PROTEOMIC PROFILING OF *DUNALIELLA SP.* FOR IDENTIFICATION OF SALT TOLERANT GENES

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BY

MRS. B. SANTHAKUMARI

UNDER THE GUIDANCE OF

DR. MAHESH J. KULKARNI

BIOCHEMICAL SCIENCES /CMC DIVISION

CSIR-NATIONAL CHEMICAL LABORATORY

PUNE - 411 008, INDIA.

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Dr. Mahesh J. Kulkarni
Scientist,
Biochemical Sciences Division,
CSIR-National Chemical Laboratory,
Pune-411 008.

+91 20 2590 2541
mj.kulkarni@ncl.res.in

CERTIFICATE

This is to certify that the work presented in the thesis entitled **"Proteomic profiling of *Dunaliella sp.* for identification of salt tolerant genes"** submitted by **Mrs. B. Santhakumari**, was carried out by the candidate at CSIR-National Chemical Laboratory, Pune, under my supervision. Such materials as obtained from other sources have been duly acknowledged in the thesis.

Date:
Place:

Dr. Mahesh J. Kulkarni
(Research Supervisor)

CANDIDATE'S DECLARATION

I hereby declare that the thesis entitled **"Proteomic Profiling of *Dunaliella sp.* for identification of salt tolerant genes"** submitted for the award of the degree of ***Doctor of Philosophy*** in Biotechnology to the 'SavitribaiPhule Pune University' has not been submitted by me to any other university or institution. This work was carried out by me at CSIR-National Chemical Laboratory, Pune, India. Such materials as obtained from other sources have been duly acknowledged in the thesis.

Mrs. B. Santhakumari **Date:**
(Researcher) **Place:**
Biochemical Sciences/CMC Division,
CSIR-National Chemical Laboratory,
Pune- 411 008, India.

Dedicated to my beloved family and friends

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Contents of Thesis

List of Abbreviations

2-DE	Two-dimensional electrophoresis
ATP	Adenosine triphosphate
CBB	Coomassie Brilliant Blue
CHAPS	3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate
Da	Dalton
DTT	Dithiothreitol
E-SEM	Environmental-Scanning Electron Microscope
FMN	Flavin mononucleotide
GA3PDH	Glyceraldehyde 3 phosphate dehydrogenase
GG	Glucosylglycerol
HDMS	High definition mass spectrometry
ID	Identification
IDA	Information dependent acquisition
IEF	Isoelectric focusing
IPG	Immobilized pH gradient
KAAS	*KEGG Automatic Annotation Server*
KEGG	*Kyoto Encyclopedia of Genes and Genomes*
LC-MS	Liquid Chromatography-Mass Spectrometry
M	Molar
m/z	Mass to charge ratio
µL	Micro litre
MS^E	MS at elevated energy
NaCl	Sodium Chloride

NCIM	National Center for Industrial Microorganisms
NDPK	Nucleoside diphosphate kinase
PCR	Polymerase chain reaction
PLGS	Protein Lynx Global Server
PPIase	Peptidyl-prolyl isomerise
ppm	Parts per million
ppt	parts per thousand
PSI	Photosystem I
PSS	Practical Salinity Scale
PSU	Practical Salinity Unit
RT	Retention Time
SAM	S-adenosylmethionine
SWATH	Sequential Window Acquisition of all Theoretical Mass Spectra
TEOS	Thermodynamic Equation of Seawater
TOF	Time of Flight
VAM	Vesicular-arbuscularmycorrhizal
XIC	Extracted ion chromatogram

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Thesis Abstract

Salinity is one of the major abiotic stresses that affect crop growth and productivity. Plants respond to salt stress by various mechanisms and these vary amongst different species. Halophillic organisms serve as excellent model systems to understand the adaptation mechanisms to salt stress. Therefore, an alga was isolated from marine water ponds of Alibagh, Raigad Dist. (Maharashtra, India), which was identified and characterized to be closely related to *Dunaliella sp. and* is referred as *Dunaliella sp.* BSK hence forth in this study. *Dunaliella* is one such model organism that can adapt to practically entire range of salt concentrations. In this study, we have used proteomic approach to compare the salt stress mechanisms between *Dunaliella sp.* BSK and *Chlorella pyrenoidosa,* a halophillic and a glycophillic alga respectively. Together 559 proteins were identified from both *Dunaliella* and *Chlorella*. These proteins belonged to various biological processes such as metabolic, cellular, single-organism and developmental process, localization, response to stimulus, cellular component organization or biogenesis, biological regulation, signal transduction, epidermal cell fate specification and meiotic cell cycle. Further the differential expression of proteins suggested that *Dunaliella* and *Chlorella* respond to salts stress with distinct mechanisms.

Chapter 1

Introduction

1.1 Introduction

Salinity is one of the major abiotic stresses that affect crop growth and productivity. Various factors such as sea water, mineral weathering and irrigation increase the soil salinity. Although irrigation is important for the crop growth and yield, excess irrigation leads to accumulation of salts in the soil. Increased soil salinity results in deterioration of soil strength affecting availability of water and nutrients to the plants. In general, salt stress interferes with the plant nutrient uptake, decreases the photosynthesis and respiration rate resulting in reduced growth and yield. High salt leads to cellular dehydration which results in the damage of proteins and cellular membranes. The decrease in photosynthetic efficiency of plants results in decreased plant growth and yield owing to reduction in central metabolic activities. Due to ever growing population, it is increasingly becoming difficult to meet the food supply. Therefore, it is necessary to develop crop varieties which can sustain and set seeds under high salt concentrations. In order to develop salt stress tolerant crop plant, it is vital to understand the plant adaption mechanism to salt stress. Plants respond to salt stress by various mechanisms and these vary amongst different species. The adaptation mechanisms include the mechanisms like control of ion uptake by roots and transport into leaves, selective accumulation or exclusion of ions, compartmentalization of ions, change in photosynthetic pathway, osmoregulation involving synthesis of compatible solutes, induction of antioxidative enzymes/plant hormones and alteration in membrane structure (Parida AK and Das AB, 2005).

Most plants adapt to low or moderate salinities, however their growth is severely limited above 200mM NaCl (Hasegawa et al., 2000). The response of plants to salt stress has previously been studied in model plant species such as Arabidopsis and rice. Differential genomic screens carried out in Arabidopsis and rice have shown that plants respond to salt stress by up-regulation of a large number of genes involved in diverse physiological functions (Kawasaki et al., 2001; Kreps et al., 2002), however these species are not well adapted to high salinity conditions. In this context halophytic organisms may serve as valuable model(s) for identification of basic mechanisms of salinity tolerance (Glenn et al., 1999). A special example of adaptation to hypersaline conditions is the unicellular algae *Dunaliella*, a

dominant photosynthetic organism are exceptional in the plant kingdom in their ability to proliferate over practically the entire range of salinities. The algae grow in NaCl media with concentrations ranging from 0.1 M to near saturation while maintaining a low intracellular ionic concentration (Avron M and Ben-Amotz A, 1992). *Dunaliella* responds to salt stress by massive accumulation of glycerol (an internal osmolyte), enhanced elimination of Na^+ ions, and accumulation of specific proteins (Pick, 2002). Thus overcomes external osmotic variations by adjusting intracellular levels of glycerol concentrations balancing the external osmotic pressure. Therefore, the cells maintain a constant volume independent of the external salinity (Avron, 1986; Sadka et al., 1991). Mechanisms governing ionic homeostasis and other aspects of salt tolerance of *D. salina* still remain largely unknown. In addition to its ability to survive in extreme environments of salt, it accumulates massive amounts of carotenoids under appropriate growth conditions. Natural carotenoids found in *Dunaliella salina* are among nature's best antioxidants, containing a variety of carotenoids including beta carotene, alpha carotene and xanthophylls like zeaxanthin, cryptoxanthin and lutein (Ben-Amotz, 1982). These carotenoids could be useful in combating oxidative stress and other abiotic stresses. Thus it serves as an excellent model organism to understand the molecular basis of adaptation to extreme salinity and abiotic stress. On the other hand, glycophillic algae *Chlorella* serves as an excellent photosynthetic model for plant systems and it was used to study salt stress mechanisms (Barry and David 1980; Ley and Mauzerall 1982; Sheekh and Demeter 1993). Comparing responses in terms of proteomic changes of these two systems to salt stress would perhaps provide molecular insight of adaptation mechanisms to salt stress. Identifying genes and proteins that enable survival at hyper-saline condition is of great importance for developing salt tolerant plants. Expression of such genes in higher plants may lead to generation of transgenic plants with improved salt-tolerance.

1.2 Objectives of the proposed project

- Isolation and characterization of a marine algae for salinity tolerance study
- To study the proteomic profile of *Dunaliella* (isolated marine algae) under different salinity conditions
- Comparative proteomic study of *Dunaliella* and *Chlorella* (a fresh water algae) to understand their salt tolerance mechanisms

1.3 Outline of the Thesis

Chapter 1: Introduction

Chapter 2: Literature Review

Chapter 3: Materials and Methods

Chapter 4: Results

Chapter 5: Discussion

Chapter 6: Summary

Chapter 2

Literature Review

2.1 Salinity

Salinity is the measure of the amount of salt dissolved in water and is defined as the grams of the dissolved salt in one kilogram of seawater. Any matter is considered to be dissolved if it can pass through a very fine filter of 0.2 μm pore size (Pawlowicz R, 2013). Normally the salinity of seawater is around 35 g/kg, whereas rivers and lakes have salinities ranging from 0.01 g/kg to a few g/kg (Eilers JM, 1990). The salinity of Dead Sea is more than 200 g/kg (Anati DA, 1999). Based upon the percentage of salinity the water bodies are classified into fresh waters, brackish waters, saline waters and brine water bodies (Table 1).

Table 1: Classification of water bodies based upon their salinity

Water body	Minimum salinity	Maximum salinity
Fresh water	< 0.05%	< 0.5 %
Brackish water	0.05 – 3%	0.5 – 30 %
Saline water	3 – 5%	30 – 50 %
Brines	> 5%	> 50%

http://en.wikipedia.org/wiki/Salinity

Historically salinity was expressed in the units of ppt (parts per thousand), by measuring the concentration of halide ions by a titration-based technique and then accounting for all the other ions with multiplication by a standard factor. Practical salinity scale (PSS-78) came into existence with the use of electrical conductivity measurements of ions. In later years the unit of PSU (practical salinity unit) was added to PSS-78. In 2010, thermodynamic equation of seawater 2010 (TEOS-10) was introduced as a standard for the measurement of various properties of sea water. Figure 1 shows salinity pattern for annual mean sea surface salinity of the World Ocean in PSU units (World Ocean Atlas 2009/Wikipedia).

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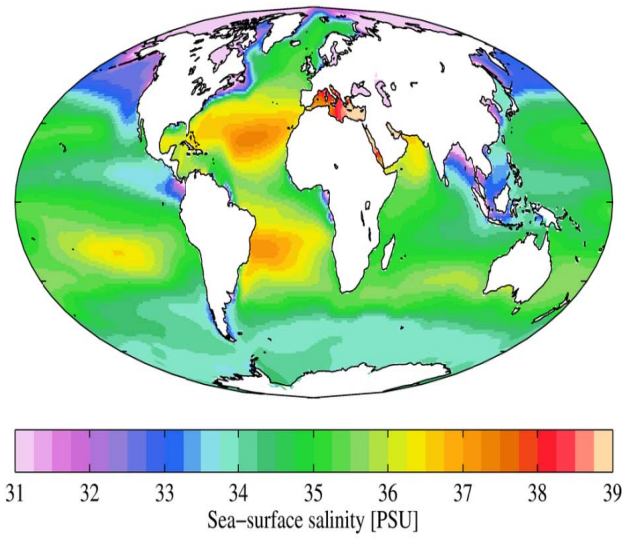


Fig. 1: Annual mean sea surface salinity for the World Ocean

The degree of salinity in oceans is a driver of the world's ocean circulation. The ocean water density varies based on the change in the factors like salinity and temperature. The changes in these factors produce changes in buoyancy which results in the sinking and rising of water bodies. Salinity changes of the oceans contribute to global changes in carbon dioxide, as it is less soluble in saline waters. Salinity is an ecological factor which influences the organisms that live in a water body and as well the plants that grow either on land or in a water body.

According to Central Soil Salinity Research Institute (CSSRI), approximately 8.6 million hectares of India's land area is afflicted with the common twin problems of salinity and alkalinity. This is seriously reducing agricultural productivity and affecting our food security system (CSSRI website). Large tracts of Indian land are becoming unsuitable for cultivation due to high salt levels. Especially, in the coastal regions, climatic changes and rise in sea level have already started threatening the sustainability of the agricultural ecosystem in Saurashtra and Thanjavur region in India (Prashant SR and Parida A. 2005).

Soil salinity is continuously affecting crop growth over the past few decades. It is estimated that over 800 million hectares of land throughout the world is salt-affected, either by salinity or the associated condition of sodicity (FAO, 2005). Salt affected soil in Asia and pacific region contributes to 14% of world's total salt affected area.

India alone constitutes about 10% of total global salt affected area. The extent of land that is salt affected poses a threat to agriculture (Flowers TJ, 2004). Statistics of major salt affected countries are provided in Table 2. To tolerate high levels of soil salinity plants with high salt-tolerance have to be grown.

Table 2: Statistics of major salt affected countries

Country	Salt affected land (%)
United States	28
China	23
Pakistan	21
India	11
Mexico	10

2.2 Causes and Effects of soil salinity on plant growth

The salinization of soil can be caused by natural processes such as gradual withdrawal of an ocean and climatic trends like mineral weathering. In addition to it salt deposition may occur via dust and precipitation. Soil salinity also caused due to landscape feature that lead to movement of water table to the soil surface which results in soil salinity (Fig. 2). Very important cause of soil salinity is human activity such as irrigation. During irrigation use of saline water, improper irrigation methods and inappropriate crops lead to soil salinity (Ezlit YD, 2010). Presently world is unable to manage food supply to the demand of the growing population. To meet the food demand the agriculture productivity is intensified, which is leading to over exploitation of the land. Salinity from irrigation occurs over time, since almost all water contains some amount of dissolved salts. The salts are left behind in the soil once the plants use the water. Increased soil salinity results in degradation of soil strength that lowers the plants ability to take up water, which results in decreased photosynthetic efficiency of plants. The decrease in photosynthetic efficiency of

plants results in decreased plant growth and yield owing to reduction in central metabolic activities (Podmore C, 2009).

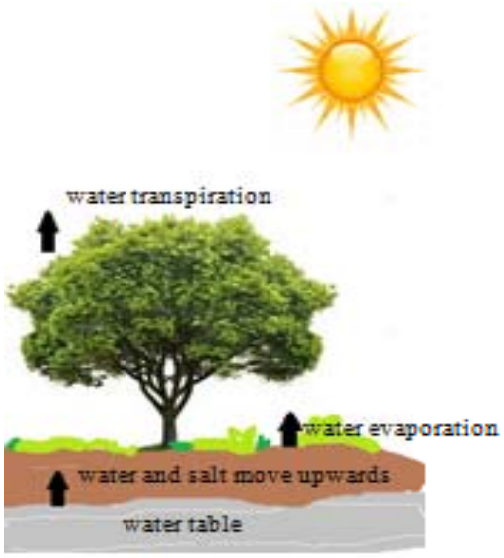


Fig. 2: Water table upward movement with transpiration by plants

The problem of excess soil-salinity in cropping systems is getting accentuated due to extension of the irrigation network. Hence it is necessary to develop crop varieties which can sustain and set seeds under high salt concentrations.

2.3 Plants salt tolerance mechanisms

Sensitivity or Resistance of plants to stress mainly depends on the species, the genotype and also the development age. Plants resistance to various stresses involve different types of mechanism like acclimation, avoidance or tolerance mechanism. Salt stress triggers various plant responses like altered cellular metabolism, gene expression, growth rate and crop yields. Initially plants recognize stress at the cellular level, this activates signal transduction pathways within the cells and then throughout the whole plant. The plants physiological, biochemical and molecular mechanisms that are involved in salt tolerance has been extensively studied, discussed and reported (Zhu J-K et.al, 1997; Yeo AR, 1998). Biochemical pathways that improve salt tolerance are thought to act additively and synergistically (Iyengar, 1996). The pathways that improve salt tolerance of plants include mechanisms like control of ion uptake by roots and transport into leaves, selective accumulation or exclusion of ions,

compartmentalization of ions, change in photosynthetic pathway, synthesis of compatible solutes, induction of antioxidative enzymes/plant hormones and alteration in membrane structure (Parida AK and Das AB, 2005). Salt stress involves ionic as well as osmotic stress (Hagemann and Erdmann, 1997; Hayashi and Murata, 1998).

Generally plants growth is limited above 200mM NaCl. Plants can adapt to low or moderate salinities, however their growth is reduced above 200mM NaCl concentrations (Hasegawa et al., 2000). Based on the salt tolerance capacity plants may be categorized as halophytes or glycophytes. Plants that can survive in high salinity and grow well are called halophytes. Relatively 2% of all the plant species are halophytes. Majority of plant species (85%) are glycophytes; these plants are not salt-tolerant and are damaged easily in the presence of high salt. The present most critical requirement is the ability of plants to develop mechanisms to detoxify radicals under salt stress conditions. Most commonly stress is caused by the presence of high concentrations of Na^+ and Cl^- in soil. High concentrations of these ions are proving to be toxic to the plants and may be hampering the absorption of other essential plant nutrients.

Salt tolerant levels of few model crops are given in Table 3. Beans and rice can tolerate about 1-3 g /L of salt. *Salicornia bigelovii* (dwarf glasswort) grows well even at 70 g/L of dissolved salts, and is a halophyte and can be considered as a promising salt tolerant crop. Plants such as date palm and the barley can be considered as marginal halophytes they tolerate about 5 g/L of salt (Glenn EP et al. 1998).

Table 3: Crops and their salt tolerance level

Highly tolerant	Moderately tolerant	Sensitive
Barley	Wheat	Peas
Sugar beet	Oats	Red clover
Cotton	Tomato	Beans
Spinach	Flax	Pear
Asparagus	Maize	Sugarcane
	Rice	Apple
	Alfalfa	Plum
	Potatoes	Prune
	Onion	Orange
	Carrot	Almond
	Cucumber	Peach
	Fig	Apricot
	Pomegranate	
	Olive	
	Date palm	
	Grape	

The major cellular processes in plants such as photosynthesis, protein synthesis, and energy and lipid metabolism are affected under stress conditions. Salt tolerance mechanisms of plants had been studied and discussed over decades (Gupta B and Huang B, 2014). To understand salt tolerance mechanisms, plants salinity response has been measured in various ways and at various stages of their growth and development.

Salt tolerant plants can regulate their intracellular solutes like amino acids, polyhydric alcohols, tertiary sulfonium compounds and quaternary compounds to adjust to osmotic perturbations. Generally plants salt tolerance ability is determined on the basis of biochemical pathways that allow easy retention of water and maintenance of ion homeostasis in plants (Parida AK and Das AB 2005). The plants with induced high antioxidant levels show good resistance to oxidative damage caused by salinity

(Dhindsa and Matowe, 1981; Spychalla and Desborough 1990). Otoch et al. (2001) reported induction of V-ATPase expression and inhibition of V-PPase in *V. unguiculata* under same salt stress conditions. Soil salinity significantly reduces the absorption of mineral nutrients in salt-stressed soils. Generally, Fungi help plants to capture nutrients such as phosphorus, sulphur, nitrogen and micronutrients from the soil. Hence fungi were used to improve plants salt tolerance. Cantrell and Linderman (2001) reported reduction of damaging effects of soil salinity on crop growth and yield by preinoculation of transplants with Vesicular-arbuscular mycorrhizal (VAM) fungi. Uptake of low mobility micro-nutrients such as P, Cu and Zn by plants was enhanced by VAM fungi (Bethlenfalvay GJ, 1992).

Possibility in the reduction of negative effects of Na^+ and Cl^- ions by maintenance of vacuolar membrane integrity in plants grown under salinity conditions by enhanced uptake of P by Arbuscular mycorrhizal fungi was reported (Rinaldelli and Mancuso, 1996).

Many plant species have adapted various mechanisms to sustain high salt stressed environmental conditions. These essential mechanisms/pathways include those that lead to synthesis of specific proteins, osmotically active metabolites and certain free radical scavenging enzymes that control ion/water flux and support scavenging of superoxide radicals. Accumulation of compatible solutes like glucosylglycerol (GG) and sucrose under salt stress by cyanobacterium (*Synechocystis* sp. PCC 6803) was observed (Hagemann M and Murata N, 2003). Salt-tolerant plant species accumulate methylated metabolites, which play dual role as osmoprotectants and radical scavengers. Salt stressed plants are also capable of accumulating organic osmolytes, like proline, valine, aspartic acid, ectoine, isoleucine, betaine, glucose, fructose, fructans, sucrose, *myo*-inositol, mannitol, and pinitol in the cytoplasm of their cells and the synthesis of these osmolytes was reported to be associated with the stress-induced enhancement of photorespiration (Parida AK and Das AB, 2005). Frequently observed organic osmolytes are sugars, sugar alcohols and complex sugars. Plants response to salt stress also has previously been studied in model plant species with sequenced genomes, such as *Rice* and *Arabidopsis*. Differential genomic screens carried out in *Rice* and *Arabidopsis* have shown up-regulation of a large number of genes involved in diverse physiological functions in plants under salt stress (Kawasaki et al., 2001; Kreps et al., 2002). However these plant species are not well

adapted to high salt stress conditions. Therefore halophytic organisms are mostly used for understanding of basic mechanisms of salt tolerance (Glenn et al., 1999). Microalgal species have been widely used and preferred as model systems for research on higher plants owing to their simple structure, ability to perform all metabolic activities, shared physiological and biochemical processes (Hicks *et al.* 2001; Harris, 2001).

Salt tolerance mechanisms were studied in various marine algae like Fucus, Cladophora, Chrysophyte flagellate Poterioochromonas, Tetraselmis, Griffithsia monilis, Lamprothamnium, Valonia utricularis, Enteromorpha intestinalis, Pelvetia canaliculata, Ascophyllum nodosum, Enteromorpha, Laminaria digitata, Chara vulgaris, Polysiphonia lanosa, Bostrychia scorpioides, Enteromorpha, Ulva lactuca, Sea Ice Algae and so on(Krist GO, 1989). Unicellular microalgae were preferred to higher plants as model organisms to study stress mechanisms owing to their simple structure and ability to perform all metabolic activities. The nature of salinity tolerance was studied in several halophytes such as *Mesembryanthemum crystallinum, Thellungiella halophila* and *Dunaliella* species. Although *Mesembryanthemum* and *Thellungiella* are equally good system to study salt tolerance, they take longer time to grow as compared to *Dunaliella*. *Dunaliella* is relatively easy to grow compared to *Mesembryanthemum* and *Thellungiella*. The unicellular alga *Dunaliella* can adapt to practically the entire range of salinities (Giordano M and Beardall J, 2006). The alga *Dunaliella* grows in media with NaCl concentrations ranging from 0.1 M to near saturation and at the same time maintains low intracellular ionic concentration (Avron, 1992). *Dunaliella* is a very important and widely studied model organism owing to its resistance to high salinity, high light intensities and other stresses, which are utmost important in plant biology. On the other hand, glycophillic alga *Chlorella* which serves as an excellent photosynthetic model for plant systems was also used to study salt stress mechanisms (Barry and David, 1980; Ley and Mauzerall, 1982; Sheekh, and Demeter, 1993). Recent development in 'Omics' approaches such as genomics, transcriptomics and proteomics technologies facilitate in better understanding of the molecular mechanisms in plants. Hence in this study *Dunaliella* was opted as a model organism and proteomics approach was followed to study salt tolerance mechanisms to identify newer genes that confers salt tolerance. And

Chlorella sp. was selected to perform the studies of comparative proteomic profiling of *Dunaliella*.

2.4 The Alga *Dunaliella*, taxonomical classification:

Domain:	Eukaryota
Kingdom:	Viridiplantae
Phylum:	Chlorophyta
Class:	Chlorophyceae
Order:	Chlamydomonadales
Family:	Dunaliellaceae
Genus:	*Dunaliella*

Genus *Dunaliella* is a unicellular and biflagellate algae belonging to the class *Chlorophyceae* and family *Dunaliellaceae*. Its cell is enclosed by a thin elastic plasma membrane and lacks a rigid cell wall; as a result osmotic variations strongly influence cell's morphology (Oren, 2005). Due to its elastic nature *Dunaliella* cells has the ability to change their shape and volume in response to changes in osmotic and other growth conditions. It reproduces with formation of a zygote by longitudinal division of the cells or by fusion of two motile cells (Fig. 3). *Dunaliella*, a red flagellate alga was first sighted in Saltern evaporation ponds of south France in 1838 by Michel Felix Dunal (Oren A, 2005b); he named it *Haematococcus salinus* and *protococcus*. During the period of 19th century, the red flagellate algae have been observed in salt lakes and other hyper saline sites in Algeria Crimea, France, Lorraine and Romania by other biologists as well. Different names were given to the organism by each investigator (Table 4). In 1905, Teodoresco named it in honour of its discoverer, Dunal.

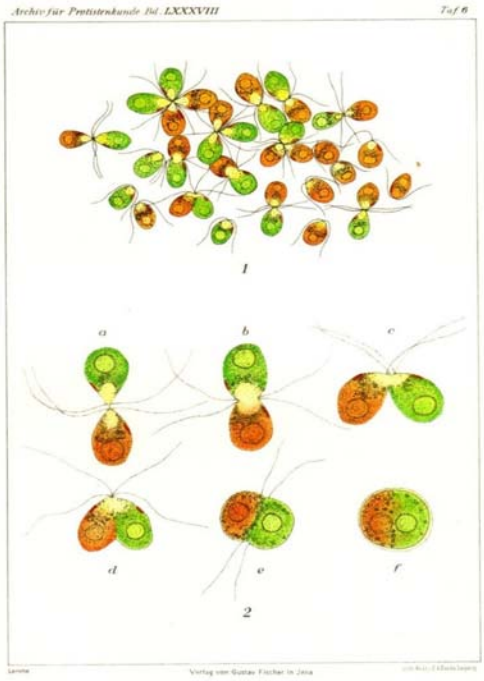


Fig. 3: Aggregation and zygote formation by green and red forms of *Dunaliella salina* (Lerche W, 1937)

Table 4: Discoverer and the given name of alga, observed in hyper saline brines during 1838–1959

Author	Name
Dunal(1838)	*Haematococcus salinus*, *Protococcus salinus*
Joly(1840); Blanchard(1888); Butschinsky(1897)	*Monas Dunalii*
Dujardin(1841)	*Diselmis Dunalii*
Cohn(1865); Blanchard(1891); Bujor(1900)	*Chlamydomonas Dunalii*
Hansgirg(1866)	*Sphaerella lacustris* var. *Dunalii*
Geleznow(1872)	*Protococcus salinus*
Teodoresco(1905, 1906)	*Dunaliella salina, Dunaliella viridis*
Baas Becking and Nicolai (1935)	*Dunaliella peircei*
Lerche (1937)	*Dunaliella Parva, Dunaliella media* *Dunaliella euchlora, Dunaliella minuta*
Butcher (1959)	*Dunaliella tertiolecta, Dunaliella quartolecta, Dunaliella primolecta, Dunaliella polymorpha*

A formal description of the two well known species of genus *Dunaliella*, *Dunaliella salina* and *Dunaliella viridis*, published in 1906 (Oren A, 2005). This study was followed by many other studies in the early years of *Dunaliella species.* Figure 4 shows cellular shapes of *Dunaliella salina* observed under various salt stress conditions and drawn by Hamburger.

