (Shariati, 2003; Hadi *et al.*, 2008). The most important role of glycerol is that at high concentrations it protects enzyme activity.

Most important bioactive compounds are carotenoids which are most commonly known for anticancer properties.

Carotenoids:

Carotenoids have commercial value owing to their color and antioxidant properties. Industrially carotenoids have an important application which includes food, feed, cosmetics and nutraceuticals. 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).

2.5 Commercial and industrial applications of *D.salina*:

Human Food:

Dunaliella has been considered as biologically active and most important candidate to be used for nutrient supplement and in feed additives also. This species is grown for photosynthetic pigment and beta-carotene. Carotene is basically used as orange dye and vitamin C supplement. The major producer of the powders from *D.salina* is Cognis Nutrition and Health (Australia). Production is 1200-1300 tones/year.

Cosmetics:

Microalgal components are constantly used in the cosmetics as thickening agents, water-binding agents and antioxidants. They are mainly used in the skin care products like for face creams etc. They are also used as sun protectants and hair care products. *D. salina* produces an important metabolite i.e. Sporopollenin to protect themselves from UV radiation and visible radiation which are used for the photosynthesis. Another microalga is *Dunaliella tertiolacta* which produces important amino acid/ UV screening compound known as Mycosporine.

Other applications are like **food colorants**, **biofuels; high-value molecules (phycobiliproteins carotenoids).** Industrially these microalgae are also used for biofertilizers, in pharmaceutical products and in aquaculture feeds (Priyadarshini and Rath, 2012)

2.6 *Dunaliella* in Molecular farming:

Dunaliella can be used as a transgenic bioreactor for production of recombinant proteins. It can be considered as good candidate for molecular farming because of having several advantages. Some of the advantages are as follows:

a) It can grow in hypersaline or other stress conditions. Halotolerant nature reduces the risk of attack with microorganisms; pathogen free product can be obtained.

b) Dunaliella lacks rigid cell wall.

c) Milking can easily be done with *Dunaliella* which leads to the constant recovery of the product from bioreactor. They act as two-phase bioreactor.

d) It contains variety of promoter like for nitrate reductase and act as essential source for protein yielding.

e) There is no need of initial elements like other fermenters. It is of autotrophic nature so can be cultured in open photobioreactors.

f) It is a haploid organism that is the big advantage for protein expression.

g) Due to salinity stress, reproduction can easily be stimulated.

h) Transformation of foreign genes is easy as it contains large chloroplast (Barzegari *et al.,* 2010).

These beneficial features of *Dunaliella* make it a promising species for the production of important/valuable products like polypeptides, vaccines and antibiotics etc.

2.7 Extracellular polymeric substances (EPSs):

Extracellular polymeric substances are bioactive metabolites which are generally released outside the cell or on the cell surface or in the medium. These EPSs play the role of protection to the cell from harsh conditions. EPSs stabilize the membrane structure of the cells and also act as the source of energy in the nutrient depletion like conditions. EPSs are the complex mixtures of the polymers like polysaccharides, lipids, proteins, nucleic acid etc. These are renewable sources that play important role in biotechnological applications. Exopolymers have been reported in the bacteria, fungi, algae and plants. Exopolymers from microorganisms have many industrial applications. Exopolymer are used as gelling agents, thickeners, carriers for controlled drug release and in specific vaccines (Kumar, Mody & Jha, 2007). EPSs are the richest source of polysaccharides basically which are important in industrial applications like sludge settling, dewatering etc. EPSs can also be exploited as biosurfectants, bioemulsifiers etc. (Mishra, Kavita, Jha, 2010). EPSs are the complex polymeric mixtures of halide groups, primary amines groups, aliphatic alkyl groups, aromatic compound, alkyl amine/cyclic amine along with polysaccharides

Green microalgae *Dunaliella salina* produce EPSs with highly complex structure and compositions. It was reported that four major monosaccharides subunits (Galactose, glucose, fructose, and xylose) were found to be released by *Dunaliella.* It was confirmed by HPLC analysis (Mishra and Jha, 2009).

2.8 Biomedical applications of bioactive metabolites of *Dunaliella*:

Bioactive compounds have functional properties in the human body. Researchers have focused on the identification of such biologically important compounds. They are physiologically active substances. These bioactive compounds can potentially be used as important ingredients such as carotenoids, polyphenols, lipids, enzymes, vitamins etc. Microalgae are known to produce various pharmacologically active compounds that can be obtained from the biomass (crude extract) or generally are released extracellularly into the medium. Some of the pharmacological activities are as follows:

1) Antioxidant
2) Antimicrobial property
3) Anti-inflammatory action
3) Potentiality over degenerative disorders
4) Health promoting functions
5) Skin protection

Antioxidant Function: Reactive oxygen species (ROS) can damage various important biomolecules in human body and may cause chronic diseases. *Dunaliella* is considered as the rich source of antioxidant compounds. Pigments, lipids, polysaccharides etc are also known for their use as antioxidants. *Dunaliella* has been recognized as the major source of β-carotene. β- carotene is a well known antioxidant. It produces more 16% of this pigment in dry biomass. *Dunaliella salina* contains rich mixture of other forms of natural carotenoids including beta-carotene, alpha-carotene, lutein, zeaxanthin and cryptoxanthin which are potent antioxidants. Microalgal polysaccharides are also known for such biomedical applications. Mostly sulfur polysaccharides are obtained from microalgae (Morais *et al.,* 2015)

Antimicrobial property: Requirement of antimicrobial compounds has been increased due to the development of antibiotic resistance in human. These microalgae have antimicrobial potential as they contain fatty acid, halogenated aliphatic compounds, terpenoids, sterols, sulfur as functional group containing heterocyclic compounds, carbohydrates, and phenols. *Dunaliella* can have antifungal activity. Very less is known about antimicrobial activity of *Dunaliella.* Together with polyunsaturated fatty acids and compounds related to carotene metabolism, such as, β-ionone contributes to its antimicrobial activity (Leon *et al.,* 2008).

Anti-inflammatory action: Inflammation is quick response produced by the body's defense mechanism against foreign agents like pathogens, toxins etc. Microalgal metabolites like polysaccharides have ability to modulate immune response. Because of anti-inflammatory action microalgae are considered as good candidate for tissue engineering also. The PUFA's (polyunsaturated fatty acids) from microalgae have been studied and applied for the treatment in the severe chronic inflammatory cases (Morais *et al.*, 2015) It has been reported by the researchers that in male non-smokers when supplemented with 15 mg/day of beta-carotene increased the percentage of those monocytes which express MHC Class II molecule HLA-DR (Stahl *et al*., 1998). *Dunaliella salina* has favorable effect on different complement levels like C3, C4 and antioxidant capacity in rainbow trout *(Oncorhynchusmykiss)* (Raja *et al.,* 2003)

Potentiality over degenerative diseases: There are several chronic diseases that caused due to the degeneration of various important biomolecules in human body. Examples of these chronic diseases are as chronic heart diseases, chronic age-related diseases, and chronic neurodegenerative diseases. Pigments derived from microalgae are neuroprotective in nature. *Dunaliella* is natural source of beta-carotene that can prevent the risk of cancer and neurodegenerative diseases .Other pigments from *Dunaliella* are cryptoxanthin, zeaxanthine that decreased the risk of Macular degeneration, stroke, cancer or other metabolic syndromes (Morais *et al.,* 2015).

Chapter-3

Objectives of study

1) Optimization of microalgal (*D.salina*) growth condition under normal and stress conditions.
2) Isolation and estimation of extracellular polymeric substances (EPSs) from *D.salina*.
3) Assessment of antioxidants activity and immunostimulating activity of extracellular polymeric substances.

Chapter-4

Materials and Methods

4.1 Materials

Table2: Chemical and reagents required during project work are as given below:

Chemicals/reagents	Make
RPMI	Himedia
DMSO	SRL
Streptomycin	Himedia
Penicillin G	Himedia
Concavalin A	Sigma Aldrich
Histopaque-1077	Sigma Aldrich
FBS	Gibco®Life technologies
Methanol	EMPARTA®Merck
Ethanol	EMPARTA®Merck
Phenol	Himedia
Sulfuric acid	LOBAchemie
DNSA	LOBAchemie
Fructose	Himedia
Glucose	Himedia
BSA	Himedia
Coomassie Brilliant Blue	Himedia
EDTA	LOBAchemie
MTT	Sigma Aldrich
Trypan Blue Dye	Himedia
DPPH	Himedia
Ascorbic acid	LOBAchemie
Sodium Bicarbonate	Himedia

Methods

4.2 Procurement of microalgae (*D. salina*) Culture:

- *Dunaliella salina*, culture used was obtained from Birla Institute of Scientific Research, Jaipur, India.

