Effect of exopolysaccharides produced under stress conditions by *Dunaliella Salina* on the growth of cancer cell lines and peripheral blood mononuclear cells.

A Dissertation

Submitted in partial fulfilment of the requirement for the award of degree of

Master of Science

In

Biotechnology

UNDER THE SUPERVISION OF

Dr.MANOJ BARANWAL

(ASSOCIATE PROFESSOR)

DEPARTMENT OF BIOTECHNOLOGY

THAPAR INSTITUTE OF ENGINEERING & TECHNOLOGY

(Deemed to be University)

PATIALA, PUNJAB (147004)

SUBMITTED BY

BANEET CHAWLA

301701006

M.Sc. BIOTECHNOLOGY (2017-19)

July 2019

Declaration

I declare that the thesis entitles "Effect of exopolysaccharides produced under stress conditions by *Dunaliella Salina* on the growth of cancer cell lines and peripheral blood mononuclear cells" is an authentic record of my own work, carried out during a period of six months. The work has been completed under the supervision and guidance of **Dr. Manoj Baranwal**, Associate Professor, Department of Biotechnology, Thapar Institute of Engineering and Technology (Patiala).

I also declare that matter presented in this thesis has not been submitted by me in any other University/Institute for the award degree.

Baneet Chawla

Place: Patiala

Baneet Chawla

Date: 02·09·2019

CERTIFICATE

This is to certify that the thesis entitled "Effect of exopolysaccharides produced under stress conditions by *Dunaliella Salina* on the growth of cancer cell lines and peripheral blood mononuclear cells" submitted by Baneet Chawla (301701006) to the Thapar Institute of Engineering and Technology (Patiala) towards partial fulfilment of the requirements for the award of the degree of Master of Science in Biotechnology. This work has been carried out under my supervision.

It is also certifying that this thesis or any part of this thesis has never been submitted, neither in part nor in full to this institute or any other institute for the award of any degree.

Dr. Manoj Baranwal

Supervisor

Department of Biotechnology

Thapar Institute of Engineering and Technology, Patiala

ACKNOWLEDGEMENT

I want to express my deepest gratitude and sincere thanks to the following people without whom my thesis could not have been possible. I thank the Almighty for His blessings in the completion of the project.

First and foremost, I am sincerely grateful from the bottom of my heart to my supervisor and guide, ***Dr. Manoj Baranwal*** *for his continuous support, trust, motivation, patience, enthusiasm, and knowledge which helped me to successfully carry out this project. I am thankful to him for resolving every problem, aspiring guidance and friendly advice in every phase of the project which helped in shaping this thesis perfectly. Apart from guiding the project, he treated me like his child and gave very important life lessons which would surely make my life beautiful to live.*

I express my thanks to ***Dr.Moushumi Ghosh****, Professor and Head, Department of Biotechnology, Thapar University, Patiala, for providing with the best laboratory facilities.*

I would also like to acknowledge the faculty of BTD, Thapar University for guiding me whenever I needed.

I express my deepest thanks to ***Ms.Neha Srivastava*** *who despite of her work was constantly resolving all my doubts and encouraging me at every step.*

I would like to express my gratitude to PhD Scholars ***Mrs Yogita kautish*** *and* ***Mr Sahil Jain*** *for their constant support, motivation and sharing helpful insights that greatly assisted the research.*

I was fortunate enough to avail help and assistance from department's laboratory staff, ***Mr.Babban****,* ***Mr.Prabhat****,* ***Mr.Lallan****,* ***Mr.Surinder*** *and* ***Mr.Phulchand****.*

I take the opportunity to acknowledge all the healthy volunteers who consented to provide blood for my research work. I would like to thank ***Dr.Akshey Jain*** *from Nitin Nursing Home, Patiala for providing us the blood needed for carrying out the experiments. .*

*Finally, I would like to thank my parents(**Mr.Guljeet Singh &Mrs.SavneetKaur)** for their blessings and for supporting me both morally as well as economically.I would also like to thank my sibling**Ms.NavreetChawla** for her corporation and cheerly assistance**.** Sincere thanks to my best friend **Ms.KirandeepKaur**for her constant motivation, support and love.A special thanks to my friends **Bimalpreet Singh, PriyankaGarg and NehaKaushal,** for their direct or indirect support and motivation throughout the project.*

I am sincerely thankful to all of you.

BaneetChawla.

Abstract

Dunaliella species is well known to produce various bioactive metabolites such as carotenoids (β-carotenoid, phytoene, phytofluene, lutein, and zeaxanthin), dried algal meal & glycerol that have been commercialized due to their antioxidant, anti-cancerous properties. In the current study, the cell growth effect of exopolysaccharide (EPS) produced under stress conditions were assessed in peripheral blood mononuclear cells (PBMC).Glucose content was found to be increased in stress conditions as compared to normal. EPS was found to be increased in stress conditons Cell growth promoting effect was observed in PBMC by EPS produced and the effect was higher in stress than normal conditions. Isolated EPS from *D.salina*with different stress conditions i.e. NaCl 3.5 M, KNO_3 (3X), KNO_3 (5X), KH_2PO_4 (5X) showed highest inhibition as compared to normal on RAW 264.7 cells. However KH_2PO_4(3X) showed highest inhibition as compared to normal on MCF-7 cells.The FTIR data confirmed the presence of alcohol, keto group, nitro group and halo functional groups.^{1}H NMR data confirmed the presence of hexose & pentose sugar with their particular chemical shifts. The obtained spectra showed hexose sugar, trisaccharides, and pentasaccharide. Hence it is concluded that enhanced glucose content in EPS produced under stress condition may be associated with increase bioactive properties.

CONTENTS

Chapter I

Introduction

Introduction

Dunaliella salina (D.salina) is an oxygen evolving microalgae and was first recognised by Teodoresco in 1905. Borowitzka and Borowitzka studied its detailed physiology in 1988 (Sathasivamet, al. 2013). It has motile biflagellate green cells. It comes in various shapes and symmetries that can adapt extreme environment such as salinity (Takouridis et al., 2015), copper (Sztrum et al., 2012), organic acids (Iwasa and Murakami, 1968), herbicide (Franqueira et al., 2000), oxidative stress (Wang et al., 2004a). *D.salina*is an autotrophic microorganism and its basic requirements are carbon source, inorganic nutrients, sunlight and water. *D.salina* can asexually reproduce by the division of motile vegetative cells and sexually through the fusion of two equal gametes into a single zygote.*D.salina* can accumulate glycerol, β-carotenoid and other carotenoids (phytoene, phytofluene, lutein, and zeaxanthin) that can resist cancer and posses many other properties such as anti-tumour, anti-coagulant and Biolubricant properties. It is commercialized due its vast bioactivities.

D. salina was grown in different stress conditions to analyze the growth of exopolysaccharides. Three different stresses were given as 3 M NaCl, 3 M NaCl& without KNO_3 and 3 M NaCl& with 3X concentration of KNO_3. Chlorophyll, carotenoid and dry mass of extracted EPS were analyzed. Isolated EPS fromdifferent sets of stress conditions was tested for the cell growth effect on peripheral blood mononuclear cells (PBMC), mouse macrophage RAW 264.7 and breast cancer MCF-7 cell lines. EPS was also analyzed for sugar content by phenol sulphuric acid test and further characterized by Fourier transform infrared spectroscopy (FTIR), NMR and mass spectroscopy (MS).

Chapter II

Review of literature

Review of literature

Algae are classified as protists under kingdom plantae based on the type of pigments present such as red (carotenoids), brown (phycobilins), and green (chlorophyll photosynthetic). Algae are divided into several types based on their habitat, Planktonic (that live in large bodies of water), neustonic (organisms that inhabit the region on or just below the surface of a body of water), cryophilic (affinity or thriving at low temperature), thermophilic (that can grow at relatively high temperature) (Madigan *et al.* 1997). The genus *Dunaliella* is one of halotolerant microalgae and is commonly found in hypersaline environments(Fig2.1).*Dunaliella* species are known for the accumulation of significant amounts of valuable fine compounds such as carotenoids, glycerol, lipids, vitamins, minerals and proteins(Sathasivam and Juntawong, 2013). *D. salina* was discovered by Michel felixDunal, 1838.

D. salina belongs to:-

Domain- Eukaryota

Kingdom- viridiplantae

Phylum- chlorophyta

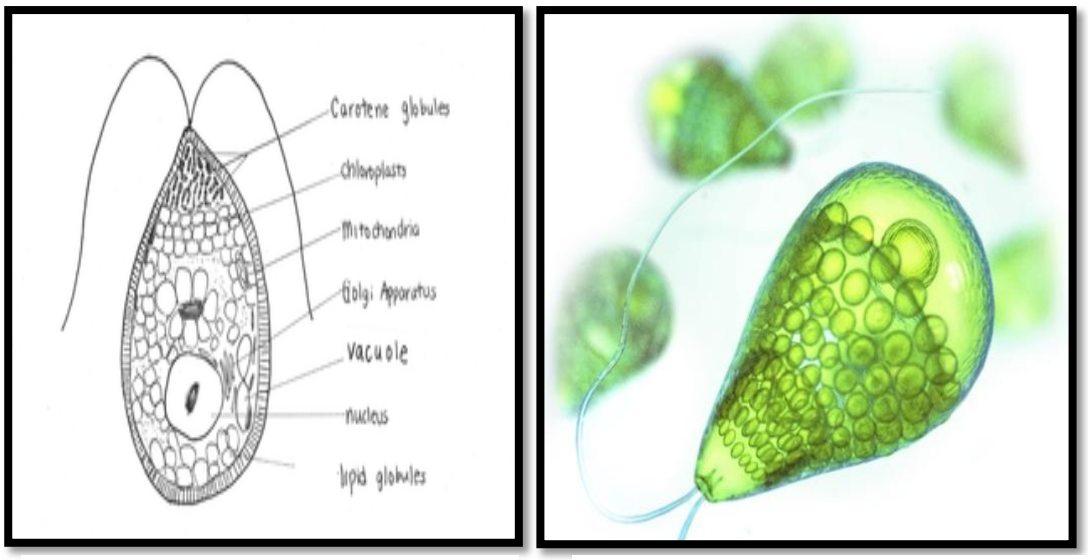
Class- Chlorophyceae

Order- Volvocales

Family- Dunalielllaceae

Genus- *Dunaliella*

Species- *Dunaliellasalina*



Schematic view of *D.salina* (Shariati et al. 2011; Heakal et al. 2010)

(https://www.algotherm.com/en/seaweed/dunaliella-salina-en/)

Fig 2.1 Schematic view & image of *D.salina*

2.1 Nutrition of *D.salina*

Primary requirements of *D.salina*include light source (natural sunlight or fluorescent lamps), temperature (25-35 ºC), nutrient source (carbon and nitrogen) (Garcia-Gonzalez et al. 2003) and pH (range of 0-11) (Ben-Amotz 1995).Growth of algae & production of metabolites is dependent on the intensity and duration of light provided. Generally, it requires 12 hours in light and 12 hours in the dark.It is always recommended to keep the pH less than 8,howeverthe optimum pH is maintained to 7.5±0.2 (Amotz and Avron 1989).

2.2 Mechanism of sustaining salt stress

Species of genus *Dunaliella*are known to resist in a wide range of salt concentrations by maintaining osmotic ion balance. The cells are known to change their volume as well as shape in response to hyper/hypo osmotic conditions (Chen & Jiang 2009). A specific mechanism is required by the cells for enabling their survival in such extreme conditions. Under salt stress Glycerol is known to accumulate in the cells and the metabolite has been commercialized as glycerine in the food and pharmaceutical industry. The osmo regulation process in *D. salina*is divided into three phases:-

1) In the first phase, cells respond with either decrease or increase in shape and size to prevent apoptosis occurred in first 10-15 min of stress.

2) After 3-4h intracellular osmotic pressure is maintained by intracellular regulation of glycerol concentration.

3) Cell activates genes in the last phase usually after 12-24h which lead to the accumulation of salt induced proteins.

2.3 Osmoregulation

Ions required for the osmotic arrangement: An intracellular level of 20-100 mM Na+ concentration is maintained by all cells, which is mostly less than outside environment (1-5 M) showing enough effectiveness of the cell to prohibit excess ions to enter the cell(Chen and Jiang, 2009). The Na^+/H^+antiportersmediumte the extrusion by ATP mediumted fashion. Likewise, K^+ionconcentrations are maintained as 100-200 mM via the K^+transporters (Fig2.2).

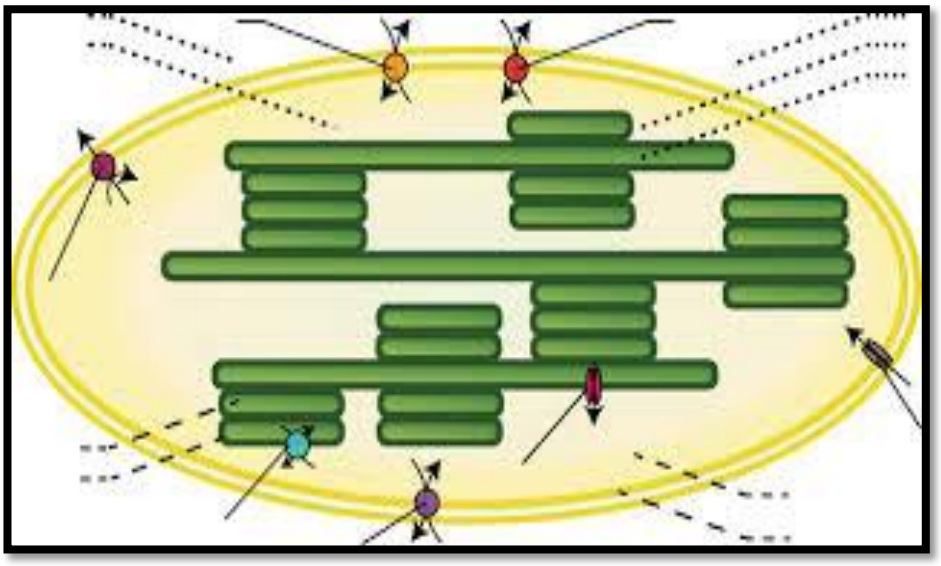


Fig2.2. Mechanism of osmoregulation by controlling the ion exchange through ion channels (Source-https://encrypted-tbn0.gstatic.com/images?q=tbn:ANd9GcSCBcge2b9fiw-m4DXPIHi6oA_71XQ1h3quQAZbTspuRMcq2QoW)

Osmoregulation is also regulated by intracellularly maintaining the glycerol levels in the algal cells. The glycerol is formed by two mechanisms i.e. by using photosynthetic product or by starch degradation (Amotz, 1980). Various enzymatic reactions follow to accumulate glycerol in the cells. Glycerol is an effective osmoregulator as it is the highly soluble end product metabolite (Fig2.3).