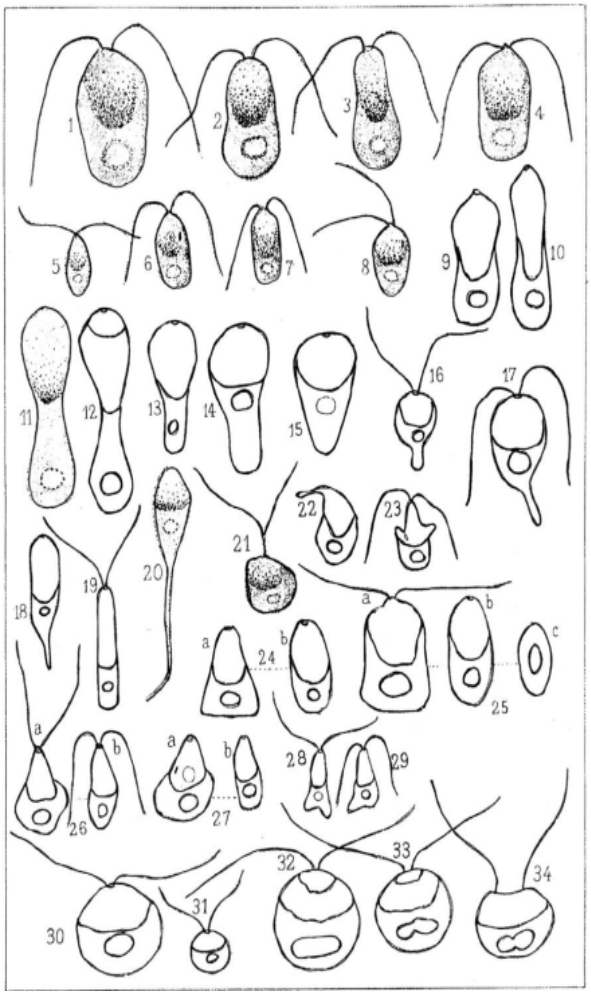


Fig. 4: Drawings of *Dunaliella salina* cells (1–4), cells of *D. viridis* (5–8), different cell shapes observed when concentrated by evaporation (9–29), upon dilution (32–34) by Hamburger (1905)

Totally around 140 species of *Dunaliella* were so far discovered and reported. Recently a new *Dunaliella* species was discovered in the Atacama Desert of Chile. Where the environment is very harsh, most life would wither. It was believed, the alga has survived drinking condensed water vapour on spider-web (Clara Moskowitz, 2010). The halophytic members of *Dunaliella* are mainly found in subtropical areas throughout the world and also in more moderate regions (Borowitzka et al. 1988). Taxonomically the genus was divided into two subgenera, *Dunaliella* and *Pascheria* (fresh water species) by Massyuk (Oren A, 2005).

2.5 Survey of work done in the research area

Unicellular green alga, *Dunaliella* is unique in its ability to adapt to some of the harshest environments on earth. Lot of research underwent on *Dunaliella* species to understand the taxonomy, distribution, cultivation, carotenoid biosynthesis, pigments, metabolism, bioactive compounds, fatty acids production, glycerol production, photosynthesis, photo damage and repair reactions, stress tolerance, iron deprivation resistance, carbon dioxide fixation, biofuels and its application to cure atherosclerosis and in ecotoxicity testing and so on. Most *Dunaliella* species are known to tolerate extreme salinities and they can survive even in The Dead Sea (U. Pick, 2006). Cultivation of these organisms is very simple and they do not cluster or clump. *Dunaliella* was the most studied organism to understand its stress tolerance mechanisms to extreme conditions of salinity, temperature, light, pH and also for the high production levels of natural carotenoids, lipids and many other bioactive compounds (Drokova IH, 1961). 9-cis-beta-carotene present in *Dunaliella* is considered to be up to ten times efficient at preventing cancer than normal beta-carotene (Hieber AD, 2000). It has high demand as a pro-vitamin A, natural food colouring agent, health food and as an additive to cosmetics (Borowitzka MA, 1986). Growth rate of *Dunaliella* isolates under various salt concentrations was studied and reported by many researchers. Baas-Becking has observed that *D. viridis* grows well over the whole range of salinities (1–4 M/6–23% NaCl) and a pH range of 6–9 (Baas-Becking, 1931). Under salt stress, *Dunaliella* accumulates massive amounts of glycerol which is its internal osmotic element), enhances elimination of Na^+ ions and accumulates specific proteins. By adjusting intracellular levels of glycerol *Dunaliella* overcomes external osmotic variations (Pick, 2002). The establishment of organic solutes to provide osmotic balance was largely based on the studies of *Dunaliella* species. *Dunaliella* adjusts to osmotic disturbances by volume regulation through a signal transduction pathway that involves G-proteins and phospholipids (Bental et al. 1990; Memon et al. 1993). In addition to its ability to survive in extreme environments of salt, it accumulates high amounts of carotenoids under suitable growth conditions and the cells are protected by this pigment from the damage of high light intensities. Carotenoids are organic pigments that are present in chromoplasts and chloroplasts of plants and some of the other photosynthetic organisms, including fungi and bacteria. Carotenoids are also produced from the building blocks (fats and

organic metabolites) of all these organisms. These are tetraterpenoids which are made of 8 isoprene molecules with 40 carbon atoms. Carotenoids of *Dunaliella salina* are considered to be among nature's best antioxidants, containing a variety of carotenoids including alpha carotene, beta carotene and xanthophylls such as zeaxanthin, cryptoxanthin and lutein (Ben-Amotz, 1982). It was estimated and reported, 13.8% of the total dry organic matter of *Dunaliella salina* in Pink Lake, Victoria, Australia, was β-carotene [Aasen AJ, 1969]. In 1966 first pilot plant for cultivation of *Dunaliella* was set in the USSR for the commercial production of β-carotene (Massyuk NP, 1968). These carotenoids could be useful in combating oxidative stress and other abiotic stresses. Presently the manipulation of microalgae species for commercial products is a rapidly expanding area of research. Molecular phylogeny techniques were applied by many researchers to the study taxonomy of *Dunaliella* (González MA, 1999; Olmos J, 2000). Using molecular phylogeny techniques Olmos et al. differentiated *D. salina*, *D. parva* and *D. bardawil* as species containing 1, 2 and 3 introns respectively within the 18S rRNA gene (Olmos et al., 2000). A wide range of research on *Dunaliella* has been carried out so far to understand its survival to different harsh environments and various applications.

Shaish A et. al. (2006) demonstrated inhibition of atherosclerosis, reduction of non-HDL plasma cholesterol concentrations and inhibition of fatty liver development and inflammation in an atherosclerosis mouse model when 9-*cis* *β*-carotene rich diet was provided. The genetic transformation and metabolic engineering of *Dunaliella* is presently thought to very interesting area of research. *Dunaliella* is being considered seriously for its use as a biological source for high-value proteins such as vaccines, antibiotics and enzymes and also for their mass-production by many researchers. This may open an interesting new phase for micro algal biotechnology in future. The enormous potentialities of different species of this useful alga are being considered for exploitation in various biotechnological areas such as biosensors, wastewater management, production of new antibiotic substances and biofuels (Hosseini AT and Shariati M, 2009).

2.6 *Dunaliella* Proteomics study

Proteomic profiling of different *Dunaliella* sp. for identification of salt tolerant genes is presently in progress to understand the stress tolerance mechanisms of *Dunaliella*.

With the advancement in proteomic techniques such as two dimensional gel electrophoresis, image analysis and mass spectrometry have opened an opportunity to look the protein complex of the whole genome. Dubey et al. (2001) opined that plant biotechnology research looks optimistically at proteomics research for newer stress tolerance genes. Only very few studies concerning the protemic profiling of *Dunaliella* were reported so far.

The major study that deals with *Dunaliella* proteomics was reported by Liska et al. (2004). In this study they have characterized proteome by a combination of chromatography, proteolytic digestion and peptide mass analysis. The identified salt induced proteins were the enzymes in Calvin cycle, redox energy production, starch mobilization, protein biosynthesis regulatory factors and bacterial Na1-redox transporters homolog and the salt concentrations selected for comparing *Dunaliella* proteome in this study were 0.5M and 3.0M.

Johnson MK et al. (1968) have studied the effects of salts on the halophillic alga *Dunaliella viridis* and revealed that the key enzymes of the algal metabolism such as ribulose diphosphate carboxylase, pentose phosphate isomerase, glucose-6-phosphate dehydrogenase and phosphohexose isomerise were strongly inhibited by low concentrations of NaCl which are far lower than NaCl concentration of the growth medium (3.75 M) and the inhibition was reported to be reversible (Johnson MK et. al., 1968). Sadka et al. reported induction of a cell surface protein (150 kDa) in halotolerant green alga *Dunaliella salina* by salt (Sadka A et al., 1991). A 60-kilodalton salt-induced plasma membrane protein that played a potential role in the extreme halotolerance of the alga *Dunaliella* was reported (Fisher M et al., 1994). A salt-resistant plasma membrane carbonic anhydrase induction by salt in *Dunaliella salina* was observed (Fisher M, 1996). Fisher also observed accumulation of a transferrin-like protein in the plasma membrane of the unicellular green alga *Dunaliella salina* which was grown under high salinities (Fisher M, 1997). Golldack reported that in low salt cells glycoprotein was produced whereas glycosylation was induced by high salt concentrations (Golldack et al., 1995). Co-migration of surface proteins at high salinity was observed on blue native gels as migration profile of native proteins which indicated oligomerization as mechanism for stabilization (Adriana Katz, 2007). Chen opined that the four enzymes of glycerol metabolic pathway work together to maintain the glycerol requirements of cells under osmotic

stress environments for the effective flow of the metabolic pathways cycle (Chen and Jiang, 2012). Shotgun proteomics approach was applied by Yanlong Jia to study the flagella proteins of *Dunaliella salina* grown in 1.5M NaCl medium and various groups of proteins were identified including intraflagellar proteins, transport proteins, tubulins, outer dynein arm subunits, central pair and axoneme (Jia Y et al. 2010). Although the *Dunaliella* research has now entered into its second century, yet it is one of the important model organisms that can be explored to understand the molecular mechanisms of salt tolerance.

Chapter 3

Materials and Methods

3.1 Materials

Required chemicals were purchased from M/S Sigma chemicals unless otherwise specified and LC-MS grade solvents Water and Acetonitrile (ACN) were from J. T. Baker.

3.2 Isolation and growth conditions of algal culture

Water samples were collected from the marine water ponds of Alibagh, Raigad Dist. (Maharashtra, India) for the isolation of microalgae. The water samples were brought to the laboratory and the initial enrichment was done by inoculation of samples in 500mL Erlenmeyer flask containing artificial sea water medium with 1.0 M NaCl, 50 mM $NaHCO_3$, 5 mM KNO_3, 5 mM $MgSO_4$, 0.2 mM $CaCl_2$, 0.2 mM KH_2PO_4, 7 µM $MnCl_2$, 5 µM EDTA, 2 µM $FeCl_3$, 1 µM $CoCl_2$, 1 µM $CuCl_2$, 1 µM $ZnCl_2$ and 1 µM $(NH_4)_6$ Mo_7O_{24} (pH 7.5). The visible and morphologically different isolates or clumps were isolated and the cultures with the highest survival potential were used for the further study. After a week of growth, the culture was again inoculated into fresh enrichment medium to obtain unialgal cultures. A loopful of this unialgal culture was streaked on to solid agar medium to purify and to get isolated colonies of single unialgal culture. The isolated organisms were morphologically motile, unicellular and rod shaped. Further 18S rRNA gene analysis was carried out to find its phylogenetic position.

3.3 Identification of the isolated strain

Extraction of genomic RNA and PCR amplification of the 18S rRNA gene were performed as described by Jayappriyan et al. (2010). 18SF1 (5´CTGCGAATGGCTCATTAAATC3´) and 18SR1 (5´ AAGGCCAGGGACGTAATCAA 3´) primers were used for amplification, PCR product was analyzed on 1% agarose gel by electrophoresis. PCR product was purified by PEG-NaCl method. PCR product was then sequenced with an ABI PRISM automatic sequencer using Big Dye terminator kit. The obtained sequence was compared with available 18S rRNA gene sequences from GenBank using the NCBI search BLAST. To determine its genomic affiliation, multiple alignments with sequences of the most closely related taxa and calculations of levels of sequence similarity were carried out using CLUSTAL_X (Tamura et al. 2007). A phylogenetic tree was constructed using the neighbour joining method and MEGA version 4.0

(Thompson et al. 1997). The topology of the phylogenetic tree was evaluated with 1,000 replicates by bootstrap resampling method.

3.4 Growth of cultures

Dunaliella sp. BSK was grown in a *Dunaliella* (CCALA) medium containing seawater soil extract, artificial seawater and the stock macro elements as mentioned in the Duna Base site (http://www.dunaliella.org/dunabase/media/ccala_dunaliella.php). The composition of minerals used for preparation of D*unaliella* medium is given in Table 5. The *Dunaliella* (CCALA) medium was prepared by mixing 910 mL of artificial seawater, 30 mL of seawater soil extract and 20 mL of the solution of stock macro elements.

Table 5: *Dunaliella* (CCALA) medium

Artificial sea water	Seawater soil extract	Stock macroelements
One litre contains 60g of NaCl, 10g of $MgSO_4X7H_2O$, 1.5g KCl and 2.0g $CaSO_4$.	Extracted from the garden soil and artificial sea water mixture	100ml contains 1.0g KNO_3, 0.1g K_2HPO_4 and 0.1g of $MgSO_4X7H_2O$

Dunaliella sp. BSK was grown in a media of various NaCl concentrations (0.2, 0.5, 0.8, 1.0, 1.5 and 2.0M NaCl) for physiological experiments. And for proteomic studies, it was grown in culture media of two different salt concentrations of 0.5 M and 2.0 M NaCl.

Chlorella pyrenoidosa procured from NCIM Resource Center of CSIR-National Chemical Laboratory, Pune (India), was grown in Fog's Medium (Table 6) containing 0.2% KNO_3.

Table 6: Fog's Medium

Agar (Difco)	12.0 g
$MgSO_4.7H_2O$	0.2 g
K_2HPO_4	0.2 g
$CaCl_2.H_2O$	0.1 g
**Fe-EDTA solution	5.0mL
*Micronutrient solution	1.0 mL
Distilled water	1L

***Micronutrient solution**	
H_3BO_3	286.0 mg
$MnCl_2.4H_2O$	181.0 mg
$Na_2MoO_4.2H_2O$	39.0 mg
$ZnSO_4.7H_2O$	22.0 mg
$CuSO_4.5H_2O$	8.0 mg
Distilled water	100.0 mL

****Fe-EDTA solution (100mL)**
745.0mg Na_2EDTA+ 557.0mg $FeSO_4.7H_2O$

Chlorella pyrenoidosa was grown in media of various salt concentrations (0, 0.05, 0.10, 0.15, 0.2 and 0.4M NaCl) for physiological experiments. And for proteomic studies, it was grown in media of two different salt concentrations; one without NaCl and the other with 0.2 M NaCl.

All cultures of *Dunaliella* sp. BSK and *Chlorella pyrenoidosa* were grown in 250mL flasks containing 100mL medium. The flasks were kept stationary under continuous illumination of white fluorescent lamps with light intensity of 1800 lumens at 28°C.

3.5 Scanning Electron Microscope (SEM) Analysis

To understand the cell morphology of *Dunaliella* and *Chlorella,* control samples of *Dunaliella* sp. BSK and *Chlorella pyrenoidosa* were analysed on E-SEM, Quanta zero-3D Scanning Electron Microscope. Prior to SEM analysis, sample were prepared by washing cells with water by slight vortex and centrifugation then few µL of samples loaded on coverslip/grid and dried in a dessicator.

3.6 General physiological studies

3.6.1 Cell growth determination

Cell density of 20 days old cultures of *Dunaliella* sp. BSK (0.2, 0.5, 0.8, 1.0, 1.5 and 2.0M NaCl grown) and *Chlorella pyrenoidosa* (0, 0.05, 0.10, 0.15, 0.2 and 0.4M NaCl grown) was estimated by optical density measurements. The optical density was measured at 750 nm by a spectrophotometer (Nguyen et al. 2014).

3.6.2 Extraction and Estimation of Glycerol content

Glycerol content was estimated spectrophotometrically as per the reported procedure (Bondioli and Bella 2005). The amount of glycerol in 20 days old cultures of *Dunaliella* sp. BSK (0.2, 0.5, 0.8, 1.0, 1.5 and 2.0M NaCl grown) and *Chlorella pyrenoidosa* (0, 0.05, 0.10, 0.15, 0.2 and 0.4M NaCl grown) was estimated using Periodate reagent and Acetylacetone reagent extraction procedure. To 200 µL of each cell culture, 120 µL of 10mM periodate reagent was added and vortexed for 30s and then 120 µL of 0.2 mM acetylacetone reagent was added and mixed. The samples were incubated at 70^0C for 1min then cooled. Optical density of all the extracted samples was determined at 410 nm on a spectrophotometer and compared to calibration standard.

3.6.3 Extraction and Estimation of Pigments

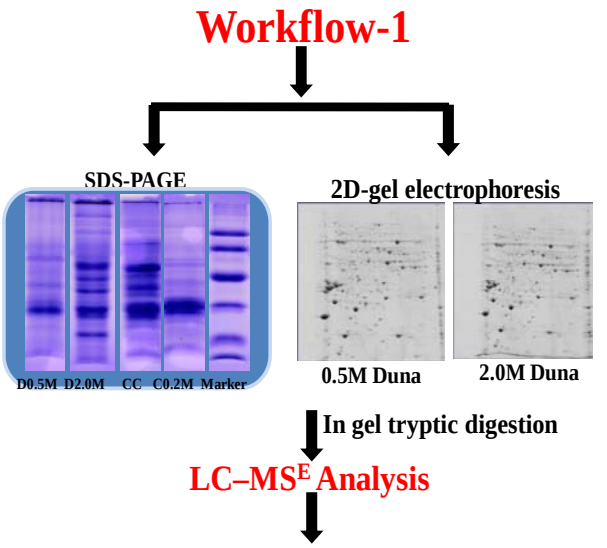
To extract pigments, 20 days old cultures of *Dunaliella* sp. BSK (0.2, 0.5, 0.8, 1.0, 1.5 and 2.0M NaCl grown) were centrifuged at 3500 rpm for 5min and the pellet was vortexed in 1 mL of *Tetrahydrofuran*. Spectrophotometric measurements were performed in the range of 350 to 700 nm, loading samples on a plate reader using a spectrophotometer (Hejazi et al. 2002). Pigment analysis of *Chlorella pyrenoidosa* culture samples was not performed owing to their low growth in presence of salt.

3.7 Protein extraction from *Dunaliella* sp. BSK and *Chlorella pyrenoidosa* culture medium

The *Dunaliella* sp. BSK and *Chlorella pyrenoidosa* cell mass separated from the culture medium by centrifugation was sonicated along with 200µL of extraction buffer (8M Urea, 2M Thiourea, 4% CHAPS and 1% DTT) and 2% protease inhibitor cocktail (sigma) and then subjected to centrifugation at 4°C for 30 minutes at a speed of 10000 rpm. To the supernatant, 800µL of cold acetone was added and kept for incubation at -40°C over night. Next day the samples were centrifuged to10000 rpm for 30 minutes at 4°C and supernatant was discarded. The pellets were solubilised in 200µL of rehydration buffer (8M Urea, 2M Thiourea, 4% CHAPS, 1% DTT. Protein was estimated following Bradford's method (Bradford, 1976) using Bradford's reagent (Bio-Rad ready mix). The extracted protein samples were subjected to proteomic analysis for their identification following two MS workflows.

1) LC-MS^{E} workflow by SYNAPT HDMS system (Fig. 5)

2) SWATH (MS/MS^{ALL}) workflow by Triple TOF™ 5600 System (Fig. 6)



Water's Synapt G1-HDMS

Fig. 5: LC-MS^E workflow

3.8 LC-MS^E workflow by Synapt HDMS system

To identify more number of proteins, we used multiple separation techniques. Prior to LC-MS^E analysis, for protein separation two experiments were carried out. In the first experiment, separation of proteins was achieved by 12% SDS-PAGE and in the second experiment protein separation was achieved by 2-dimensional electrophoresis.

3.8.1 Separation of protein by 12% SDS-PAGE

To achieve one-dimensional separation equal amount of protein samples (12µg) were loaded and run on a 12% SDS-PAGE, and proteins were visualized by CBB (coomassie brilliant blue) staining.

3.8.2 Separation of proteins by 2-dimensional electrophoresis

Isoelectric focusing (IEF) was carried out using IPG strips (11 cm, pH 3-10, Bio-Rad) for first dimensional separation of proteins on Protean Isoelectric focusing system (Bio-Rad, CA, USA). Rehydration buffer containing 8M Urea, 2% CHAPS, 50mM DTT and 0.2% ampholytes (pH 3-10) premixed with the 200µg of protein sample was used for rehydration of IPG strips. IEF was carried out setting voltage to 250V for 1 h, ramped to 10,000 V in 3h and keeping 10,000V as end voltage the experiment was continued until it reached to 72,000Vh. Then the IPG strips were equilibrated with 10mM DTT and 55mM iodoacetamide followed by a second-dimensional electrophoresis on a 12% SDS-PAGE (11cm). To visualize protein spots gels were stained with CBB. Gel images were acquired on GS-800 (Bio-Rad) densitometer and image analysis was carried out using PDQuest advanced software of M/S Bio-Rad. Normalization factor was calculated from the total optical density of the gel, for small variations in staining or protein loads. Statistical analysis was performed by student t-test, differences were considered to be significant if $p \leq 0.05$.

3.8.3 In-gel Trypsin digestion

Gel spots were excised, cut into small pieces and destained with destaining solution containing acetonitrile and 50mM ammonium bicarbonate (1:1 v/v) then gel pieces were dehydrated with 100% acetonitrile. Proteins were reduced and alkylated with 10mM DTT and 55mM iodoacetamide respectively. Proteins were subjected to in gel-digestion for 16 h by incubating with trypsin (sigma) in the ratio of 10:1 at 37°C; peptides were extracted in buffer containing 50% acetonitrile and 5% formic acid and then reconstituted in 10 µL of 0.1% formic acid.

3.8.4 LC-MS^E analysis of proteins for their identification by Synapt HDMS system

Tryptic peptides obtained from in-gel digestion were analyzed by nano LC-MS^E work flow (MS at elevated energy) using a Nano Acquity UPLC system (Waters, Milford, MA, USA) coupled to SYNAPT-HDMS (Waters, Milford, MA, USA). The instrument was calibrated with MS/MS spectra of Glu-fibrinopeptide B (600 fmol/ µL). Tryptic peptides were reconstituted in 3% ACN and 0.1% formic acid, preconcentrated and desalted using a Symmetry C18 trapping column (20 mm x 180 µm) (Waters, Milford, MA, USA) online at a flow rate of 5µL/min with 0.1% ACN.

Further, the peptide sample separation was achieved on a C18 reversed phase BEH 130 column (1.7µm, 150mm x75µm, Waters, Milford, MA, USA) with a gradient elution of 5-40% of the mobile phase system, consisting of water with 0.1% formic acid as mobile phase A and acetonitrile with 0.1% formic acid as mobile phase B with a flow rate of 300 nL / min. The eluted peptides were allowed to ionize using NanoLock Spray ion source. MS analysis was performed in positive V mode in the mass range of *m*/*z* 50 to 2000 with a scan time of 1.0s at a resolution of about 9000 FWHM (full width half maximum). MS^E was performed by acquiring the data at constant low collision energy of 4 eV to analyse intact peptides and the high collision energy of 15 to 35 eV to generate product ions. For label free quantification 100 fmol of yeast enolase was spiked. Three most intense peptides of the spiked protein were considered for quantification.

3.8.5 Data Processing and Database Searching

Since the number of reviewed proteins from *Dunaliella* and *Chlorella* were scarce, the MS^E data was searched against the Chlorophyceae protein databank downloaded from UniProt database for protein identification using PLGS (Protein Lynx Global Server 2.4, Waters, Milford, MA, USA) as described earlier (Barvkar et al. 2012). The search parameters considered were one missed cleavage, carbamidomethylation of cysteines and possible oxidation of methionine with peptide tolerance of 20 ppm and fragment tolerance of 0.05D.

Workflow-2

Protein sample

↓

In-solution tryptic digestion

↓

IDA and SWATH analysis

↓

AB Sciex Triple TOF 5600 with Eksigent microLC 200

Fig. 6: SWATH (MS/MS^{ALL}) workflow

3.9 SWATH (MS/MS^{ALL}) workflow by Triple TOF™ 5600 System

3.9.1 Protein samples preparation

The extracted protein samples were subjected to in-solution tryptic digestion for 16 h by incubating with trypsin (sigma) in the ratio of 10:1 at 37°C, tryptic peptides were extracted in buffer containing 50% acetonitrile and 5% formic acid and then reconstituted in 10 µL of 0.1% formic acid.

3.9.2 Peptides sample preparation prior to MS analysis by Triple TOF™ 5600 System

Millipore's Zip-Tip C_{18} tips were used for desalting and enrichment of tryptic peptides. The tips were equilibrated by washing with 100% acetonitrile, 5 to 6 times and then with 0.1% of TFA, 5 to 6 times. In solution digested peptide samples were allowed to bind to Zip-Tips by aspiration. Zip-Tips were desalted by washing with 0.1%TFA. Bound peptides were eluted with 50% acetonitrile containing 0.5% formic

acid. Collected eluate was dried by speed vac and reconstituted in 20µL of 3% acetonitrile before analysing on AB SCIEX Triple TOF 5600 Mass Spectrometer.

3.9.3 IDA and SWATH analysis of peptides for the identification and quantification of proteins by Triple TOF™ 5600 System

For IDA run and SWATH run, 4µg and 1.25µg of peptide digest were loaded respectively on the column and analysed using micro LC system coupled to AB SCIEX Triple TOF™ 5600 system in triplicates. After each injection syringe was washed twice. Separation of peptides was achieved on micro-LC system by using a C18 reverse phase column (3µm, 100mmx0.3mm, 120 Å, AB SCIEX, Singapore) with a gradient elution (3-50%) of the mobile phase system consisting of water with 0.1% formic acid as mobile phase A and acetonitrile with 0.1% formic acid as mobile phase B at a flow rate of 8 µL/min. Analyst TF V1.6 software was used for instrument control, data acquisition and data processing.