4.3 Production of *Dunaliella*:

4.3.0 Culture conditions:

Axenic culture of *Dunaliella* was grown in A-100 media. The chemical composition of media is given below in table. The culture was maintained in 250ml of Erlenmeyer flask containing 100 ml of medium provided controlled laboratory conditions at temperature 25±2 ºC and light for 12:12 h (Light/Dark cycle) for 20 days.

Cultures were manually shaken thrice in a day. Sub culturing was done after every 20th day.

Table3: Chemical Composition of AS-100 medium:

Chemicals	Concentration(in Moles)
NaCl	1.7M
$MgCl_2.6H_2O$	7.3mM
$MgSO_4.7H_2O$	2.0mM
KCl	2.6mM
$CaCl_2$	2.3mM
KNO_3	4.9mM
Tris-buffer	4.9mM
EDTA di-Na salt	8.5µM
$ZnCl_2$	0.58µM
H_3BO_3	19µM
$CoCl_2$	0.12µM
$CuCl_2$	0.46µM
$MnCl_2$	6.3µM

$FeCl_3$	9.2µM
KH_2PO_4	1.4mM

Media was autoclaved at 121ºC 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.

4.4 Modification of Culture Conditions for Production of Extracellular polymeric substances (EPSs):

One important parameter is nutrient stress (nitrogen concentration and phosphate concentration) which was considered as the best parameters to find out the accumulation of extracellular polymeric substances on *Dunaliella salina* cells in the media.

To know about the effects of nutrients on growth of cells and production of EPSs, *Dunaliella* cells were cultivated under nutrient stress associated with a macronutrient i.e. Nitrogen and phosphate excess conditions. The cells were grown in AS-100 medium containing different nitrogen (KNO_3) concentrations such as (9.8mM, 24.5mM) and Phosphate (KH_2PO_4) concentrations such as (2.8mM, 7mM).

4.5 Measurement of Growth and productivity:

4.5.1 Cell Count Measurement:

Growth of *Dunaliella* was measured in terms of cell number and counted after every two days interval for 20 days using Neubauer hemocytometer. Cells of *Dunaliella* are motile so the cells were fixed by diluting it with Lugol's Iodine solution (Mohan *et al.,* 2012). The cell count was expressed as cells $ml^{-1.}$

4.5.2 Optical density measurement:

Specific growth rate was calculated by measuring the absorbance at 560 nm by spectrophotometer. Specific growth rate μ_{max} (divisions per day) was estimated with the help of 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 at 560nm; N_o is the optical density of Initial reading at 560nm; t is the time interval (number of days).

4.6 Downstream processing of Extracellular polymeric substances (EPSs):

Extraction procedure:

Axenic culture of microalgae *Dunaliella salina* was grown in 100ml of A-100 media under controlled and fixed laboratory conditions at 25±2 ºC under 12:12 h (Light/Dark cycle). *D. salina* culture was grown for twenty days and centrifuged at 10,000 g for 30min at 4 ºC to remove microalgal cells and debris. Filtration of supernatant was done twice with whatmann filter paper and concentration was done up to one fifth or sixth volume on hot plate magnetic stirrer at 70ºC for 18-20 h or till it gets concentrated. Equal volume of cold methanol was gradually added to the concentrated supernatant for precipitation of EPSs and was kept at 4ºC for 12 to 15 h. Centrifugation was done to remove the residual methanol from precipitates. Precipitates were then washed with absolute ethanol and redissolved in Millipore water. Gradual heating was provided to the precipitates to dissolve properly in water. Again filtration was done to remove any type of clumps formed in exopolymer solution (Mishra et. al., 2008).

4.6.1 Dialysis: To remove ions and salts, dissolved EPSs were dialyzed against tap water for 2 days followed by double distilled water for 1 day. Dialysis membrane tubing and membrane activation was done before dialysis. After membrane activation sample was loaded in it.

4.6.1.1 Dialysis membrane tubing preparation and membrane activation:

Dialysis tubing was done by cutting the membrane into desired length. Tubing was immersed into 1 L of 2% sodium bicarbonate and boiled for 10 min. Tubes were then rinsed thoroughly with Double distilled (dd) water. Boiling was done in (dd) water for 10 min then water was decanted. 50% ethanol was added into the beaker containing membranes and submerged completely. Storage was done at 4 ºC. Every time membrane tubing was rinsed before use.

4.6.2 Lyophilization: After dialysis, EPSs were frozen at -20 ºC and lyophilization was done at -60 ºC for 10-12 hours to form powder. Powdered EPSs were dissolved in sterile double distilled water for further estimations.

4.7 Analysis of Extracellular polymeric substances (EPSs):

Total sugar content, total protein content and nucleic acid estimation were done by various estimation assays.

4.7.1 Estimation of Total carbohydrate content by Phenol-sulfuric acid method (A determination with microtiter format):

The easiest and most reliable colorimetric method known for carbohydrate estimation is phenol sulfuric acid method. It has been used for the detection and measurement of neutral sugars like glycoproteins, glycolipids, oligosaccharides, proteoglycans etc. 96-well plate microtiter method is high throughput method and very sensitive one. It can measure 1-150 nmol of sugars in a sample. Originally, this estimation requires 50-450 nmoles of monosaccharides which is inadequate for precious samples. This microtiter procedure takes 15-20 minutes to complete (Masuko Tatsuya *et. al.*, 2004)

Phenol-sulfuric estimation was done in microplate format. Method was convenient and has taken less time (<20mins) to complete every time. To every well of 96-well microtiter plate, 50 µl sample of EPSs was added. 150 µl Sulfuric acid was rapidly added after sample for proper mixing. Immediately 5% phenol prepared in water was added. Incubation was given for 10 min at 70 ºC by floating microplate carefully. Glucose standards were also subjected to this assay in the same way. Plate was then cooled at room temperature and absorbance was observed at 492 nm.

4.7.2 Estimation of Total protein content by Bradford method (A quantitation of microgram quantity of proteins):

The most sensitive and rapid method for the protein quantitation is Bradford method. It's the protein determination method in which Coomassie brilliant blue G-250 dye is used to bind with protein. Dye gets bind to the protein and leads to the shift in the absorption maximum of dye from 465 to 595 nm. This increase in absorption at 595 nm is generally monitored. Assay takes very less time to complete virtually complete in 2 min. Assay is very reproducible as well. There is no interference in color of dye with carbohydrates or other cationic species. Interferences can only be caused be strong alkaline buffering agents. Care must be taken during handling as detergents like SDS, TritonX-100 or commonly used detergents can interfere in color (Bradford Marion, 1976).

Bradford reagent preparation: 100 mg of Coomassie Brilliant Blue G-250 dye was dissolved in 100 ml of distilled water/absolute ethanol. Then added 100 ml of 88% phosphoric acid mixed well and volume was made up to 500 ml with double distilled water. Reagent prepared was then filtered with whatmann filter paper (UK). Dissolved it with 1:1 water and absorbance was taken at A_{550} against water blank. Blank was recorded as 1.1 nm (standard).

It was performed by two ways: By spectrophotometric estimation and colorimetric estimation.

Spectrophotometric estimation: BSA stock solution (0.2 mg/ml) was prepared by pipetting out into clean dry test tubes in increasing concentration (2 to 20 µl) and amounts (10-100 µl).Volume of each test tube was make up to 100 µl with distilled water. Blank was also kept aside. 1.5 ml of dye solution to each test tube was added and mixed gently. Tubes were left for 5 min at room temperature. Optical density was observed at 595 nm.

Colorimetric estimation: Stock solution of BSA (0.2 mg/ml) was prepared as for spectrophotometric analysis. Volume was then made up to 0.5 ml with double distilled water and blank was also kept aside. 4.5 ml of the dye reagent was added and mixed gently. Absorbance was read at 595 nm after 5 min at room temperature.

Color of this solution remains stable generally for 30 min after that precipitation of dye-binding complex occurs. Sensitivity of this assay is 1-20 µg.