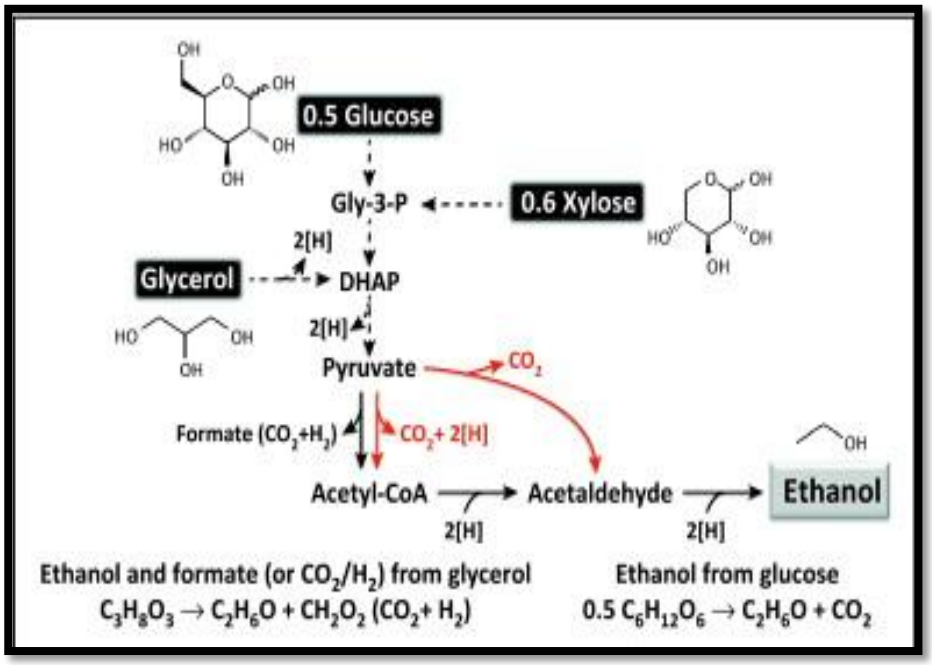


Fig2.3. Mechanism of production of glycerol

(Source-https://ars.els-cdn.com/content/image/1-s2.0-S0167779912001862-gr3.jpg)

2.4 Commercialisation of *D. salina*

Commercial production of *D.salina*are carried out extensively either in unstirred open ponds or intensively in paddle wheel ponds. There are two producers of the algae on a commercial basis; Western Biotechnology Ltd, Perth, Australia (5-hectare area) and Betatene ltd, Melbourne, Victoria, Australia (50-hectare area)(Curtain 2000). Alternatively, the biomass of the culture is increased by using paddlewheel ponds commercially used by companies in Israel and the UK (Tafreshi and Shariati 2009). Closed cultures pose several advantages of preventing the contamination with other protozoans but as they are expensive than open lagoons, it is not preferred although, better designs are awaited. The specific increase or decrease in nutrient stresses leads to either increase or decrease in the production of a specific metabolite thus, it becomes important to know the growth requirements of the algal cells. When any product is commercialised, cost of production plays a vital role. In any bioprocess, downstream processing (extraction and purification) mostly adds up to the cost of the final product. β-carotene and glycerol being intracellular products pose a lot of difficulty in extraction and purification as different down streaming processes leads to the destabilization of the product and high shear

stress caused leads to the non-reusability of the culture. Despite these problems, β-carotene demand is increased and is sold for $300 per kg (Amotz and Avron 1989).

D. salina is also marketed as a whole organismunder a trading name of Algotene® by Inter Clinical laboratories Pvt Ltd. Australia (UltraHealth*et al*.,).Different varieties of algae have been commercialized as food such as *Porphyra*, *spirogyra*, and *laminaria*. Other species of algae such as *chlorella, Dulse,* and *undaria* are also consumed in Canada, Korea, and china extensively as an integral part of their diet. Many algal species have been used as multivitamin and mineral supplements which are consumed widely as chewing tablets (Chu, 2012).

D. salina is the most halotolerant eukaryotic algae and known for survival even in the saturated salt concentrations. *D. salina* is commercialized for glycerol, a primary metabolic product which is required for its stabilization in hyper salt environment & β- carotene.

Apart from intracellular carotenoids and glycerol, algae are also explored for their extracellular secretions and it has been found that majorly, the secretions are composed of polysaccharides. It was reported that various industrial applications are associated with extracellular polysaccharides (Raposo. *et al*.,2015). One of the great advantages of these secretions is that being extracellular; the cost of extraction and purification is decreased in contrast with the intracellular productions. Also, the culture is reproducible as no damage to the cells occurs in the downstream processing

2.5 Polysaccharides isolated from various algae

Polysaccharides have glycosidic linkage between their monosaccharide units and therefore known as glycans. Glucose, fructose, manose and galactose are simple sugars that are found in polysaccharides. Theglycosidic bond consists of oxygen molecule with carbon rings. Polysaccharides are classified as homopolymers (such as glycogen and starch) and heteropolymers (such as hyaluronic acid). Their main function is to store energy. Polysaccharides show various properties as anti-tumour, anti-adhesives, nutraceuticals, soil aggregation etc.

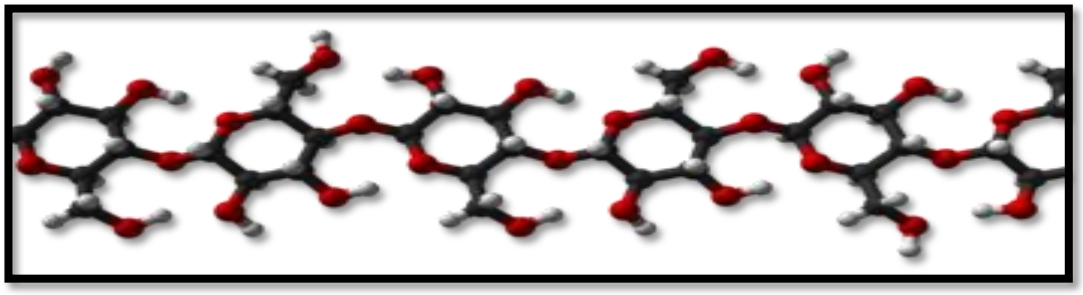


Fig2.4. General structure of polysaccharide (Source- https://en.wikipedia.org/wiki/Polysaccharide)

Exopolysaccharides are reported to be present in algae species (Table: 2). They are generally secreted by microorganisms into the surrounding environments (Angelaalincy*et al.* 2017). They are also known to protect pathogenic bacteria against desiccation and predation, and contribute to their pathogenicity.Through symbiotic relationships they can facilitate the attachment of nitrogen-fixing bacteria to plant roots and soil particles.

Table 2.1: Extracellular polysaccharides secreted by various micro algae(de Jesus Raposo*et al.* 2015)

Species	Group of Algae	Type of polysaccharide	Monosaccharide units
Phaeodactylumtricornutum	Diatoms	sEPS	Glucose, xylose, mannose, rhamnose
Chlorella stigmatophora	Chlorophytes	sPS	Glucose, xylose fucose
Chlorella. Vulgaris	Chlorophytes	PS (β-1,3 glucan)	Rhamnose, arabinose, galactose, 2-O methyl rhamnose
Dunaliellasalina	Chlorophytes	EPS	Galactose, glucose, xylose, fructose
Porphyridium	Rhodophytes	sPS	Xylose, galactose, glucose
Gyrodiniumimpudicum	Dinoflagellates	sPS	Mannose, galactose, glucose
Aphanothece Halophytica	Cyanophytes	EPS	Glucose, fucose, arabinose, mannose

Polysaccharides extracted from microalgae are known to have different bioactive properties which includesanti-tumour, anti-oxidant, anti-thrombotic,anti-lipidemic, and anti-glycemic properties.

2.6 Biological activityof *Dunaliella salina*

Dunaliella is a rich source of natural products and have wide range of applications in the food and therapeutic industries. With the emergence of new technologies in the study of biological activity of compounds from various algae, *D. salina* is drawing attention for its promising properties. Various bioactive compounds derived from algae are attractive assests for screening of drugs, providing with tremendous structural variations and bioactivity.

β-carotene: *D. salina* is the best source of β-carotene. It is well known to inhibit the growth of tumors and controlling cardiovascular problems. β-carotene can also be obtained from vegetables, but it gives its best result (i.e around 1000X) from microalgae *D. salina*. Other carotenoids such as phytoene, phytofluene, lutein, and zeaxanthin have cancer preventing and anti-oxidant activities. Lutein is also reported to inhibit several chronic diseases such as cataract (PulzandGross., 2004).

Dried algal meal: The dried algal consist of 40% protein after the removal of β-caroteneand glycerol. The amino acid composition of the proteins is low in cysteine, isoleucine, methionine and tryptophan but relatively high in lysine. *D. tertiolecta*has been extensively used as a source of single cell protein. Along with proteins, a high concentration of total lipids rich in ώ-polyunsaturated fatty acids are also reported in the dried algal mass which is an excellent source of anti-oxidants (Tafreshi and Shariati 2009). Apart from the bioactive compounds which are either anti-cancer or possess good anti-oxidant activity, other valuable compounds such as the enzymes and vitamins are also isolated from *D. salina*. Dihydroxyacetonereductase enzyme is commercially sold which have been isolated from *D. salina* (Ben-Amotz 1980). Other species of genus *Dunaliella* such as *D. tertiolecta* has shown various bioactive properties such as analgesic, antihypertensive, angioedema, bronchodilator, polysynaptic block and muscle relaxant activities (de Morais et al. 2015). *D. primolecta* has effectively shown inhibition against *Staphylococcusaureus*, *Bacillus cereus*, *Bacillus subtilis* and *Enterobacter acrogens*. A study concluded that the 15 different volatile compounds and the fatty acids are responsible for the anti-microbial effect observed in the case of *D. salina*(Herrero*et al*., 2006).

2.7 Immunomodulation

Immunomodulation refers to the process in which immune response is altered (involving induction, amplification, expression or inhibition) to the desired level. Immunomodulators may be categorized as immune suppressants and immunostimulants based on their effects on the

modulation of immune system. Polysaccharides have been reported to act as potential immunomodulators and various microalgae are explored for these biopolymers (de Jesus Raposo*et al.* 2015). Soluble polysaccharides (sPS) isolated from *Chlorella stigmatophora*have shown immunosuppressive effect on macrophage cells, whereas the immunostimulant effect was observed for the exopolysaccharides isolated from *Phaeodactylumtricornutum*(Guzman *et al*. 2003). Although, many microalgae have confirmed for their bioactive extracellular polysaccharide secretions, still, no algal polysaccharide is commercially exploited.

Chapter III

Objectives

Objectives

- Isolation of exopolysaccharides (EPS) from Dunaliellasalina grown under stress condition
- Assessment of effect of isolated EPS on growth of cancer cell lines and peripheral blood mononuclear cells
- Characterization of the exopolysaccharide by Fourier Transform Infrared Spectroscopy, Nuclear Magnetic Resonance spectroscopy and Liquid Chromatography- Mass spectrometry.

Chapter IV

Materials &Methodology

4.1Materials

Table4.1: List of reagents

Reagents/ Chemical	Company
ABTS (2,2'-Azinobis[3-ethylbenzothiazoline-6-sulfonicacid]-diammonium salt) substrate	ThermoFisherScientific, USA
Amphotericin B	Sigma Aldrich, USA
Bovine serum albumin	Sigma Aldrich
Concanavalin A	Sigma Aldrich
Dimethyl Sulphoxide	Merck, Germany
Foetal bovine Serum	Gibco®Life Technologies, USA
Glutamine	Himedium, India
HEPES buffer	Sigma Aldrich
Histopaque® -1077	Sigma Aldrich
Human IFN-γ Mini ABTS ELISA Development Kit	PeproTech, USA
MTT(3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide)	Sigma Aldrich
Penicillin Sodium	Himedium
Potassium Chloride (KCl)	Himedium
Potassium phosphate monobasic (KH2PO4)	Himedium
Rosewell Park Memorial Institute (RPMI)-1640 medium	Sigma Aldrich
Sodium Bicarbonate (NaHCO3)	Himedium
Sodium Chloride (NaCl)	Himedium

Sodium phosphate dibasic (Na2HPO4)	Himedium
Streptomycin	Sigma Aldrich
Trypan blue	Himedium
Tween 20	Sigma Aldrich

4.2 Methodology

4.2.1 Procurement of algae

The culture of microalgae *Dunaliellasalina*(*D.salina*) was procured from Birla Institute of Scientific Research, Jaipur, India.

4.2 .2 Culture of microalgae

Prepared medium: The procured medium was established using AS100 medium which was composed with different salts as mentioned in Table (Starr 1987). With the specific defined molarities, salts were added; the final volume was made with distilled water and was autoclaved for sterlization at 20 psi, 121°C temperature with the holding time of 15 min.

KH_2PO_4 and $FeCl_3$ were autoclaved separately to avoid precipitation and filter sterilised $NaHCO_3$ was added in the medium. pH was finally adjusted to 7.5. 10 % of the medium with the cell density of $3\text{-}5\times10^6$ cells/mL was used as the volume for sub-culturing for the production of metabolites.

The culture was grown in 2000 ml Erlenmeyer flask containing 1000 ml medium. AS 100 medium was used and the composition of medium is mentioned in table 4.2.The initial 1000 ml of the culture served as the mother culture. The working culture volume was kept 500 ml. The culture was grown in controlled laboratory conditions i.e., 25 ± 2°C temperature and 12/12 h light and dark period for 20 days. The cultures were constantly shaken. Sub culturing was done after every 20 days (Mishra &Jha., 2009).

4.2.3Stress conditions

D. salina is known to survive in wide range of salt and nutrient concentrations although that puts the organism into stress(Mishra &Jha 2009). This leads to either increase or decrease in the production of the metabolites (β-carotene, glycerol and EPS). To increase the production of exopolysaccharides, nutrient (KNO_3)and salt stress (NaCl)) were used. There were three stress condition were used which are NaCl = 3 M& KNO_3 = 4.9mM (normal condition of KNO3), NaCl = 3 M & KNO_3 = 0, NaCl = 3 M & KNO_3 = 14.7 mM).

Table4.2 Medium composition

Salts	**Concentration(molarity)**
NaCl	1.7 M
$MgCl2._6H_2O$	7.3 mM
$MgSO_4._7H2O$	2.0 mM
KCl	2.6 mM
$CaCl_2$	2.3 mM
KNO_3	4.9 mM
Tris- buffer	4.9 mM
EDTA-di-Na salt	8.5 µM
$ZnCl_2$	0.58 µM
H_3BO_3	19 µM
$CoCl_2$	0.12 µM
$CuCl_2$	0.46 µM
$MnCl_2$	6.3 µM
$FeCl_3$	9.2 µM
KH_2PO_4	1.4 mM

4.2.5. Measurements of growth

4.2.5.1 Cell count

The growth of cell culture was measured in the terms of cell number which was counted every three days for 20 days using nauberg haemocytometer. As the cells of *D. salina*are motile in nature, the lugol's iodine solution was used to fix the cells for counting (Auinger*et al.* 2008).

Cell count (mL^{-1}) = [(Number of cells counted in the four squares / 4) X 10^4 X Dilution factor]

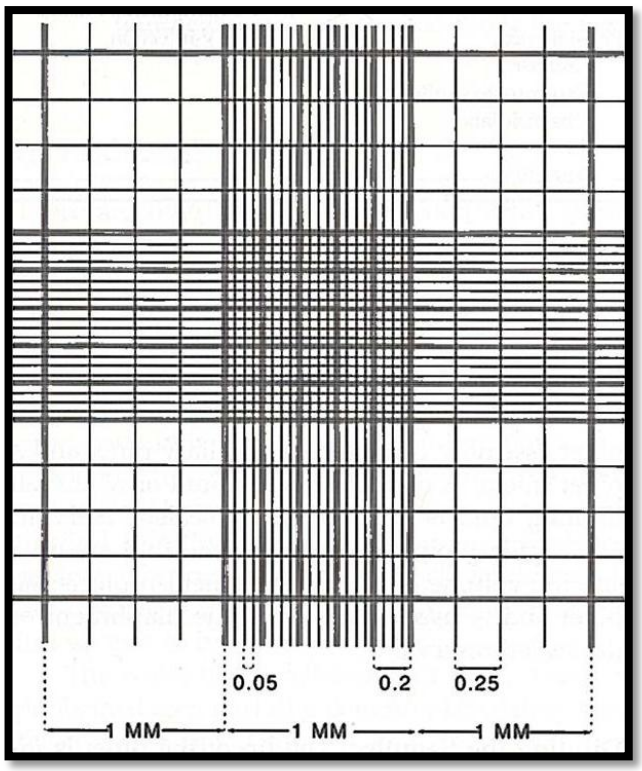


Fig4.1- schematic view of haemocytometer

4.2.5.2 Specific growth rate

Specific growth rate was measured by absorbance at 560 nm after every 2-3 days by spectrophotometer. The specific growth μ (max) was calculated by the standard understated equation(García-González *et al.* 2005).