Mass spectrometric analysis of IDA run and SWATH run were carried out in high sensitive positive ionization mode in the mass range of *m/z* 350 to 1250Da, 120cps with accumulation times of 0.249999s (IDA run) and 0.050001s (SWATH run). The MS/MS analysis was also carried out in high sensitive positive ionization mode and in the mass range of m/z 100 to 1600Da with accumulation time of 0.070028s (IDA run) and 0.073099s (SWATH run). The charge state was 2 to 5.

3.9.4 Data Processing and Database Searching

The Triple TOF IDA and SWATH analysis data were searched against Chlorophyceae protein databank downloaded from UniProt. IDA data was processed using Protein Pilot software 4.0 (AB SCIEX) to generate spectral library for SWATH based quantification. Using this spectral library, SWATH data analysis was performed using PeakView (AB SCIEX) software (Lambert et al. 2013). The data was further analysed using Markerview software V 1. 2. 1 (AB SCIEX) to obtain statistical data. Statistical analysis was performed by student t-test and protein ids with probability (p) value less than 0.05 were considered to be significant.

3.10 Microarray Data Analysis of *Dunaliella*

Raw Data from GEO database with series GSE10271 was taken for analysis using GeneSpring GX software from Agilent. The Microarray experiment carried for

GSE10271 belongs to two color experiment. Therefore, normalization of the data was done in GeneSpring GX using the Lowess normalization method. In two-color experiments, where two fluorescent dyes (red and green) have been used, intensity-dependent variation in dye bias may introduce spurious variations in the collected data. Lowess normalization merges two-colour data, applying a smoothing adjustment that removes such variation. The fold expression values obtained for all the genes are in terms of logbase2. Differential expression patterns were identified among the samples. Significant genes up regulated fold > 0.8 (logbase2) and down regulated <-0.8 (logbase2) in the test samples with respect to control sample were identified. Statistical student T-test p-value among the replicates was calculated based on volcano plot algorithm. Differentially regulated genes were clustered using hierarchical clustering based on Pearson coefficient correlation algorithm to identify significant gene expression patterns. The Significant Functional classification of differentially regulated genes was performed using GeneSpring GX software gene ontology.

Chapter 4

Results

4.1 Identification and Characterization of *Dunaliella* sp. BSK

The isolated marine algae collected from marine water ponds of Alibagh was identified based on 18s rRNA sequence analysis. The 18S rRNA sequence of isolated strain was a continuous stretch of 1349 bp and identified as *Dunaliella* on the basis of NCBI search blast. BLAST analysis suggested that the isolated alga is closely related to *Dunaliella* sp. and shows 99% sequence similarity. The18s rRNA sequence has been submitted to NCBI with accession number KF899830.1. Phylogenetic tree analysis provided the information about its relatedness to different species of *Dunaliella* (Fig. 7). The isolated alga is referred as *Dunaliella* sp. BSK.

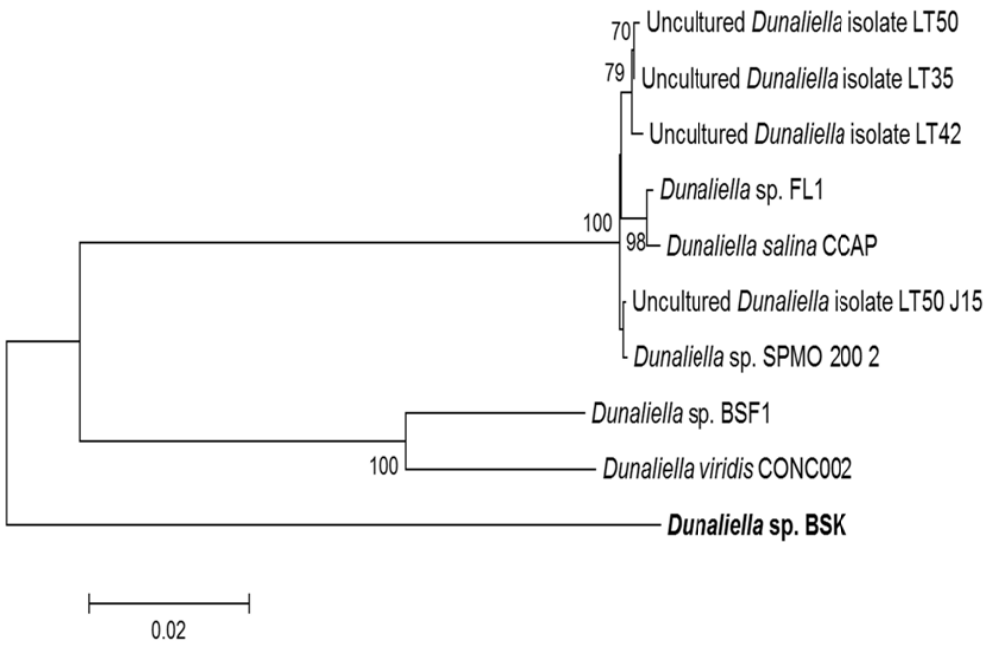


Fig. 7: Phylogenetic tree of *Dunaliella* sp. BSK with distance similarity

4.2 Scanning Electron Microscope analysis

Scanning Electron Microscope analysis has shown that *Dunaliella* sp.BSK is a unicellular, rod shaped biflagellate alga and *Chlorella pyrenoidosa* is a unicellular green alga which is spherical in shape (Fig. 8).

(1) (2)

Fig. 8: Scanning Electron Microscope images of (1) *Dunaliella* sp.BSK and (2) *Chlorella pyrenoidosa* (2) grown in their respective control media (analysed on E-SEM, Quanta zero-3D)

4.3 General physiological response to salt stress

4.3.1 Growth and glycerol content of *Dunaliella* sp.BSK and *Chlorella pyrenoidosa*

The *Dunaliella* sp. BSK has shown significant increase in glycerol content and cell growth with increasing salt concentration (0.2, 0.5, 0.8, 1.0, 1.5 and 2.0M NaCl) (Fig. 9). However, the levels of cell growth and glycerol content decreased considerably with increasing NaCl concentration (0, 0.05, 0.10, 0.15, 0.2 and 0.4M) in case of *Chlorella pyrenoidosa* (Fig. 10).

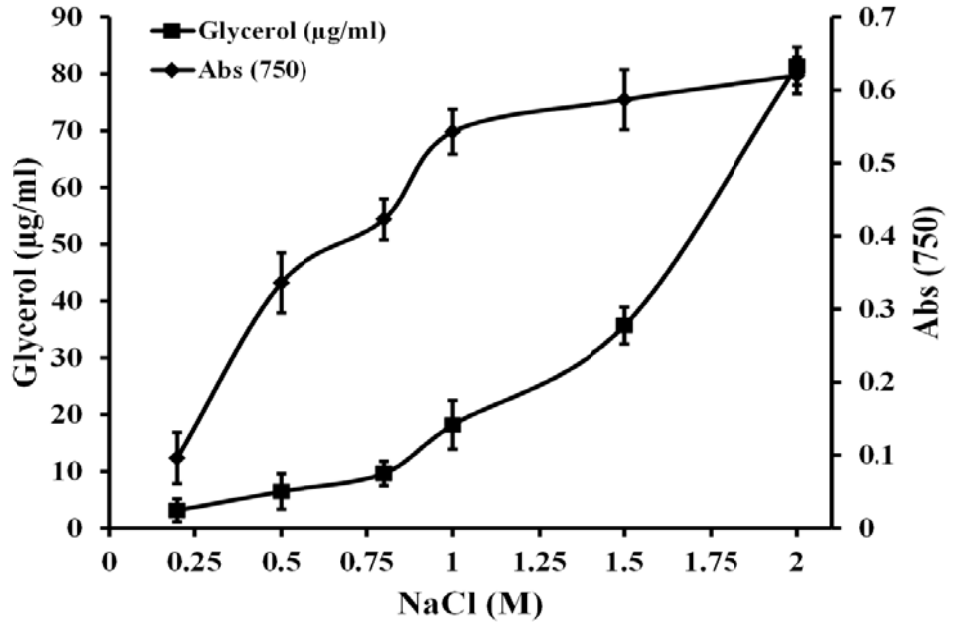


Fig. 9: Growth and Glycerol content of *Dunaliella* sp. BSK whole cells grown in the presence of various salt concentrations for a period of 20days.

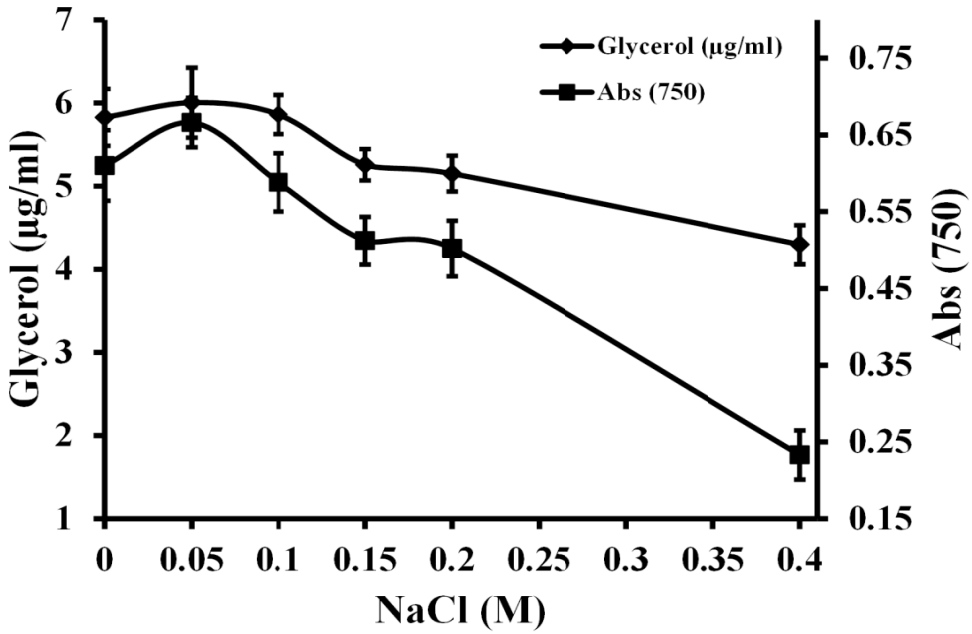


Fig. 10: Growth and Glycerol content of *Chlorella pyrenoidosa* whole cells grown in the presence of various salt concentrations for a period of 20 days.

4.3.2 Pigment analysis of *Dunaliella* sp.BSK

The pigment analysis of 20 days old cultures of *Dunaliella* sp. BSK grown in different NaCl concentrations (0.2, 0.5, 0.8, 1.0, 1.5 and 2.0M NaCl grown) has shown increasing trend in the contents of chlorophylls and carotenoids (Fig. 11) with increasing salt concentration.

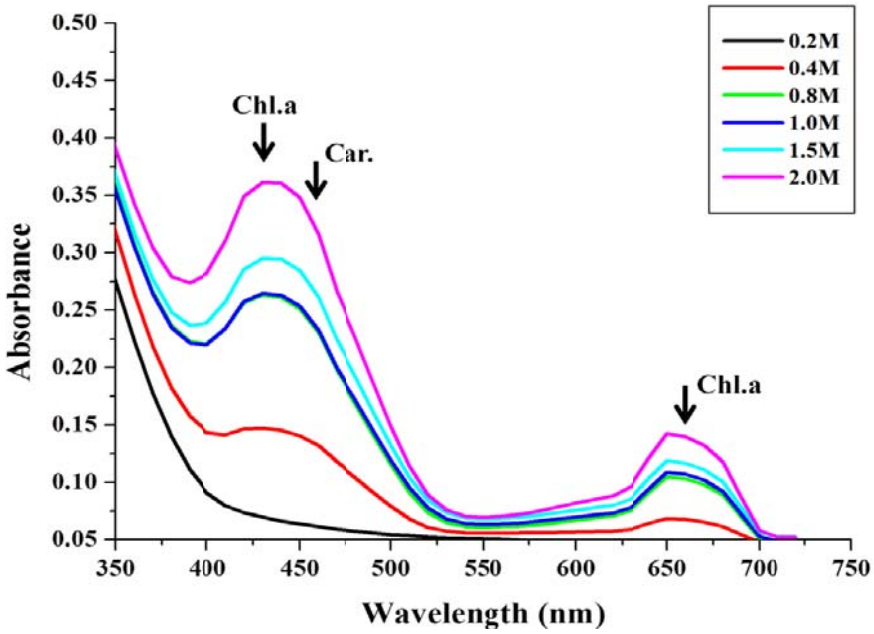


Fig. 11: Spectra of the extracted pigments in THF from *Dunaliella* sp. BSK, whole cells grown in the presence of various salt concentrations for 20 days.

4.4 Proteomic analysis for the Identification of total proteins and differentially expressed proteins of *Dunaliella* sp. BSK and *Chlorella pyrenoidosa* under salt stress

In order to compare the adaptation mechanisms to salt stress in *Dunaliella* sp. BSK and *Chlorella pyrenoidosa* a proteomic study was carried out. Together 559 proteins were identified from both D*unaliella* sp. BSK and *Chlorella pyrenoidosa* by following LC-MS^{E} and SWATH work flows and the identified protein data is provided in annexure as mentioned in the Table 7.

1) LC-MS^E work flow by Synapt HDMS system

2) SWATH (MS/MS^{ALL}) workflow by Triple TOF™ 5600 System.

Table 7: Protein Identification data

Annexure	ID's
I	Total protein ID's of *Dunaliella* sp. BSK, which were separated by SDS-PAGE and identified by Synapt HDMS
II	Total protein ID's of *Chlorella pyrenoidosa*, which were separated by SDS-PAGE and identified by Synapt HDMS
III	Protein ID's of *Dunaliella* sp. BSK, which were separated by 2-dimensional electrophoresis and identified by Synapt HDMS
IV	Total protein ID's of *Dunaliella sp. BSK* by SWATH analysis on Triple TOF MS
V	Total protein ID's of *Chlorella pyrenoidosa* by SWATH analysis on Triple TOF MS

4.4.1 Functional Annotation of identified proteins of *Dunaliella* sp. BSK and *Chlorella pyrenoidosa*

The identified proteins were functionally annotated as per gene ontology terms at UNIPROT site. The bar diagrams Fig. 12 and Fig. 13 show the distribution of GO annotation in the three functional categories of Molecular functions, cellular components and biological processes for *Dunaliella* sp. BSK and *Chlorella pyrenoidosa* respectively. These proteins comprised of central metabolic pathways, such as metabolic, cellular, single-organism and developmental process, localization, response to stimulus, cellular component organization or biogenesis, biological regulation, signal transduction, epidermal cell fate specification and meiotic cell cycle.

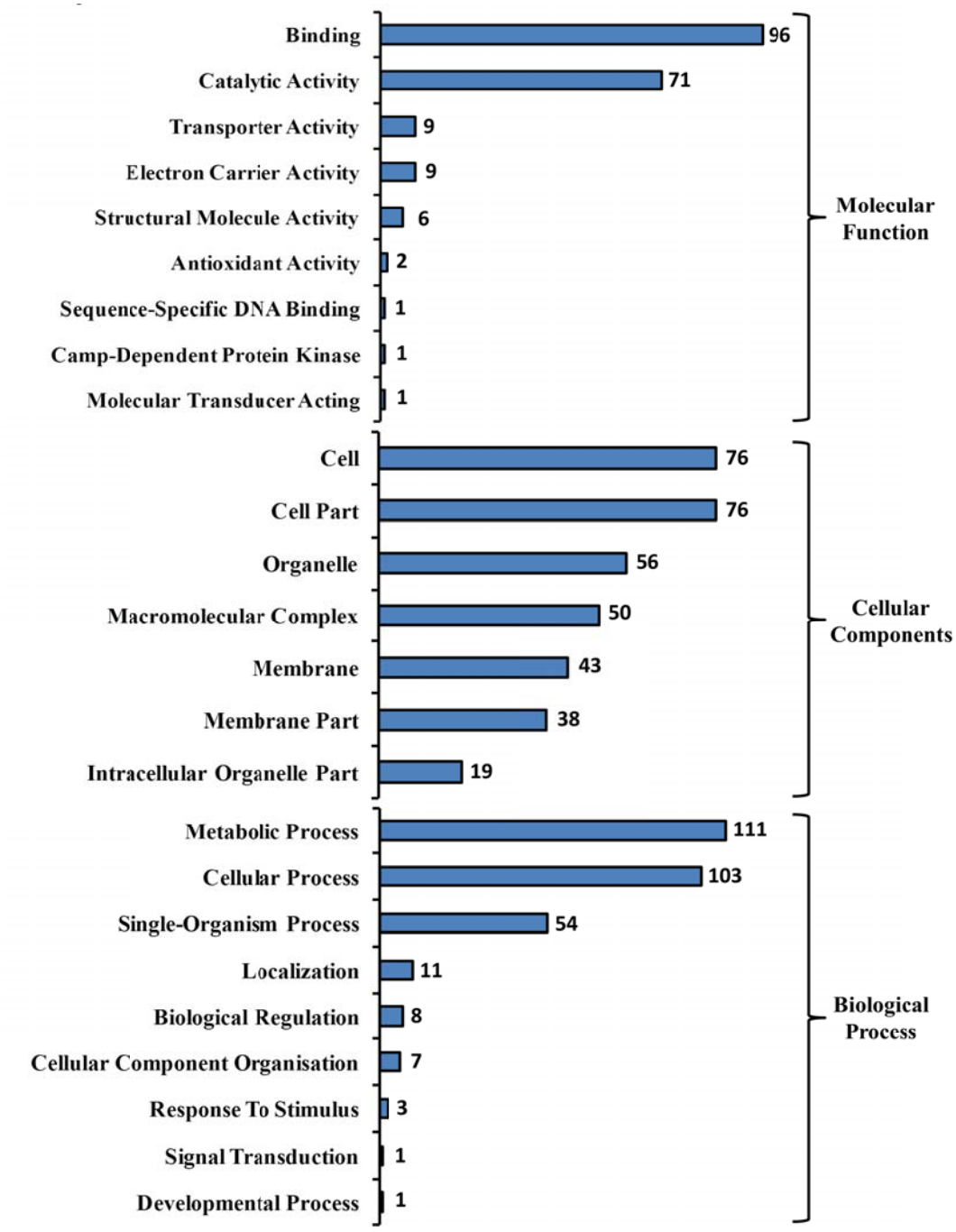


Fig. 12: Gene Ontology analysis of proteins identified in *Dunaliella sp. BSK*: The GO categories are Molecular functions, Cellular components and Biological processes.

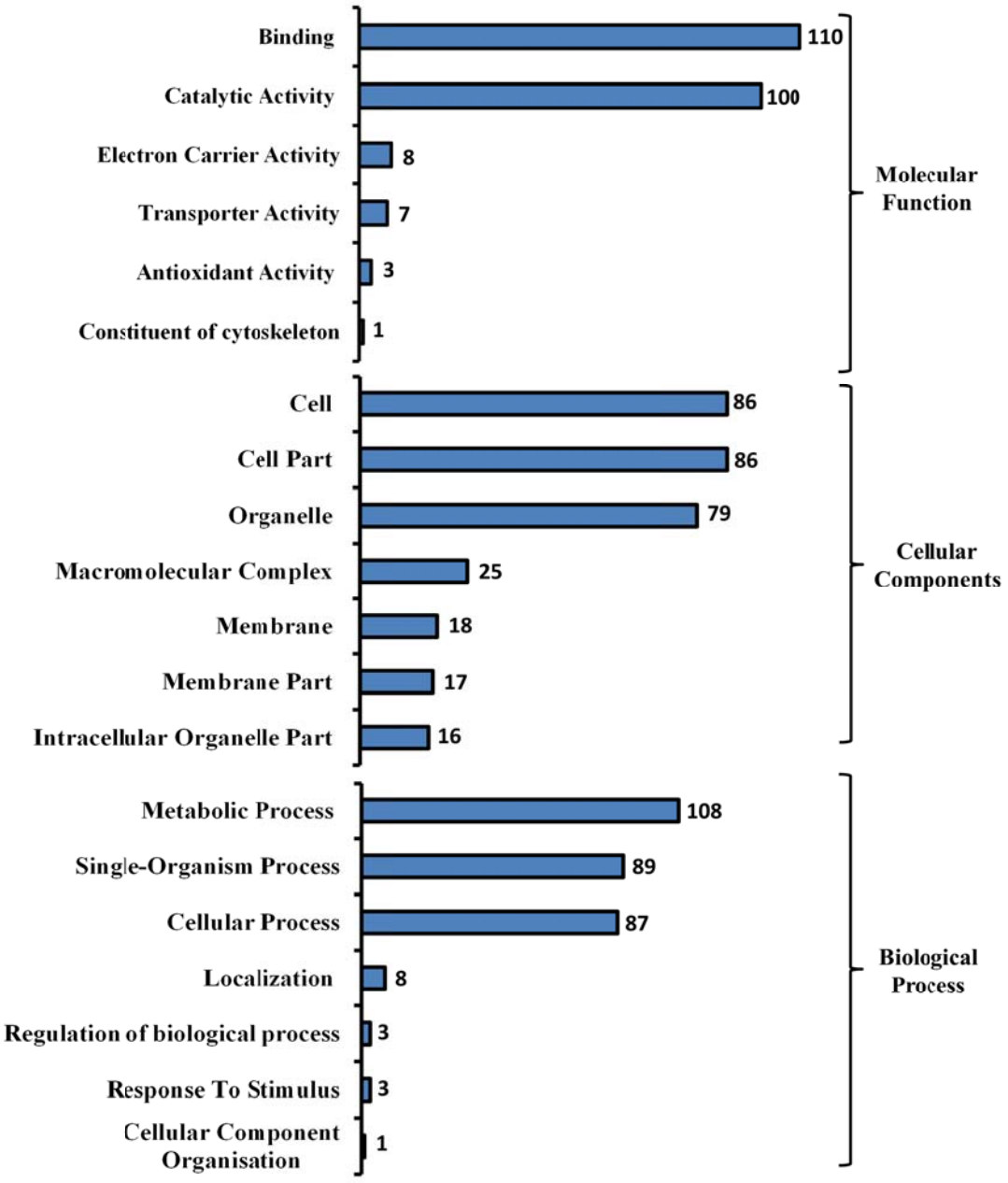


Fig. 13: Gene Ontology analysis of proteins identified in *Chlorella pyrenoidosa*: The GO categories are Molecular functions, Cellular components and Biological processes.

4.4.2 Differential expression studies of *Dunaliella* sp. BSK by SWATH analysis

Proteomic analysis of *Dunaliella* sp. BSK grown in 0.5 M NaCl and 2.0 M NaCl was performed by SWATH and the differentially expressed proteins are listed in Table 8. Totally 27 proteins were found to be differentially expressed. Among these seven proteins were up regulated and 20 proteins were down regulated.

Table 8: Differentially expressed proteins of *Dunaliella* sp. BSK under salt stress (0.5M→2.0M)

Accession	Protein	Fold Change	Function
K7NRF9	Photosystem I iron-sulfurcenter *(Dunaliella tertiolecta)*	9.98	Photosynthesis
F4ZL30	Peroxiredoxin type II (*Dunaliella viridis)*	6.99	Oxidative stress
V5J1A2	Carotene globule protein (*Dunaliella bardawil*)	4.59	Stress response
G4WEL1	Ribulosebisphosphate carboxylase small chain (*Dunaliella parva*)	4.50	Carbon fixation
Q8VY41	Heat shock protein 70B (*Dunaliella salina*)	2.44	Stress response
D0FXY0	ATP synthase subunit beta, chloroplastic (*Dunaliella salina*)	2.06	ATP synthesis
Q8HDD9	ATP synthase subunit beta (Fragment) (*Dunaliella parva)*	1.94	ATP synthesis
D0FXZ7	Ribulose bisphosphate carboxylase large chain (*Dunaliella salina*)	0.53	Carbon fixation

C1K004	Photosystem I light-harvesting chlorophyll-a/b protein 3 (*Dunaliella salina*)	0.52	Photosynthesis
G4WUV7	Alpha-tubulin protein (*Dunaliella salina*)	0.48	Structural protein
G4WUW2	Ribulose-phosphate 3-epimerase (*Dunaliella salina*)	0.47	CO2 fixation
A7Y228	Histone H4 (Fragment) (*Dunaliella salina*)	0.44	Structural ptotein
Q39708	Calmodulin-like protein (*Dunaliella salina*)	0.42	Signalling
C5H3W1	Glycerol-3-phosphate dehydrogenase (*Dunaliella viridis)*	0.41	Lipid biosynthesis
A8BDJ0	Major light-harvesting chlorophyll a/b protein 3 (*Dunaliella salina*)	0.40	Photosynthesis
Q1HVA0	Chloroplast glyceraldehyde-3-phosphate dehydrogenase (*Dunaliella viridis*)	0.39	Carbon metabolism
B1PL92	Glyceraldehyde-3-phosphate dehydrogenase (*Dunaliella salina*)	0.38	Carbon metabolism
A1XKU7	Major light-harvesting chlorophyll a/bprotein2.2 (*Dunaliella salina*)	0.36	Photosynthesis
E2E6H3	Vacuolar H+-pyrophosphatase (*Dunaliella viridis*)	0.32	ATP synthesis
Q9ATJ1	Fructose-bisphosphate aldolase (*Dunaliella salina*)	0.29	CO2 fixation
K7NU01	Photosystem I P700 chlorophyll a apoprotein A1 (Fragment) (*Dunaliella tertiolecta*)	0.27	Photosynthesis

K7NU68	Photosystem I P700 chlorophyll a apoprotein A2 (*Dunaliella tertiolecta*)	0.23	Photosynthesis
A7Y226	Histone H3 (Fragment) (*Dunaliella salina*)	0.23	DNA binding, nucleosome assembly
Q8RY44	Heat shock protein 70a (*Dunaliella salina*)	0.22	Stress response
G4WUW1	Chloroplast minor chlorophyll a-b binding protein of photosystem II (Fragment) (*Dunaliella salina*)	0.21	Photosynthesis
K7NSQ0	Elongation factor Tu (*Dunaliella tertiolecta*)	0.20	Protein biosynthesis
K7NU65	Cytochrome b6 (*Dunaliella tertiolecta*)	0.04	Respiration

The up regulated proteins of *Dunaliella* sp. BSK comprised of mainly stress responsive proteins such as peroxiredoxin, heat shock protein, carotene globular protein, and proteins associated with photosynthesis and carbon fixation namely Photosystem I iron-sulfur protein, Rubisco small subunit and ATP synthase (Fig. 14). While the down regulated proteins mainly comprised of proteins involved in light reaction of photosynthesis such as Major Light Harvesting complex proteins, Photosystem I P700 Chlorophyll a apolipoprotein 1 and Photosystem I P700 Chlorophyll a apolipoprotein 2 Chloroplast minor chlorophyll a-b binding protein of photosystem II (Fragment); enzymes involved in Calvin cycle such as Rubisco large subunit, glyceraldehyde 3 Phosphate dehydrogenase and Fructose-bisphosphate aldolase and Cytochrome b6, which is involved in respiration (Fig. 15).