4.8 Antioxidant activity of EPSs extracted from *Dunaliella salina*:

Free radicals mainly reactive oxygen species (ROS) can disturb the metabolic functions and can also damage the structure of various building blocks/ biomolecules which can lead to cell death or dysfunctioning of cells. This increased oxidative stress can leads to various health issues like cancer, cardiovascular diseases etc. There are many types of natural compounds with antioxidant properties which are known to minimize the harmful effects of such free radical species. DPPH assay is used generally for the detection of free radical scavenging potential of an antioxidant compound. 2, 2-diphenyl-1-picrylhydrazyl (DPPH) is actually a stable radical in solutions. It gives purple color when dissolved in methanol and absorbing at wavelength 517 nm. DPPH basically accepts a hydrogen atom from the antioxidant molecule and reduces from DPPH to DPPH2. Color change occurs from purple to pale yellow along with decrease in absorbance at 517 nm. Color change is monitored by spectrophotometer. (Mishra Krishnanand *et. al.*, 2011)

DPPH assay was performed to assess the antioxidant activity of extracellular polymeric substances (EPSs) extracted from *D. salina* under normal and stress conditions. After lyophilization, powdered EPSs were prepared by dissolving it in double distilled water. Dissolved EPSs (50 µl each) of varying concentrations (500 µg/ml to 2000 mg/ml) was added into each well. And then 150 µl of DPPH (100 µM) in methanol immediately was added to each well containing EPSs. Ascorbic acid (100 µg/ml) was used as positive control (Standard antioxidant). The plate was wrapped in aluminum foil and kept at 30 ºC for 45 min in dark. Color change was (from deep violet to pale yellow) was measured at 517 nm using ELISA microplate reader.

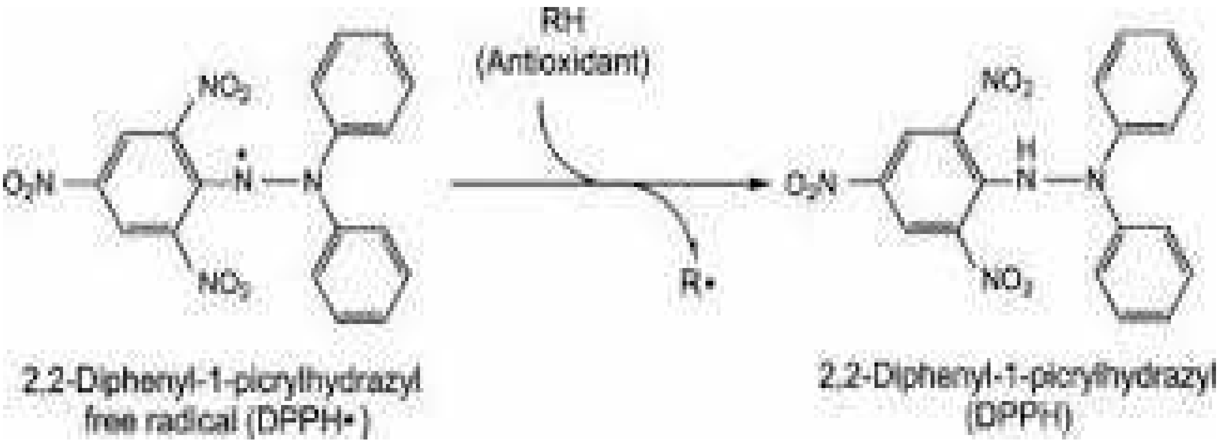


Fig5: DPPH accept one hydrogen atom from antioxidant and get reduced.

Free radical scavenging activity was expressed as the inhibition percentage which was calculated using formula:

$$\text{Free radical scavenging activity} = \{(A_{control} - A_{sample})/A_{control}\} \times 100$$

4.9 Effect of Extracellular polymeric substances (EPSs) on the growth of peripheral blood mononuclear cells (PBMCs):

Effects of EPSs were evaluated against healthy human PBMCs. Blood (5 ml) was drawn by the trained technicians of Nitin Hospital, Patiala (India). All the volunteers gave their informed consent to donate blood for the experiments and the study was approved by institutional ethical committee (IEC).

PBMC isolation was carried out using Histopaque-1077(Density gradient). To a 15ml falcon tube 5 ml of Histopaque-1077 was added at room temperature. 5ml of healthy donor blood was layered carefully onto the Histopaque-1077 and centrifugation was done at 450× g for 30 min at 25ºC. After centrifugation, top layer of plasma (pale color) was carefully removed slowly and buffy coat containing mononuclear cells was collected in sterilized falcon .Cells were then washed by adding 8ml of 1×PBS and centrifugation was done at 250 ×g for 10-15 minutes. After centrifugation supernatant was taken carefully with micropipette and discarded. Then washed cell pellet was resuspended with 8ml of sterile 1×PBS buffer. Again centrifugation was done at 250×g for 10minutes. Finally cell pellet was resuspended in 1ml of complete media (RPMI-1640 media+Antibiotic+10% Fetal Bovine Serum). Cell counting was done using hemocytometer.

4.9.1 Cell counting: 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. This diluted suspension with Trypan blue was loaded on Hemocytometer. Hemocytometer was focused by using the 10X objective lens of the microscope then was set at 40X and cells were counted in all 4 sets of squares of hemocytometer using 40X objective of the microscope. Cells were counted using the formula given below:

$$\text{Cell count} = \frac{\text{Total number of cells counted in 4 chambers x Dilution Factor x } 10^4 \text{ cells ml}^{-1}}{\text{Number of chambers counted}}$$

4.10 Assessment of effect of EPSs on PBMCs cell growth using MTT assay:

Most commonly used assay for cell viability, proliferation and cytotoxicity is MTT assay. It's a colorimetric assay of tetrazolium salt thiazolyl blue or methyl-thiazolyl-tetrazolium (MTT). MTT appears yellowish in color (aqueous solution). It undergoes for reduction by the enzyme called dehydrogenases present in cells which are metabolically active. It was reported that succinate dehydrogenase enzyme present in mitochondria of viable cells reduced MTT into its formazan product. This on reaction yields formazan product of blue violet color. Formazan is known for its lipid solubility. This can be extracted with the help of organic solvents. Estimation is done spectrophotometerically. It has been reported by the researchers that MTT formazan is directly proportional to the number of living cells in the sample (Stockert Juan *et. al.*, 2012).

Seeding of freshly isolated PBMCs was done at a density of 2×10^5 cells $well^{-1}$ of 96-well microtiter plate and Concanavalin A mitogen (2 µl) was added after 2 h. Complete media in required volume was then added to each well for cell culture. EPSs samples in varying concentration were prepared (500 µg/ml to 2500 µg/ml) and added to each well in triplicates. Total volume of each well was made 200 µl (cells, media, sample) along with 2 µl mitogen. Positive control (cells and media) were also added in triplicates. After 48 h of incubation in CO_2 incubator, 20 µl of MTT reagent was added to each well and again incubation was provided for 4 hours. Microtiter plate was centrifuged at 200 rpm for 10min.160µl of media was removed from each well and purple formazan crystals formed after 4 hours of incubation were dissolved in 100 µl DMSO in each well and absorbance was observed at 570 nm taking 620 nm as reference wavelength on ELISA plate reader. Then difference between the two wavelengths was retrieved by the ELISA microplate reader.

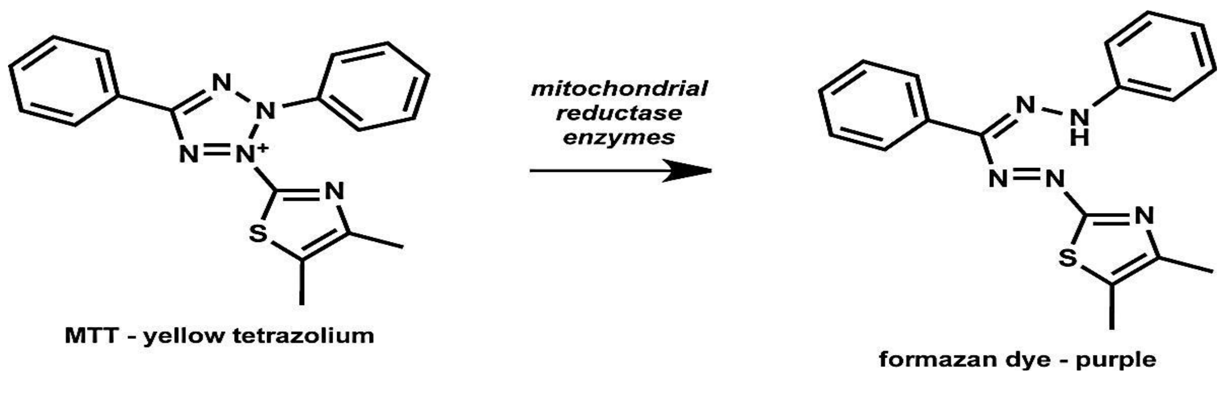


Fig 6: Reduction MTT into formazan with the help of mitochondrial reductase enzyme.

Chapter-5

Results and Discussions

Results and Discussions

5.1 Maintenance and production of *Dunaliella salina*:

Present study is concerned with isolation of Extracellular polymeric substances from *D.salina* and was analyzed for various bioactive properties of EPSs. 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 production of extracellular polymeric substances (EPSs).