μ (max) = 3.3(log N – Log No) / t

Where,

μ (max) = specific growth

N = Final reading at 560 nm

No = Initial spectrophotometer reading at 560 nm

t = Time

4.3 Pigment estimation

4.3.1 Estimation of Carotenoid content

D.salina cells in their log phase were taken in micro-centrifuge tubes and centrifuged at 8000 rpm/ 15 min. The pellet obtained after discarding the supernatant was mixed with 80% acetone diluted with autoclaved distilled water. Then, the mixture was vortexed for 40-50 sec and the tubes were kept in dark for 24 h. After 24 h tubes were taken out and centrifuged again at 3000 rpm/15 min. The supernatant was taken and absorbance was measured at 450 nm by spectrophotometer. Carotenoid content was evaluated by davis formula and extinction coefficient (2500).

Carotenoid (mg/ml) = (O.D. 450 * Volume of sample taken)/2500

4.3.2 Estimation of Chlorophyll content

Chlorophyll content in the algal cells were also estimated in 80% acetone using same protocol as mentioned for carotenoid content and analyzed via spectrophotometer by measuring absorbance at 645 & 661.5 nm. Chlorophyll a, b and total chlorophyll concentration was measured by using equations (Singh et al. 2014).

Chl a (mg/mL) = 11.24*O.D661.5-2.04*O.D645.0

Chl b (mg/mL) = 20.13*O.D645-4.19*O.D661.5

Total chlorophyll content (mg/mL) = Chl a + Chl b

= 7.05* O.D 661.5 + 18.09 * O.D 645.0

4.3.3 Estimation of dry weight of cells

To determine the total dry weight of algal cells, the cells were collected after centrifugation in fresh pre-weighed tube after washing it twice with the distilled water to remove extra chemicals, salts and debris) and were put in the oven for drying at 80^{o}C until the constant weight was obtained.

4.4 Extraction of Exopolysaccharides from *Dunliellasalina*

4.4.1 Extraction

After 20 days of growth, the algal culture was taken and centrifuged at 10000 rpm/30 min to remove separate the debris and cells. The supernatant was collected to a fresh tube and filtered with whattman filter paper and heated on hot plate at 50-80^{o}C to reach 1/6th of the original volume. Equal volume of cold methanol was used for organic precipitation of EPS and stored for 16-18 hrs in 4^{o}C. Centrifugation was done again at 10000 rpm/10 min to remove methanol and pellet and was dissolved in MQ (Milli Q) water after washing thrice with ethanol. The dissolved pellets were heated to dissolve clumps and filtered (Mishra et al., 2011).

4.4.2 Dialysis

The dissolved pellets were dialyzed to remove the salts and chemicals against double distilled water for 48 h. Water were changed thrice a day. The dialysis membrane were activated beforehand (marusyk&sergeant, 1980).

Dialysis membrane activation and tubing preparation: The dialysis membrane of 12 kDa was taken and tubing of 7-8 cm was cut. In 2% sodium bicarbonate solution tubings were immersed and boiled for activation for 10 min. The solution was discarded and tubes were boiled on distilled water. The activated membranes were stored in 50% ethanol at 4ºC.

Fig4.2. Dialysis on magnetic stirrer without hotplate.

4.4.3 TCA (Trichloro- acetic acid) precipitation for proteins

The dialysed liquid was subjected to TCA precipitation to remove the proteins present in the sample (Link &LaBaer., 2011). From the stock of 100% TCA, the working concentration of 20% TCA was freshly prepared. TCA was added to liquid and incubated for 1-2 h and centrifuged at 14000 rpm/10 min. The proteins containing pellet was discarded.

4.4.4 Lyophilisation

The TCA precipitated samples was lyophilised. For lyophilisation samples were frozen to -80ºC beforehand. In lyophiliser, vacuum was created by vacuum pump for evaporation of the liquid to obtain the powdered sample (Coppa et al. 2001).

4.5 Analysis and Estimation ofbioactive compounds (Biomolecules) in extracellular polymeric substances

4.5.1 Estimation of glucose in exopolysaccharides content

The polysaccharides content in extracted extracellular polymeric substances was estimated by phenol sulphuric acid assay (Masuko et al, 2005). The principal of this assay is that sulphuric acid hydrolyses polysaccharides in monosaccharide units thus their reducing ends are exposed. The hydrolysed monosaccharide units react with phenols and develops yellow golden colour.

Standard preparation: Glucose was taken as standard and prepared in the range of 10-100 µg/mL in autoclaved distilled water.

Sample preparation: 250 µg/mL of the powdered sample was prepared in autoclaved distilled water.

Estimation of glucose content on microtitre plate: The glucose standards and prepared samples were added in 96 well microtitreplates and volume was made upto 230µL. Following the steps 150 µL of sulphuric acid was added and 30 µL of 5% of phenol (prepared in distilled water) was added in each well. The plate was incubated at 70ºC/15 min in water bath then cooled to room temperature. The absorbance was taken at 492 nm. The amount of glucose was calculated by the obtained curve equation.

4.6 Cell Proliferation assay

The effects of extracted sample from *D.salina* were evaluated on peripheral blood mononuclear cells **(PBMC)**, mouse macrophage (**RAW 264.7) &** breast cancer **MCF-7 cell lines.**

4.6.1 Preparation for experimentation

Medium preparation: RPMI 1640 is a best suited medium for the suspension cultures as it containshigh amount of glutathione and vitamin (vitamin B12, biotin, choline and inositol). For thepresent work, RPMI 1640 medium was used for the culturing of PBMC (peripheral bloodmononuclear cells).The medium was prepared as per the instructions by manufacturer. Briefly, 9.6 g of powder mediumwas dissolved in 900 mL of MQ water. The pH of the medium was adjusted to 4 before adding 20mL of 200 mM glutamine solution and 26.5 mL of 4% sodium bicarbonate solutionto the medium.Then, final pH of the medium was kept at 7.4 using 1M HCl and 1N NaOH. 10 mL of antibioticssolution containing 100 µg/mL streptomycin, 100 IU/mL penicillin and 2.5 µg/mL amphotericinwas also added to the medium. The final volume of medium was adjusted to 1000 mL with MQ water. Then, medium was subjected to filtration under vacuum conditions using 0.22 µm filtermembrane. Similarly, DMEM medium was prepared as per manufacturer instruction,

Preparation of phosphate buffer saline (PBS): One liter (1x) PBS was prepared by mixing 0.2gKCl, 8 g NaCl, 0.24 g of KH_2PO_4 and 1.44 g Na_2HPO_4 in 900 mL of autoclaved DW and thepH of the solution was adjusted to 7.4 using 1N HCl and 1N NaOH. Final volume was raised to1000 mL and was autoclaved.

4.6.2 Cell enumeration

Cell counting was done with the help of trypan blue exclusion assay and haemocytometer.

4.6.3 Isolation of peripheral blood mononuclear cells (PBMC)

Five ml blood of healthy donor was taken by veni puncture by the trained technicians from Lifeline blood bank, Patiala in EDTA coated tubes (BD vacutainer® Tubes). The informed conset was taken by all the donors for the experiment work and the study was approved by institutional ethical committe.

Isolation of PBMC: PBMC were isolated with the help of ficoll density gradient method(English & Andersen 1974). Blood was layered on histopaque 1577 in 1:1 ratio in a falcon. Then

centrifugation at 450 g at 28^{o} C/30 min was done in a swinging bucket rotor (Thermo Scientific BiofugeStratos). After centrifugation plasma was discarded and buffy coat containing PBMC cells was collected very carefully. Cells were washed twice with 5 mL of 1x PBS and centrifugation was done at 350 g for 10 min. The pellet was resuspended in prepared 1 mL complete RPMI 1640 medium containing 10% (v/v) FBS. MTT assay for PBMC

The effect of extracted EPS on PBMC cell proliferation was estimated by calorimetric basedMTT (3- 4, 5 dimethylthiazolyl-2-2-5, diphenyltetrazolium) assay (Borenfreund*et al.* 1988). Theprinciple behind this assay is that on the reduction of MTT, a purple colored product, formazan isproduced by the presence of an enzyme (succinate dehydrogenases), which is secreted by themetabolically viable active cells from mitochondria (Figure 4.4). The crystals produced aredissolved in dimethyl sulfoxide (DMSO). This assay accurately quantifies the changes in the rateof cell proliferation, as it gives the linear relationship between cell number and the signalproduced.

Sample preparation: EPS was prepared as a stock of 5mg/mL, out of which it was diluted andprepared with the concentration range from 100-1000 µg/mL in autoclaved DW.

Positive control preparation: Concanavalin A (ConA) with the concentration of 10 µg/mLserved as the positive control.

MTT assay in Microtitre plate: Isolated PBMC cells were seeded in 96 well microtitreplate atthe fixed density of 2×10^{5}cells/well. Then, the prepared EPS extract and ConA was added ineach well and volume was made upto 200 µL using complete RPMI 1640 (by adding 10% FBS).Only medium served as the control for the experiment. The experiments were performed intriplicates. The plate was incubated for 48 h at 37ºC provided with 5% CO2 and after theincubation, 20 µL MTT (5 mg/mL in PBS) was added in each well. Then, the plate was again incubated for 4h for the reaction to occur. 150 µLmedium was discarded from each well and100µL of DMSO was added. The final absorbance was taken at 570nm taking 620nm as thereference wavelength on ELISA plate reader (iTecan Infinite Pro ELISA reader). The cell proliferative index was calculated as the function of absorbance taken by the equationbelow,

$$\text{Proliferative Index} = \text{Abs}_{\text{sample}}/\text{Abs}_{\text{control}}$$

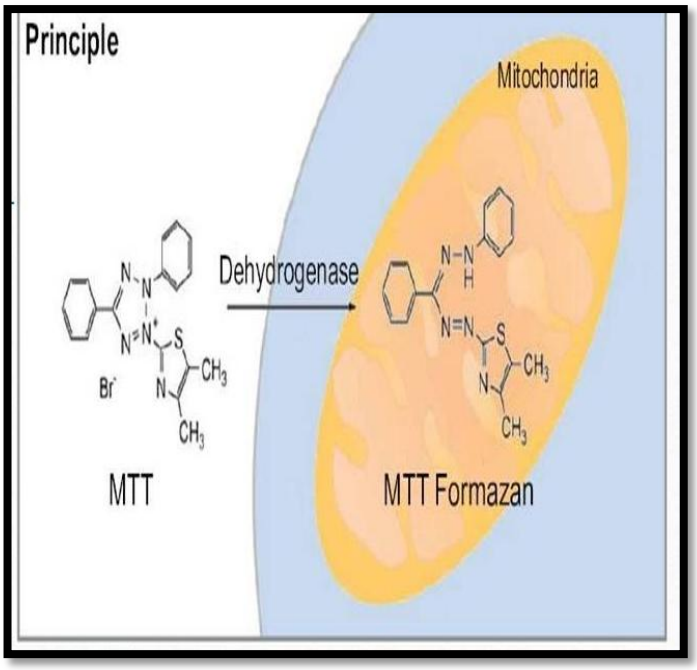


Figure4.4: Principle of MTT assay (Source-https://i.ytimg.com/vi/ExAGdK6dpF8/maxresdefault.jpg)

4.6.4 MTT assay on RAW 264.7 & MCF-7 cell lines

Prior to the test, the culture flask with 70-80% confluence was trypsinised and cells were counted. Then, the cell culture microtitre plate was seeded at the density of 10^4 cells per well. The volume was made up to 200 µL by complete medium (DMEM). The seeding was followed by overnight incubation before adding the sample. 2 µg/mLDoxorubicinwas served as positive control for RAW 264.7 cells and MCF-7. After 48h of incubation at 37ºC in CO2 Incubator with 5% CO2, 20 µL MTT was added in each well. Following the addition, the plate was again incubated for 4h. 170 µL of medium was discarded and 100 µL of DMSO was added in each well. The absorbance was recorded at 570nm taking reference wavelength at 620nm on ELISA plate reader (Tecan Infinite Pro ELISA reader). Proliferative index was calculated as mentioned in section 4.6.3

4.7 Mass Spectroscopy

Mass spectroscopy analyzes the mass of different molecules within the sample by the measurement of mass to charge ratio of ions. Molecules are vaporized, converted to ions for analyses then ions are sorted into acceleration and deflection then finally ions reaches to the detector on the basis of increasing masses and spectrum was formed.

Liquid Chromatography- Mass spectroscopy (LC-MS)

LCMS is the technique which combines the principle of separation of chemical compounds usingliquid chromatography and with their mass analysis. In this technique the compound ionizes intoions which are sorted on the basis of m/z (mass to charge) ratio (Herbert &Johnstone 2002).

Liquid chromatography

In LC, the fixed amount of sample is directly injected into the mobile phase which is deliveredthough a high pressure pump. The mobile phase containing the analytical sample (analytes)moves to the stationary phase column. The components in the compound mixture are separatedon the basis of their affinity with both the phases.

After separation though LC, the analytes moves to the MS section which measures m/z ratio of the ions (charged particles).The basic components of MS include the ion source, mass analyzer, detector and the vacuum systems (Figure 4.5). The ion source provides the components of a sample in a MS system which is ionized by the beam of electrons, photons and laser. In the case of electrospray ionization (ESI), the ion source converts the liquid analytes into gaseous phase. And detector gives the spectrum of the compound. Thus, the spectrum obtained is helpful to measure the molecular mass and structure of the compound.

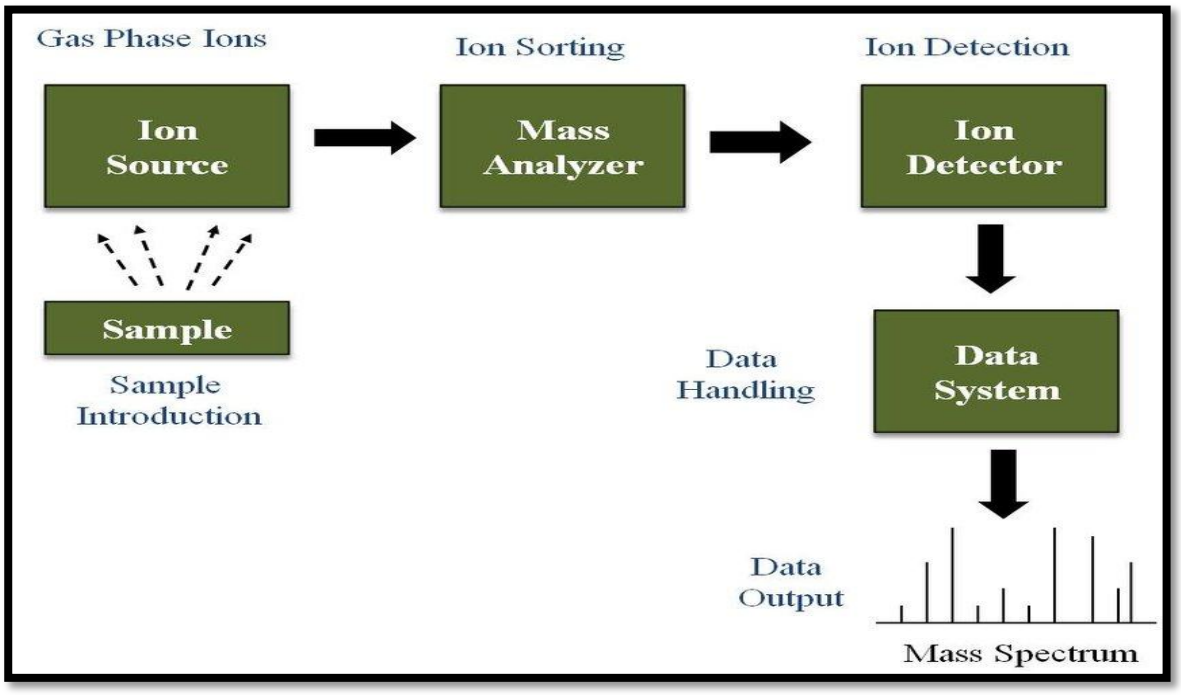


Fig4.5.Components of mass spectrophotometer (Source-https://www.researchgate.net/figure/Components-of-a-mass-spectrometer_fig2_6033979)

Mass spectroscopy was carried out in SAIF labs, Punjab University, Chandigarh. The details of the used techniques are given inthe Table 4.3 & 4.4.