These differential expressions of *Dunaliella* sp. BSK proteins were manually validated by the extracted ion chromatograms (XIC's) of peptides. Figures 14a, 14b,

14c, and 14d represent peptide XIC's of up regulated proteins and figures 15a, 15b, and 15c are the peptide XIC's of down regulated proteins.

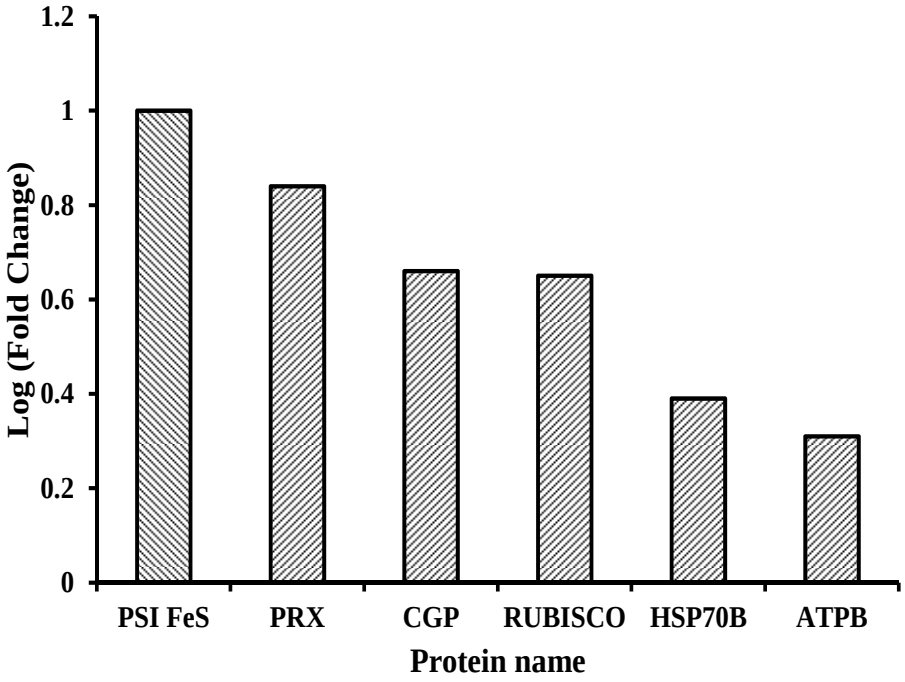


Fig. 14: The up regulated proteins of *Dunaliella* sp. BSK and their Log (Fold) change

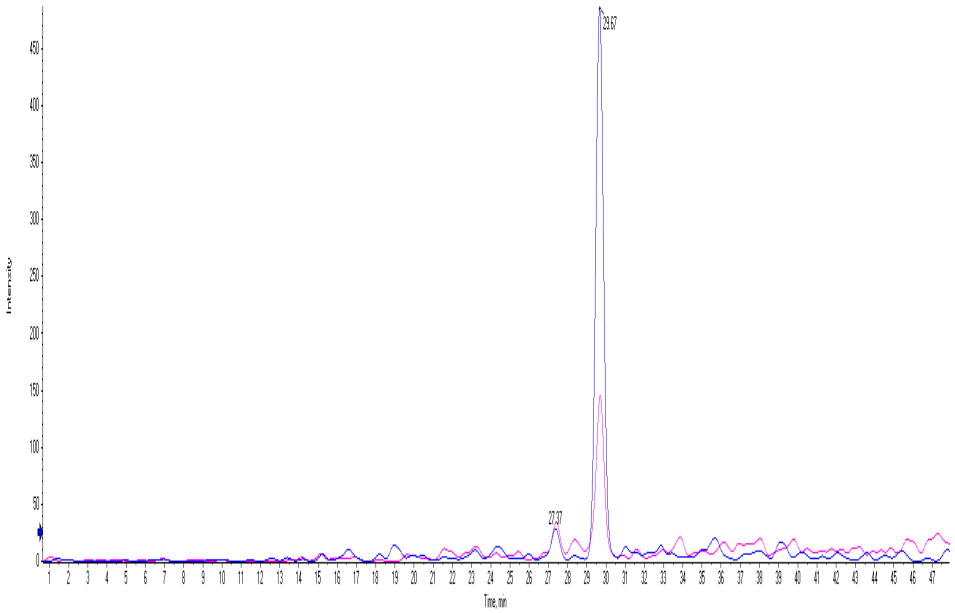


Fig. 14a: Peptide XIC of Photosystem I iron-sulfurcenter (m/z=778.3476, Z=2, RT=29.67), pink chromatogram (low salt) and blue chromatogram (high salt)

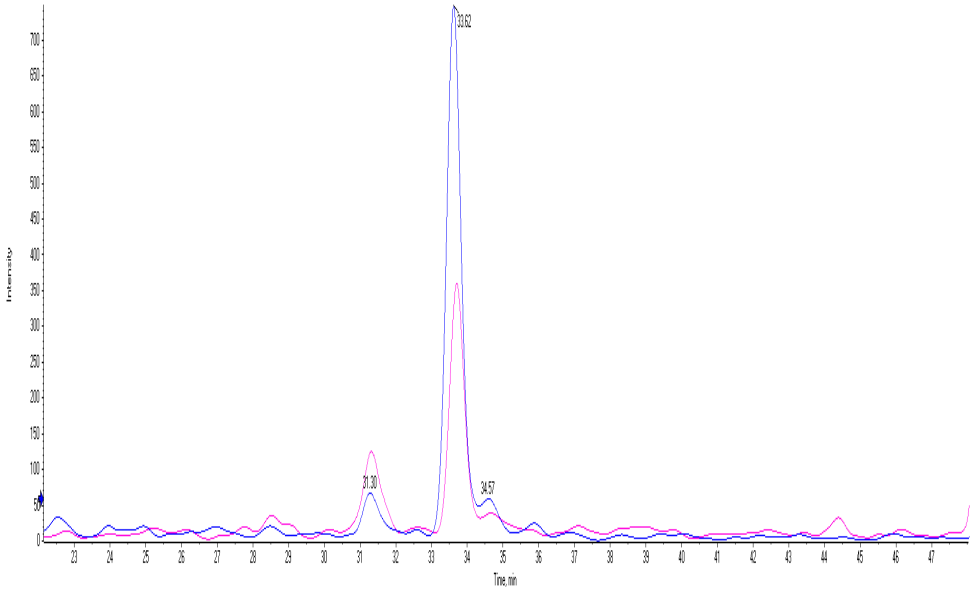


Fig. 14b: Peptide XIC of Ribulosebisphosphate carboxylase small chain (m/z=865.4722, Z= 2, RT=33.62), pink chromatogram (low salt) and blue chromatogram (high salt)

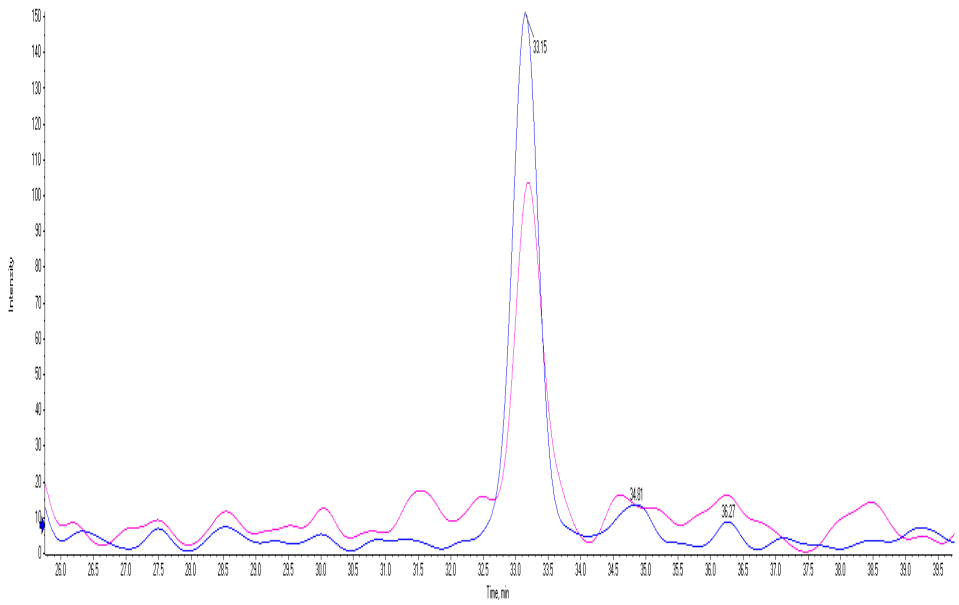


Fig. 14c: Peptide XIC of Heat shock protein 70B (m/z=505.8215, Z= 2, RT=33.15), pink chromatogram (low salt) and blue chromatogram (high salt)

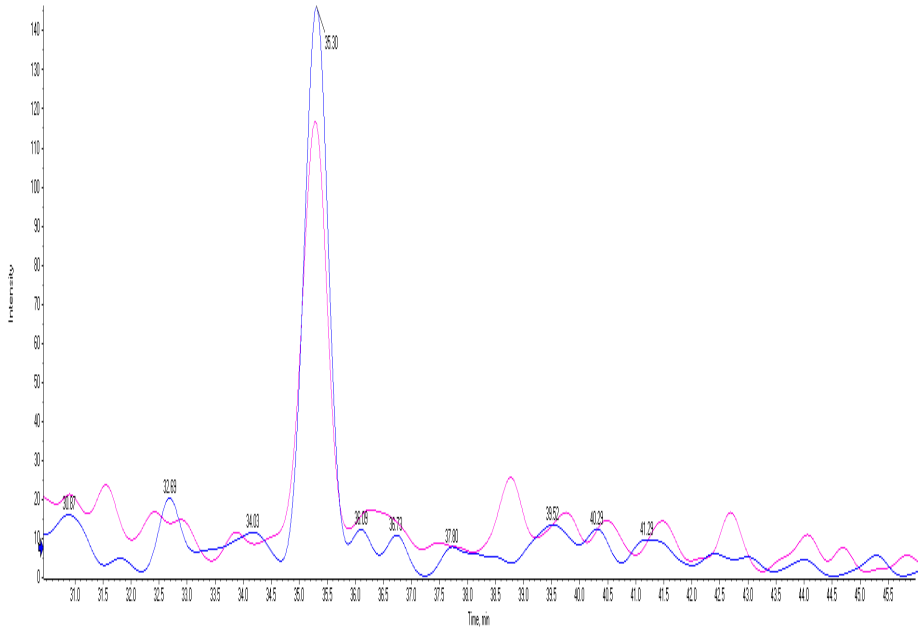


Fig. 14d: Peptide XIC of ATP synthase subunit beta (Fragment) (m/z=770.4021, Z= 3, RT=35.30), pink chromatogram (low salt) and blue chromatogram (high salt)

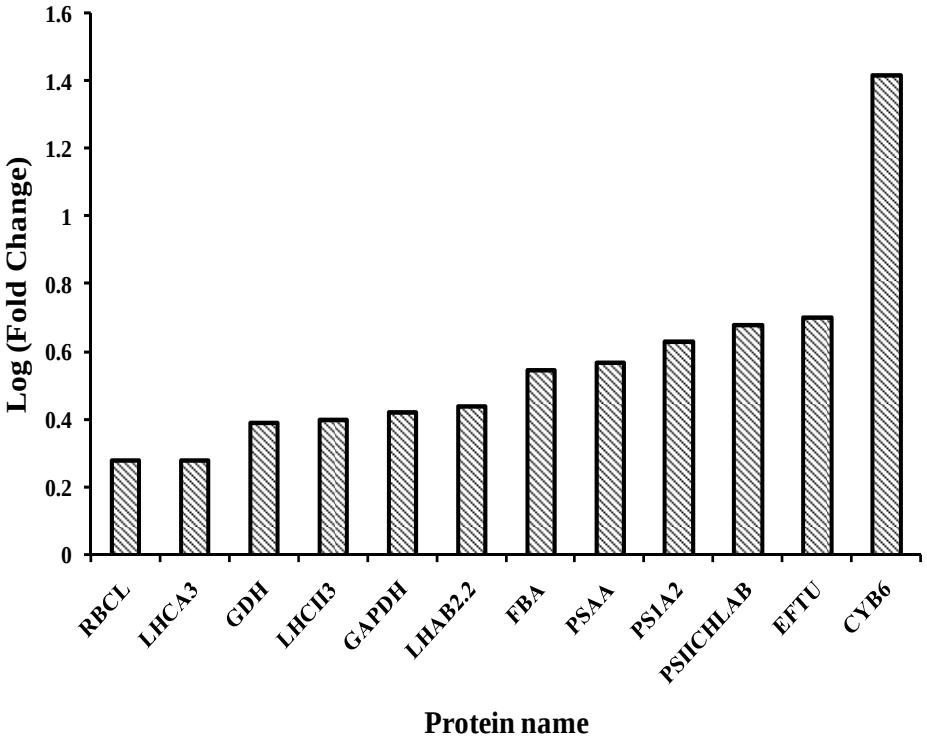


Fig. 15: The down regulated proteins of *Dunaliella* sp. BSK and their Log (Fold) change

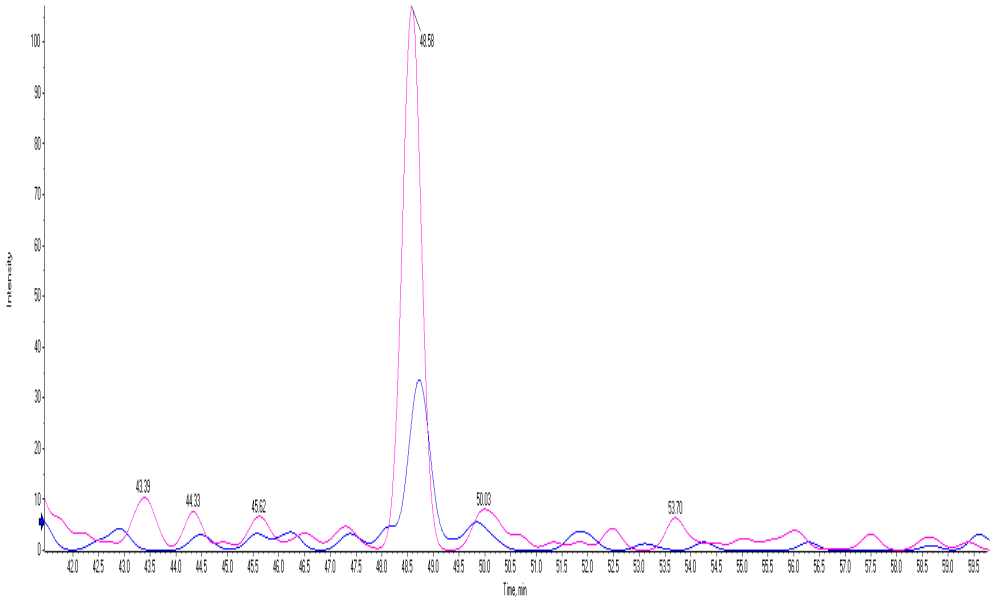


Fig. 15a: XIC of Vacuolar H+-pyrophosphatase peptide (m/z=686.3719, Z=2, RT=48.58), pink chromatogram (low salt) and blue chromatogram (high salt)

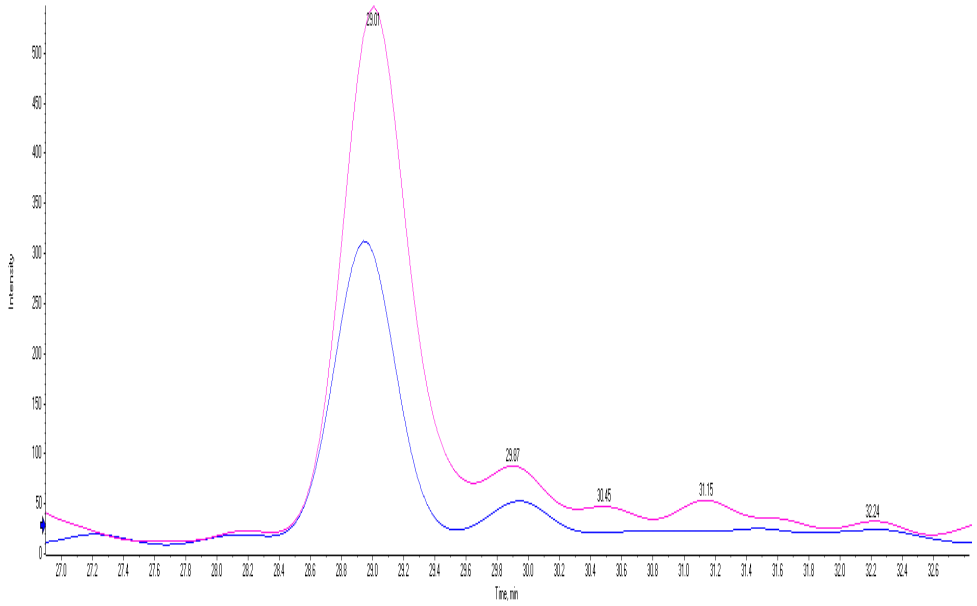


Fig. 15b: Peptide XIC of Major light-harvesting chlorophyll a/b protein 3 (m/z= 522.2902, Z=2, RT=29.07), pink chromatogram (low salt) and blue chromatogram (high salt)

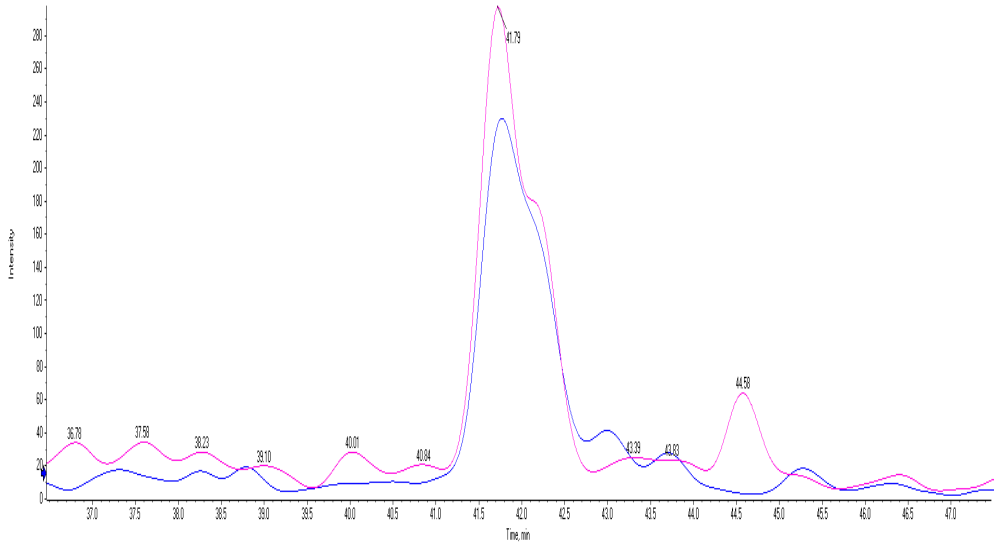


Fig. 15c: Peptide XIC of Calmodulin-like protein (m/z=681.8321, Z=2, RT=41.79), pink chromatogram (low salt) and blue chromatogram (high salt)

4.4.3 Differential expression studies of *Dunaliella* sp. BSK identified by LC-MS^E analysis from 2-D tryptic peptides

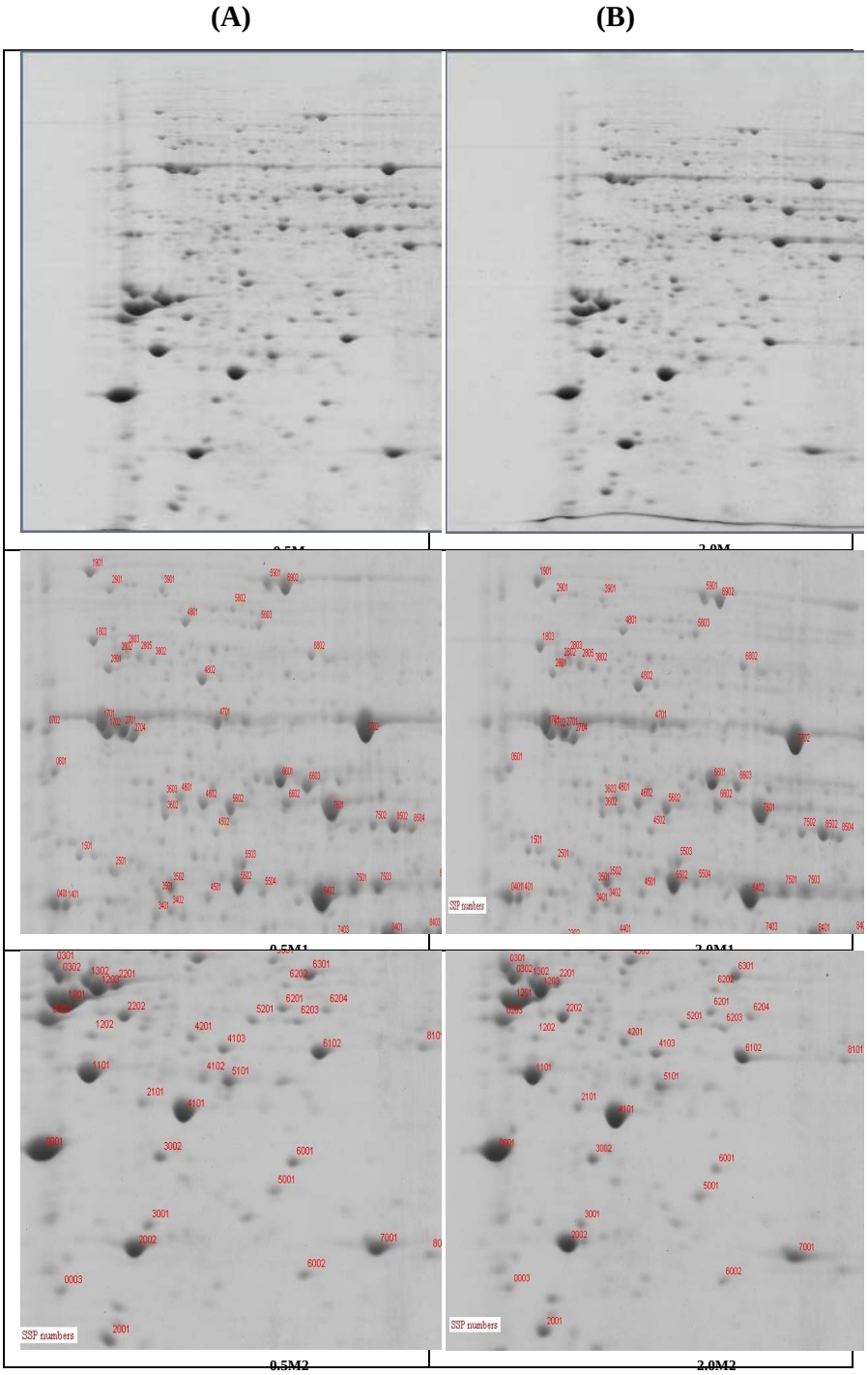


Fig. 16: Representative 2DE gel patterns of *Dunaliella* sp. BSK (A) 0.5MNaCl, (B) 2.0MNaCl

The representative 2DE gel images and their spot labelled images are provided in Figure 16. The two bottom images below (A) and (B) are the spot labelled images which were divided into two parts for convenience. Proteomic analysis of 2-D tryptic peptides of *Dunaliella* sp. BSK grown in 0.5 M NaCl and 2.0 M NaCl was performed by LC-MS^{E} analysis. From the identified proteins twelve proteins found to be differentially expressed, among them four were up regulated and eight our down regulated. The differentially expressed proteins, quantity graph and gel protein spot images are provided in Table 9a and 9b.

Table 9a: Upregulated proteins of *Dunaliella* sp. BSK identified by 2D (LC-MS^{E}) analysis (0.5M→2.0M)

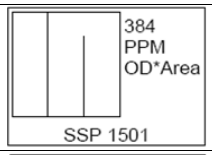
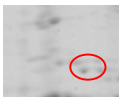
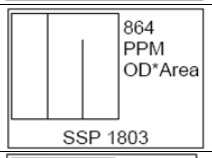
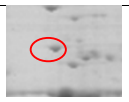
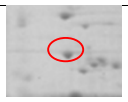
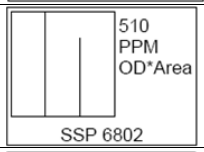
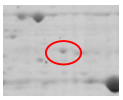
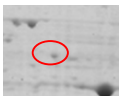
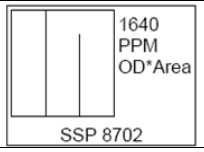
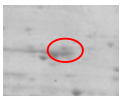
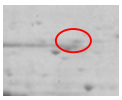
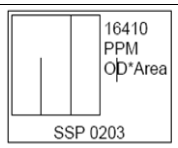
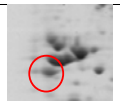
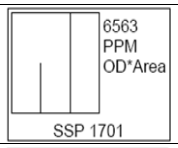
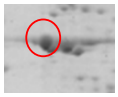
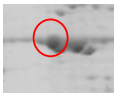
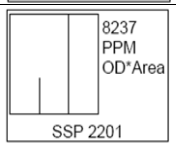
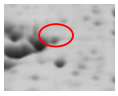
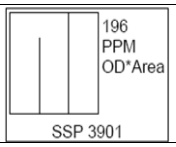
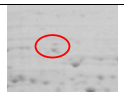
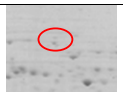
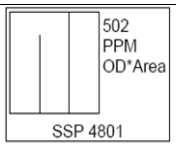
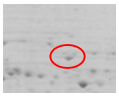
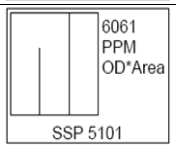
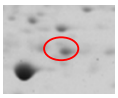
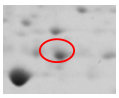
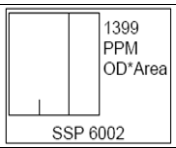
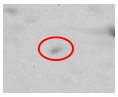
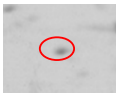
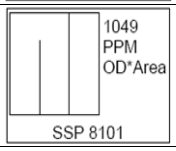
Sl no	Protein Identified	Quantity in 2.0M	Quantity in 0.5M		2.0M	0.5M
1	Magnesium chelatase subunit I A8IMZ5 CHLRE	384.5↑	295.5			
2	ATP synthase subunit beta A0ZW41 9CHLO	863.7↑	648.5			
3	Transketolase A8IAN1 CHLRE	510.5↑	381.5			
4	Ribulose 1 5 bisphosphate carboxylase oxygenase large D0FXZ7 DUNSA	1640.1↑	1261.0			

Table 9b: Down-regulated proteins of *Dunaliella* sp. BSK identified by 2DE (LC-MS^{E}) analysis

Sl no	Protein Identified	Quantity in 2M	Quantity in 0.5M		2M	0.5M
1	Major light harvesting chlorophyll a b protein 2 A1XKU6 DUNSA	9228.6↓	16410.4			
2	Beta tubulin Fragment Q4JJJ6 DUNSA	3245.2↓	6562.5			
3	Major light harvesting chlorophyll a b protein 2 1 A1XKU6 DUNSA	2931.9↓	8236.9			
4	Heat shock protein 70a Q8RY44 DUNSA	145.3↓	195.5			
5	Chloroplast heat shock protein 70B Fragment B7TJI1 DUNSA	385.2↓	502.4			
6	Major light harvesting chlorophyll a b protein 3 A8BDJ0 DUNSA	3940.3↓	6061.1			
7	Nucleoside diphosphate kinase A8J9H8 CHLRE	211.0↓	1398.6			
8	Global transcription factor Fragment A8HQJ4 CHLRE	765.1↓	1049.5			

4.4.4 Differential expression studies of *Chlorella pyrenoidosa* by SWATH analysis

Proteomic analysis of *Chlorella pyrenoidosa* grown in Fog's Medium without NaCl or with NaCl (0.2M) was performed by SWATH analysis and the differentially expressed proteins are listed in Table 10. From the identified proteins, 10 proteins were found to be differentially expressed, among them 6 proteins were up regulated and 4 proteins were down regulated.