Hence in this section, experiments were conducted to evaluate the influence of nutritional stress on growth rate and accumulation of bioactive metabolites (EPSs) of *D.salina.*

Initially the suspension culture of *D. salina* was maintained in 250ml Erlenmeyer flask containing 100 ml of AS-100 media respectively. This mother cultures was perpetuated at 28 °C under continuous light provided by white lamps (Philips, India). Cell growth was monitored and after 20 days, mother cultures were subcultured and various stress conditions were given with $3\text{-}5\times10^6$ cells/ml of *D.salina* as inoculums.

5.2 Optimization of *D.salina* under normal conditions:

Specific growth rate

Under the normal growth conditions (1.4 mM KH_2PO_4 and 4.9 mM KNO_3), the specific growth rate i.e. divisions per day was calculated. Specific growth rate decreased on the 1st day and thereafter it increased. The exponential phase was obtained approximately on the 5th or 6th day and the growth entered stationary phase on the 17th day. After the 22nd day a sharp decline in the growth was seen (Figure). Maximum growth rate was seen i.e. 0.8039 (Table 2).

Table 4: Specific Growth rate (μ_{max}) of *Dunaliella* cells:

Days	Specific growth rate
2	0.007
4	0.021
6	0.7872
8	0.8039
10	0.59068
12	0.4966
14	0.4323
16	0.3752
18	0.330
20	0.310
22	0.280
24	0.120

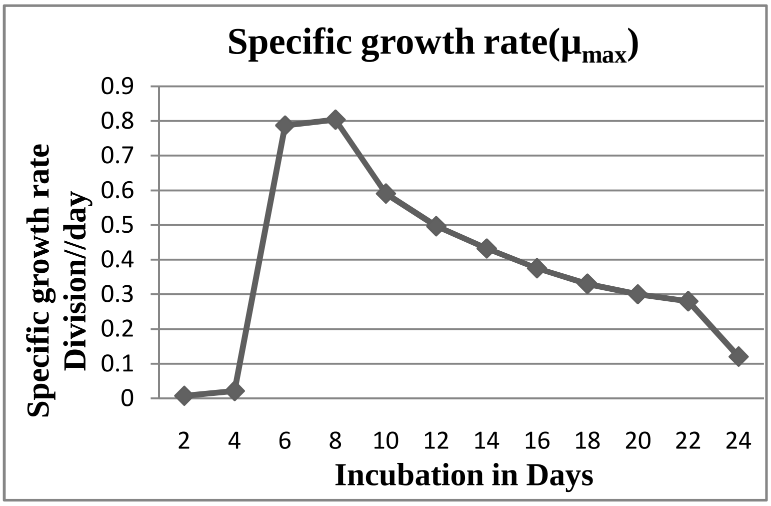


Fig7: Specific growth rate of *D.salina* in normal conditions.

Cell count

Under the normal growth conditions, the cell count of *D.salina* was increased linearly and attained a log phase on the 6th day. The maximum cell count was observed on the 20th day i.e. 15.12×10^6 cells/ml. Thereafter it was decreasing.

Table 5: Cell count of *D.salina* cells per ml.

Days	Cells per ml (in million)
2	1.36
6	3.8
10	7.92
14	11.4
18	13.60
20	15.12
24	14.8
26	13.8

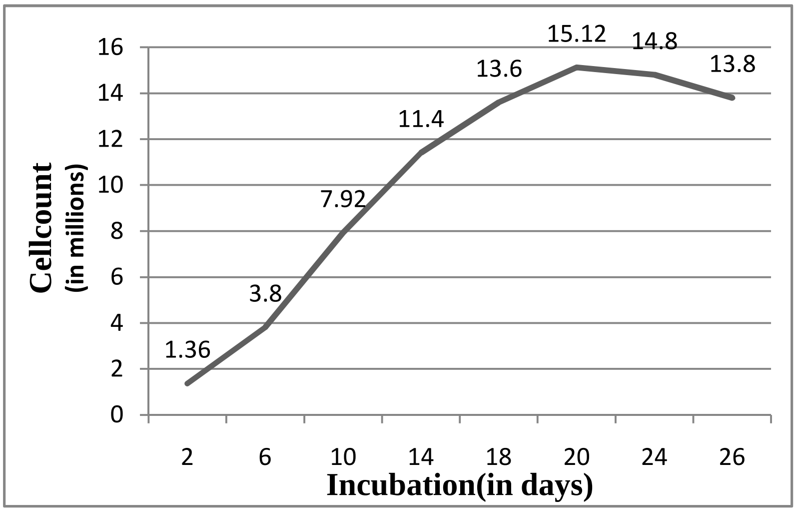


Fig 8: Cell count of *D.salina* under normal conditions.

5.3 Measurements of growth under stress conditions:

Under optimum conditions, concentrations of KNO_3 and KH_2PO_4 were 4.9 mM and 1.4 mM and under stress conditions, concentrations of these were 9.8 mM (two times), 24.5 mM (5 times) for KNO_3 and 2.8mM (2 times), 7mM (5 times) for KH_2PO_4. Cell growth was measured based on absorbance at 560. Cell growth and cell count was measured on 20th day to compare between stress conditions and normal conditions. It was observed that cell growth and count were increased as compared to normal condition and 5× concentration at 9.8 mM KNO_3 (Table 4). While at 24.5 mM KNO_3, cell growth is comparable to normal but cell count is lower. Both cell growth and count has lower value as compared to normal in KH_2PO_4 stress.

Table 6: Cell growth of *D.salina* under normal and stress conditions (Excess KH_2PO_4 and KNO_3) after 20 days

Concentration of KNO_3	**Optical Density**	**SD**
4.9mM (Normal)	0.65	0.07
9.8mM	0.965	0.02
24.5mM	0.69	0.042

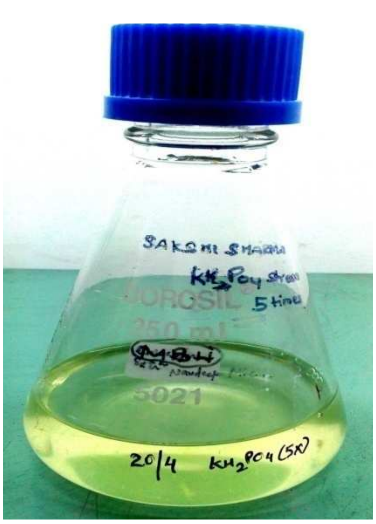
Concentration of KH_2PO_4	**Optical Density**	**SD**
1.4mM(Normal)	0.65	0.07071
2.8mM	0.306	0.03111
7mM	0.2335	0.01

(a)

(b)

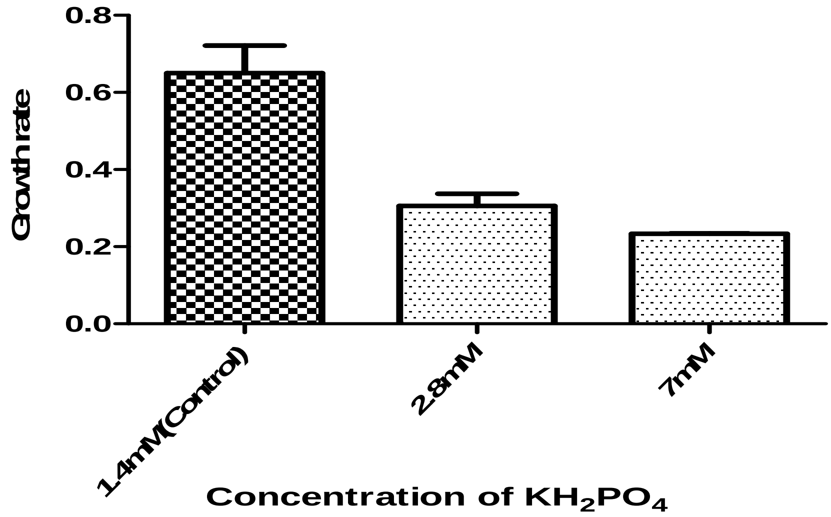
(c)

(d)



(e)

Fig 9: (a) *D.salina* incubation under normal conditions (NC). Green colored culture of healthy cells. **(b)** KNO_3 (2 times) stress provided to the *D.salina.* There was no change in color. **(c)** KNO_3 (5 times) stress to the *D. salina* cells. Pale yellow color was seen due to stress on cells. **(d)** KH_2PO_4 (2 times) stress provided to cells **(e)** KH_2PO_4 (5 times) stress provided to the cells. Light green/ pale yellow color was seen due to stress condition.