Table 4.3: Details of the instrument used

Instrument	Waters, Micromass Q-TOF micro
Separation Module	Waters Alliance 2795
Ionization	Electro spray Positive (ES+)
Acquisition	MRM, unit resolution
Injection Volume	20 micro litres
Flow rate	0.4 ml/min

Table 4.4: Parameters used of performing mass spectroscopy

Desolvation Gas	550 L/h
Cone Gas	30 L/h
Desolvation Temperature	300 ºC
Source Temperature	110 ºC
Capillary Voltage	3000V
Cone Voltage	30V
Collision energy	4ev
Gasesused	N2 and Ar
Mobile Phase used	20% H2O and 80% Methanol
N2 supply pressure	6-7 bar(90-100psi)
Argon	5-6 Bar

4.8 Fourier transform infrared spectroscopy

FTIR expanded as Fourier Transform Infrared Spectroscopy is the method of passing infrared rays through the sample and the absorbance and transmittance patterns of any compound is imprinted (Nicolet & All 2001). Each molecule has its own pattern of arrangements of the chemical bonds having specific vibrational energies. These molecular bonds absorb light at particular wavelength and transmit light at different wavelength which results in the generation of spectra where peaks at particular wavelength corresponds to a particular chemical bond and the length of peak

corresponds to the abundance. The spectrum obtained is unique to every compound as no two molecules can share the exact same infrared spectrum. Thus, this technique forms very important in identification of the particular compound. Basic instrumentation flow in FTIR includes a source, interferometer, sample, detector and computer (Figure 4.6). Briefly, the infra-red light is emitted from a source which after passing through interferometer, passes through the sample and the transmittance is measured by the detector. The signals produced are input to a computer where fourier transformation is applied onto the spectra and the actual spectrum is obtained which is readable by the user.

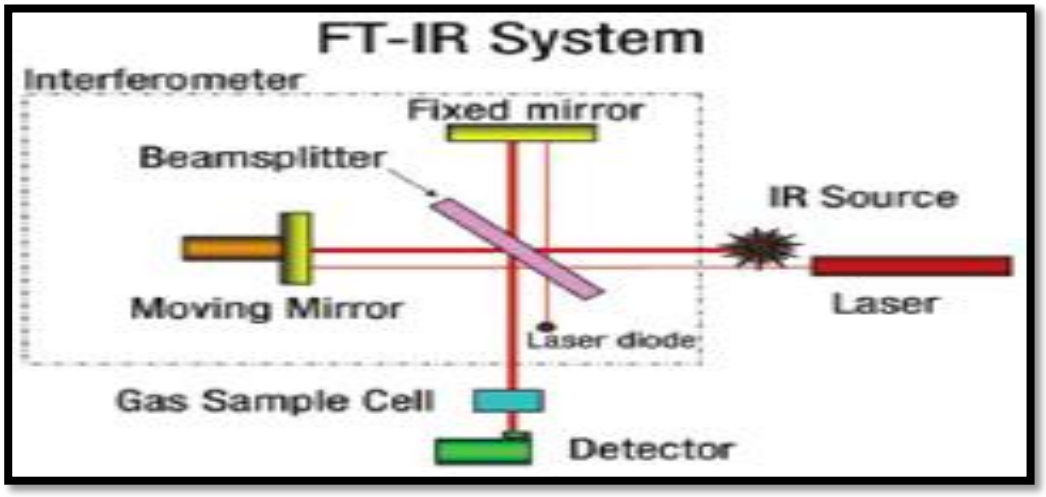


Figure 4.6: Flow of working of fourier transform infrared spectroscopy (Source-https://www.iitk.ac.in/dordold/images/facilities/021.png)

4.9 *^{1}H Nuclear Magnetic Resonance (NMR) spectroscopy*

NMR spectroscopy is a technique which is done to determine the structure of a compound. It characterizes the carbon and hydrogen bonds of an organic compound. This method along with other methods like, IR and mass spectrometry, the entire structure of a molecule can be determined.

The fact behind this method is that the spinning charged atomic nucleus generates a magnetic field. Without the influence of an applied external magnetic field (B0), the nuclear spins are arranged in random direction. But, when B0 is applied, the nuclear spins align themselves in the direction or against the direction of applied magnetic field. The working model of NMR contains radio frequency transmitter, sample tube holder, magnet, detector and the computer (Figure 4.7).

Briefly, the sample in a tube is placed in the applied magnetic field and the NMR signal is generated by excitation of the nuclear spin from radio waves into nuclear magnetic resonance,

which gets detected by the sensitive radio receivers. Thus, it leads to the change in the resonance frequency of the intramolecular magnetic field of atom and details about the functional group of a molecule and its electronic structure determined. The NMR spectrum tells about the no. of protons and positions of them (chemical shift) (HolgerFörsterling 2010).

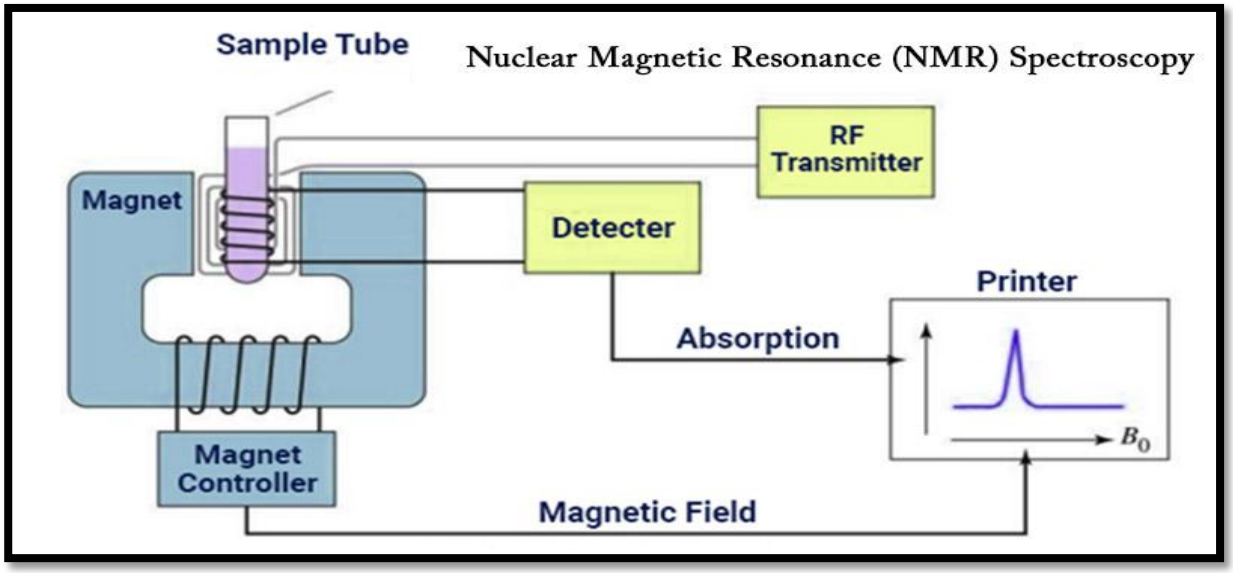


Fig:4.7Working principle of NMR (Source- https://microbenotes.com/nuclear-magnetic-resonance-nmr-spectroscopy/)

Chapter V

Results

5.1.1 Maintenance and Production of *D.salina*

The suspension cultures of *D.salina*were initially maintained in 1000ml Erlenmeyer flask containing 500 ml of medium (Fig5.1). These flasks served as the mother cultures and were incubated at 28±2°C under photoperiodism of light and dark conditions (12/12 h). Cell growth was monitored for 30 days and the mother cultures were sub cultured and further various nutritional stress conditions were incorporated in the medium with *D. salina*(2-3 x 10^5 cells/ml) as inoculum.

Fig5.1. Mother Culture of *D. salina*

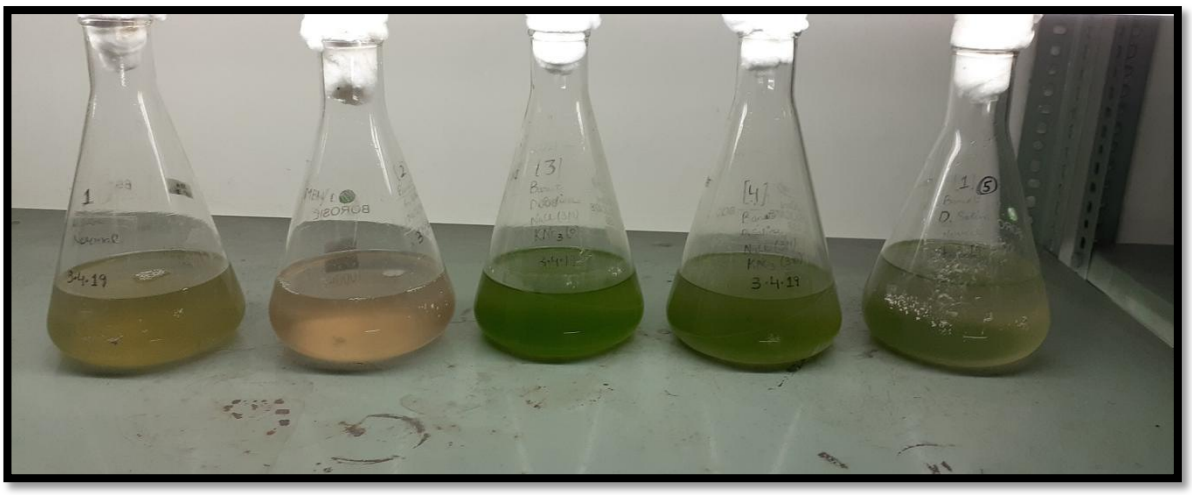


Fig5.2. Different stress conditions

5.2 Measurement of growth and productivity

5.2.1 Study of cell growth under microscope

Growth of *D.salina*was observed under Nikon Eclipse E200 microscope after every couple of days.

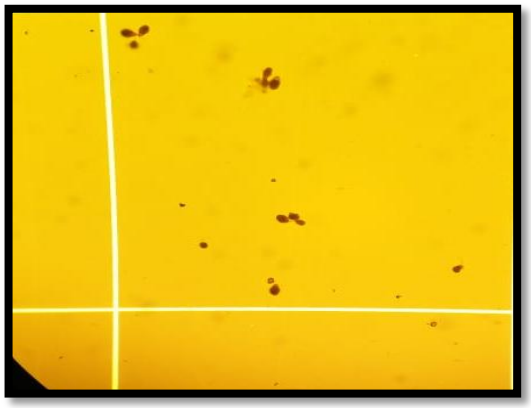


Fig5.3. Unicellular *D. salina cells* fixed in lugol's iodine solution

5.2.2 Study of growth curve

The growth curve of *D.salina*cells on AS100 medium was estimated spectrophotometrically at optical density at 680 nm. The growth curve of *D.salina*was studied for 20 days till the stationary phase was achieved (Table5.1&Fig5.4). It was observed that cells attained stationary phase approximately after 20-22 days of the inoculation.

Table5.1: Growth of *D.salina*

Days	**A680nm**(Mean)
3	0.1095
6	0.1222
9	0.1315
12	0.1425
15	0.1564
18	0.1657

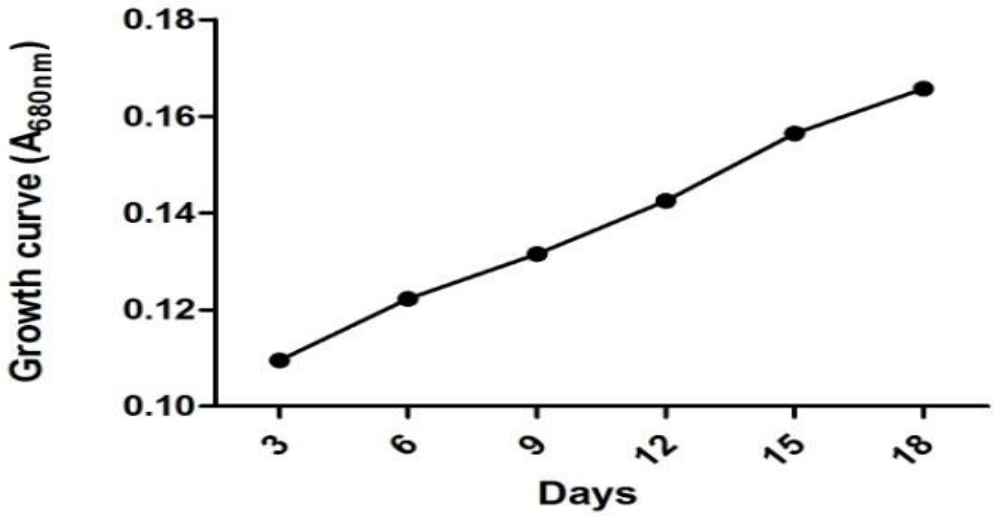


Fig5.4: Growth curve study of *D.salina*

5.2.3 Measurement of the Cell Count

Under optimum growth conditions, the cell count and density of *D.salina*was observed. The maximum cell count was seen to be at 18th day (Fig5.5&Table 5.2).

Table5.2: Cell count of *D.salina*

10^6 cells /ml(Mean)	
Days	Cells/ml
3	0.145
6	0.2633
9	0.3547
12	0.5123
15	0.688
18	0.7753

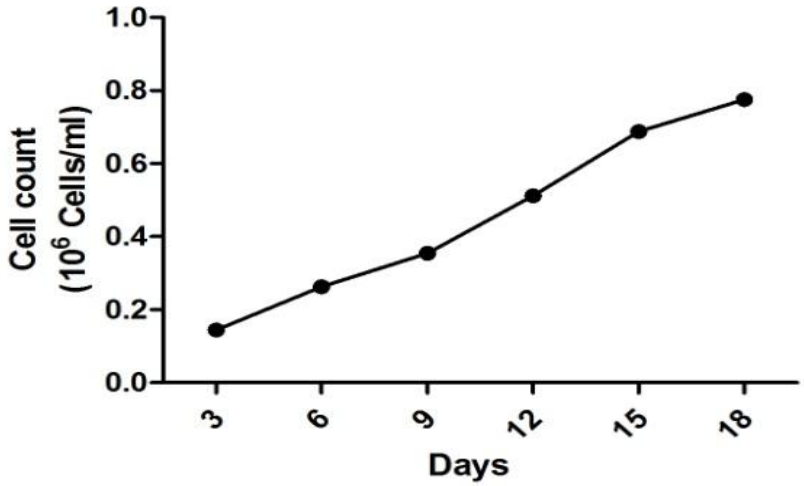


Fig5.5: Cell count study of *D.salina*

5.2.4 Measurement of dry weight of *D.salina*cells

The dry weight of the algal cells was taken and the weight of biomass accumulation was recorded (Table 5.3)

Table5.3: Dry weight biomass of *D.salina*

	Dry biomass(mg/ml)(Mean± SD)
Total Dry weight	0.92±0.03

5.2.5 Pigment Estimation

Chlorophyll and carotenoid content of *D.salina*were estimated. The carotenoid content was found to be less than the chlorophyll content. The accumulated data of chlorophyll and carotenoid is shown in Table 5.4 and5.5.

Table 5.4: Total chlorophyll content accumulation in *D.salina*growing in AS100 medium

Total Chlorophyll Content (mg/ml) (Mean± SD)	
Chl a content	1.33±0.008
Chl b content	2.096±0.023
Total Chl content	3.42±0.032

Table 5.5: Total carotenoid content accumulation in *D.salina*growing in AS100 medium

Total Carotenoid Content (mg/ml) (Mean± SD)	
Total carotenoid content	0.0685±0.00651

5.3 Extraction of exopolysaccharides from*D.salina*

After the optimization of growth and cultivation conditions, the culture of *D.salina*was found to be suitable for extraction on 20th day and the exopolysaccharides**(EPS)** were extracted by alcoholic precipitation and protein was removed by TCA. The obtained liquid was processed and lyophilised to get the powdered form of extracellular polysaccharides.