Table 10: Differentially expressed proteins of *Chlorella pyrenoidosa* under salt stress

Accession	Protein	Fold change	Function
E1ZJM1	Tubulin beta chain (*Chlorella variabilis*)	34.90	Microtubule –based process, protein polymerization
E1Z345	Histone H2A (*Chlorella variabilis)*	20.57	Nucleosome assembly
E1ZE06	S-adenosyl methionine synthase (*Chlorella variabilis*)	4.42	S-adenosyl methionine biosynthetic process
L0N3V0	Ribulose-bisphosphate carboxylase large subunit (Fragment) (*Chlorococcum sp.*)	3.28	Carbon fixation
Q0H702	Superoxide dismutase OS=*Chlorella pyrenoidosa*	1.63	catalytic activity
E3SC29	Oxygen-evolving enhancer protein (Fragment) OS=*Parachlorella kessleri*	0.71	Photosystem

F2YGK8	Photosystem I iron-sulfur center OS=Chlorella variabilis	0.69	Photosystem
E1ZSK9	Peptidyl-prolyl cis-trans isomerase (Fragment) (*Chlorella variabilis*)	0.47	Protein folding
Q9S8L3	Flavodoxin I (Fragment) (*Chlorella fusca*)	0.05	FMN binding Oxido-reductase activity

The up regulated proteins (Fig. 17) of *Chlorella pyrenoidosa* were found to be mainly involved in carbon fixation, protein polymerization, nucleosome assembly and oxidative stress. Rubisco, an important protein involved in carbon fixation was up regulated. In addition, Tubulin beta chain and Histone H2A were up regulated. S-adenosylmethionine synthase (SAM), a rate limiting enzyme involved in methionine biosynthesis cycle was up regulated. Superoxide dismutase (SOD) was up regulated, involved in reducing the oxidative stress. While the down regulated proteins were mainly involved in photosynthesis, protein folding and FMN binding. Proteins such as Flavodoxin I, peptidyl-prolyl cis-trans isomerase, Photosystem I iron-sulfur center , and Oxygen-evolving enhancer protein were down regulated (Fig. 18).

The differentially expressed proteins of *Chlorella pyrenoidosa* were manually validated by the peptide XIC's (extracted ion chromatograms). Figures 17a, 17b and 17c are the peptide XIC's of up regulated proteins and figures 18a, 18b, 18c and 18d are the peptide XIC's of down regulated proteins.

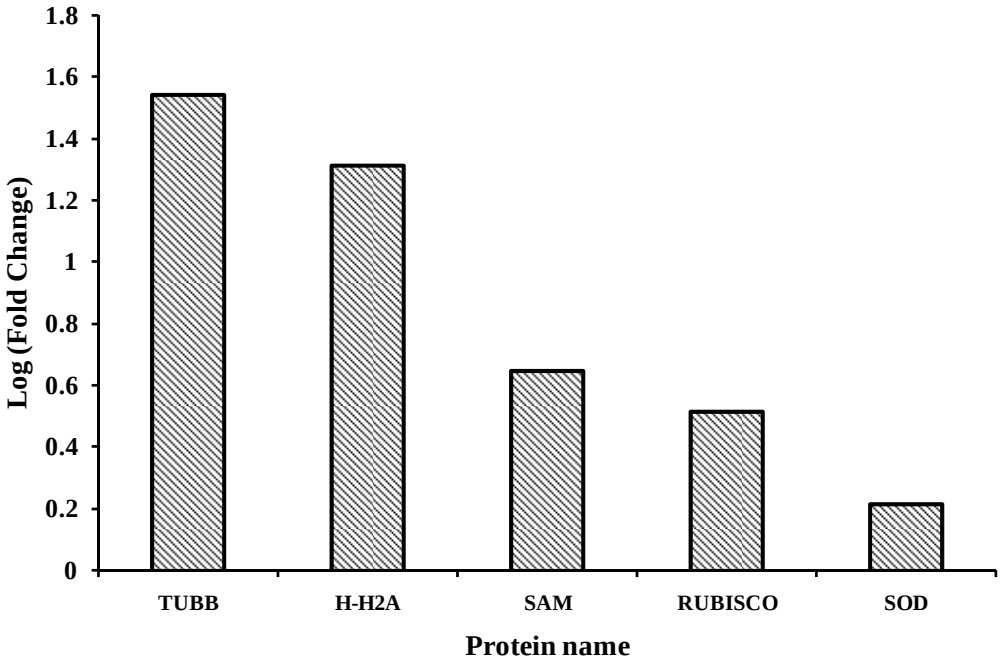


Fig. 17: The up regulated proteins of *Chlorella pyrenoidosa* and their Log (Fold) change

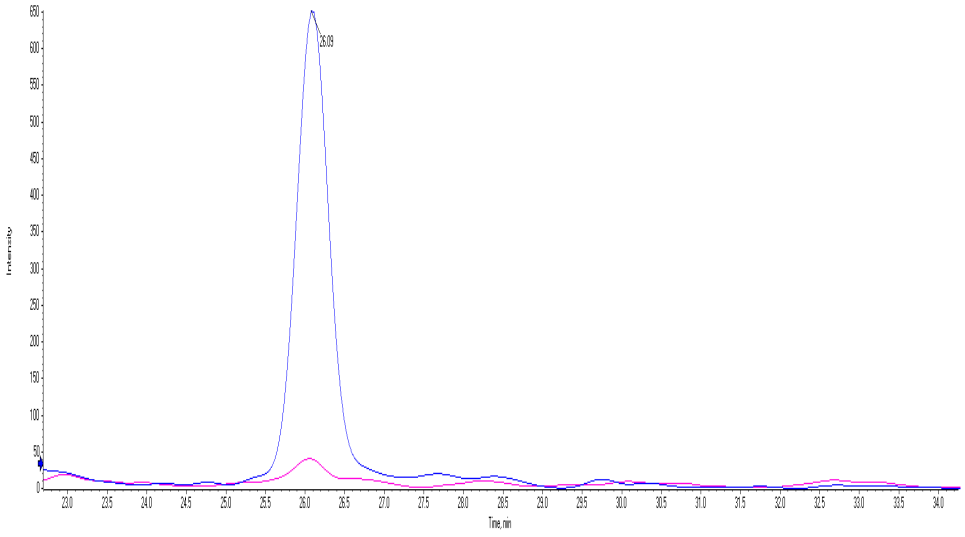


Fig. 17a: Peptide XIC of Histone H2A (m/z=582.3283, Z=2, RT= 26.09), pink chromatogram (low salt) and blue chromatogram (high salt)

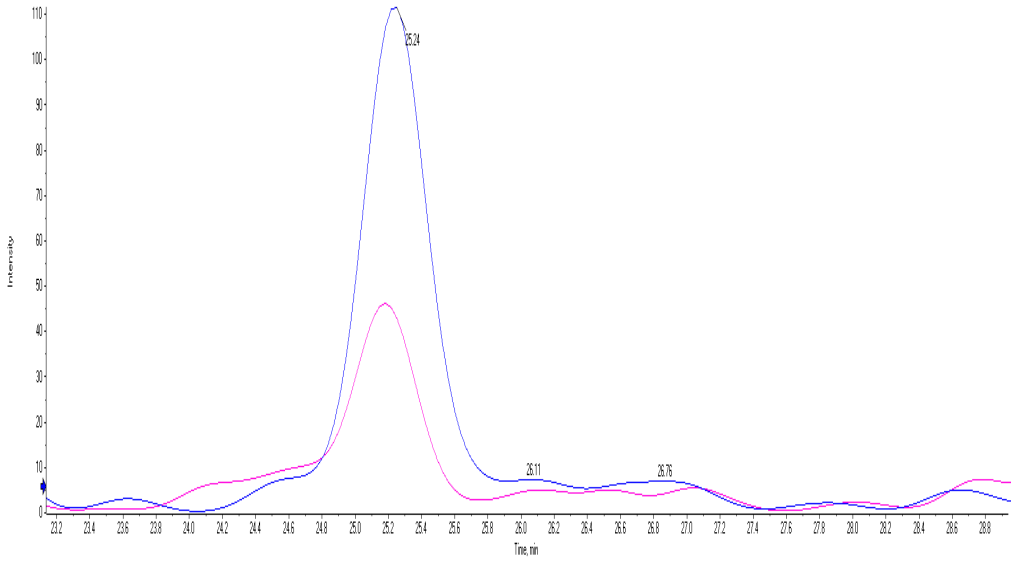


Fig. 17b: Peptide XIC of S-adenosyl methionine synthase (m/z=583.5495, Z=4, RT=25.24), pink chromatogram (low salt) and blue chromatogram (high salt)

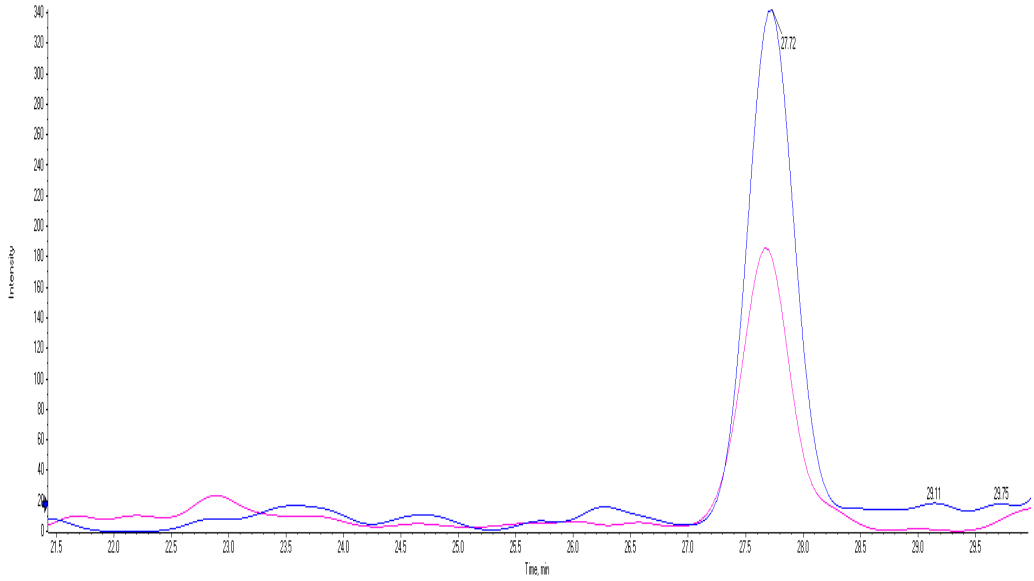


Fig. 17c: Peptide XIC of Ribulose-bisphosphate carboxylase large subunit (Fragment) (m/z= 681.3530, Z=2, RT=27.72), pink chromatogram (low salt) and blue chromatogram (high salt)

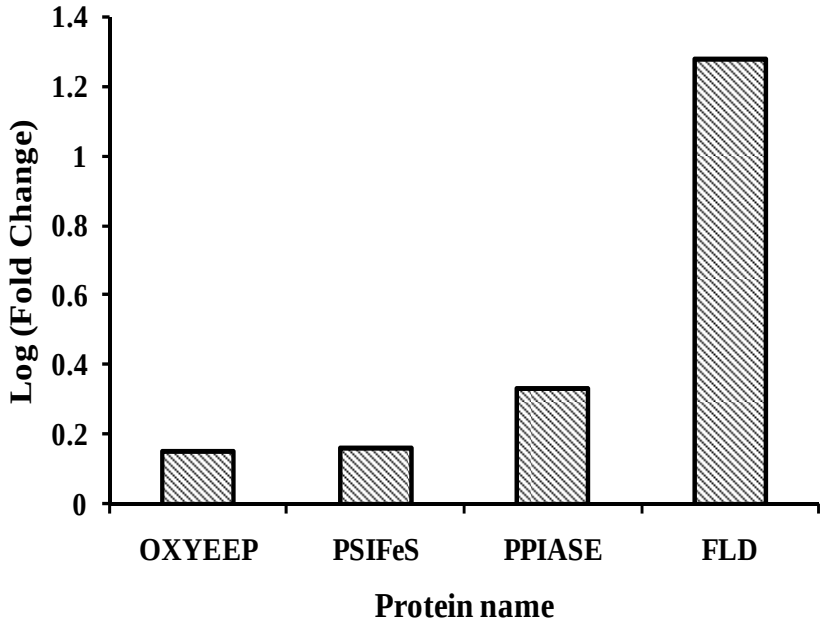


Fig. 18: The down regulated proteins of *Chlorella pyrenoidosa* and their Log (Fold) change

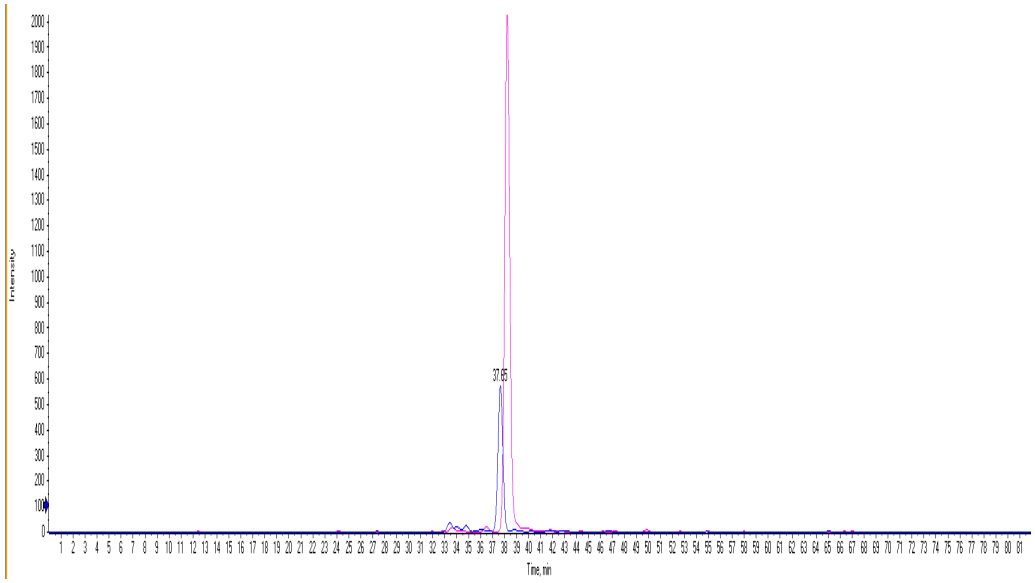


Fig. 18a: Peptide XIC of Oxygen-evolving enhancer protein (m/z=858.9207, Z=2, RT=37.63), pink chromatogram (low salt) and blue chromatogram (high salt)

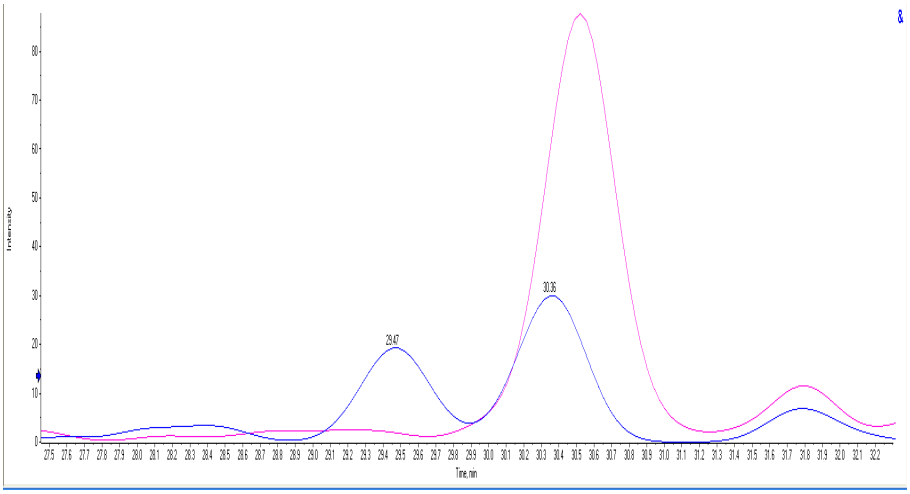


Fig. 18b: Peptide XIC of Photosystem I iron-sulfurcenter (m/z=778.3476, Z=2, RT=30.36), pink chromatogram (low salt) and blue chromatogram (high salt)

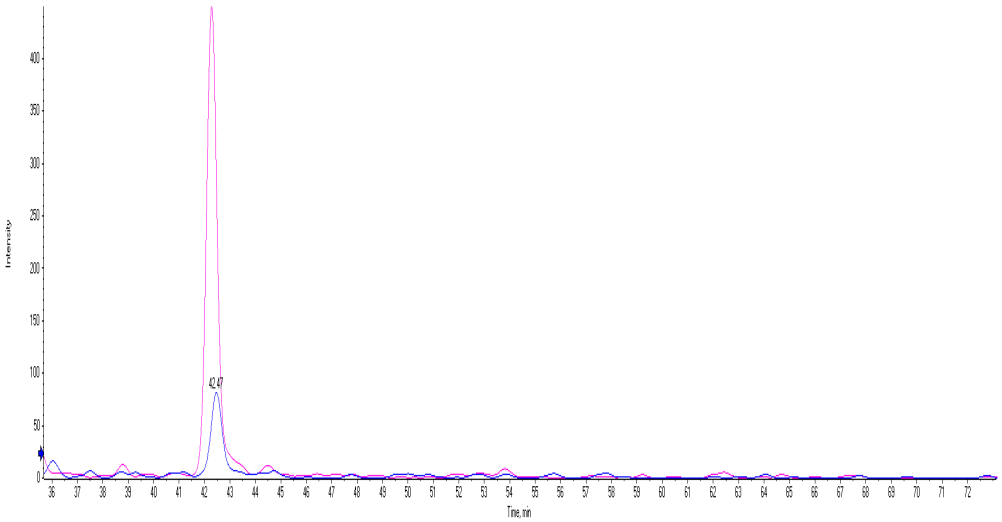


Fig. 18c: Peptide XIC of Peptidyl-prolyl cis-trans isomerase (m/z=808.9616,Z=2, RT=42.47), pink chromatogram (low salt) and blue chromatogram (high salt)

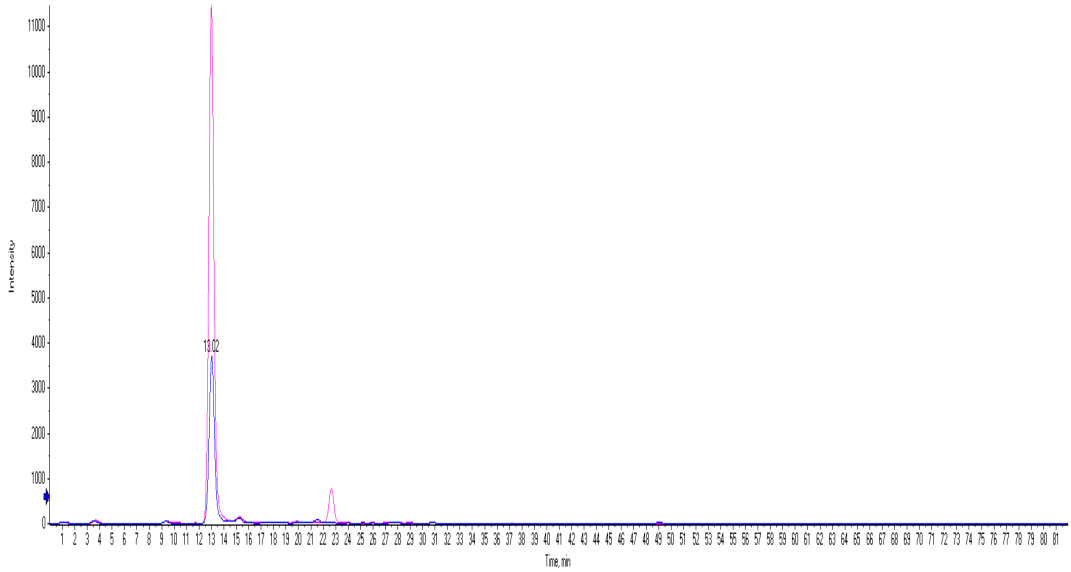
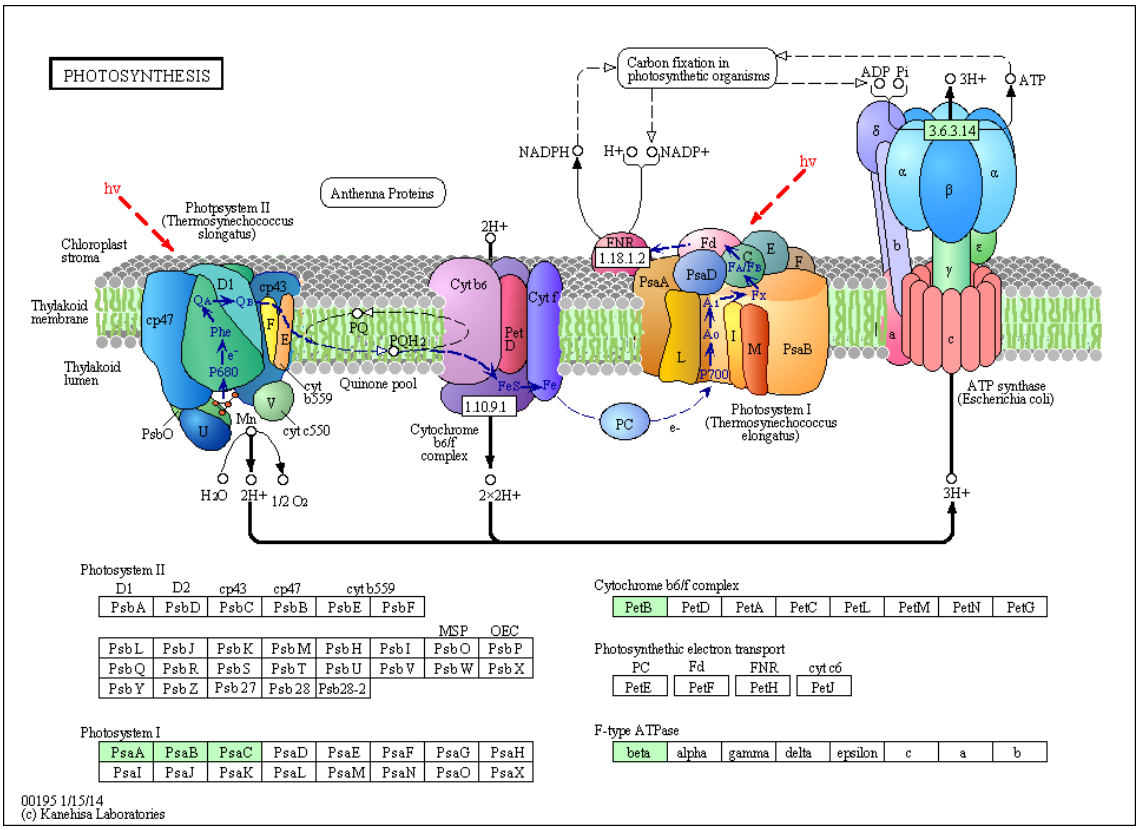


Fig. 18d: Peptide XIC of Flavodoxin I (Fragment) (m/z=615.8219, Z=2, RT=13.02), pink chromatogram (low salt) and blue chromatogram (high salt)

4.4.5 Gene Pathway analysis

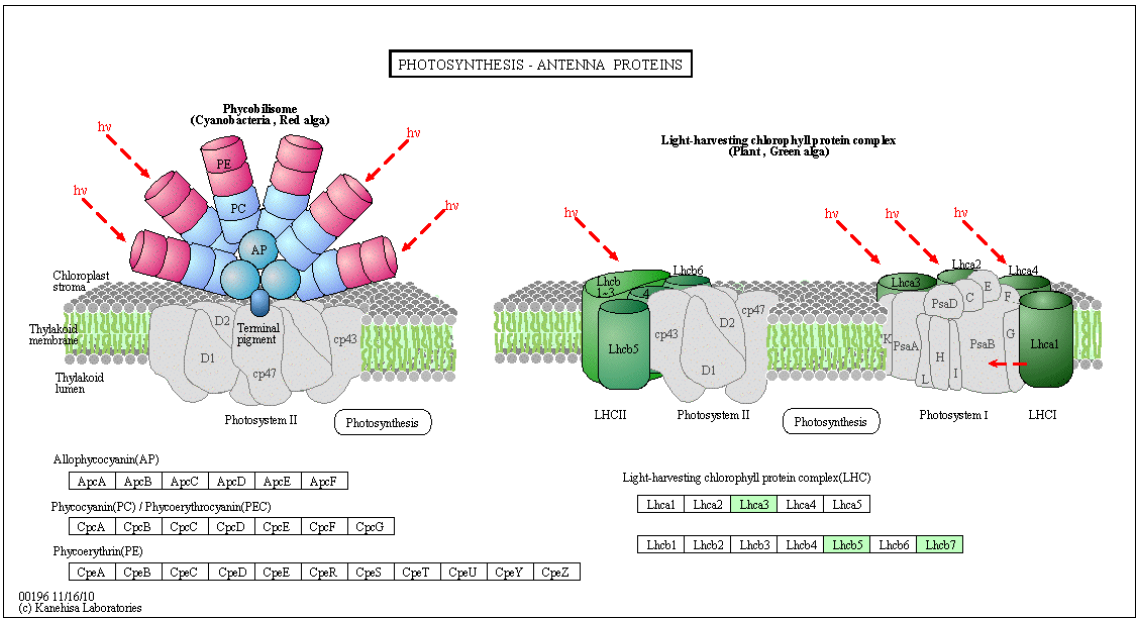
Pathway analysis was performed for the identified salt tolerant genes of *Dunaliella* sp BSK using web-based server KAAS (KEGG automatic annotation server). The sequences were uploaded in a FASTA format and BLAST analysis was carried out against all the sequences of green algae for generating pathways. BBH assignment method was selected for computation of the data.