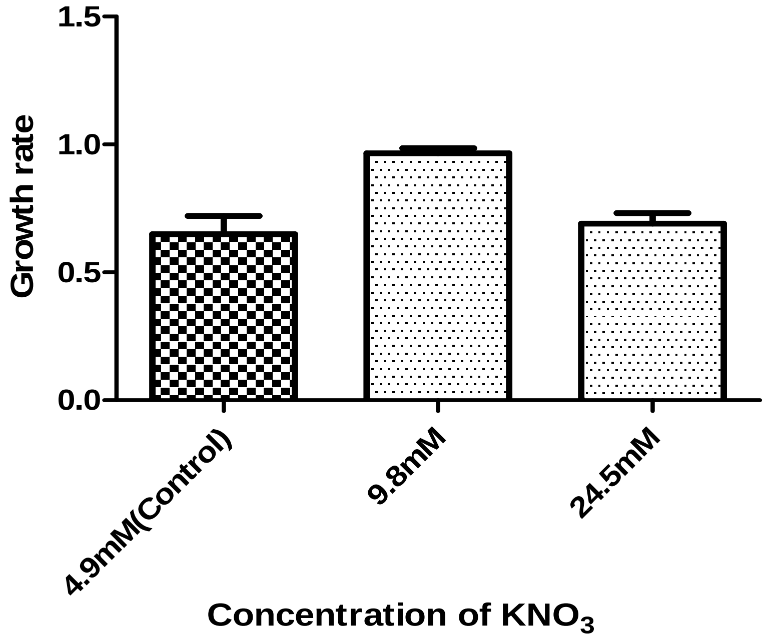


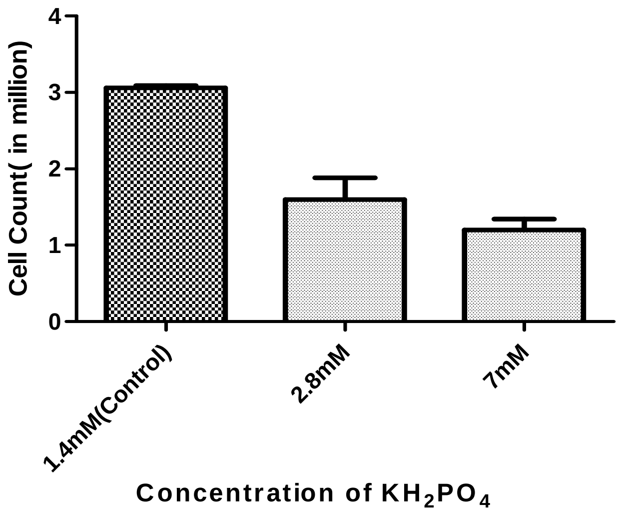
Fig 10: Cell growth after 20 days under stress conditions.

Cell count:

Cell count was measured after 20 days and it was observed that in case of KNO3, on increasing its concentration to 2 times, cell count was also increased to 4.4 million. Under optimum conditions and 5 times increase in concentration, cell count was 2.5 million and 3.08 million. In case of KH_2PO_4, cell count was decreasing on increasing the concentration of KH_2PO_4 i.e. 1.4 million for 2 times and 1.1 million for 5 times concentration.

Table 7: *D. salina* cell count (mean of duplicates) after 20 days under normal and stress conditions.

Concentration of KNO_3	Cell count (in million)
4.9mM (Normal)	3.06 ±0.02828
9.8mM	4.48 ± 0.1131
24.5mM	2.35 ± 0.2121
Concentration of KH_2PO_4	**Cell count (in million)**
1.4mM	3.06 ± 0.02828
2.8mM	1.6 ±0.2828
7mM	1.2 ± 0.1414



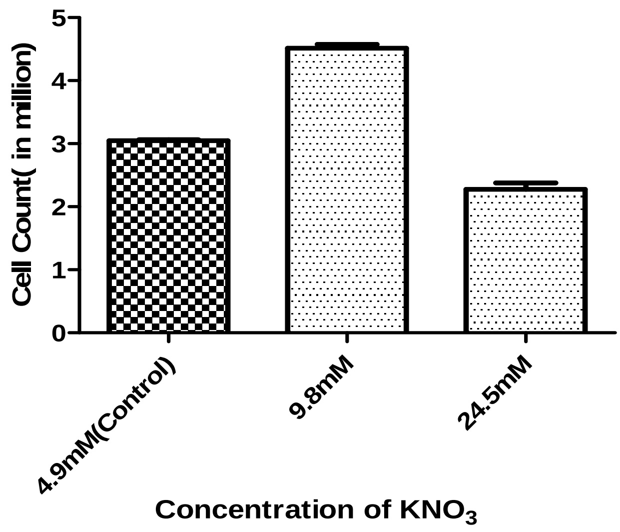


Fig 11: Cell counts under different stress and normal conditions.

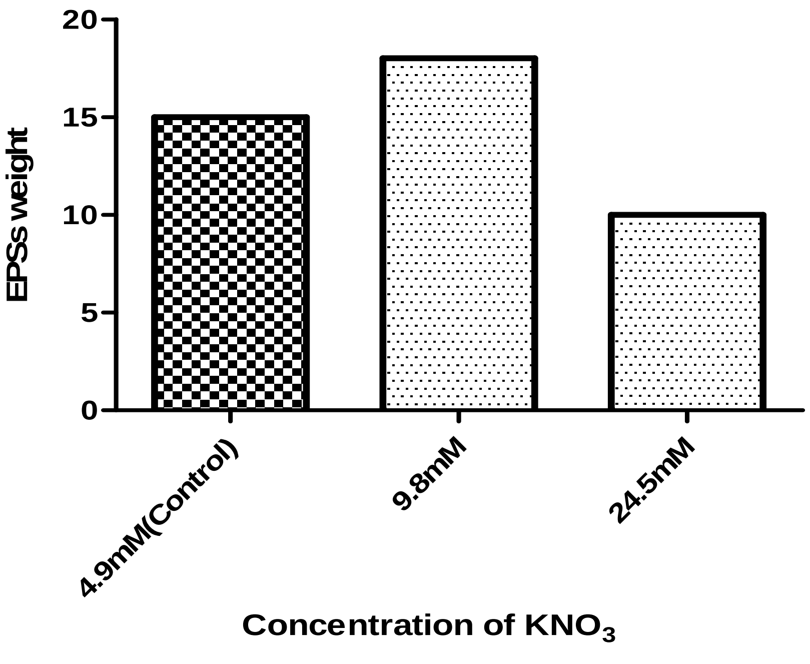
5.4 EPSs production under normal and stress conditions:

Extraction of EPSs was done after 20 days of incubation from each of the flasks containing 100ml of culture under different nutrient stress conditions and it was observed that highest weight of EPSs i.e. 57.4 mg was obtained from 2 times concentration of KNO_3. Whereas lowest weight of EPSs i.e. 3.5 mg was obtained from 5 times concentration of KH_2PO_4.

Table 8: Production of EPSs (wt.) under normal and stress conditions

Concentration of KNO_3	EPSs weight (in mg)
4.9mM (Normal)	15
9.8mM	18
24.5mM	10

Concentration of KH_2PO_4	EPSs weight (in mg)
1.4mM(Normal)	15
2.8mM	4
7mM	3.5



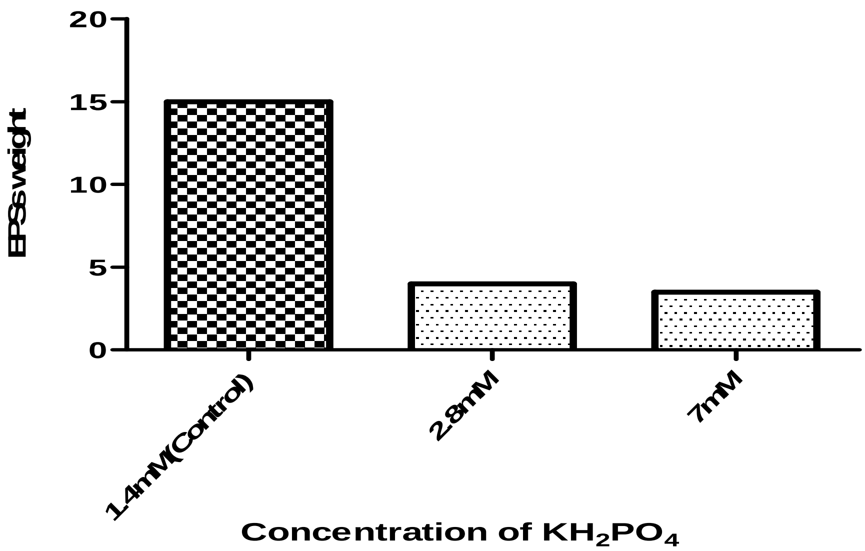


Fig 12: EPSs Production (weight) under stress conditions.