5.4 Biomolecules content in extracellular polymeric substances

The extracted sample was analyzed for the presence of sugar using phenol-sulphuric acid method in which glucose was taken as the standard. The sugar was estimated quantitatively by the equation obtained from the standard graph (Fig 5.6).The obtained results showed the presence of sugar in the EPS (Table5.6).

Table5.6: Glucose Standard Curve

Concentration	Absorbance A_{492}
10	0.1503
20	0.2446
40	0.2999
60	0.4421
100	0.6995

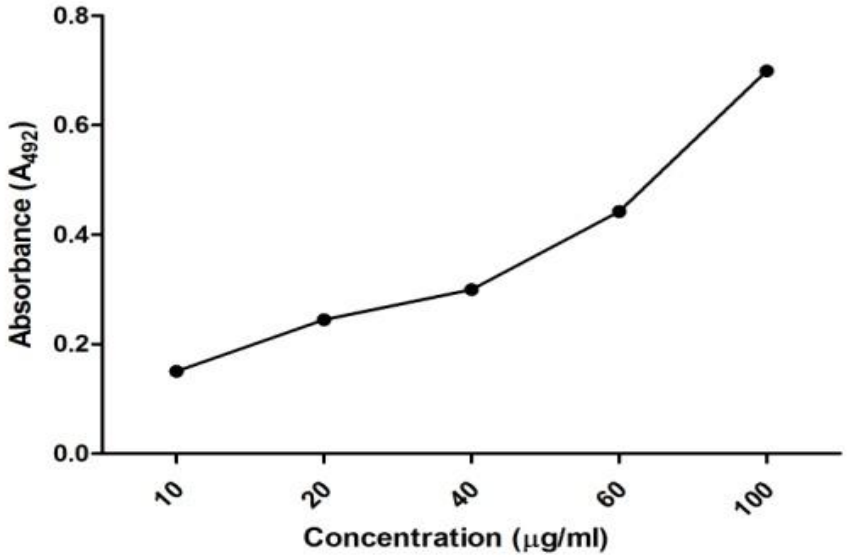


Fig5.6: Glucose Standard Curve

Table5.7: Presence of Glucose equivalents.

Aborbance A_{492nm}	Glucose equivalents(μg) /EPS (mg)
0.313	2.851163±0.04035

5.5 Modification of Culture Conditions of AS 100 medium for the effect on growth of *D.salina*

To enhance the yield of the EPS, different nutritional stress were introduced to study the effect of stress on cell growth and EPS yield using AS 100 medium (Fig 5.7& Table5.8).

The effect of stress condition was studied where stress of NaCl and KNO_3 was given to AS-100 medium. Cell growth rate was found to be less in different stress conditions as compared to normal conditions (Fig 5.7).

Table 5.8: Comparative study of Growth curve in different stress conditions of EPS extracted from *D.salina*grown in AS100 Medium.

Days	Sample 1 Mean±SD	Sample 2 Mean±SD	Sample 3 Mean±SD	Sample 4 Mean±SD
3	0.145±0.003	0.1433±0.0351	0.1567±0.0351	0.1847±0.0035
6	0.2633±0.0404	0.2643±0.0040	0.2567±0.0416	0.1997±0.0050
9	0.3547±0.0045	0.336±0.0026	0.51±0.00300	0.3523±0.0035
12	0.5123±0.0049	0.3653±0.0045	0.6713±0.0042	0.5553±0.0040
15	0.688±0.0046	0.4527±0.0050	0.7727±0.0060	0.8847±0.0035
18	0.7753±0.0035	0.5827±0.0050	0.851±0.0046	0.9163±0.0040

*Sample1- Normal, Sample2- 3M NaCl, Sample3- 3M NaCl& Without KNO_3, Sample4- 3M NaCl& With KNO_3.

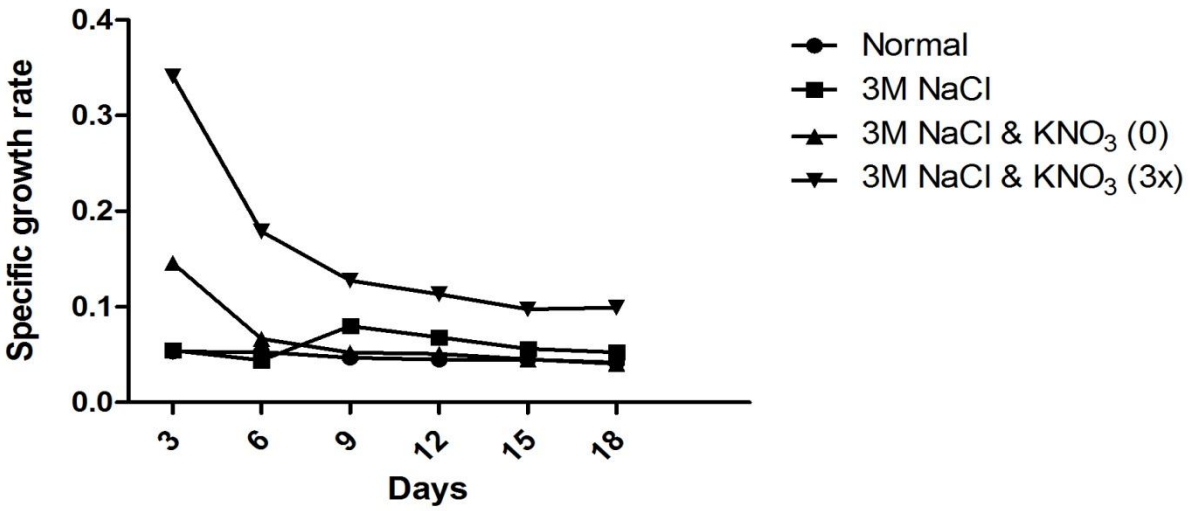


Fig5.7: Growth curve in different stress conditions

KNO_3 (3X): It represents three times nitrate from normal condition which is 14.7mM

5.6 Cell count study

The cell count study was measured and the comparative results were compiled in Fig5.8 and Table4.9. After 12 days, there is clear decrease in cell count of all stress conditions as compared to normal condition (Fig 5.8).

Table4.9: Comparative study of Cell count in different stress conditions of EPS extracted from *D.salina*grown in AS100 Medium.

Days	Sample 1	Sample 2	Sample 3	Sample 4
3	0.1095	0.1076	0.1343	0.2083
6	0.1222	0.1155	0.1307	0.2156
9	0.1315	0.1584	0.1374	0.2271
12	0.1425	0.1697	0.1513	0.2627
15	0.1564	0.1726	0.1585	0.2827
18	0.1657	0.1855	0.1647	0.3545

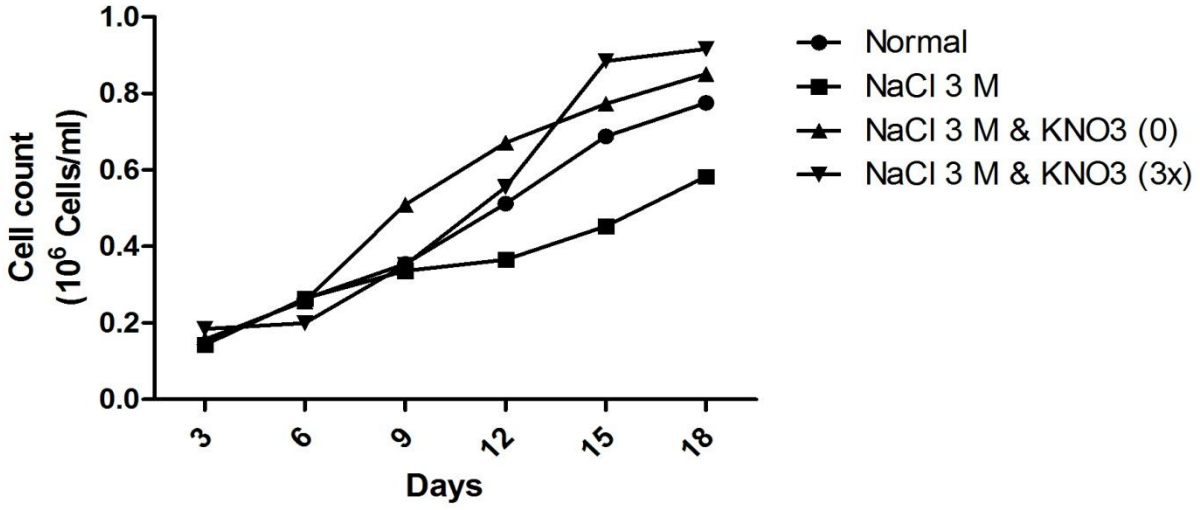


Fig5.8: Cell Count in different stress conditions in AS-100 medium.

5.7 Pigment Estimation

Chlorophyll and carotenoid content of *D.salina*with different stress conditions were estimated (Table 5.10 and 5.11) & (Fig 5.9 and 5.10). Chlorophyll and carotenoid content was found be increased in two stress condition i.e. NaCl (3M) & KNO_3 (0) and NaCl (3M) & KNO_3 (14.7 mM) as compared to normal conditions (Fig 5.9 and 5.10).

Table5.10: Total chlorophyll content accumulation in *D.salina*with different stress conditions growing in AS100 medium.

Total Chlorophyll Content (mg/ml) (Mean± SD)			
Samples	Chl a content	Chl b content	Total Chl content
Sample 1	1.336±0.008	2.0778±0.023	3.414±0.0317
Sample 2	1.247±0.0134	2.0702±0.0255	3.317±0.039
Sample 3	2.329±0.446	2.7016±0.212	5.031±0.659
Sample 4	1.6567±0.098	2.2953±0.113	3.952±0.211

Table 5.11: Total carotenoid content accumulation in *D.salina*with different stress conditions growing in AS100 medium.

Total Carotenoid Content (mg/ml))	
Samples	Carotenoid Content
Sample 1	0.0750
Sample 2	0.0659
Sample 3	0.1142
Sample 4	0.0842

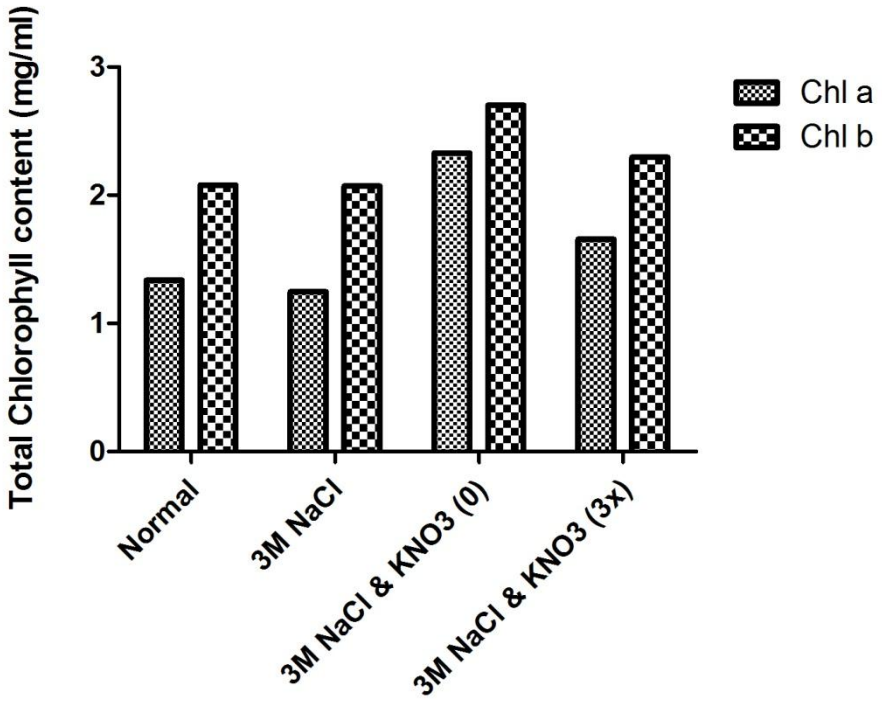


Fig5.9.Total Chlorophyll Content in different stress conditions of *D.salina*grown in AS100 medium.

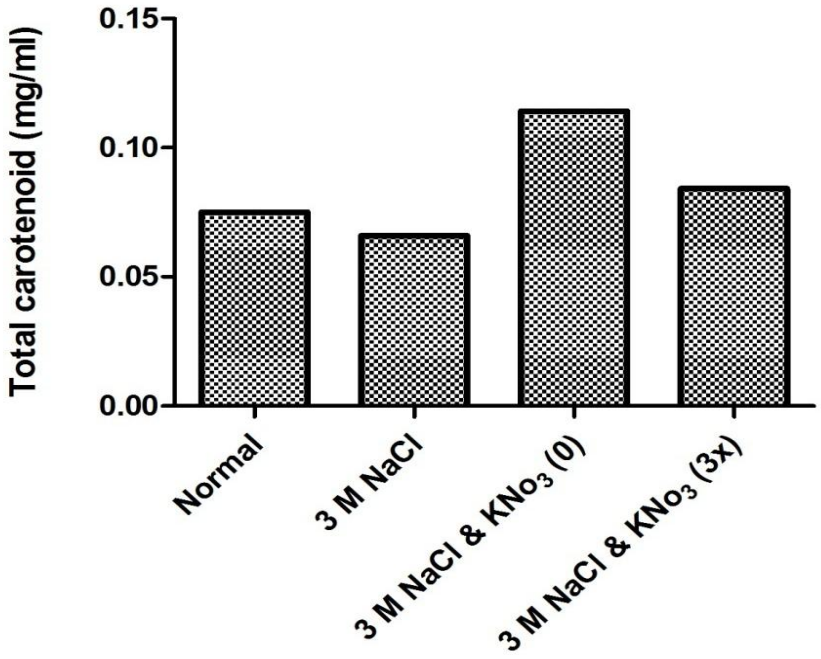


Fig5.10.Total Carotenoid Content in different stress conditions of *D.salina*grown in AS100 medium.

5.8 Biomolecules content in extracellular polymeric substances extracted from stress conditions of *D.salina*

Table5.12: Glucose estimation in samples of *D. salina in* different stress conditions.

Samples	Aborbance A_{492nm}	Glucose equivalents(µg)/EPS(mg)
Sample 1	0.31395±0.04035	2.8512
Sample 2	0.3468±0.0169	2.8512
Sample 3	0.4007±0.01825	3.2694
Sample 4	0.1744±0.0112	1.5147

5.9 Measurement of dry weight of *D.salina*extracted from cultures with different stress conditions

The dry weight of the algal cells was taken and the weight of biomass accumulation was recorded(Table5.13 &Fig 5.11). In stress condition NaCl (3M) & KNO_3 (0), the dry mass was higher than other two stress condition and normal condition (Fig 5.11).