As per KAAS (KEGG automatic annotation server) pathway analysis majority of differential regulated genes of *Dunaliella* sp. BSK were involved in photosynthesis and carbon fixation, which are highlighted in green colour in the Fig: 19, 20 and 21.



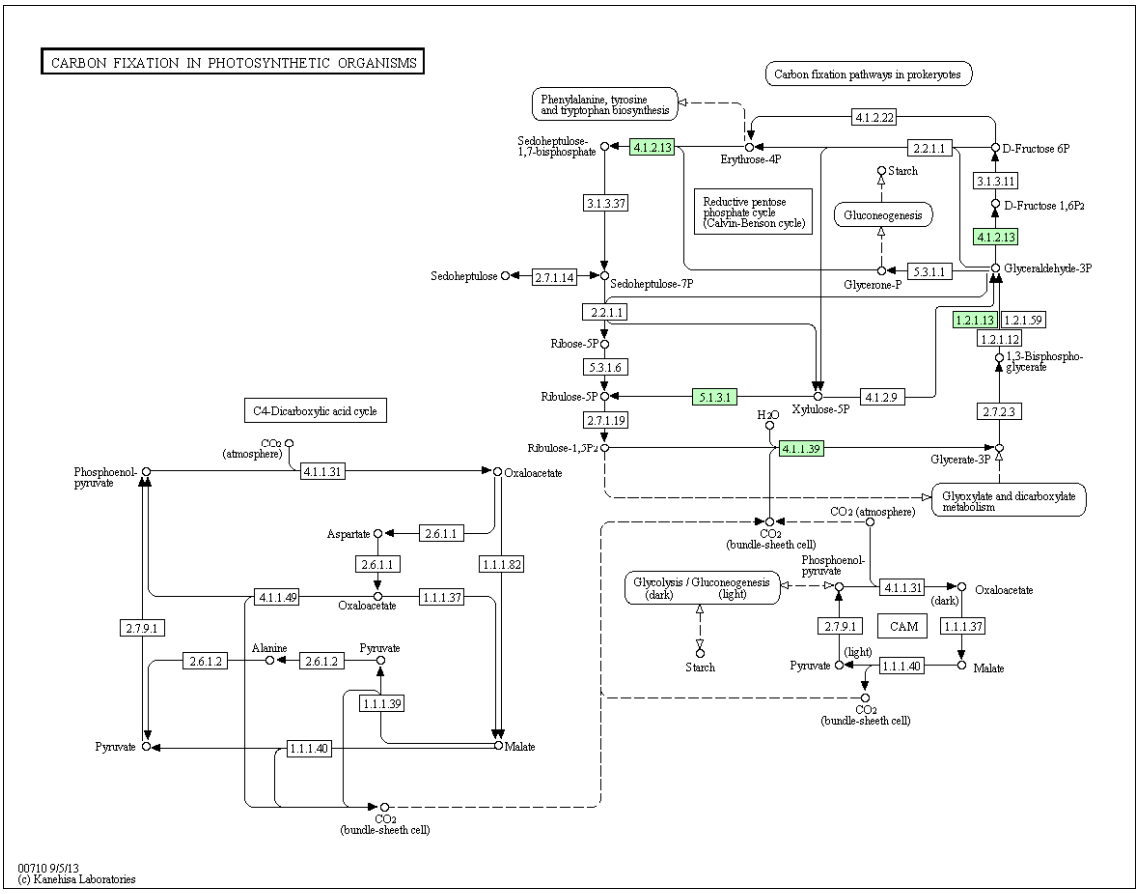
- ko00195 Photosynthesis (5)
-
- ko:K02112 ATPF1B; F-type H+-transporting ATPase subunit beta [EC:3.6.3.14]
- ko:K02635 petB; cytochrome b6
- ko:K02689 psaA; photosystem I P700 chlorophyll a apoprotein A1
- ko:K02690 psaB; photosystem I P700 chlorophyll a apoprotein A2
- ko:K02691 psaC; photosystem I subunit VII

Fig. 19: Pathway analysis depicting proteins involved in photosynthesis.



- ko00196 Photosynthesis - antenna proteins (3)
-
- ko:K08909 LHCA3; light-harvesting complex I chlorophyll a/b binding protein 3
- ko:K08916 LHCB5; light-harvesting complex II chlorophyll a/b binding protein 5
- ko:K14172 LHCB7; light-harvesting complex II chlorophyll a/b binding protein 7

Fig. 20: Pathway analysis depicting proteins involved in Light harvesting complex of photosynthesis



- ko00710 Carbon fixation in photosynthetic organisms (5)
-
- ko:K01601 rbcL; ribulose-bisphosphate carboxylase large chain [EC:4.1.1.39]
- ko:K01602 rbcS; ribulose-bisphosphate carboxylase small chain [EC:4.1.1.39]
- ko:K01623 ALDO; fructose-bisphosphate aldolase, class I [EC:4.1.2.13]
- ko:K01783 rpe; ribulose-phosphate 3-epimerase [EC:5.1.3.1]
- ko:K05298 GAPA; glyceraldehyde-3-phosphate dehydrogenase (NADP+) (phosphorylating) [EC:1.2.1.13]

Fig. 21: Pathway analysis depicting proteins involved in carbon fixation

4.5 Microarray Data Analysis of *Dunaliella*

Microarray data was downloaded from GEO Database to analyze differentially expressed genes under salt stress. Data was normalized by GeneSpring GX using the Lowess normalization method. Differential expressed genes were identified among the samples. Significant genes up regulated fold> 0.8 (logbase2) and down regulated <-0.8 (logbase2) in the test samples with respect to control sample were identified. Statistical student T-test p-value among the replicates was calculated based on volcano plot algorithm. Differentially regulated genes were clustered using hierarchical clustering based on Pearson coefficient correlation algorithm to identify significant gene expression patterns a total of 161 genes were upregulated and 145 genes were down regulated.

Further we have shortlisted the salt tolerance genes by selecting those genes which were found to be up regulated in high salt concentration and down regulated in low salt concentration. The list of these genes is represented in Table 11. The expression of glycerol 3 phosphate dehydrogenase showed similar trend with proteomic analysis.

Table 11: Differentially expressed genes of *Dunaliella* from microarray data analysis

Probe ID	HIGH SALT	LOW SALT	Gene	Description
884	1.32	-0.93	PTOX2	*Dunaliella salina* strain UTEX200 plastid terminal oxidase (PTOX2) mRNA, partial cds
165	1.63	-1.53	*Dunaliella salina* PsaG-like protein mRNA, complete cds	*Dunaliella salina* PsaG-like protein mRNA, complete cds
443	1.11	-1.81	*Dunaliella salina* alpha-tubulin protein mRNA, complete cds	*Dunaliella salina* alpha-tubulin protein mRNA, complete cds
1015	1.4	-1.55	ttf1	*Dunaliella salina* triplicated transferrin Ttf-1 (ttf1) mRNA, complete cds
197	1.93	-1.61	*Dunaliella salina* PsaG-like protein mRNA, complete cds	*Dunaliella salina* PsaG-like protein mRNA, complete cds
337	1.67	-1.07	*Dunaliella salina* 14-3-3 protein gene, complete cds	*Dunaliella salina* 14-3-3 protein gene, complete cds
95	1	-2.23	HDR	*Dunaliella salina* 4-hydroxy-3-methylbut-2-enyl diphosphate reductase (HDR) mRNA, complete cds
202	2.36	-1.32	NR	*Dunaliella bardawil* nitrate reductase (NR) mRNA, complete cds
316	0.91	-0.95	CDPK	*Dunaliella salina* calcium-dependent protein kinase (CDPK) mRNA, complete cds
745	-1.87	1.33	GPDH1	*Dunaliella salina* glycerol-3-phosphate dehydrogenase (GPDH1) mRNA, complete cds

Chapter 5

Discussion

5.1 General physiological response to salt stress

5.1.1 Growth and glycerol content of *Dunaliella* sp. BSK and *Chlorella pyrenoidosa*

The *Dunaliella* sp. BSK and *Chlorella pyrenoidosa* show much difference in terms of growth and glycerol content of their cells with increase in the salt concentration of culture medium. With increase in salt concentration(0.2, 0.5, 0.8, 1.0, 1.5 and 2.0M NaCl), good overall growth and increase in glycerol content was observed in case of *Dunaliella* sp. BSK cultures (Fig. 7). Pick et al. (2002) reported that *Dunaliella* responds to external osmotic stress by adjusting intracellular levels of glycerol. The role of glycerol in osmotic balance as a compatible solute under salinity stress was studied and established (Amtoz and Avron 1973; Borowitzka and Brown 1974; Ahmed and Zidan 1987). It was reported (Brown et al. 1982; Gimmler and Hartung 1988) because of very low permeability of membrane to glycerol the *Dunaliella* cells are capable of retaining glycerol inside them.

However, the levels of cell growth and glycerol content decreased considerably in case of cultures of *Chlorella pyrenoidosa* with increasing concentration of NaCl (0, 0.05, 0.10, 0.15, 0.2 and 0.4M) (Fig. 8) which is in agreement with previous reports (Pietryczuk 2008; Rai and Abraham 1993). It is a well established fact that high salinities reduce cell growth and chlorophyll content of the cells as salt toxicity limits the assimilation rate (Bohnert et al. 1995; Hasegawa et al. 2000). In case of *Chlorella pyrenoidosa* reduction in cell growth may be due to reduced photosynthesis under salinity conditions which is also reflecting in the reduction of glycerol content.

5.1.2 Pigment analysis of *Dunaliella* sp. BSK

The pigment analysis of 20 days old cultures of *Dunaliella* sp. BSK grown in different salt concentrations (0.2, 0.5, 0.8, 1.0, 1.5 and 2.0M NaCl grown) has shown increasing trend in the contents of chlorophylls and carotenoids (Fig. 9) with increasing NaCl concentration.

Carotenoids accumulation was reported in *Dunaliella* when exposed to high salt and high light intensities (Coesel et al. 2008). The accumulation levels of carotenoids by high salinity was well studied and established in *D. salina* and *D. bardawil* by Borowitzka et al. (1990); Cifuentes et al. (1996a). Wang et al. (2003) opined carotenoid accumulation may provide protection to the cells against various abiotic stresses.

The increase in chlorophyll, glycerol and cell growth of *Dunaliella* sp. BSK suggests that increasing salt concentration does not affect the growth and physiology of the cells.

5.2 Proteomic analysis for the Identification of total proteins and differentially expressed proteins of *Dunaliella* sp. BSK and *Chlorella pyrenoidosa* under salt stress

Proteomic analysis of *Dunaliella* sp. BSK and *Chlorella pyrenoidosa* by work flows of LC-MS^E and SWATH identified 559 proteins. From gene ontology analysis it was observed that majority of identified proteins of *Dunaliella* sp. BSK and *Chlorella pyrenoidosa* were involved in metabolic, cellular and single-organism processes.

5.2.1 Differential expression studies of *Dunaliella* sp. BSK

From the identified proteins of *Dunaliella* sp. BSK by 2D (LC-MS^E) and SWATH work flows, 39 were differentially expressed among them 11 proteins were up regulated and 28 proteins were down regulated.

Photosystem I iron-sulfurcenter, apoprotein for the two 4Fe-4S centers of photosystem I, which is essential for photochemical activity as multifunctional electron carriers in diverse redox systems, was found to be highly up-regulated under high NaCl concentration. The other three up regulated stress responsive proteins peroxiredoxin, heat shock protein and carotene globule protein were reported to be induced in response to stress earlier (Tavender and Bulleid 2010; Immenschuh and Baumgart-Vogt 2005; Weids and Grant 2014; Feder and Hofmann 1999; Seki et al. 2002; Zhao and Jones 2012; Liska et al. 2004; Katz et al. 1995). Peroxiredoxin type II protein which is upregulated seven folds under salt stress belongs to the proteins of peroxiredoxin family; which are antioxidant enzymes that reduce peroxides and protect cells from oxidative stress (Tavender and Bulleid 2010; Yuan et al. 2011) and play an important role in cell signalling by regulating H_2O_2 levels (Rhee SG, 2005). During photosynthesis they protect cell membrane from photo oxidative damage (Baier M and Dietz KJ, 1999). Proteins of this family are familiar as contributors to salt tolerance (Lewis et al. 1995; Lahav et al. 2004; Liang et al. 2007). Heat shock protein (HSP-70) is a stress-related protein involved in protein stabilization was

reported to be upregulated by high salt in *Dunaliella salina* (Katz et al 2007). Davidi et al. (2014, 2015) observed induction of carotene globule protein with accumulation of β-carotene at high salt in *Dunaliella.* Proteins associated with photosynthesis such as Rubisco small subunit and photosystem 1 were up regulated under salinity. Up regulation of ATP synthase subunit beta, chloroplastic and ATP synthase subunit beta (Fragment), the proteins associated with ATP generation support the increased growth rate of cells. The up-regulation of chlorophyll biosynthetic enzyme Mg-chelatase was also observed at high NaCl levels, Mg-chelatase catalyses the insertion of magnesium ion into Protoporphyrin IX (precursor to chlorophyll). Transketaolase, which is an enzyme involved in pentose phosphate pathway in all organisms and the Calvin cycle of photosynthesis has also shown up-regulation with increase in NaCl concentration.

While the down regulated proteins of *Dunaliella* sp. BSK, mainly comprised of proteins involved in light reaction of photosynthesis such as Major Light Harvesting complex proteins, Photosystem I P700 Chlorophyll a apolipoprotein 1 and Photosystem I P700 Chlorophyll a apolipoprotein 2 Chloroplast minor chlorophyll a-b binding protein of photosystem II (Fragment); enzymes involved in Calvin cycle such as Rubisco large sub unit, glyceraldehyde 3 Phosphate dehydrogenase, Fructose-bisphosphate aldolase and Cytochrome b6, which is involved in respiration (Fig. 5b). Rubisco is well known to be inhibited by oxidative stress (Cohen et al. 2005; Ozturk ZN et al. 2002; Liska et al. 2004). The proteins involved in photosynthesis and respiration are known to differentially regulate under salt stress (Kim et al. 2005; Bandehagh et al. 2011; Parker et al. 2006). Several earlier studies have reported that the salt stress affects the photosynthetic activity of the many of the enzymes including Rubisco, GAPDH, Fructose 6 phosphate aldolase as well the expression of these proteins are down regulated in plants under salt stress (Seki et al. 2002; Sobhanian et al. 2010). Inhibition of Rubisco by NaCl was reported by Johnson et al. (1968). The down regulation of vacuolar H+ -pyrophosphatase is in contrast to its up regulation in some plants at higher salinity (Meng et al. 2011). The down-regulation of vacuolar H+ -pyrophosphatase at high salt suggests that it may not be involved in proton gradient generation to drive vacuolar sequestration of sodium. EF-Tu (elongation factor Tu) was observed to be down regulated with increasing NaCl. Its expression levels has been studied and reported in plants under different environmental stress conditions like temperature, salinity and drought. In plants its induction was reported

under heat, heat and drought combination, cold and salt stress (Ristic Z et al. 1999, 2004, 2007; Dunn et al. 1993; Berberich et al. 1995; Shin et al 2009). Salekdeh et al. (2002) reported its up-regulation in rice leaves under drought stress. EF-Tu is known for its dual role as a chaperone and in protecting proteins against misfolding. The other protein Nucleoside diphosphate kinase (NDPK) which catalyzes the exchange of phosphate groups was found to be down regulated. NDPK has shown up-regulation in sugar beet leaves under drought stress (Hajheidari et al. 2005) and mtNDPK role as a possible modulator in heat stress response was reported (Galvis et al. 2001).

5.2.2 Differential expression studies of *Chlorella pyrenoidosa*

Proteomic analysis of *Chlorella pyrenoidosa* grown in Fog's Medium without salt and 0.2M salt medium was performed by SWATH analysis and the differentially expressed proteins are listed in Table 3. Totally 13 proteins were found to be differentially expressed. Five proteins were up regulated and 8 proteins were down regulated.

The up regulated proteins of *Chlorella pyrenoidosa* were found to be mainly involved in carbon fixation, protein polymerization and nucleosome assembly. Rubisco, an important protein involved in carbon fixation was up regulated. In addition the structural proteins Tubulin beta chain and Histone H2A were up regulated. S-adenosylmethionine synthase (SAM), a rate limiting enzyme involved in methionine biosynthesis cycle was up regulated (Fig. 6a). Up regulation of SAM was observed in tomato seedling subjected to salt stress (Espartero et al. 1994) and also in wheat (Caruso et al.2008). SAM is a precursor of metabolites like betaine, glycine and polymines which are stress responsive metabolites. Catalyzing formation of SAM under salt stress was reported in barley (Witzel et al. 2009) and its differential expression was reported by Schröder et al. (1997) in *Catharanthus roseus*.

While the down regulated proteins of *Chlorella pyrenoidosa* were mainly involved in photosynthesis and carbon fixation. Earlier studies have reported reduction in photosynthetic related gene expression in *Chlorella* in presence of NaCl (Rashad et al. 2014). Proteins such as plastocyanin, Light harvesting complex, ATP synthase were down regulated (Fig. 6b). In photosynthesis, plastocyanin functions as a mobile electron carrier between the membrane-bound cytochrome *b6f* complex and P-700, the reaction center of photosystem I (PSI) in the thylakoid lumen of photosynthetic

organisms. With addition of salt, reduction in concentration of electron donors to P700 was reported (Tamura et al. 1981). In addition Flavodoxin I and peptidyl-prolyl cis-trans isomerase were down regulated. Flavodoxin is a bacterial protein and acts as electron-transfer protein. Induction of Flavodoxin by salt was reported in *Cyanobacterium Synechocystis* sp. PCC 680 (Fulda and Hagemann 1995). Its induction was also reported under oxidative and other stress conditions. Reduction of Flavodoxin was reported in iron-replete condition (Inda and Peleato 2011). The other down regulated protein, Peptidyl-prolyl isomerise (PPIase) is known to accelerate protein folding. In cotton plant PPIase was induced upon salt stress (Wang et al. 2014). Reduction in PPIase activity was observed in endosperm (cold stress) and whereas it's increased activity was observed in shoot and endosperm (salt stress) of *Sorghum bicolour* seedling (Sharma et al. 2003/4).

5.3 Microarray Data Analysis of *Dunaliella*

Microarray data downloaded from GEO Database was analyzed to find differentially expressed genes under salt stress. Differentially expressed genes data was further shortlisted to get salt tolerant genes and ten genes were found to be salt tolerant. Among those GPDH (glycerol 3 phosphate dehydrogenase) showed similar trend with proteomic analysis.

Chapter 6

Summary

6.1 Isolation, identification and charachterization of a marine alga

In this study for the identification of salt tolerant genes a marine alga was isolated from marine water ponds of Alibagh, Raigad district, Maharashtra, which was identified and characterized. The isolated marine alga was found to be closely related to *Dunaliella* sp. and is referred as *Dunaliella* sp. BSK in this study.

6.2 Scanning Electron Microscope Analysis

To understand the cell morphology of *Dunaliella* and *Chlorella,* SEM analysis of control samples of *Dunaliella* sp. BSK and *Chlorella pyrenoidosa* were analysed on Environmental Scanning Electron Microscope. SEM images have shown that *Dunaliella* sp. BSK is a unicellular, rod shaped biflagellate alga and *Chlorella pyrenoidosa* is a unicellular green alga and is spherical in shape.

6.3 General physiological response to salt stress

To understand the effect of salinity on cell physiology, general physiological studies were carried out to measure cell growth, glycerol production and pigments. For these studies *Dunaliella* sp. *BSK* was grown in culture media of 0.2, 0.5, 0.8, 1.0, 1.5 and 2.0M NaCl and *Chlorella pyrenoidosa* was grown in culture media of 0, 0.05, 0.10, 0.15, 0.2 and 0.4M NaCl for 20 days. The *Dunaliella* sp. BSK and *Chlorella pyrenoidosa* have shown much difference in terms of growth and glycerol content of their cells with increase in the salt concentration of culture medium. *Dunaliella* sp.BSK cells growth and glycerol content increased considerably with increase in salt (NaCl) concentration whereas cell growth and glycerol content of *Chlorella pyrenoidosa* decreased.

While increasing salt concentration of culture media of *Dunaliella* sp. BSK, cells have shown increasing trend in the contents of chlorophylls and carotenoids. Pigment analysis of *Chlorella pyrenoidosa* culture samples was not performed owing to their low growth in presence of salt.

6.4 Proteomic analysis of *Dunaliella* sp. BSK and *Chlorella pyrenoidosa*

A proteomic approach was followed for the identification of salt tolerance genes of *Dunaliella* and for comparative proteomic study of *Dunaliella* a glycophilic alga *Chlorella pyrenoidosa* was selected. To study salt tolerance mechanisms *Dunaliella*

sp. BSK was grown in culture media of two different salt concentrations of 0.5 M and 2.0 M NaCl and *Chlorella pyrenoidosa* was grown in culture media of two different salt concentrations; one without NaCl and the other with 0.2 M NaCl.

Proteomic analysis of *Dunaliella sp.* BSK *and Chlorella pyrenoidosa* was carried out by following below mentioned two Mass Spectrometry workflows

1) LC-MS^E workflow by Synapt HDMS system

2) SWATH (MS/MS^{ALL}) workflow by Triple TOF™ 5600 System

By following work flows of LC-MS^E and SWATH, totally 559 proteins were identified from both *Dunaliella* sp. BSK and *Chlorella pyrenoidosa*.

The identified proteins were functionally annotated as per gene ontology terms and found to be involved in central metabolic pathways, such as metabolic, cellular, single-organism and developmental process, localization, response to stimulus, cellular component organization or biogenesis, biological regulation, signal transduction, epidermal cell fate specification and meiotic cell cycle.

Proteomic profiles of *Dunaliella* and *Chlorella* has shown that their response to high salinity is different. In case of *Dunaliella*, stress responsive proteins such as peroxiredoxin, HSP, carotene globular proteins were up regulated, and this was not observed in *Chlorella*. While SAM, a rate limiting enzyme was up regulated only in *Chlorella*. Expression of Photosystem I iron-sulfur center was found to be up regulated in *Dunaliella* whereas in *Chlorella* it was down regulated. Flavodoxin I and peptidyl-prolyl cis-trans isomerase have shown down regulation in *Chlorella.* Together these differences in proteomic profile could contribute to differences in their salt tolerance responses.

6.4.1 Gene pathway analysis

Pathway analysis was performed for the identified salt tolerant genes of *Dunaliella* sp. BSK using web-based server KAAS (KEGG automatic annotation server). As per KAAS pathway analysis majority of differential regulated genes of *Dunaliella* sp. BSK were involved in photosynthesis and carbon fixation.

6.5 Microarray Data Analysis of *Dunaliella*

Series GSE10271 raw data was taken from GEO database for analysis using GeneSpring GX software from Agilent. The Microarray experiment carried for GSE10271 belongs to two color experiment. The Significant Functional classification of differentially regulated genes was performed using GeneSpring GX software gene ontology, to identify significant gene expression patterns, further the gene list was shortlisted by selecting those genes which were found to be up regulated in high salt concentration and down regulated in low salt concentration vice versa to get salt tolerance genes. Among salt tolerance genes only the expression of glycerol 3 phosphate dehydrogenase showed similar trend with proteomic analysis.