5.5 Estimation of Polymeric substances (Total carbohydrates and protein content):

5.5.1 Total carbohydrates content under normal conditions and stress conditions:

Phenol- Sulfuric acid method was carried out to estimate carbohydrates in EPSs sample. A standard curve was plotted by taking glucose as standards (Fig 15). Carbohydrates concentration in EPSs isolated in normal and stress conditions were calculated from equation obtained in standard curve and its contents is given in table. The carbohydrate contents were found to higher at KH_2PO_4 than normal conditions.

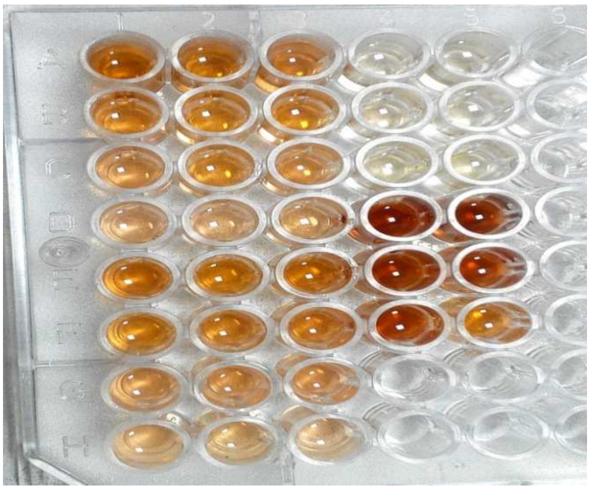


Fig 13: 96-well plate containing different glucose standard along with EPSs sample in dark brown and pale color.

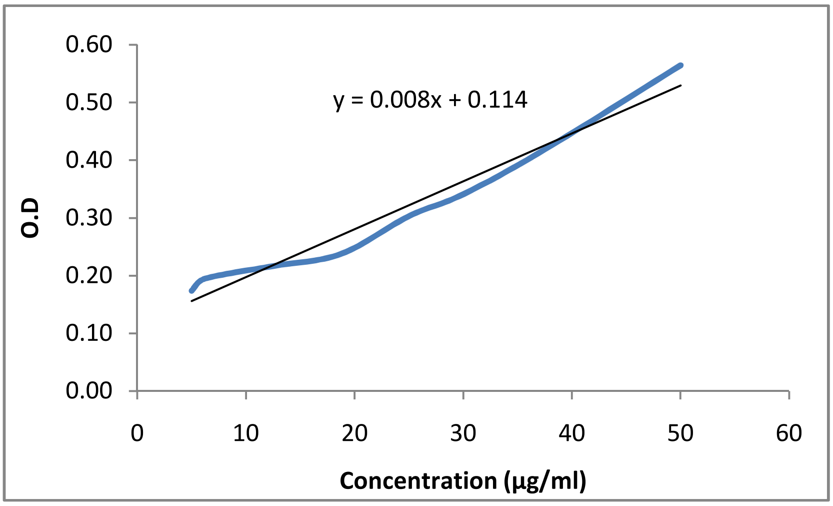


Fig 14: Standard curve obtained from glucose (500µg/ml) standards used.

Table 9: Total Carbohydrates obtained in per mg of EPSs sample (40 mg/ml)

EPSs sample concentration(µg/ml)	**Optical density**	**Sugar concentration(µg/ml)**	**Sugar concentration (per mg)**
1000	0.2069	11.6	11.17 µg
2000	0.2577	17.96	8.98 µg

Estimation under Stress conditions

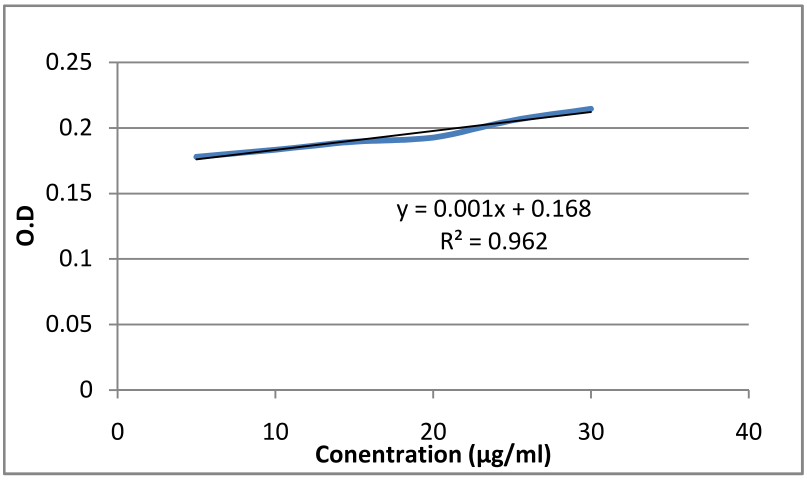
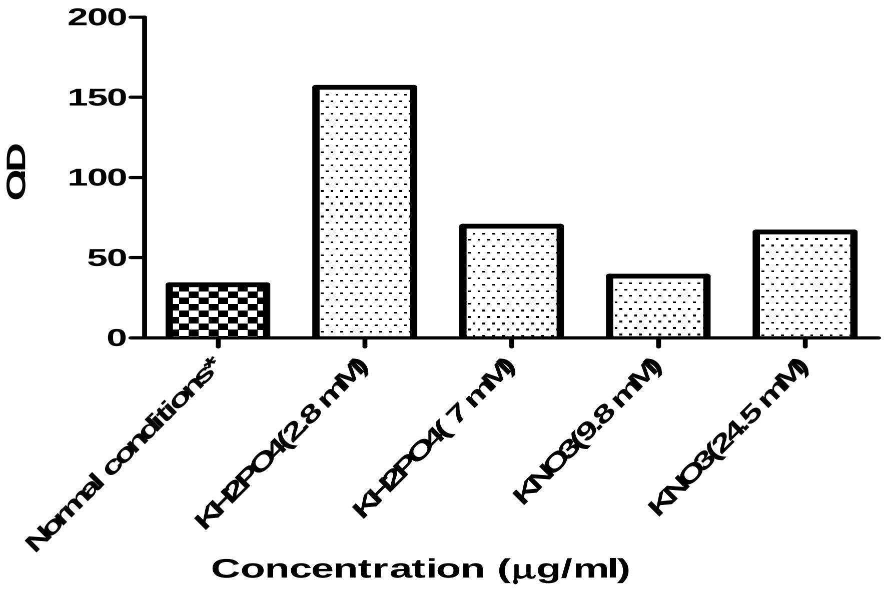


Fig 15: Standard curve obtained from glucose standards.

Table 10: Carbohydrates concentration in per mg of EPSs obtained in different stress conditions.

Type of Conditions	EPSs sample concentration(µg/ml)	O.D	Sugar concentration(µg/ml)	Sugar concentration (per mg)
Normal conditions	1000	0.2011	33.1	33.1
KH2PO4(2.8 mM)	1000	0.2575	156.3	156.3
KH2PO4(7 mM)	1000	0.2376	69.66	69.66
KNO3(9.8 mM)	1000	0.2065	38.5	38.5
KNO3(24.5 mM)	1000	0.2341	66.1	66.1



*****Normal conditions -4.9 mM KNO_3 and 1.4 mM KH_2PO_4

Fig 16: Total carbohydrate concentration in EPSs under different stress conditions.

5.5.2 Protein estimation under normal conditions:

Bradford method was carried out to estimate proteins in EPSs sample. A standard curve was plotted by taking BSA as standard (Fig 17). Proteins concentration in EPSs isolated in normal and stress conditions were calculated from equation obtained in standard curve and its contents is given in table. The protein contents were found to be higher in KNO_3 (24.5mM) than normal conditions

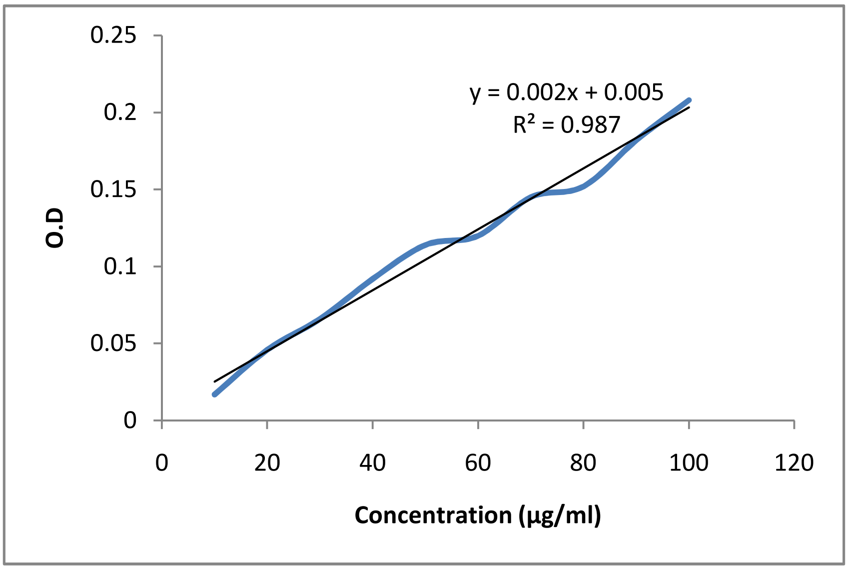


Fig 17: Standard curve obtained for BSA-1 mg/ml.