Table5.13: Dry weight biomass of *D.salina*on AS100 medium

	Dry biomass(mg/ml)(Mean± SD)
Sample 1	0.9200±0.030
Sample 2	0.7233±0.045
Sample 3	1.2167±0.067
Sample 4	0.8167±0.040

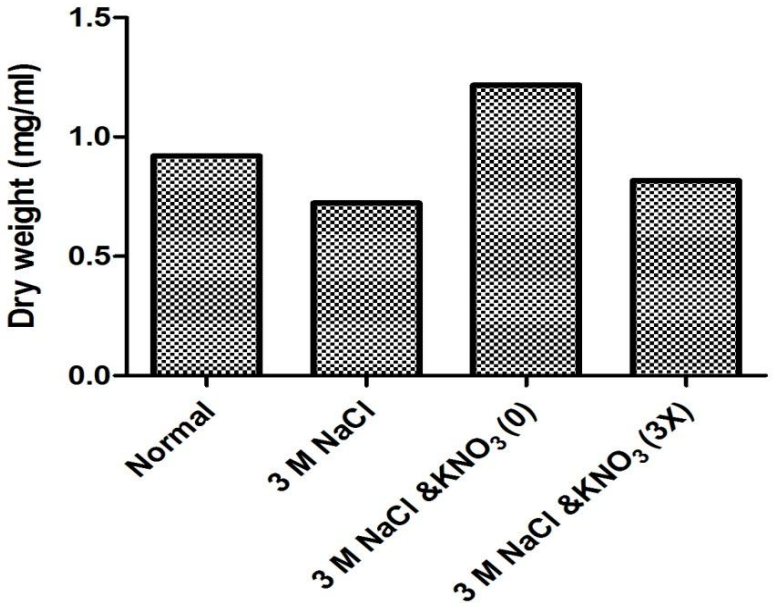


Fig5.11 Comparative study of dry weight of cultures

5.10 Effect of EPS on peripheral blood mononuclear cells (PBMC)

Isolated EPS was assessed on PBMC using MTT assay). The EPS isolated from some of the stress condition of potassium nitrate and potassium dihydrogen phosphate was included from previous study for the comparative purpose. EPS isolated EPS isolated under normal and stress conditions have shown proliferative index more than one indicating enhanced cell proliferation.It was found that the proliferation was increased by providing nutritional stress conditions i.e. Sodium chloride salt(3M &3.5 M), Potassium nitrate (3X & 5X) and Potassium dihydrogen phosphate (3X & 5XEPS with 3X& 5XKH_2PO_4 stress condition shows highest proliferation as compared with other stress conditions (Fig5.12, 5.13, 5.14).

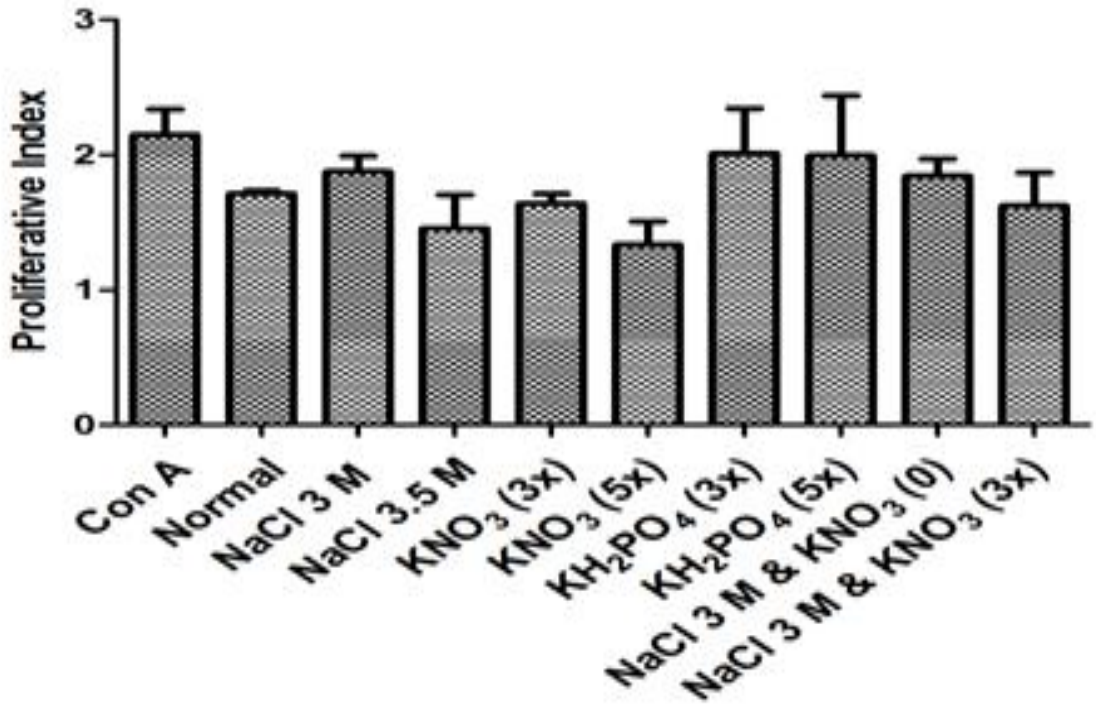


Fig5.12.Cell proliferation on PBMC by obtained EPS from different stress conditions Concanavalin A (10µg/mL) was used as positive control. Proliferation index is the absorbtion of ratio of absorbance of the Con A/ Cells with EPS & without EPS.

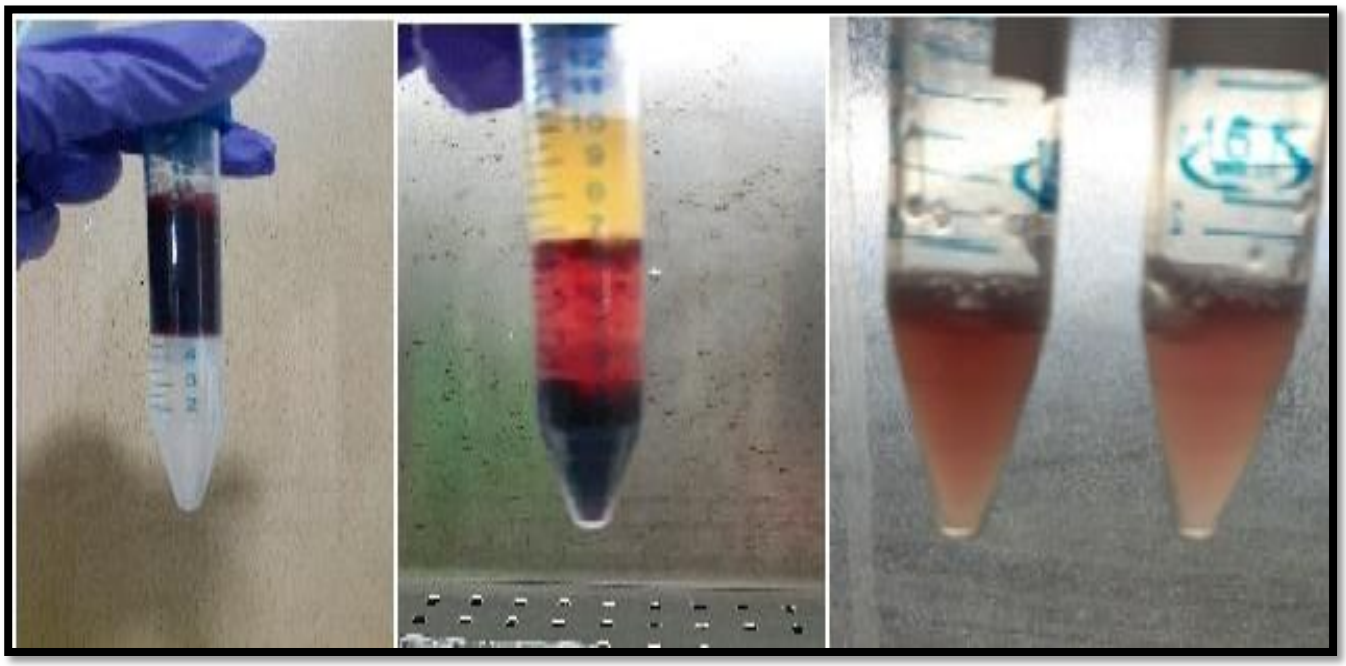


Fig5.13. PBMC isolation

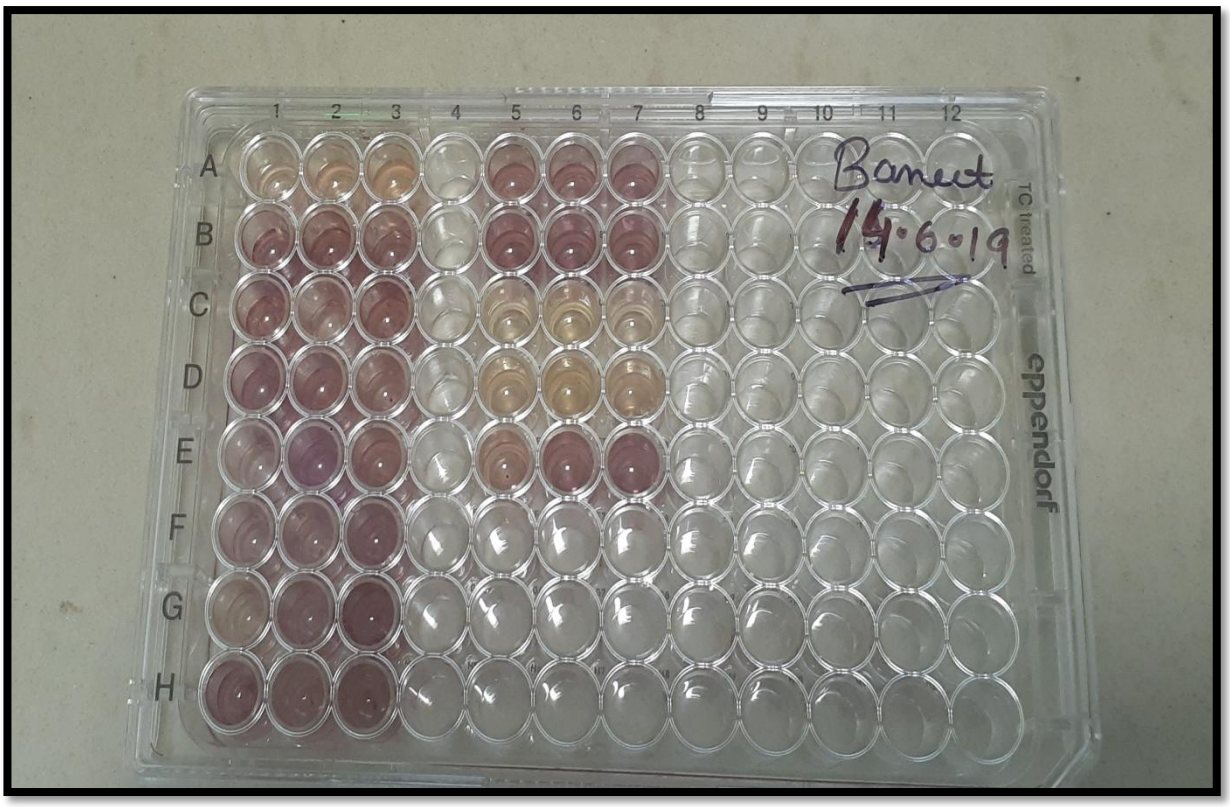


Fig5.14. 96 well ELISA plate with MTT assay.

5.11 Effect of EPS on RAW 264.7Cells &MCF-7 (Michigan Cancer Foundation-7) Cells.

The extracted EPS was assessed for their effect on RAW 264.7 and MCF-7 cells and it was observed that cell growth inhibition by EPS isolated under normal and stress conditions. Isolated EPS from *D.salina*with different stress conditions i.e. NaCl 3.5 M, KNO_3 (3X), KNO_3 (5X), KH_2PO_4 (5X) showed highest inhibition as compared to normal on RAW 264.7 cells(Fig5.15). However KH_2PO_4(3X) showed highest inhibition as compared to normal on MCF-7 cells (Fig5.16).

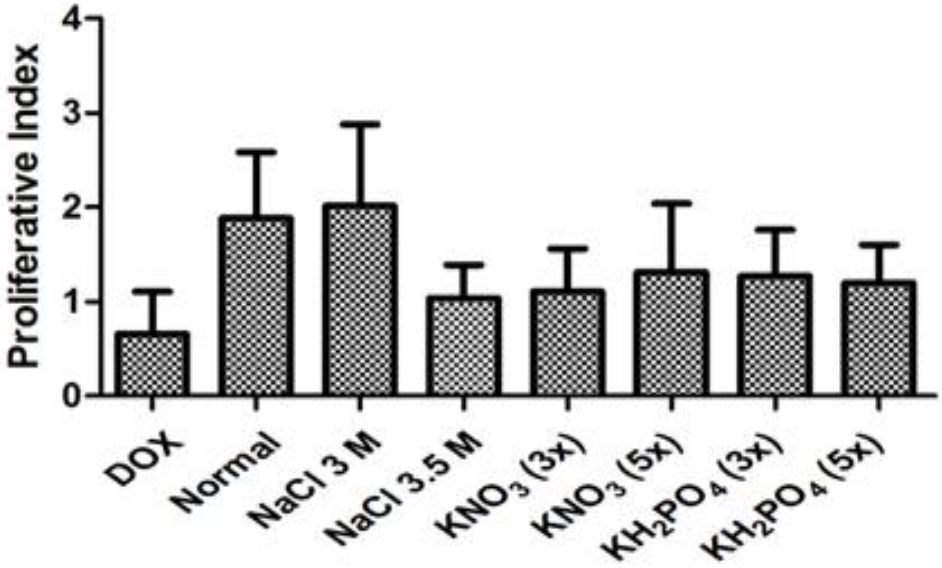


Fig5.15.Cell growth inhibition by EPS with different stress conditions on RAW 264.7 cells.DOX was used as a positive control.

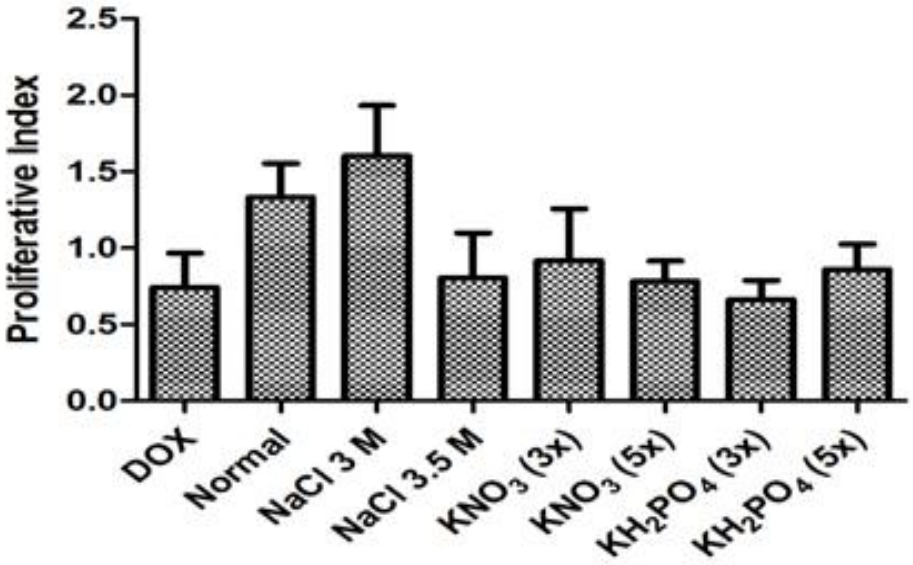


Fig5.16.The cell growth inhibition by EPS with different stress conditions on MCF-7 cells. DOX (Doxorubicin) was used as a positive control.

5.12 Characterization of isolated EPS

The final objective of the study was to characterize the exopolysaccharides isolated from *D.salina* hence in this section the results of various methods (FTIR, NMR and LC-MS) have been explained.

5.12.1. FTIR spectroscopy

The FTIR spectra of the EPS extracted from *D. salina* was carried out to identify the presence of functional groupsin the sample. The acquired spectra display peaks at specific wavelength which correspond to the particular chemical group. FTIR spectra showed the intense characteristic absorbtion of polysaccharides between 1633.8 cm^{-1} & 1749.9 cm^{-1} and O-H stretching band at 3636.6 cm^{-1}.

Another functional group corresponds, such as stretching C-H at 2967.1 and Carboxylic acid bending peak at 2603.0 cm^{-1}. Further halo stretching was also noticed at 680.9, 846.3 &1240.5 and Nitro group at 1347.8, Showed the presence of halide & nitro group along with the polysaccharides (Table 5.14&Fig5.17).