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Annexure

Dunaliellasp. BSK total protein Ids by Synapt HD-MS

Accession	Protein Name	Species	Function	PLGS Score	Coverage (%)	Amount (ng)
A1E905	AtpB Fragment	9CHLO	plasma membrane ATP synthesis coupled proton transport	411.90	19.47	0.00
A1XKU6	Major light harvesting chlorophyll a b protein 2 1	DUNSA	photosynthesis, light harvesting	1142.51	39.92	9.44
A8BDJ0	Major light harvesting chlorophyll a b protein 3	DUNSA	photosynthesis, light harvesting	223.17	12.65	6.23
A8HP67	Exostosin like glycosyltransferase Fragment	CHLRE	Enzyme that transfers a chemical group	35.19	27.78	0.22
A8HPA3	Predicted protein	CHLRE	cell regulatory protein-zinc ion binding	12.67	10.75	0.57
A8HPB8	Peptide methionine sulfoxidereductase	CHLRE	oxidation reduction, protein folding, protein modification process	36.22	10.28	0.97
A8HQA2	Predicted protein	CHLRE	NA	18.63	9.54	1.63
A8HRA8	Predicted protein	CHLRE	NA	22.05	4.71	1.09
A8HWL8	Predicted protein	CHLRE	chloroplast organization, pentose-phosphate shunt	103.59	13.51	8.23
A8HZS1	Predicted protein	CHLRE	NA	10.61	5.90	1.83
A8I029	Predicted protein	CHLRE	karyogamy	16.94	4.72	7.98
A8I388	Predicted protein	CHLRE	response to abscisic acid, response to water deprivation	21.99	8.12	2.68
A8IDN7	Pre mRNA splicing factor PRP38 family protein	CHLRE	nuclear-transcribed mRNA catabolic process, response to salt stress	25.93	13.48	0.80
A8ITH8	Chaperonin 60B2	CHLRE	protein refolding	84.08	12.65	2.91
A8IXU7	Phototropin	CHLRE	protein phosphorylation, regulation of transcription, DNA-dependent	25.76	9.47	0.00
A8J0Q2	Predicted protein	CHLRE	NA	54.22	8.45	2.15
A8J5F7	6 phosphogluconate dehydrogenase decarboxylating	CHLRE	oxidation reduction, pentose-phosphate shunt	55.03	9.36	0.00
A8J5Z4	Predicted protein	CHLRE	NA	19.49	11.72	2.38
A8J7H5	Predicted protein	CHLRE	NA	23.44	10.50	1.12
A8J7K8	Predicted protein	CHLRE	NA	47.53	22.03	0.23
A8J8A7	Predicted protein Fragment	CHLRE	NA	17.81	10.51	3.29

Accession	Protein Name	Species	Function	PLGS Score	Coverage (%)	Amount (ng)
A8J944	NAD dependent epimerasedehydratase	CHLRE	carbohydrate metabolic process, cellular metabolic process	47.52	17.87	0.69
A8JCA1	Predicted protein	CHLRE	NA	26.36	9.46	1.85
A8JD08	DNA topoisomerase 2 Fragment	CHLRE	DNA topological change, regulation of transcription, DNA-dependent	20.50	9.17	1.95
A8JEZ7	Predicted protein	CHLRE	NA	42.31	9.94	3.61
A8JHR9	Glyceraldehyde 3 phosphate dehydrogenase dominant splicing variant	CHLRE	glucose metabolic process, oxidation reduction	48.93	11.05	1.12
A9ZPM6	Elongation factor 1 alpha like protein	GONPE	protein biosynthesis	97.32	15.01	4.30
B1PL92	Glyceraldehyde 3 phosphate dehydrogenase	DUNSA	glucose metabolic process, oxidation reduction	7607.71	42.02	4.71
B1PL92	Glyceraldehyde 3 phosphate dehydrogenase	DUNSA	glucose metabolic process, oxidation reduction	5592.03	38.83	2.92
B2X1W8	Photosystem II CP43 chlorophyll apoprotein	OEDCA	photosynthetic electron transport in photosystem II, protein-chromophore linkage	259.58	10.41	0.41
B7TJI1	Chloroplast heat shock protein 70B Fragment	DUNSA	response to stress	433.78	46.88	0.08
B8R185	Photosystem II CP47 chlorophyll apoprotein	VOLCA	Electron transport, Photosynthesis, Transport	25.34	10.43	1.41
C1K004	Photosystem I light harvesting cholrophyll a b protein 3	DUNSA	photosynthesis, light harvesting	721.23	14.60	5.67
C5H3W1	Glycerol 3 phosphate dehydrogenase	9CHLO	glycerol-3-phosphate catabolic process, oxidation reduction	196.86	9.49	2.78
D0FXV4	Cytochrome f Fragment	DUNSA	oxidation reduction, photosynthesis	175.99	15.51	1.08
D0FXX3	ATP synthase subunit alpha chloroplastic	DUNSA	ATP synthesis, Hydrogen ion transport, Ion transport,Transport	145.14	15.48	3.61
D0FXY0	ATP synthase subunit beta chloroplastic	DUNSA	ATP synthesis, Hydrogen ion transport, Ion transport,Transport	415.21	22.71	0.29
D0FXZ7	Ribulose 1 5 bisphosphate carboxylase oxygenase large subunit	DUNSA	Photosynthesis	2976.47	41.05	5.62

Accession	Protein Name	Species	Function	PLGS Score	Coverage (%)	Amount (ng)
D0UDM8	Alpha tubulin	HAEPL	microtubule-based	1751.67	35.75	0.00

	Fragment		movement, protein polymerization			
D0VMX7	Ribosomal protein S3	VOLCA	tranlation	19.84	9.70	3.57
D3WFN7	Phosphoglucomutase 1	DUNSA	carbohydrate metabolic process	71.87	8.61	0.39
D8TI31	Putative uncharacterized protein	VOLCA	NA	16.29	4.69	0.79
D8TIU2	Putative uncharacterized protein	VOLCA	cyclic nucleotide biosynthetic process	13.98	6.01	7.25
D8TKJ3	Putative uncharacterized protein jtpnB9 2b	VOLCA	NA	19.69	10.98	1.52
D8TKS2	Putative uncharacterized protein Fragment	VOLCA	oxidation reduction	24.60	17.57	3.41
D8TLI5	Putative uncharacterized protein	VOLCA	NA	25.42	6.38	0.96
D8TMF8	Putative uncharacterized protein	VOLCA	NA	30.75	10.68	12.36
D8TND3	Putative uncharacterized protein	VOLCA	NA	24.17	12.47	0.61
D8TNT0	Putative uncharacterized protein	VOLCA	NA	15.67	7.26	1.36
D8TR30	Putative uncharacterized protein	VOLCA	protein phosphorylation	41.48	21.81	3.11
D8TRK5	Putative uncharacterized protein	VOLCA	photosynthesis, light harvesting	559.98	26.19	0.00
D8TTX9	Putative uncharacterized protein Fragment	VOLCA	protein phosphorylation	21.94	4.21	23.35
D8TUG4	Putative uncharacterized protein	VOLCA	porphyrin biosynthetic process	168.91	9.27	1.44
D8TVL4	Putative uncharacterized protein	VOLCA	NA	16.21	9.00	1.11
D8TVM5	Putative uncharacterized protein	VOLCA	oxidation reduction	178.90	9.95	1.49
D8TW00	ATP energized ABC transporter	VOLCA	ATP binding	19.63	8.40	1.96
D8TXC8	Putative uncharacterized protein	VOLCA	NA	33.21	10.79	32.33
D8TXT2	Putative uncharacterized protein	VOLCA	tetrahydrobiopterin biosynthetic process	15.11	7.58	0.40
D8TYC9	Putative uncharacterized protein	VOLCA	RNA binding	21.53	6.82	2.36

Accession	**Protein Name**	**Species**	**Function**	**PLGS Score**	**Coverage (%)**	**Amount (ng)**
D8TZ54	Putative uncharacterized protein	VOLCA	NA	12.15	2.48	69.60
D8TZ67	Putative	VOLCA	GTP binding	12.65	6.31	2.33

	uncharacterized protein					
D8TZD7	Putative uncharacterized protein	VOLCA	protein refolding	44.33	12.31	1.05
D8TZY5	Putative uncharacterized protein	VOLCA	RabGTPase activator activity	38.32	11.23	4.40
D8U219	Dynamin related GTPase	VOLCA	GTP binding	44.63	12.02	5.23
D8U4Q1	Putative uncharacterized protein	VOLCA	transketolase activity	45.85	8.11	3.51
D8U637	Putative uncharacterized protein Fragment	VOLCA	ATP binding, DNA binding	37.25	15.38	45.74
D8U6P0	Putative uncharacterized protein	VOLCA	NA	28.69	10.54	47.75
D8U8M7	Putative uncharacterized protein	VOLCA	NA	14.39	6.35	5.75
D8UBJ0	Putative uncharacterized protein	VOLCA	NA	38.65	9.85	1.98
D8UCX5	Putative uncharacterized protein	VOLCA	NA	29.24	23.63	1.75
D8UJ57	Putative uncharacterized protein	VOLCA	nucleic acid binding	24.08	5.18	1.94
D8UKM3	Putative uncharacterized protein	VOLCA	NA	17.79	8.09	15.67
D8ULK1	Putative uncharacterized protein	VOLCA	NA	211.89	36.61	2.63
O04386	Tubulin beta chain	CHLIN	microtubule-based movement, protein polymerization	2212.47	33.92	11.71
O22113	HCR2	CHLLO	oxidation reduction	24.41	8.34	3.71
O22661	Alpha tubulin	CHLLO	microtubule-based movement, protein polymerization	1923.03	34.42	10.62
O98932	ATP synthase subunit beta Fragment	CHLMO	ATP synthesis, Ion transport,Transport	716.18	37.77	0.00
Q1HVA0	Chloroplast glyceraldehyde 3 phosphate dehydrogenase	CHLLO	glucose metabolic process, oxidation reduction	6844.94	45.01	12.68
Q1XIR4	Large subunit of ribulose 1 5 bisphosphate carboxylase oxygenase Fragment	CHLLO	Photosynthesis	4672.94	55.28	13.55

Accession	Protein Name	Species	Function	PLGS Score	Coverage (%)	Amount (ng)
Q20FC3	Glyceraldehyde 3	SPESI	glucose	6056.23	14.54	0.22

	phosphate dehydrogenase subunit A Fragment		metabolic process, oxidation reduction			
Q52ZA0	Glycerol 3 phosphate dehydrogenase	DUNSA	glycerol-3-phosphate catabolic process, oxidation reduction	193.77	14.55	3.80
Q5VBK0	Ribulose 1 5 bisphosphate carboxylase oxygenase small subunit n 1 Tax DunaliellatertiolectaRepID Q	DUNTE	carbon fixation	390.06	25	0.15
Q5VBK1	Ribulosebisphosphate carboxylase small chain	DUNTE	Carbon dioxide fixation, Photorespiration, Photosynthesis	337.34	20.63	1.01
Q5XR40	Ribulosebisphosphate carboxylase small chain	DUNSA	carbon fixation, oxidation reduction, photorespiration, photosynthesis	488.72	25.79	1.96
Q6QNV1	Ribulose 1 5 bisphosphate carboxylase oxygenase large subunit Fragment	DUNSA	Photosynthesis	4662.33	54.57	0.08
Q8HDG3	ATP synthase subunit beta Fragment	PEDDU	ATP synthesis coupled proton transport	750.19	39.89	7.27
Q8RY44	Heat shock protein 70a	DUNSA	Stress response	18.54	7.69	2.37
Q8S4W6	Coiled coil flagellar protein	CHLRE	motile	13.65	5.00	11.35
Q9ATJ1	Fructose bisphosphatealdolase	DUNSA	Glycolysis	238.25	18.19	4.57
Q9M452	Heat shock protein	DUNSA	Stress response	88.89	17.61	1.71

*Chlorella pyrenoidosa*total protein Ids by Synapt HD-MS

Accession	Protein Name	Species	Function	PLGS Score	Coverage (%)	Amount (ng)
A2SY33	Light harvesting chlorophyll a b binding protein LhcbM2	photosynthesis, light harvesting	ACUOB	3418.96	31.33	8.43
A2SY35	Light harvesting chlorophyll a b binding protein LhcbM4	photosynthesis, light harvesting	ACUOB	1593.93	30.39	1.27
A2SY50	Light harvesting chlorophyll a b binding protein Lhcb5 Fragment	photosynthesis, light harvesting	ACUOB	134.81	9.85	1.29
A8HPB8	Peptide methionine sulfoxidereductase	oxidation reduction, protein folding, protein modification process	CHLRE	26.21	19.54	0.84
A8HV42	Predicted protein	NA	CHLRE	11.41	4.02	2.50
A8HX38	Eukaryotic translation elongation factor 1 alpha	Protein biosynthesis	CHLRE	844.81	21.71	4.23
A8HXN9	Subunit of GARP complex	NA	CHLRE	19.44	11.33	1.30
A8HXV5	Predicted protein	NA	CHLRE	4.90	7.0578	19.67
A8I3Z0	Predicted protein Fragment	NA	CHLRE	32.29	10.29	1.34
A8I5K1	Dynamin related GTPase	Aminotransferase	CHLRE	51.91	13.09	6.83
A8I9U4	Centriole proteome protein	regulation of RabGTPase activity	CHLRE	10.87	7.3459	28.55
A8IDK3	Predicted protein	NA	CHLRE	48.13	24.21	3.26
A8ISE2	SNF2 superfamily protein	ATP binding, helicase activity, nucleic acid binding	CHLRE	11.09	6.70	2.07
A8IYK8	Predicted protein	NA	CHLRE	49.68	24.19	0.22
A8J128	Flagellar associated protein Fragment	Motile cilium	CHLRE	18.36	12.89	1.19

Accession	Protein Name	Species	Function	PLGS Score	Coverage (%)	Amount (ng)
A8J1E1	Predicted protein Fragment	oxidation reduction	CHLRE	210.17	6.3973	3.09
A8J238	Predicted protein	oxidation reduction	CHLRE	10.37	2.28	3.14
A8J426	Subunit of GARP complex Fragment	NA	CHLRE	13.18	11.87	0.38
A8J682	Protein translocase subunit secA	Protein transport, Transport	CHLRE	10.16	17.30	0.91
A8J7H5	Predicted protein	NA	CHLRE	22.38	9.29	1.24
A8J7W6	Predicted protein	NA	CHLRE	11.49	3.74	1.51
A8J8V6	Predicted protein	NA	CHLRE	24.54	14.01	0.55
A8JA75	Predicted protein	NA	CHLRE	35.20	6.8884	2.26
A8JAA3	Predicted protein Fragment	NA	CHLRE	10.24	7.43	2.77
A8JBW8	Predicted protein Fragment	Catalytic activity	CHLRE	22.68	7.28	0.27
A8JC04	Phosphoglycerate kinase	glycolytic process	CHLRE	170.75	18.18	0.00
A8JC04	Phosphoglycerate kinase	glycolysis	CHLRE	149.77	7.5758	0.00
A8JC04	Phosphoglycerate kinase	glycolysis	CHLRE	160.26	12.88	0.00
A8JEG8	Predicted protein	NA	CHLRE	18.33	18.82	0.13
A8JEU4	Heat shock protein 70A	response to stress	CHLRE	175.84	15.78	2.74
A9CM95	33kDa oxygen evolving protein of photosystem II	photosynthesis, photosystem II stabilization	CHLO	36.86	23.02	2.23
A9ZPM7	Elongation factor 1 alpha like protein Fragment		GONPE	90.02	12.6904	1.09
B1PL92	Glyceraldehyde 3 phosphate dehydrogenase	glucose metabolic process, oxidation reduction	DUNSA	3571.76	12.37	0.00
B2X1W8	Photosystem II CP43 chlorophyll apoprotein	photosynthetic electron transport in photosystem II, protein-chromophore linkage	OEDCA	364.86	12.91	0.99
B2X1W9	Photosystem II D2 protein	photosynthetic electron transport in photosystem II	OEDCA	100.22	5.29	0.00
B2X290	Photosystem I iron sulfurcenter	photosynthetic electron transport in photosystem I	CHLMO	487.14	43.21	0.28
B2X2D0	Elongation factor Tuchloroplastic	Protein biosynthesis	CHLMO	150.85	12.86	1.64

Accession	Protein Name	Species	Function	PLGS Score	Coverage (%)	Amount (ng)
B7U1J0	ATP synthase subunit alpha chloroplastic 1	ATP synthesis, Hydrogen ion transport, Ion transport,Transport	CHLRE	334.36	12.40	0.57
C0LL60	Translation elongation factor like protein Fragment	protein biosynthesis	ACUOB	326.94	32.19	0.84
D0FXV5	DNA directed RNA polymerase	Transcription	DUNSA	5.01	4.8808	9.02
D0FY05	Photosystem II CP47 chlorophyll apoprotein	photosynthesis, Transport	DUNSA	46.07	7.48	0.75
D0VMZ1	ATP synthase subunit alpha	ATP synthesis, Ion transport,Transport	VOLCA	531.25	16.83	0.92
D2KAH9	Ribulosebisphosphate carboxylase large chain Fragment	oxidation reduction, reductive pentose-phosphate cycle	9CHLO	661.30	22.92	3.70
D5L2W2	Chloroplast thioredoxin peroxidase Fragment	cell redox homeostasis	VOLCA	22.55	10.19	0.10
D8TH51	Putative uncharacterized protein	NA	VOLCA	48.76	8.63	1.36
D8TI16	ATP synthase gamma chain	ATP synthesis coupled proton transport	VOLCA	85.81	5.42	0.78
D8TIF7	Putative uncharacterized protein	NA	VOLCA	7.03	3.15	10.93
D8TIQ0	Putative uncharacterized protein	NA	VOLCA	28.53	8.82	1.91
D8TKY4	Putative uncharacterized protein	glycolysis	VOLCA	588.32	15.38	3.03
D8TNN3	Eukaryotic translation elongation factor 1 alpha 2	protein biosynthesis	VOLCA	310.46	24.62	0.88
D8TNT0	Putative uncharacterized protein	NA	VOLCA	8.47	5.94	7.23
D8TP82	Putative uncharacterized protein	post-translational protein modification, regulation of protein metabolic process, ubiquitin-dependent protein catabolic process	VOLCA	13.87	4.03	12.83
D8TRR7	Putative uncharacterized protein	carbohydrate metabolic process	VOLCA	80.26	8.51	0.00

Accession	Protein Name	Species	Function	PLGS Score	Coverage (%)	Amount (ng)
D8TS47	Putative uncharacterized protein	NA	VOLCA	14.39	9.16	0.38
D8TSJ3	Putative uncharacterized protein	NA	VOLCA	15.83	12.34	13.10
D8TTI2	Putative uncharacterized protein	NA	VOLCA	19.57	12.52	0.99
D8TTT8	Putative uncharacterized protein	NA	VOLCA	18.46	6.72	8.44
D8TTX0	Putative uncharacterized protein	DNA binding	VOLCA	17.96	5.22	3.83
D8TTZ4	Putative uncharacterized protein	NA	VOLCA	10.94	7.30	6.20
D8TUM0	UV damaged DNA binding complex subunit 1 protein Fragment	nucleic acid binding	VOLCA	22.15	11.96	3.10
D8TUP1	Dihydrolipoamideacetyltransferase	transferase activity	VOLCA	79.36	3.21	0.87
D8TVC7	Putative uncharacterized protein	NA	VOLCA	68.46	18.70	0.86
D8TVS7	Putative uncharacterized protein	NA	VOLCA	15.58	6.18	2.17
D8TZP4	Putative uncharacterized protein	NA	VOLCA	11.61	23.96	0.19
D8TZR1	Putative uncharacterized protein	heme oxidation, oxidation reduction	VOLCA	27.19	15.15	0.22
D8U096	Putative uncharacterized protein	voltage-gated potassium channel activity	VOLCA	14.45	5.18	4.73
D8U0N8	Lipoxygenase	oxidation reduction, oxylipin biosynthetic process	VOLCA	23.08	7.9545	3.91
D8U219	Dynamin related GTPase	GTPase activity, GTP binding	VOLCA	27.76	7.0064	3.66
D8U2M6	Putative uncharacterized protein	protein phosphorylation	VOLCA	3.39	2.8875	0.80
D8U3J2	Putative uncharacterized protein	NA	VOLCA	13.30	4.8528	1.19

Accession	Protein Name	Species	Function	PLGS Score	Coverage (%)	Amount (ng)
D8U5E6	Putative uncharacterized protein	NA	VOLCA	22.57	13.01	0.49
D8U665	Putative uncharacterized protein	NA	VOLCA	20.61	16.1787	5.40
D8U8C9	Putative uncharacterized protein	NA	VOLCA	15.54	6.11	1.95
D8U8F9	Putative uncharacterized protein	NA	VOLCA	12.70	8.92	2.79
D8U9J4	Putative uncharacterized protein	glucose metabolic process, oxidation reduction	VOLCA	3578.15	16.58	2.18
D8U9J4	Putative uncharacterized protein	glucose metabolic process, oxidation reduction	VOLCA	1121.30	11.4973	0.96

D8UA77	Putative uncharacterized protein	NA	VOLCA	18.09	12.67	5.79
D8UB06	Putative uncharacterized protein	microtubule-based movement	VOLCA	38.12	17.70	1.75
D8UBR4	Histone H3 methyltransferaseTrithorax	catalytic activity	VOLCA	3.18	4.9778	2.99
D8UC27	Putative uncharacterized protein	NA	VOLCA	4.43	5.9744	2.60
D8UD54	Kinesin II motor protein	microtubule-based movement	VOLCA	19.15	9.31	1.65
D8UEF4	Putative uncharacterized protein	ATP binding	VOLCA	25.81	12.91	9.29
D8UFA0	Putative uncharacterized protein	retrograde transport, endosome to Golgi	VOLCA	14.83	4.69	0.78
D8UHM8	Putative uncharacterized protein	chlorophyll biosynthetic process, oxidation reduction, photosynthesis	VOLCA	20.08	12.80	0.66
D8UJT6	SF assemblin	structural constituent of cytoskeleton	VOLCA	12.67	18.35	0.17
D8UMV3	Putative uncharacterized protein Fragment	transmembrane transport	VOLCA	355.39	13.2979	9.66

Accession	Protein Name	Species	Function	PLGS Score	Coverage (%)	Amount (ng)
D9CJ53	S adenosylmethionine synthase	one-carbon metabolic process	VOLCA	43.57	20.26	0.44
D9CJ92	PRX1m	cell redox homeostasis	VOLCA	221.24	29.94	1.07
E2DSM8	CP43 chlorophyll apoprotein of photosystem II	photosynthetic electron transport in photosystem II, protein-chromophore linkage	9CHLO	326.17	11.93	1.11
O04386	Tubulin beta chain	microtubule-based movement, protein polymerization	CHLIN	173.46	17.3815	1.53
O98928	ATP synthase subunit beta Fragment	ATP synthesis, Hydrogen ion transport, Ion transport,Transport	9CHLO	579.92	28.7234	0.00
P00300	Plastocyanin	Electron transport, Transport	CHLFU	513.55	18.37	1.25
P50362	Glyceraldehyde 3 phosphate dehydrogenase A chloroplastic	Calvin cycle	CHLRE	2784.97	17.65	0.40
Q1HVA0	Chloroplast glyceraldehyde 3 phosphate dehydrogenase	glucose metabolic process, oxidation reduction	9CHLO	4453.72	17.55	1.26
Q1KVS9	Elongation factor Tuchloroplastic	Protein biosynthesis	SCEOB	97.69	3.58	1.24
Q1KVT0	ATP synthase subunit beta chloroplastic	ATP synthesis, Hydrogen ion transport, Ion transport,Transport	SCEOB	828.42	28.65	2.41
Q1KVU0	ATP synthase subunit alpha chloroplastic	ATP synthesis, Hydrogen ion transport, Ion transport,Transport	SCEOB	304.68	24.95	2.94
Q1WLV2	Chloroplast oxygen evolving enhancer protein 1	photosynthesis, photosystem II stabilization	CHLIN	118.79	15.12	1.26
Q20FC3	Glyceraldehyde 3 phosphate dehydrogenase subunit A Fragment	glucose metabolic process, oxidation reduction	SPESI	1160.67	8.7452	0.50
Q39582	Tubulin gamma chain	microtubule-based process, protein polymerization	CHLRE	26.12	17.74	0.55
Q8HD99	Ribulosebisphosphate carboxylase large chain Fragment	Calvin cycle, Carbon dioxide fixation, Photosynthesis	SCEQU	759.93	17.82	4.11
Q8HDF3	ATP synthase subunit beta Fragment	ATP synthesis, Hydrogen ion transport, Ion transport,Transport	9CHLO	819.99	27.66	0.00

Accession	Protein Name	Species	Function	PLGS Score	Coverage (%)	Amount (ng)
Q8VXQ8	Glyceraldehyde 3 phosphate dehydrogenase cytosolic Fragment	Glycolysis	SCEVA	258.23	20.35	1.15
Q8VXQ9	Glyceraldehyde 3 phosphate dehydrogenase A chloroplastic Fragment	Calvin cycle	SCEVA	2421.69	23.65	2.63
Q946Z4	Enolase Fragment	Glycolysis	9CHLO	296.77	20	0.56
Q96550	ATP synthase subunit alpha	ATP synthesis, Hydrogen ion transport, Ion transport,Transport	CHLRE	82.06	10.0176	4.04
Q9FEK6	CP26	Photosynthesis	CHLRE	250.59	9.34	1.75
Q9GGY5	ATP synthase subunit beta Fragment	ATP synthesis, Hydrogen ion transport, Ion transport,Transport	9CHLO	741.77	30.05	0.20

Protein ID's of *Dunaliella*sp. BSK, which were separated by 2-dimensional electrophoresis and identified by Synapt HDMS

S. No	SSP No	Accession no	Protein Name	Function
1	0203, 0302, 2201	A1XKU6	Major light harvesting chlorophyll a b protein (*Dunaliellasalina*)	photosynthesis, light harvesting
2	0702,3401, 6201,8101,9601	A8HQJ4	Global transcription factor Fragment (*Chlamydomonasreinhardtii*)	Regulation of DNA-templated transcription
3	1501	A8IMZ5	Magnesium chelatase subunit (*Chlamydomonasreinhardtii*)	Chlorophyll biosynthetic process
4	1701	Q4JJJ6	Beta tubulin Fragment (*Dunaliellasalina*)	Protein polymerization
5	1803	A0ZW41	ATP synthase subunit beta (*Polytomella*)	photosynthesis
6	1901	Q8VY41	Heat shock protein 70B (*Dunaliellasalina*)	stress respone
7	2002	Q5XR40	Ribulosebisphosphate carboxylase (*Dunaliellasalina*)	carbon fixation
8	2510	A8JIB7	Chaperonin 60A (*Chlamydomonasreinhardtii*)	protein refolding
9	2901	A8IL08	Membrane AAA metalloprotease (*Chlamydomonasreinhardtii*)	ATP binding
10	3301,5301	C1K004	Photosystem I light harvesting cholrophyll a b protein 3 (*Dunaliellasalina*)	photosynthesis
11	3602	D8TK19	Actin like protein (*Volvoxcarteri f nagariensis*)	multi cellular process
12	3603	ACT	Actin (Volvoxcarteri)	multi cellular process
13	3901	Q8RY44	Heat shock protein 70a (*Dunaliellasalina*)	stress respons
14	4801	B7TJI1	Chloroplast heat shock protein 70B Fragment (*Dunaliellasalina*)	stress response
15	4802	A8JH98	Enolase (Chlamydomonasreinhardtii)	_
16	5101	A8BDJ0	Major light harvesting chlorophyll a b protein (*Dunaliellasalina*)	photosynthesis
17	5201	Q6PSL5	Fe hydrogenase assembly protein (*Chlamydomonas*)	catalytic activity
18	5504	A8JE10	Fructose 1 6 bisphosphatealdolase (*Chlamydomonas*)	CO2 fixation
19	6002	A8J9H8 A8IXU7	Nucleoside diphosphate kinase (*Chlamydomonas*) Phototropin (*Chlamydomonas*)	CTP biosynthetic process
20	6102	A5X458	Superoxide dismutase (*Haematococcuspluvialis*)	oxidoreductase activity

S. No	SSP No	Accession no	Protein Name	Function
21	6402,8505	Q9ATJ1	Fructose bisphosphatealdolase (*Dunaliellasalina*)	glycolysis
22	6802	A8IAN1	Transketolase (*Chlamydomonas*)	photosynthesis
23	7001	D8U454	Malate dehydrogenase (*Volvox*)	carbohydrate metabolic process
24	7702,8702	D0FXZ7	Ribulose 1 5 bisphosphate carboxylase oxygenase large subunit (*Dunaliellasalina*)	carbon fixation
25	8702	A8HS14	Glucose 1 phosphate adenylyltransferase (*Chlamydomonas*)	carbon fixation

Dunaliellasp. BSK total protein Ids by Triple TOF MS

Accession	Protein Name	Species	% Coverage (95%)	Peptides (95%)	Function
K7NU72	Photosystem II 44 kDa protein	DUNTE	15.4	4	Photosynthetic electron transport in photosystem II
C1K004	Photosystem I light-harvesting cholrophyll-a/b protein 3	DUNSA	26.6	4	Photosynthesis, Light harvesting
K8EQC6	Uncharacterized protein	9CHLO	36.2	4	microtubule-based proces
Q9XFT4	Photosystem I chain IV	9CHLO	53.6	4	Photosystem I reaction center
J7KEQ3	Photochlorophyllidereductase subunit L	9CHLO	34.2	4	chlorophyll biosynthetic process
D0FXX3	ATP synthase subunit alpha, chloroplastic	DUNSA	26.6	4	ATP hydrolysis coupled proton transport ,plasma membrane ATP synthesis coupled proton transport
H2DRI9	Glutamine synthetase	9CHLO	35.1	3	glutamine biosynthetic process
A8IL08	Membrane AAA-metalloprotease	CHLRE	15.5	3	ATP binding
D0FXZ7	Ribulosebisphosphate carboxylase large chain	DUNSA	27	3	photo respiration
A5YKI8	Elongation factor-1 alpha-like protein (Fragment)	9CHLO	18.2	3	Protein biosynthesis
P54213	Caltractin	DUNSA	32	3	mitotic nuclear division
K7NRG3	Photosystem II D2 protein	DUNTE	17.1	3	Electron transport
K7NSQ0	Elongation factor Tu	DUNTE	40	3	Protein biosynthesis
E3SC38	Oxygen-evolving enhancer protein (Fragment)	OSTTA	35.8	3	Photo synthesis
Q00Y36	Polyubiquitin (ISS)	OSTTA	41.7	3	ubiquitination
K7NVH0	ATP synthase subunit beta (Fragment)	DUNTE	33.7	3	ATP hydrolysis coupled proton transport