Table 11: Protein concentration in per mg of EPSs obtained under normal conditions.

EPSs sample concentration(µg/ml)	**Optical density**	**Protein concentration(x)**	**Protein concentration (per mg)**
1000	0.01754	6.27	6.27

Protein estimation under Stress conditions:

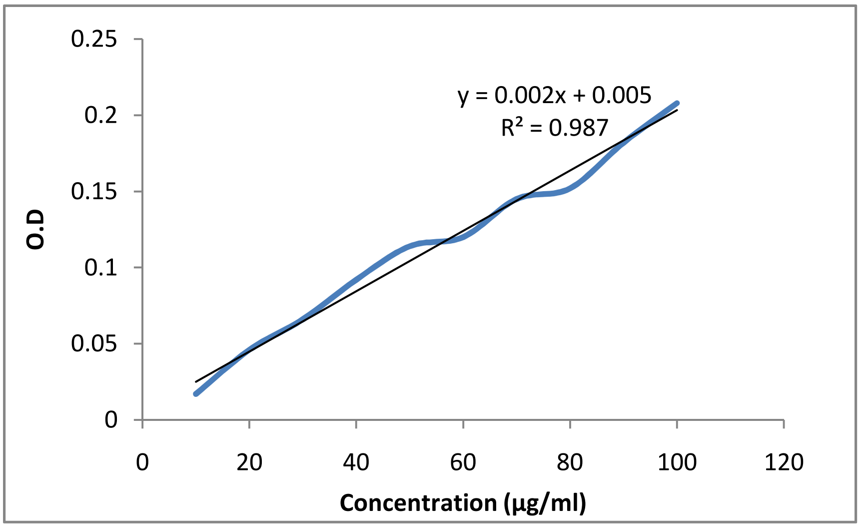


Fig 18: Standard curve obtained from BSA- 1mg/ml.

Table 12 : Protein concentration in per mg of EPSs obtained under normal conditions.

Types of Conditions	EPSs sample concentration(µg/ml)	O.D	Protein concentration(µg/ ml)	Protein concentration (per mg)
Normal conditions	1000	0.025	10	10
KH2PO4(2.8 mM)	1000	0.0802	37.5	37.5
KH2PO4(7 mM)	1000	0.095	45	45
KNO3(9.8 mM)	1000	0.045	20	20
KNO3(24.5 mM)	1000	0.106	50.5	50.5

5.6 Assessment of antioxidant effects of PBMCs under normal conditions:

To check the antioxidant effects of EPSs (15 mg/ml) for different concentration were used. Ascorbic acid was used as positive control. It was observed that on increasing the concentration of EPSs percentage inhibition was also increased. After a particular high concentration there was no antioxidant effect was observed.

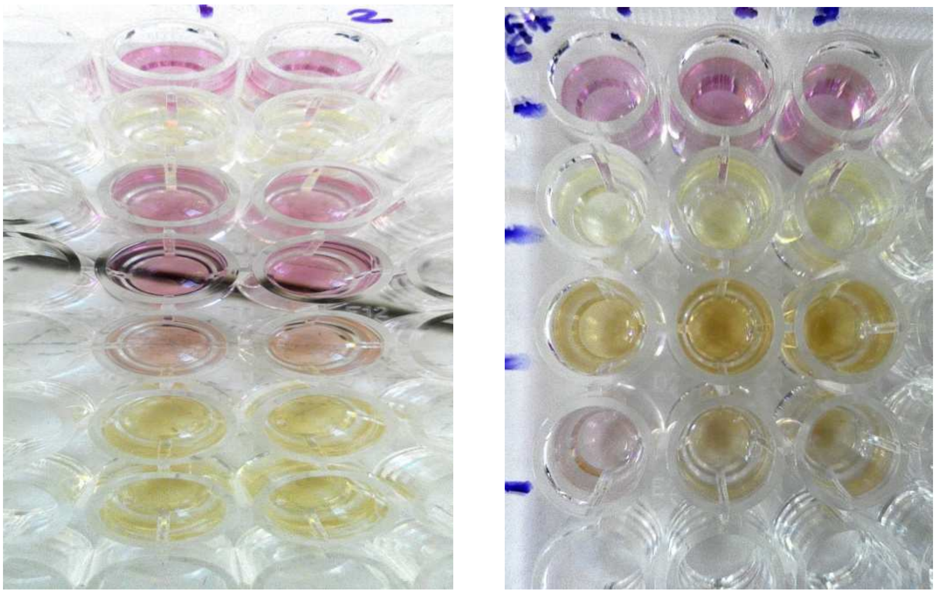
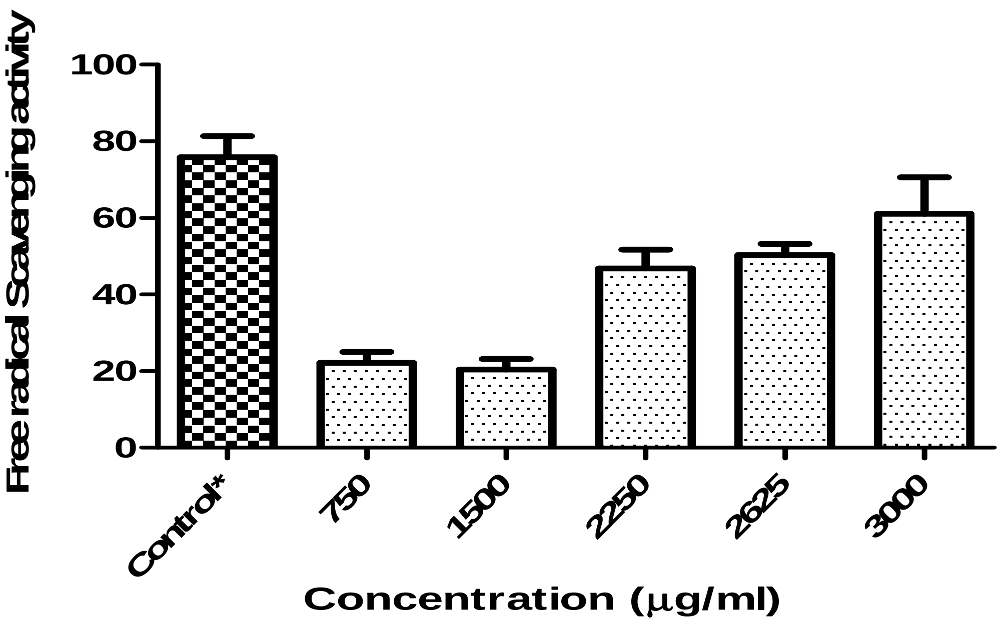


Fig 19: In above pictures, both 96-well plate show ascorbic acid control in pale yellow color in second position and yellow color change in samples shows their antioxidant nature.

Table 13: **Free radical scavenging activity by EPSs shown for different concentrations of EPSs**

Concentration (µg/ml)	Experment1	Experiment2	Experiment3	Mean
Control*	82.22	72.67	72.79	75.89±5.479
750	19	23.33	24.34	22.0±2.836
1500	21.72	22.31	17.26	20.43±2.7611
2250	51.53	47.07	41.77	46.79±4.886
2625	53.48	49.75	47.66	50.3±2.948
3000	71.83	57.86	53.69	61.13±9.501



*Control- Ascorbic acid (50µl)

Fig 20: Free radical scavenging activity of EPSs (All data presented are mean of triplicate experiments).

5.7 Assessment of effects of EPSs on PBMCs by MTT method:

Activity under normal conditions:

Different concentrations (500, 1000, 1500 µg/ml) of EPSs were used to check the effect on stimulated (Concanavalin A treated) PBMC's. It was appeared that on 1 mg/ml concentration

EPSs was showing very good proliferation in cells. Cell growth was observed on this particular concentration. After this on increasing concentration up to 1.5 mg/ml, no proliferation was observed.