Table5.14:FTIR analysis of extracted EPS

Wavelength (cm^{-1})	**Range**	**Functional Group**	**Intensity**
3636.6	3700-3500	O-H (Alcohol)	Strong, Broad
2967.1	3000-2850	N-H, C-H (Alkanes)	Medium
2603.0	3300-2500	COOH (Carboxylic acid)	Strong, Broad
3548.3	3700-3500	O-H (Alcohol)	Strong, Broad
1749.9	1750-1730	C=O	Strong
1633.8	1648-1630	C=O (α,β unsaturated ketone)	Medium
1347.8	1350-1300	N-O(Nitro)	Strong, Medium
952.2	1000-700	C=C (Alkene,bending)	Strong
680.9	690-515	C-Br (halo compound strechting)	Strong
753.5	1000-700	C=C (Alkene,bending)	Strong
846.3	850-550	C-Cl (halo compound strechting)	Strong
1240.5	1400-1000	C-Fl (halo compound strechting)	Strong
447.7	<600	C-Br(halo compound strechting)	Strong
428.8	<600	C-I (halo compound strechting)	Strong

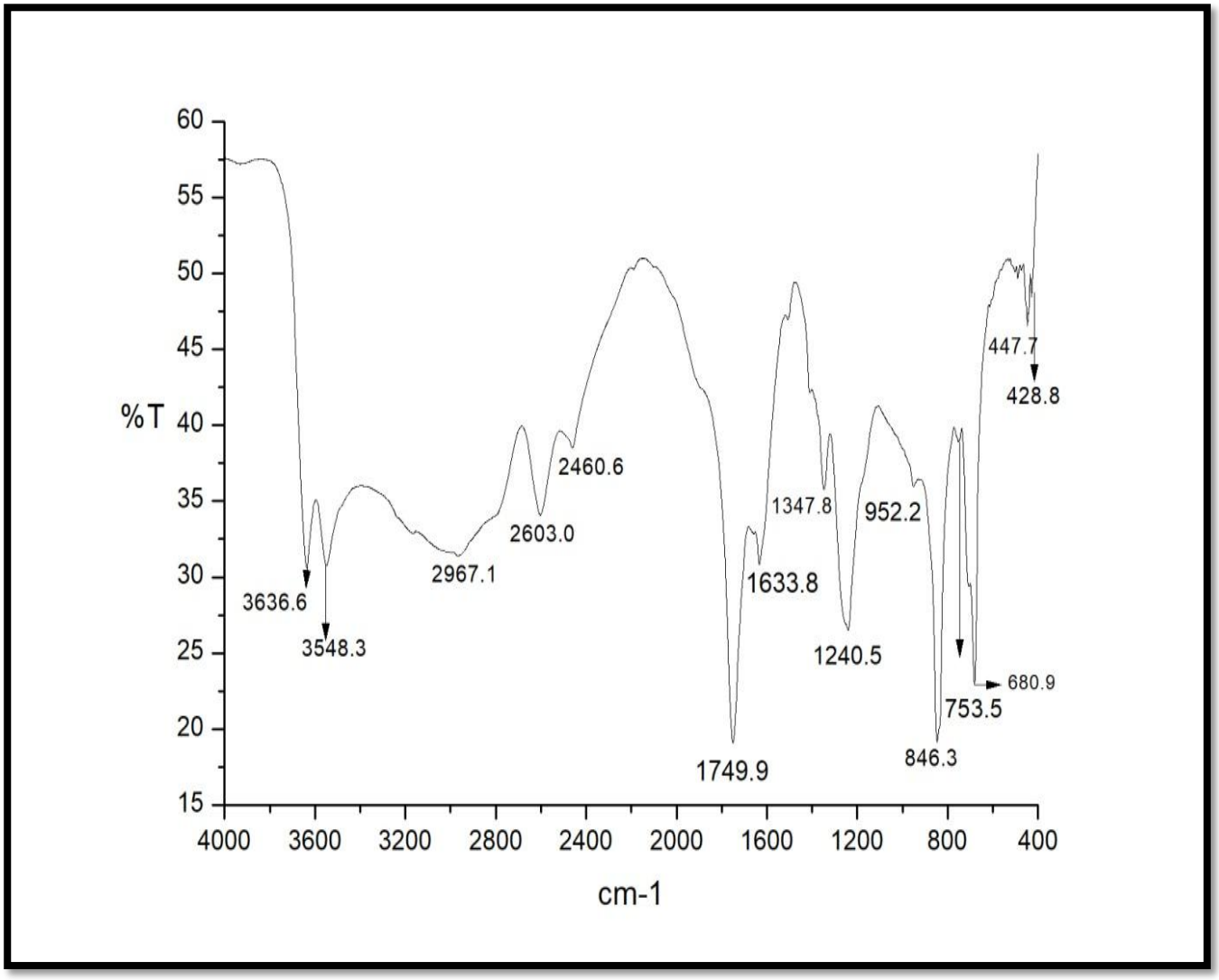


Fig 5.17: FTIR Spectrometery Results

5.12.2 Liquid chromatography-Mass spectroscopy (LC-MS)

LC-MS analyzes the data on the basis of m/z ratio that corresponds to relative intensity. The data (Table5.15 &Fig5.18) shows that the extracted EPS contains hexose sugars (206.89), Disachharides(379.21),Trisachharides(576.70,578.71,580.68),Pentasaccharides(822.55)&Octasach harides(1376.25).

Table 5.15: LCMS analysis of extracted EPS

m/z Ratio	Intensity	Probable compound
206.89	251	Hexose Sugar(Glu/Fruc/Galac/Mann) associated with magnesium
379.21	370	Disaccharides (Maltose/ Sucrose/ Lactose)
576.70	1476	Trisaccharides
578.71	1816	Trisaccharides
580.68	1411	Trisaccharides
636.66	258	Tetrasaccharides
822.55	259	Pentasaccharides
1376.25	152	Octasaccharides

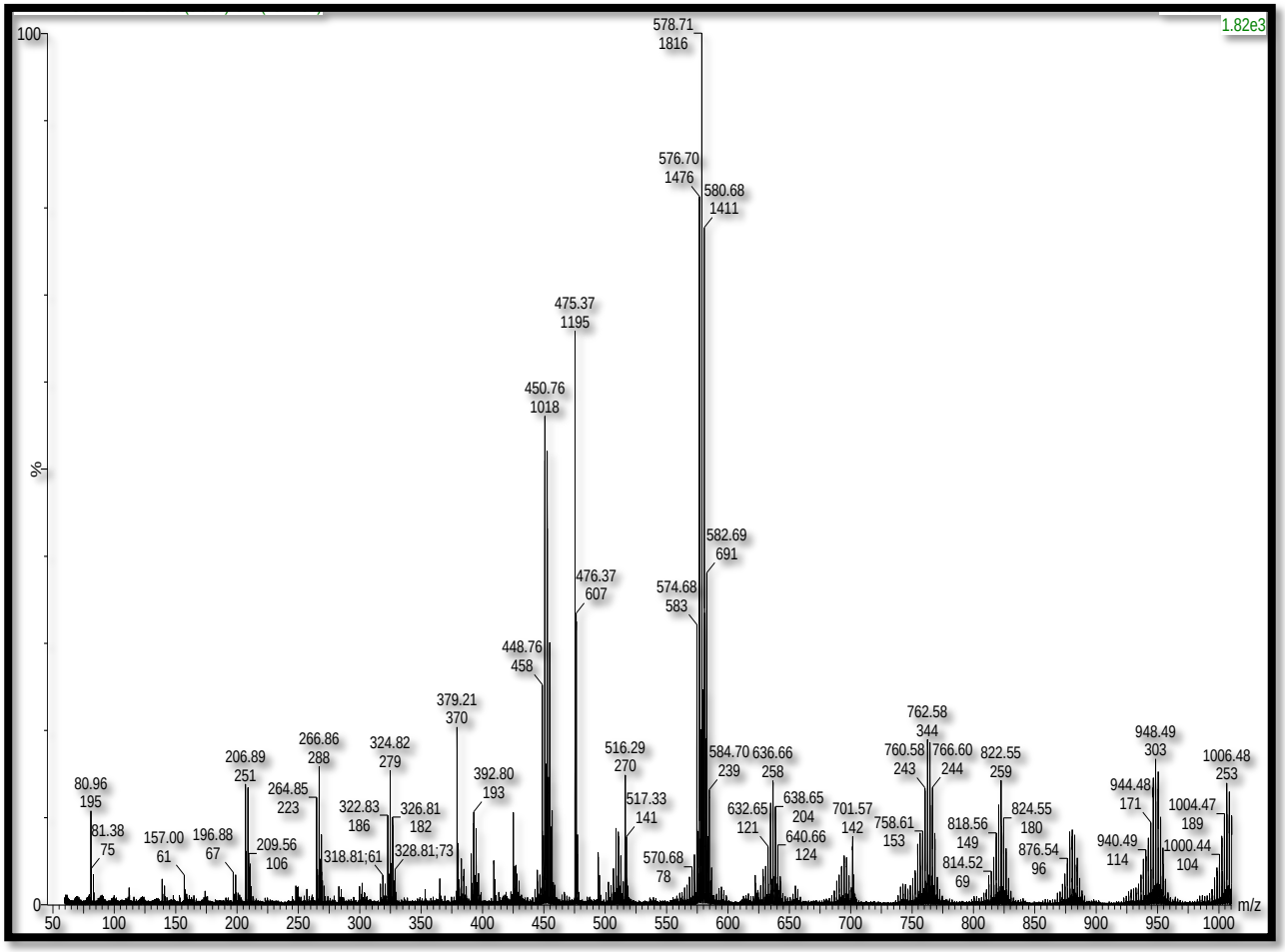


Fig 5.18: LC-MS Spectrometery Results

5.12.3.NMR

The NMR spectroscopy gives an idea about the structure (protons) of the compound. The 1H NMR spectra of the EPS isolated from *D.salina* showed the chemical shifts (ppm) to their corresponding functional groups. The chemical shift between 2.5-3.2 ppm confirmed the presence of alkyl halide and acetyl amine groups CH (NH) similarly the spectrum from 4.0 – 5.6 ppm assigned the presence of β-anomeric (4.0-4.9) and α-anomeric carbon of pentose and hexose sugars, respectively (Fig5.18).

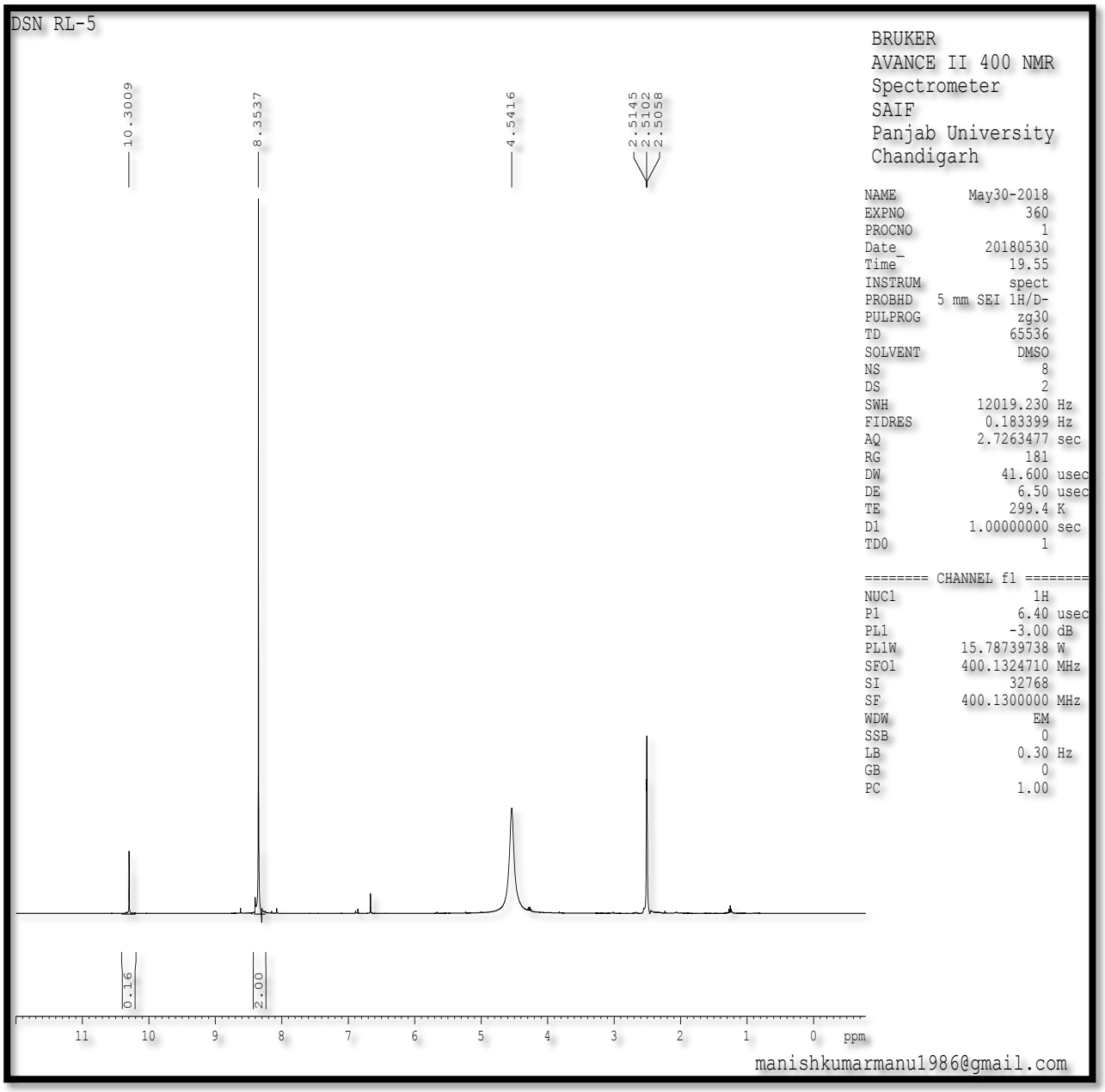


Fig 5.19 : NMR Spectrometery Results

Chapter VI

Discussion & Conclusion

Discussion

In the present study, EPS have shownthe cell growth promoting effect inPeripheral blood mononuclear cells (PBMC) while cell growth inhibition effect on RAW 264.7 & MCF-7 cell linesThis study also showed that providing nitrate, phosphate & sodium salt nutritional stress conditions influence the growth of *D.salina*, which effects the production of exopolysaccharides.

Various characterization techniques like Fourier transform infrared spectroscopy, Liquid chromatography mass spectrometry, and Nuclear magnetic resonance spectroscopy were also analyzed to detect the functional groups, molecular masses, and structure of EPS containing polysaccharides, respectively. FTIR spectra determine the functional groups present in the compound which are estimated by the correlation of the absorption spectra of the unknown compound to known compounds. The FTIR data showed peaks of O-H, N-H, C-H, N=O, C=O, C=C,C-Br groups.

LCMS gives an idea about m/z ratio, i.e., the molecular weight of the compound. Higher the molecular weight of the polysaccharides, the higher are m/z ratio. However, NMR spectra determine the type of hydrogen atom in EPS along with the ppm peaks that showed the chemical shifts associated with the likely sugars and few other groups associated with them. The LC-MS data showed presence of Monosaccharide, trisaccharides and octasaccharides with m/z ratio 206.89, 578.71 & 1376.25 respectively. The NMR spectra, which defined chemical shifts in ppm, were analyzed the type of hydrogen atom in EPS isolated from D *salina* in different stress conditions. The chemical shift obtained in the present study corresponds to beta and alpha anomeric carbon of pentose and hexose sugars along with alkyl halide, acetyl amine.

For the production of β-carotene, *Dunaliellasalina* is a very common microalga. Saha et al., 2018, tested the carotenogenic ability of *Dunaliellasalina* CCAP 19/20 strain under sixteen stress conditions and several biochemical alterations in response to specific stress. The production of carotenoid was maximum due to the removal of Cu and KNO3 from the stress growth conditions under high irradiance.*D. salina* is a valuable source of natural antioxidants (β-carotene and lutein) that have utilized in food, feed, and cosmetic and health industries.