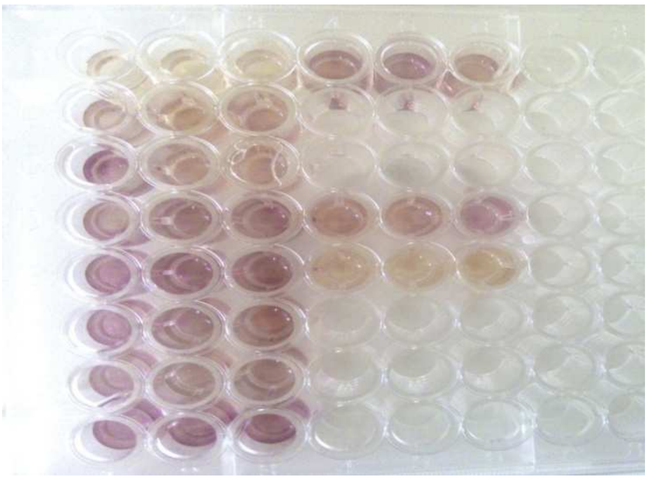


Fig 21: Purple and yellow color in wells show viable or non-viable cells.

Table 14: Percentage proliferation shown for different concentrations of EPSs under normal conditions:

Concentration	Experiment1	Experiment2	Experiment3	Mean
500 µg/ml	5.43	6.6	2.6	4.876±2.05
1000 µg/ml	11.81	17.49	22.32	17.2067±5.26
1500 µg/ml	-15.2	-10.42	-30.42	-18.67±10.44

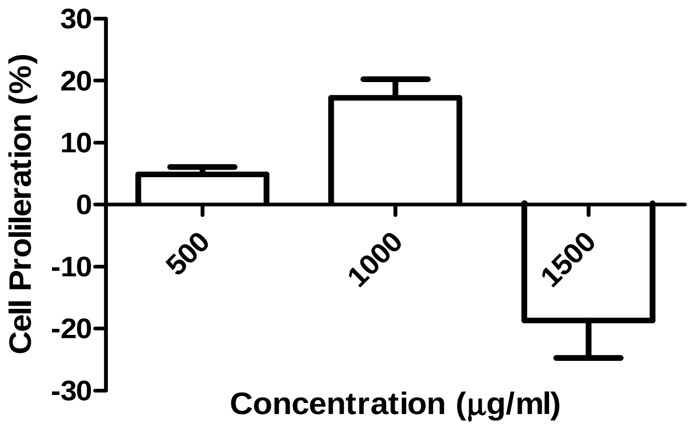


Fig 22: Effect of EPSs under normal conditions at different concentrations (All presented are mean of triplicate experiments).

Assessment of proliferative effect of EPSs produced under stress (Phosphate and Nitrate) conditions:

1 mg/ml EPSs of all stress and normal conditions were used to study its effect on conA stimulated PBMCs. Interestingly proliferation of conA stimulated PBMCs was observed in all stress conditions and normal conditions. As compare to normal conditions, nitrate (5 times) and phosphate ((2 times) has shown higher proliferations. Low proliferation was observed at (KNO_3-2 times and KH_2PO_4-5 times) stress conditions.

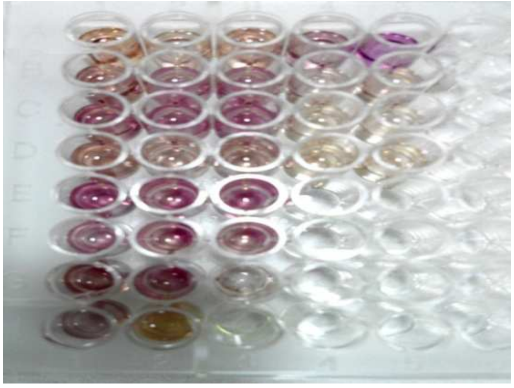


Fig 23: Effect of EPSs samples shown on 96-well plate along with their controls

Table 15: Cell proliferation (%) under stress conditions (Concentration-1 mg/ml for all)

Type of stress	Experiment1	Experiment2	Mean
Normal conditions	18.71	35.25	26.98±11.69
KH2PO4(2.8 mM)	33.75	44.95	39.35±7.919
KH2PO4(7 mM)	29.74	3.47	16.60±18.57
KNO3(9.8 mM)	4.475	31.57	18.02±19.15
KNO3(24.5 mM)	49	37.78	43.39±7.93

.

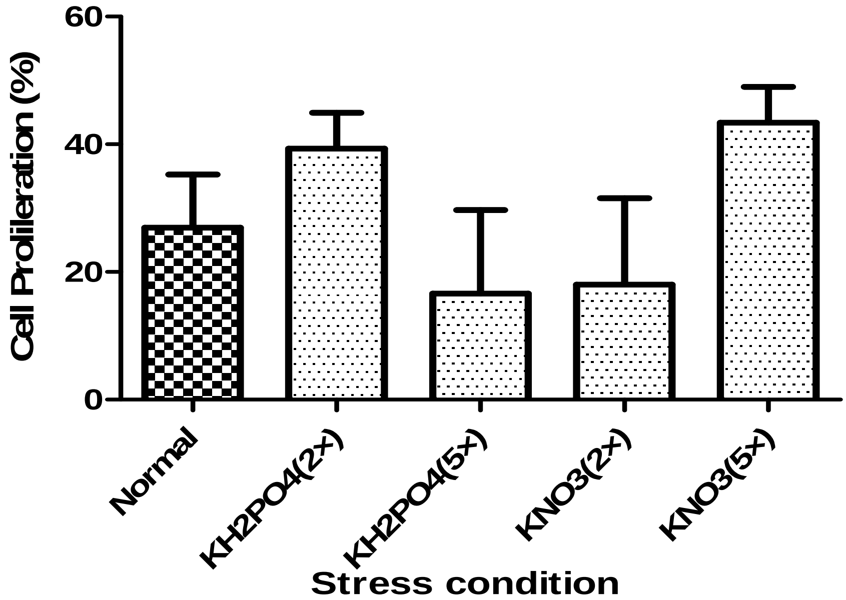


Fig 25: Effect of EPSs (1mg/ml) produced under different under different stress conditions on cell proliferation of PBMCs

5.8 Discussions

D.salina was able to grow at different nutritional stress conditions and produce EPSs in culture media that showed antioxidant and immunostimulating activities. In this work, efforts were made to optimize the concentration of various components of algal growth media to enhance the bioactive EPSs productions. In present work, extracellular polymeric substances were extracted from *D.salina* and their antioxidant and immunostimulating activity were studied. Further, stress condition of phosphate (2.8 mM and & 7 mM) and nitrate (9.8 mM and 24.5 mM) as compared to normal conditions (1.4 mM KH_2PO_4 and 4.9 mM KNO_3) were given to *D. salina* to study its growth, EPSs production and bioactive properties. Cell count and specific growth rate was found to increase on doubling the concentration of KNO_3 (9.8 mM) $_{as}$ compare to normal conditions. On the other hand, cell count and specific growth rate decreased under phosphate stress conditions. Chemical analysis of EPSs extracted from *D. salina* confirms the presence of protein and carbohydrate contents. Yield of EPSs also varied according to stress condition provided. Highest EPSs production was observed in KNO3 (9.8mM). Also, it was observed that under certain stress condition (), protein and carbohydrates concentrations in EPSs were found to higher as compare to normal conditions.

Antioxidant activity was assessed under normal conditions and it was observed that this effect increased with increase in concentration of EPSs. Highest antioxidant activity was found at concentration 3 mg/ml.

PBMCs were incubated with the EPSs in order to assess the immunostimulating activity of EPSs. 1 mg/ml concentration of EPSs extracted under normal conditions shown increase in proliferation of mitogen stimulated PBMCs. On the other hand, under stress conditions, highest cell proliferation was observed in EPSs isolated from alga grown at KNO_3 (24.5 mM).

Present study suggests that production of EPSs was enhanced under certain stress condition. Further. EPSs extracted under stress and normal condition has shown the immunostimulating and antioxidant properties, which shows the medicinal importance of EPSs extracted from *D. Salina*.

Conclusion

Dunaliella salina is considered as best model organism to study stress conditions as it can grow in harsh/ adverse condition and known for the production of high-value bioactive compounds. *D. salina* is found in salt lakes, saltern ponds and crystallizer pond at high salt concentration i.e. above saturation where other oxygenic phototrophs are not actually able to grow.

In present study, *D.salina* was subjected to different nutritional stresses during growth and extracellular polymeric substances (EPSs) were isolated and purified. This preparation was screened for antioxidant and immunostimulating activity in PBMC *in vitro*. It was observed that EPSs extracted from *D.salina* in normal as well as stress conditionswere immune-stimulating in nature. Immunostimulating activity i.e. cell proliferation increased under stress conditions. The concentration of protein and carbohydrates in EPSs was observed to increase under stress conditions. The present work has provided as insight to the bioactive properties of EPSs of *D. salina* and the nutritional stress conditions for enhancing production of EPSs.

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