Conclusion

In the present study one high sugar containing sample (3M NaCl without KNO_3) of EPS.In conclusion, there is increase in glucose concentration was observed in exopolysaccharides produced under some stress conditions and the cell growth promoting (PBMC) and cell growth inhibition properties (RAW 264.7 & MCF-7 cell lines)was also found to be increasein different stress conditions. Itshows the association of enhanced glucose concentration with increase bioactive of EPS understress conditions. Further, FTIR, NMR and LCMS analysis confirm the polysaccharide nature of the isolated EPS. Hence, it is suggested that these stress conditions can be used to enhance the production of EPS.

Appendix

List of Tables

Lists of figures

REFERENCES

1 Auinger, B.M., Pfandl, K. and Boenigk, J., 2008. Improved methodology for identification of protists and microalgae from plankton samples preserved in Lugol's iodine solution: combining microscopic analysis with single-cell PCR. *Appl. Environ. Microbiol.*,*74*(8), pp.2505-2510.

2 Amaro, H.M., Barros, R., Guedes, A.C., Sousa-Pinto, I. and Malcata, F.X., 2013. Microalgal compounds modulate carcinogenesis in the gastrointestinal tract. Trends in Biotechnology, 31(2), pp.92-98.

3 Abd El Baky, H.H. and El-Baroty, G.S., 2013. Healthy benefit of microalgal bioactive substances. J. Aquat. Sci, 1(1), pp.11-23.

4 Ben-Amotz, A., 1980. Glycerol production in the alga Dunaliella. *Biochemical and Photosynthetic aspects of energy production*, pp.191-208.

5 Borowitzka, M.A., 1992. Algal biotechnology products and processes—matching science and economics. *Journal of applied phycology*, *4*(3), pp.267-279.

6 Ben-Amotz, A., 1995. New mode of Dunaliella biotechnology: two-phase growth for β-carotene production. *Journal of applied phycology*, *7*(1), pp.65-68.

7 Chen, B., You, W., Huang, J., Yu, Y. and Chen, W., 2010. Isolation and antioxidant property of the extracellular polysaccharide from Rhodellareticulata. *World Journal of Microbiology and Biotechnology*, *26*(5), pp.833-840.

8 Chen, M., Tang, H., Ma, H., Holland, T.C., Ng, K.S. and Salley, S.O., 2011. Effect of nutrients on growth and lipid accumulation in the green algae *Dunaliella*tertiolecta. Bioresource technology, 102(2), pp.1649-1655.

9 Clomburg, J.M. and Gonzalez, R., 2013. Anaerobic fermentation of glycerol: a platform for renewable fuels and chemicals. Trends in biotechnology, 31(1), pp.20-28

10 de Jesus Raposo, M., de Morais, A. and de Morais, R., 2015. Marine polysaccharides from algae with potential biomedical applications. *Marine drugs*, *13*(5), pp.2967-3028.

11 Delattre, C., Pierre, G., Laroche, C. and Michaud, P., 2016. Production, extraction and characterization of microalgal and cyanobacterialexopolysaccharides. *Biotechnology advances*, *34*(7), pp.1159-1179.

12 *Dunaliellasalina* modified from Shariati and Hadi and Heakel, Hefny and El-Tawab

13 Franqueira, D., Orosa, M., Torres, E., Herrero, C. and Cid, A., 2000. Potential use of flow cytometry in toxicity studies with microalgae. *Science of the total environment*, *247*(2-3), pp.119-126

14 Fu, W., Guðmundsson, Ó.,Paglia, G., Herjólfsson, G., Andrésson, Ó.S., Palsson, B.Ø. and Brynjólfsson, S., 2013. Enhancement of carotenoid biosynthesis in the green microalga *Dunaliellasalina* with light-emitting diodes and adaptive laboratory evolution. Applied microbiology and biotechnology, 97(6), pp.2395-2403.

15 Fu, W., Nelson, D.R., Yi, Z., Xu, M., Khraiwesh, B., Jijakli, K., Chaiboonchoe, A., Alzahmi, A., Al-Khairy, D., Brynjolfsson, S. and Salehi-Ashtiani, K., 2017. Bioactive compounds from microalgae: Current development and prospects. In Studies in Natural Products Chemistry (Vol. 54, pp. 199-225). Elsevier.

16 Goyal, M., Kaur, H., Bhandari, M., Rizvanov, A.A., Khaiboullina, S.F. and Baranwal, M., 2018. Antioxidant and Immune Effects of Water Soluble Polysaccharides Isolated from Cinnamomumverum Bark. BioNanoScience, 8(3), pp.935-940

17 Heakal, F.E.T., Hefny, M.M. and El-Tawab, A.A., 2010. Electrochemical behavior of 304L stainless steel in high saline and sulphate solutions containing alga *DunaliellaSalina* and β-carotene. Journal of Alloys and Compounds, 491(1-2), pp.636-642.

18 https://www.toppr.com/guides/biology/biomolecules/polysaccharides/

19 http://pubs.sciepub.com/ajfn/5/2/2/index.html

20 https://www.algotherm.com/en/seaweed/*Dunaliella-salina*-en/

21 https://encryptedtbn0.gstatic.com/images?q=tbn:ANd9GcSCBcge2b9fiwm4DXPIHi6oA_71XQ1h3quQAZbTspuRMcq2QoW

22 https://en.wikipedia.org/wiki/Polysaccharide

23 http://supervisit.tk/noxab/rbc-count-by-hemocytometer-30-30-dyh.php

24 mdpi.com/1420-3049/19/4/4409/html

25 https://i.ytimg.com/vi/ExAGdK6dpF8/maxresdefault.jpg

26 https://www.researchgate.net/figure/Components-of-a-mass-spectrometer_fig2_6033979

27 https://www.iitk.ac.in/dordold/images/facilities/021.png

28 https://microbenotes.com/nuclear-magnetic-resonance-nmr-spectroscopy

29 Wei, S., Bian, Y., Zhao, Q., Chen, S., Mao, J., Song, C., Cheng, K., Xiao, Z., Zhang, C., Ma, W. and Zou, H., 2017. Salinity-induced palmella formation mechanism in halotolerant algae Dunaliellasalina revealed by quantitative proteomics and phosphoproteomics. *Frontiers in plant science*, *8*, p.810.

30 Marusyk, R. and Sergeant, A., 1980. A simple method for dialysis of small-volume samples. *Analytical biochemistry*, *105*(2), p.403.

31 Masuko, T., Minami, A., Iwasaki, N., Majima, T., Nishimura, S.I. and Lee, Y.C., 2005. Carbohydrate analysis by a phenol–sulfuric acid method in microplate format. *Analytical biochemistry*, *339*(1), pp.69-72.

32 Markou, G. and Nerantzis, E., 2013. Microalgae for high-value compounds and biofuels production: a review with focus on cultivation under stress conditions. Biotechnology advances, 31(8), pp.1532-1542.

33 Pulz, O. and Gross, W., 2004. Valuable products from biotechnology of microalgae. *Applied microbiology and biotechnology*, *65*(6), pp.635-648.

34 Plaza, M., Santoyo, S., Jaime, L., Reina, G.G.B., Herrero, M., Señoráns, F.J. and Ibáñez, E., 2010. Screening for bioactive compounds from algae. Journal of pharmaceutical and biomedical analysis, 51(2), pp.450-455.

35 Strober, W., 1997. Trypan blue exclusion test of cell viability. Current protocols in immunology, 21(1), pp.A-3B

36 Shariati, M. and Hadi, M.R., 2011. Microalgal biotechnology and bioenergy in Dunaliella. In *Progress in Molecular and Environmental Bioengineering-From Analysis and Modeling to Technology Applications*. IntechOpen.

37 Sun L., Wang L. & Zhou Y. (2012) Immunomodulation and antitumor activities ofdifferent-molecular-weight polysaccharides from Porphyridiumcruentum. Carbohydrate polymers87, 1206-10.

38 Sztrum, A.A., Sabatini, S.E. and Rodríguez, M.C., 2012. Isocitratelyase activity and antioxidant responses in copper-stressed cultures of Chlamydomonasreinhardtii (Volvocales, Chlorophyceae). *Phycologia*, *51*(2), pp.135-143.

39 Sathasivam, R. and Juntawong, N., 2013. Modified medium for enhanced growth of *Dunaliella* strains. Int J CurrSci, 5, pp.67-73.

40 Singh, P., Baranwal, M. and Reddy, S.M., 2016. Antioxidant and cytotoxic activity of carotenes produced by Dunaliellasalina under stress. *Pharmaceutical biology*, *54*(10), pp.2269-2275.

41 Sharma, S., Baranwal, M.G. and Reddy, M.S., 2016. Extracellular polymeric substances (EPSs) from *Dunaliellasalina* having antioxidant and immunostimulating activity.

42 Saha, S.K., Kazipet, N. and Murray, P., 2018. The carotenogenicDunaliellasalina CCAP 19/20 produces enhanced levels of carotenoid under specific nutrients limitation. *BioMed research international*, *2018*.

43 Truc Mai, Phuc Nguyen, Trung Vo, Hieu Huynh, Son Tran, Tran Nim, Dat Tran, Hung Nguyen, Phung Bui, "Accumulation of lipid in *Dunaliellasalina*under Nutrient Starvation Condition"

44 Takouridis, S.J., Tribe, D.E., Gras, S.L. and Martin, G.J., 2015. The selective breeding of the freshwater microalga Chlamydomonasreinhardtii for growth in salinity. *Bioresource technology*, *184*, pp.18-22.

45 Varki, A., Cummings, R.D., Esko, J.D., Freeze, H.H., Stanley, P., Bertozzi, C.R., Hart, G.W. and Etzler, M.E., 2009. *Nematoda--Essentials of Glycobiology*. Cold Spring Harbor Laboratory Press.

46 Van Meerloo, J., Kaspers, G.J. and Cloos, J., 2011. Cell sensitivity assays: the MTT assay. In *Cancer cell culture* (pp. 237-245). Humana Press.

47 Wang, S.B., Chen, F., Sommerfeld, M. and Hu, Q., 2004. Proteomic analysis of molecular response to oxidative stress by the green alga Haematococcuspluvialis (Chlorophyceae).*Planta*, *220*(1), pp.17-29.

48 Wei, S., Bian, Y., Zhao, Q., Chen, S., Mao, J., Song, C., Cheng, K., Xiao, Z., Zhang, C., Ma, W. and Zou, H., 2017. Salinity-induced palmella formation mechanism in halotolerant algae Dunaliellasalina revealed by quantitative proteomics and phosphoproteomics. *Frontiers in plant science*, *8*, p.810.

49 Yan, R., Cao, Y. and Yang, B., 2014. HPLC-DPPH screening method for evaluation of antioxidant compounds extracted from Semen Oroxyli. Molecules, 19(4), pp.4409-4417.

Master Thesis

ORIGINALITY REPORT

9%	6%	6%	%
SIMILARITY INDEX	INTERNET SOURCES	PUBLICATIONS	STUDENT PAPERS

PRIMARY SOURCES

1	Mehendi Goyal, Harloveleen Kaur, Maitri Bhandari, Albert A. Rizvanov, Svetlana F. Khaiboullina, Manoj Baranwal. "Antioxidant and Immune Effects of Water Soluble Polysaccharides Isolated from Cinnamomum verum Bark", BioNanoScience, 2018 Publication	1%
2	A. Hosseini Tafreshi. "*Dunaliella* biotechnology: methods and applications", Journal of Applied Microbiology, 07/2009 Publication	1%
3	Sushanta Kumar Saha, Naresh Kazipet, Patrick Murray. " The Carotenogenic CCAP 19/20 Produces Enhanced Levels of Carotenoid under Specific Nutrients Limitation ", BioMed Research International, 2018 Publication	1%
4	baadalsg.inflibnet.ac.in Internet Source	<1%
5	en.wikipedia.org Internet Source	

		<1%
6	pdxscholar.library.pdx.edu Internet Source	<1%
7	Shivpoojan Kori, Ankush Parmar, Jony Goyal, Shweta Sharma. "Cloud point extraction coupled with microwave-assisted back-extraction (CPE-MABE) for determination of Eszopiclone (Z-drug) using UV–Visible, HPLC and mass spectroscopic (MS) techniques: Spiked and in vivo analysis", Journal of Chromatography B, 2018 Publication	<1%
8	www.frontiersin.org Internet Source	<1%
9	www.ncbi.nlm.nih.gov Internet Source	<1%
10	docplayer.net Internet Source	<1%
11	Xuan, G.S.. "Development of an electrochemical immunosensor for alanine aminotransferase", Biosensors and Bioelectronics, 20031215 Publication	<1%
12	Kumar Satish, Neeraj, Krishna Mishra Viraj, Kr. Karn Santosh. "Biodegradation of phenol by free	<1%

	and immobilized Candida tropicalis NPD1401", African Journal of Biotechnology, 2018 Publication	
13	www.tandfonline.com Internet Source	<1%
14	core.ac.uk Internet Source	<1%
15	krishikosh.egranth.ac.in Internet Source	<1%
16	id.scribd.com Internet Source	<1%
17	ijpsr.com Internet Source	<1%
18	zomemory.blogspot.com Internet Source	<1%
19	onlinelibrary.wiley.com Internet Source	<1%
20	Aparna Singh, Samer Singh, Syed Mohsin Waheed, Puneet Khandelwal, Rakesh Bhatnagar. "Expression of anthrax lethal factor gene by osmolyte induction", FEMS Microbiology Letters, 2002 Publication	<1%
21	www.tsijournals.com Internet Source	<1%

22	www.pnnl.gov Internet Source	<1%
23	ddd.uab.cat Internet Source	<1%
24	sevgiligiyim.com Internet Source	<1%
25	Pooja Sharma, Varsha Agarwal, M. Krishna Mohan, Sumita Kachhwaha, S. L. Kothari. "Isolation and Characterization of Dunaliella Species from Sambhar Lake (India) and its Phylogenetic Position in the Genus Dunaliella Using 18S rDNA", National Academy Science Letters, 2012 Publication	<1%
26	pubs.rsc.org Internet Source	<1%
27	www.ege.fcen.uba.ar Internet Source	<1%
28	S. Ueno. "Biomagnetic Imaging: Principles of Magnetic Resonance Imaging and Emerging Techniques in Progress", Sensors Applications, 05/05/2005 Publication	<1%
29	Gabi Drochioiu, Karin Popa, Doina Humelnicu, Manuela Murariu, Ion Sandu, Alexandru Cecal. "Comparison of various sensitive and selective	<1%

spectrophotometric assays of environmental cyanide", Toxicological & Environmental Chemistry, 2008
Publication

30	www.eurekaselect.com Internet Source	<1%
31	www.jesusking.info Internet Source	<1%
32	F. O. Ochai-Ejeh, D. Y. Momodu, M. J. Madito, A. A. Khaleed, K. O. Oyedotun, S. C. Ray, N. Manyala. "Nanostructured porous carbons with high rate cycling and floating performance for supercapacitor application", AIP Advances, 2018 Publication	<1%
33	repository.up.ac.za Internet Source	<1%
34	dspaces.uok.edu.in Internet Source	<1%
35	"Expression analysis of photosynthesis genes in Dunaliella salina grown at different NaCl concentrations", Journal of Applied Biology & Biotechnology, 2015 Publication	<1%
36	Senthil Nagappan, R. Rajendra Kumar, J. Rupesh Balaji, Shachi Singh, Sanjay Kumar	<1%

Verma. "Direct saponification of wet microalgae by methanolic potassium hydroxide using acetone as co-solvent", Bioresource Technology Reports, 2019
Publication

37	academic.oup.com Internet Source	<1%
38	www.dest.gov.au Internet Source	<1%

Exclude quotes	On	Exclude matches	< 10 words
Exclude bibliography	On		