Accession	Protein Name	Species	% Coverage (95%)	Peptides (95%)	Function
M1VB00	ATP synthase subunit beta, chloroplastic	GONPE	19.8	3	ATP hydrolysis coupled proton transport Source
A8HX38	Eukaryotic translation elongation factor 1 alpha 1	CHLRE	33.9	3	plasma membrane ATP synthesis coupled proton transport
E1Z7C4	Putative uncharacterized protein	CHLVA	15.3	2	pentose-phosphate shunt, non-oxidative branch
D8UDZ4	Adenylate kinase	VOLCA	18.1	2	adenylate kinase activity, ATP binding
K7NTZ9	Component of cytochrome b6/f complex (Fragment)	DUNTE	37.3	2	photosynthesis
M1VME9	Photosystem II CP47 chlorophyll apoprotein	GONPE	12.6	2	photosynthetic electron transport chain
D8TZZ8	Putative uncharacterized protein	VOLCA	16.2	2	protein folding
D8U4U4	Peptidyl-prolylcis-trans isomerase	VOLCA	14.9	2	protein folding
C1K003	Photosystem I light-harvesting cholrophyll-a/b protein 2	DUNSA	26.3	2	Photosynthesis, Light harvesting
E2E6H3	Vacuolar H+-pyrophosphatase	9CHLO	9.2	2	proton transport
A7Y228	Histone H4 (Fragment)	DUNSA	40	2	nucleosome assembly
G4WUW1	Chloroplast minor chlorophyll a-b binding protein of photosystem II (Fragment)	DUNSA	18.4	2	Photosynthesis, Light harvesting
A8I7T8	Binding protein 1	CHLRE	13.9	2	ATP binding
Q84KQ4	Elongation factor-1alpha (Fragment) OS=Pleodorina sp. 2000-602-P14 GN=EF-1alpha PE=2 SV=1	9CHLO	25.9	2	protein biosynthesis

Accession	Protein Name	Species	% Coverage (95%)	Peptides (95%)	Function
D8U5K1	Putative uncharacterized protein	VOLCA	12.7	2	protein folding
S5QBM3	Photosystem I iron-sulfur center	OSTTA	63	2	photosynthetic electron transport in photosystem I
K8ENF9	Molecular chaperone DnaK	9CHLO	19	2	Protein folding
C5H3W1	Glycerol-3-phosphate dehydrogenase	9CHLO	13.7	2	carbohydrate metabolic process, glycerol-3-phosphate catabolic process, L-serine biosynthetic process
D8TLJ4	Oxygen-evolving enhancer protein 3	VOLCA	13.4	2	Photosynthesis
O64424	Elongation factor Tu (Fragment)	9CHLO	21.4	2	Protein biosynthesis
Q8RY44	Heat shock protein 70a	DUNSA	12	2	Stress response
K9MIA6	Elongation factor tu (Fragment)	9CHLO	26.1	2	Protein biosynthesis
I0Z918	Binding protein 1	9CHLO	10.6	2	ATP binding
Q39708	Calmodulin-like protein	DUNSA	38.4	1	Calcium ion binding
M9P835	Photosystem Q(B) protein	9CHLO	14.5	1	Catalytic activity
D8TNF1	Histone H2B	VOLCA	34.4	1	DNA binding
D8THE1	Photosystem I reaction center subunit psaK, chloroplast	VOLCA	16.5	1	Photosynthesis
D8UIE7	Putative uncharacterized protein	VOLCA	23.3	1	NA
E1ZGR1	Putative uncharacterized protein	CHLVA	8.5	1	Catalytic activity
C1MPQ3	Ribulosebisphosphate carboxylase/oxygenaseactivase, chloroplast	MICPC	10.3	1	ATP binding
E1ZPP6	Putative uncharacterized protein	CHLVA	3.4	1	ATP binding
D8TRA2	ATP synthase subunit beta	VOLCA	10.7	1	ATP hydrolysis coupled proton transport
K8EHR6	Uncharacterized protein	9CHLO	14.6	1	Photosynthesis
I2FKQ9	Mitochondrial chaperonin 60	CHLRE	4.9	1	Protein refolding

Accession	Protein Name	Species	% Coverage (95%)	Peptides (95%)	Function
E1Z2A2	Putative uncharacterized protein	CHLVA	6.9	1	Protein refolding
D8U3Y2	Putative uncharacterized protein	VOLCA	9.7	1	NA
D8TV83	Putative uncharacterized protein	VOLCA	9.5	1	NA
M9P842	Cytochrome b6	9CHLO	6	1	Photosynthesis
K7NU68	Photosystem I P700 chlorophyll a apoprotein A2	DUNTE	5.6	1	Photosynthesis
Q01FA5	Histone H2A	OSTTA	18.2	1	DNA binding
E1Z349	Malate dehydrogenase	CHLVA	9.5	1	cellular carbohydrate metabolic process, malate metabolic process
E1Z2A2	Putative uncharacterized protein	CHLVA	6.9	1	Protein refolding
C1EGG2	Predicted protein	MICSR	10	1	NA
I0YRT4	Putative arginine biosynthesis bifunctional protein argJ 1	9CHLO	3	1	arginine biosynthetic process
K8ECL1	Uncharacterized protein	9CHLO	2.6	1	NA
D8TSE1	Putative uncharacterized protein (Fragment)	VOLCA	16.9	1	Protein transport
A8J3C3	Chaperonin 11	CHLRE	22.7	1	protein folding
K8FHN0	REVERSED Uncharacterized protein	9CHLO	4.2	1	NA
Q946Z5	Enolase (Fragment)	CHLRE	13	1	glycolytic process
D8UF22	Malate dehydrogenase	VOLCA	3.6	1	cellular carbohydrate metabolic process Source, malate metabolic process
Q1WLW2	Chloroplast inorganic pyrophosphatase (Fragment)	CHLIN	6.3	1	phosphate-containing compound metabolic process
Q1KVS9	Elongation factor Tu, chloroplastic	ACUOB	23.6	1	protein biosynthesis

Accession	Protein Name	Species	% Coverage (95%)	Peptides (95%)	Function
D8TKY4	Putative uncharacterized protein	VOLCA	9.8	1	-
E1ZRV3	Putative uncharacterized protein	CHLVA	9.1	1	protein folding
A4RY81	Predicted protein	OSTLU	2.9	1	chlorophyll biosynthetic process, cytokinin metabolic process, isopentenyldiphosphate biosynthetic process, methylerythritol 4-phosphate pathway, photosynthesis
D8TQZ8	Plastid/chloroplast ribosomal protein L7/L12	VOLCA	16.1	1	translation
C1FDU0	Aaa-metalloprotease chloroplast	MICSR	8.4	1	ATP binding, metalloendopeptidase activity
K7NU01	Photosystem I P700 chlorophyll a apoprotein A1 (Fragment)	DUNTE	6.2	0	photosynthesis
E1ZD21	Putative uncharacterized protein	CHLVA	12.9	0	NA
Q9AXG9	PsaG-like protein	DUNSA	18.2	0	photosynthesis
Q019Q8	WGS project CAID00000000 data, contig chromosome 05 (Fragment)	OSTTA	13.9	0	Obsolete
D8TP03	Putative uncharacterized protein	VOLCA	13	0	proteinfolding
E1ZIH8	REVERSED Putative uncharacterized protein	CHLVA	3.8	0	NA
C1MN70	Predicted protein	MICPC	4.2	0	NA
Q9SBM9	Light harvesting complex a protein	VOLCA	7	0	photosynthesis, light harvesting, protein-chromophore linkage
Q9FNR7	Plastidic NADP-dependent malate dehydrogenase	DUNBI	2.3	0	carbohydrate metabolic process, malate metabolic process
Q01G76	Chloroplast ascorbate peroxidase (ISS)	OSTTA	2.8	0	obsolete
K8EIU3	REVERSED Uncharacterized protein	9CHLO	0.7	0	NA
K8EF58	Ribulose-phosphate 3-epimerase	9CHLO	3	0	pentose-phosphate shunt
C1MVP3	Heat shock protein 70, chloroplast	MICPC	22.7	0	protein folding
Q8HDA6	P700 chlorophyll a-apoprotein A2 (Fragment)	9CHLO	6.3	0	photosynthesis

Accession	Protein Name	Species	% Coverage (95%)	Peptides (95%)	Function
D8TTK4	Putative uncharacterized protein	VOLCA	10.8	0	ATP binding
C1N664	Predicted protein	MICPC	14	0	protein folding
K7TEQ9	Chloroplast photosystem II subunit S	K7TEQ9	9	0	photosynthesis
D8TVM5	Putative uncharacterized protein	VOLCA	2.8	0	protochlorophyllidereductase activity
Q5IWX9	Plastid 5,10-methylene-tetrahydrofolate dehydrogenase (Fragment)	PROWI	8.1	0	folic acid-containing compound biosynthetic process
C1DY02	Predicted protein	MICSR	5.1	0	NA
A8J390	REVERSED Flagellar/basal body protein (Fragment)	CHLRE	3.2	0	motile cilium
K8EJ98	Kinesin K39	9CHLO	5.1	0	NA
E1ZF27	Phosphoribulokinase (Fragment)	CHLVA	2.5	0	carbohydrate metabolic process
Q9ZRY8	Fructose-bisphosphatealdolase	SCHDU	5.9	0	glycolytic process
D8TKN4	REVERSED Putative uncharacterized protein mot51	VOLCA	2.5	0	NA
C1EAZ5	REVERSED Predicted protein	MICSR	2.8	0	metal ion binding
Q018N5	Histones H3 and H4 (ISS)	OSTTA	9.4	0	obsolete
A8HXX8	REVERSED Predicted protein	CHLRE	5.1	0	NA
D8TS40	REVERSED Putative uncharacterized protein	VOLCA	2	0	NA
K8F3W2	Uncharacterized protein	9CHLO	2.4	0	Ubl conjugation pathway
A8JCE1	Predicted protein (Fragment)	CHLRE	8	0	gluconeogenesis
Q8LPE0	Putative blue light receptor	CHLRE	5.7	0	catalytic activity
I0Z537	Thioredoxin dependent peroxidase	9CHLO	9	0	peroxidase activity
D8THE2	Proteasome subunit alpha type	VOLCA	10	0	ubiquitin-dependent protein catabolic process
D8TUG4	Putative uncharacterized protein	VOLCA	4.5	0	tetrapyrrole biosynthetic process

*Chlorella pyrenoidosa*total protein Ids by Triple TOF-MS

Accession	Protein Name	Species	% Coverage (95%)	Peptides (95%)	Function
Q9S8L3	Flavodoxin I (Fragment)	CHLFU	77.78	2	FMN binding, oxidoreductase activity
F2YGK8	Photosystem I iron-sulfur center	CHLVA	51.85	4	photosynthetic electron transport in photosystem I
C7BF11	Photosystem I iron-sulfur center	PARKE	51.85	4	photosynthetic electron transport in photosystem I
U5U7Z9	Photosystem I iron-sulfur center	9CHLO	51.85	3	photosynthetic electron transport in photosystem I
E1ZP76	Putative uncharacterized protein	CHLVA	51.18	5	Electron transport chain
Q0H702	Superoxide dismutase	CHLPY	36.45	6	catalytic activity
E1ZQR2	Putative uncharacterized protein	CHLVA	34.68	10	photosynthesis
E1Z5I7	Putative uncharacterized protein	CHLVA	27.95	4	NA
L7QK48	Chloroplast cytochrome b6-f complex iron-sulfur subunit (Fragment)	AUXPR	25	2	2 iron, 2 sulfur cluster binding, metal ion binding, ubiquinol-cytochrome-c reductase activity
E1ZHZ0	Putative uncharacterized protein (Fragment)	CHLVA	20.83	3	NA
E3SC29	Oxygen-evolving enhancer protein (Fragment)	PARKE	20.28	2	photosynthesis, photosystem II stabilization
E1Z9G7	Putative uncharacterized protein	CHLVA	20.27	3	protein folding
E1ZSK9	Peptidyl-prolylcis-trans isomerase (Fragment)	CHLVA	19.23	1	protein folding
E1ZCE0	Putative uncharacterized protein	CHLVA	17.88	3	transport

Accession	Protein Name	Species	% Coverage (95%)	Peptides (95%)	Function
U5U4S6	Elongation factor Tu, chloroplastic	9CHLO	14.91	4	protein biosynthesis
E1Z406	Putative uncharacterized protein	CHLVA	14.71	2	CTP biosynthetic process, GTP biosynthetic process, UTP biosynthetic process
E1ZDF1	Expressed protein	CHLVA	13.1	1	NA
F2YGM8	Elongation factor Tu	CHLVA	11	3	protein biosynthesis
C7BEW9	Elongation factor Tu, chloroplastic	PARKE	10.02	3	protein biosynthesis
E1ZA99	Putative uncharacterized protein	CHLVA	9.821	1	oxidation-reduction process
E1Z7C4	Putative uncharacterized protein	CHLVA	9.818	2	pentose-phosphate shunt, non-oxidative branch
V9XTN3	Fe-superoxide dismutase	CHLPY	9.322	3	metal ion binding, superoxide dismutase activity
L0N3V0	Ribulose-bisphosphate carboxylase large subunit (Fragment)	9CHLO	8.289	3	carbon fixation
E1Z514	Putative uncharacterized protein	CHLVA	8.095	1	oxidoreductase activity
E1ZGZ5	Histone H2A	CHLVA	7.965	1	DNA binding
E1ZUC1	REVERSED Putative uncharacterized protein (Fragment)	CHLVA	7.965	1	NA
E1ZRB7	Histone H2A	CHLVA	7.143	1	DNA binding
E1ZRA5	Histone H2A	CHLVA	7.143	1	DNA binding
E1Z4G5	Putative uncharacterized protein	CHLVA	7.046	2	glucose metabolic process
E1ZCE3	Histone H2A	CHLVA	6.87	1	DNA binding
K4RLK1	Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (Fragment)	9CHLO	6.865	3	catrbon fixation
Q6DTW2	Chloroplast light-harvesting complex II (Fragment)	CHLPY	6.704	1	photosynthesis, light harvesting, protein-chromophore linkage
E1Z2X3	Histone H2A	CHLVA	6.667	1	DNA binding
V5REV5	Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (Fragment)	CHLSO	6.471	1	magnesium ion binding, ribulose-bisphosphate carboxylase activity
Q08GP1	Ribulosebisphosphate carboxylase large chain (Fragment)	AUXPR	6.364	2	reductive pentose-phosphate cycle
E1ZG17	Putative uncharacterized protein (Fragment)	CHLVA	6.349	2	peroxiredoxin activity
E1Z345	Histone H2A	CHLVA	6.122	1	DNA binding

Accession	Protein Name	Species	% Coverage (95%)	Peptides (95%)	Function
E1ZE06	S-adenosylmethionine synthase	CHLVA	5.598	1	one-carbon metabolic process, S-adenosylmethionine biosynthetic process
E1ZBK2	Putative uncharacterized protein	CHLVA	5.4	2	GTP activity
L0N4A0	Ribulose-bisphosphate carboxylase large subunit (Fragment)	9CHLO	5.319	2	carbon fixation
L0N319	Ribulose-bisphosphate carboxylase large subunit (Fragment)	9CHLO	5.319	2	carbon fixation
L0N0Y5	Ribulose-bisphosphate carboxylase large subunit (Fragment)	9CHLO	5.319	2	carbon fixation
L0N3V5	Ribulose-bisphosphate carboxylase large subunit (Fragment)	9CHLO	5.319	2	carbon fixation
L0N321	Ribulose-bisphosphate carboxylase large subunit (Fragment) OS=Chlorella sp. M10 GN=rbcL PE=3 SV=1	9CHLO	5.319	2	carbon fixation
L0N1W3	Ribulose-bisphosphate carboxylase large subunit (Fragment)	9CHLO	5.319	2	carbon fixation
A0FJ39	Ribulosebisphosphate carboxylase large chain (Fragment)	9CHLO	5.319	2	reductive pentose-phosphate cycle
B3F313	Ribulosebisphosphate carboxylase large chain (Fragment)	CHLVU	5.305	2	reductive pentose-phosphate cycle
A1E8S9	Ribulosebisphosphate carboxylase large chain (Fragment)	CHLSO	5.291	2	reductive pentose-phosphate cycle
H6TI63	Ribulosebisphosphate carboxylase large chain (Fragment)	9CHLO	5.102	2	reductive pentose-phosphate cycle
A9ZM88	Ribulosebisphosphate carboxylase large chain (Fragment)	9CHLO	4.926	2	reductive pentose-phosphate cycle

Accession	Protein Name	Species	% Coverage (95%)	Peptides (95%)	Function
A9ZM86	Ribulosebisphosphate	9CHLO	4.866	2	reductive pentose-

	carboxylase large chain (Fragment)				phosphate cycle
A1E8S7	Ribulosebisphosphate carboxylase large chain (Fragment)	CHLEL	4.854	2	reductive pentose-phosphate cycle
S4VV46	Ribulosebisphosphate carboxylase large chain (Fragment)	9CHLO	4.603	1	reductive pentose-phosphate cycle
E1Z453	Phosphoglycerate kinase	CHLVA	4.511	1	glycolytic process
E1ZKK0	Putative uncharacterized protein	CHLVA	4.478	1	glucose metabolic process
Q85YT9	Ribulose 1,5-bisphosphate carboxylase/oxygenase large subunit (Fragment)	CHLVU	4.425	2	carbon fixation
A8TKT4	Ribulosebisphosphate carboxylase large chain	CHLVU	4.421	2	photorespiration
C7BF02	Ribulosebisphosphate carboxylase large chain	PARKE	4.421	2	photorespiration
S4VNL4	Ribulosebisphosphate carboxylase large chain (Fragment)	CHLSO	4.297	1	reductive pentose-phosphate cycle
H6X2F7	Ribulosebisphosphate carboxylase large chain (Fragment)	CHLSO	4.247	1	reductive pentose-phosphate cycle
S4VUT6	Ribulosebisphosphate carboxylase large chain (Fragment)	CHLPY	4.231	1	reductive pentose-phosphate cycle
Q33BU6	RuBisCO large subunit	CHLPY	4.211	2	carbon fixation
D6R453	Ribulosebisphosphate carboxylase large chain	CHLSO	4.211	2	photorespiration
A9ZM92	Ribulosebisphosphate carboxylase large chain	9CHLO	4.211	2	photorespiration
A9ZM91	Ribulosebisphosphate carboxylase large chain	9CHLO	4.211	2	photorespiration
A8TKS7	Ribulosebisphosphate carboxylase large chain	AUXPR	4.211	2	photorespiration
A8TKR9	Ribulosebisphosphate carboxylase large chain	CHLPY	4.211	2	photorespiration
A8TKR2	Ribulosebisphosphate carboxylase large chain	CHLPY	4.211	2	photorespiration
U5U809	Ribulosebisphosphate carboxylase large chain	9CHLO	4.211	2	photorespiration
Q39463	Ribulose 1,5-bisphosphate carboxylase large subunit	9CHLO	4.211	2	carbon fixation
A9ZM90	Ribulosebisphosphate carboxylase large chain	CHLVU	4.211	2	photorespiration
A9ZM82	Ribulosebisphosphate carboxylase large chain	9CHLO	4.211	2	photorespiration
A9ZM80	Ribulosebisphosphate carboxylase large chain	CHLVA	4.211	2	photorespiration
F2YGL1	Ribulosebisphosphate carboxylase large chain	CHLVA	4.211	2	reductive pentose-phosphate cycle
I3QP02	Ribulosebisphosphate carboxylase large chain (Fragment)	CHLPY	4.074	1	reductive pentose-phosphate cycle

Accession	**Protein Name**	**Species**	**% Coverage (95%)**	**Peptides (95%)**	**Function**
S4VR69	Ribulosebisphosphate	9CHLO	4.059	1	reductive

	carboxylase large chain (Fragment)				pentose-phosphate cycle
E1ZBM4	Putative uncharacterized protein	CHLVA	4.037	1	oxidoreductase activity
E1ZIJ3	REVERSED Putative uncharacterized protein	CHLVA	4.018	1	nucleic acid binding, nucleotide binding
S4VNN3	Ribulosebisphosphate carboxylase large chain (Fragment)	9CHLO	3.957	1	reductive pentose-phosphate cycle
I3UMQ5	Ribulosebisphosphate carboxylase large chain (Fragment)	9CHLO	3.957	1	reductive pentose-phosphate cycle
H6X2F3	Ribulosebisphosphate carboxylase large chain (Fragment)	CHLSO	3.943	1	reductive pentose-phosphate cycle
E1ZPB0	Putative uncharacterized protein	CHLVA	3.927	1	ubiquitin-dependent protein catabolic process
H6X2F5	Ribulosebisphosphate carboxylase large chain (Fragment)	9CHLO	3.873	1	reductive pentose-phosphate cycle
H6X2E6	Ribulosebisphosphate carboxylase large chain (Fragment)	CHLVU	3.741	1	reductive pentose-phosphate cycle
H6X2F2	Ribulosebisphosphate carboxylase large chain (Fragment)	CHLSO	3.667	1	reductive pentose-phosphate cycle
A9ZM89	Ribulosebisphosphate carboxylase large chain (Fragment)	9CHLO	3.607	1	reductive pentose-phosphate cycle
A9ZM93	Ribulosebisphosphate carboxylase large chain (Fragment)	PARKE	3.022	1	reductive pentose-phosphate cycle

Accession	**Protein Name**	**Species**	**% Coverage (95%)**	**Peptides (95%)**	**Function**
E1ZPZ4	REVERSED Putative uncharacterized protein	CHLVA	2.98	1	nucleic acid binding, nucleotide binding
B3F315	Ribulosebisphosphate carboxylase large chain (Fragment)	CHLEL	2.918	1	reductive pentose-phosphate cycle
Q08GN5	Ribulosebisphosphate carboxylase large chain (Fragment)	CHLSA	2.88	1	reductive pentose-phosphate cycle
Q08GN8	Ribulosebisphosphate carboxylase large chain (Fragment)	9CHLO	2.88	1	reductive pentose-phosphate cycle
K4RI49	Ribulosebisphosphate carboxylase large chain (Fragment)	9CHLO	2.813	1	reductive pentose-phosphate cycle
E1ZJM1	Tubulin beta chain	CHLVA	2.752	1	microtubule-based process
K4RL54	Ribulosebisphosphate	9CHLO	2.723	1	reductive pentose-

	carboxylase large chain (Fragment)				phosphate cycle
U6E337	Ribulosebisphosphate carboxylase large chain (Fragment)	CHLSA	2.689	1	reductive pentose-phosphate cycle
A5HLW4	Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (Fragment)	9CHLO	2.6	1	carbon fixation
K4RMF3	Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (Fragment)	9CHLO	2.535	1	carbon fixation
E1ZIW5	Putative uncharacterized protein	CHLVA	2.524	1	arginine biosynthetic process
K4RM74	Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (Fragment)	9CHLO	2.517	1	carbon fixation
A8TKU1	Ribulosebisphosphate carboxylase large chain	CHLEL	2.316	1	photorespiration
E1ZE86	REVERSED Putative uncharacterized protein	CHLVA	2.278	1	metal ion binding, nucleotide binding, RNA binding
E1ZK85	Putative uncharacterized protein	CHLVA	1.951	1	ATP binding
E1ZMD2	Putative uncharacterized protein	CHLVA	1.946	1	protein folding
E1ZD08	REVERSED Putative uncharacterized protein	CHLVA	1.674	1	NA
E1Z4W2	Putative uncharacterized protein	CHLVA	1.117	1	ATP binding, ATP-dependent helicase activity, nucleic acid binding
E1ZGC8	Putative uncharacterized protein (Fragment)	CHLVA	1.085	1	NA
E1Z482	Putative uncharacterized protein	CHLVA	0	0	chromatin binding, DNA binding
E1Z3B4	Putative uncharacterized protein	CHLVA	0	0	regulation of transcription, DNA-templated

Accession	**Protein Name**	**Species**	**% Coverage (95%)**	**Peptides (95%)**	**Function**
E1ZN67	Proteasome subunit alpha type	CHLVA	0	0	ubiquitin-dependent protein catabolic process
Q39660	Late embryogenesis abundant protein homolog	CHLVU	0	0	NA
Q1ZZT3	Antifreeze protein	CHLVU	0	0	NA
G4WUD3	Antifreeze protein	CHLVU	0	0	NA
G4WUD2	Antifreeze protein	CHLVU	0	0	NA
E1Z7R4	Heat shock protein 70	CHLVA	0	0	response to stress
E1Z5Y7	Putative uncharacterized protein	CHLVA	0	0	NA

E1ZBC1	Peptidyl-prolylcis-trans isomerase (Fragment)	CHLVA	0	0	protein folding
U5U4Y4	ATP synthase subunit beta, chloroplastic	9CHLO	0	0	ATP hydrolysis coupled proton transport, plasma membrane ATP synthesis coupled proton transport
F2YGR0	ATP synthase subunit beta	CHLVA	0	0	ATP hydrolysis coupled proton transport, plasma membrane ATP synthesis coupled proton transport
C7BET5	ATP synthase subunit beta, chloroplastic	PARKE	0	0	ATP hydrolysis coupled proton transport, plasma membrane ATP synthesis coupled proton transport
B9TU67	ATP synthase subunit beta (Fragment) OS=Chlorella ellipsoidea GN=atpB PE=3 SV=1	CHLEL	0	0	ATP hydrolysis coupled proton transport, ATP synthesis coupled proton transport
A1E8Z7	ATP synthase subunit beta (Fragment)	CHLSO	0	0	ATP hydrolysis coupled proton transport, ATP synthesis coupled proton transport
A1E8Z5	ATP synthase subunit beta (Fragment)	CHLEL	0	0	ATP hydrolysis coupled proton transport, ATP synthesis

Accession	**Protein Name**	**Species**	**% Coverage (95%)**	**Peptides (95%)**	**Function**
E1ZN42	Putative uncharacterized protein	CHLVA	0	0	protein folding
E1ZPM4	REVERSED DNA polymerase	CHLVA	0	0	DNA replication initiation
E1ZM24	Putative uncharacterized protein	CHLVA	0	0	NA
E1ZFI9	REVERSED Putative uncharacterized protein	CHLVA	0	0	NA
E1ZHK8	REVERSED Putative uncharacterized protein	CHLVA	0	0	DNA binding

E1ZAV4	REVERSED Putative uncharacterized protein	CHLVA	0	0	NA
E1Z4S5	REVERSED Expressed protein	CHLVA	0	0	NA
E1Z2N0	Putative uncharacterized protein	CHLVA	0	0	protein repair, response to oxidative stress
E1ZAQ6	REVERSED Putative uncharacterized protein	CHLVA	0	0	NA
F2YGI6	Cell division protein OS=Chlorella variabilis GN=ftsH PE=4 SV=1	CHLVA	0	0	cell division
E1ZBV9	Putative uncharacterized protein	CHLVA	0	0	NA
E1ZCH0	Peptidyl-prolylcis-trans isomerase	CHLVA	0	0	protein folding
E1ZQ43	Putative uncharacterized protein	CHLVA	0	0	NA
M1HK84	REVERSED Uncharacterized protein	9PHYC	0	0	NA
M1HBU4	REVERSED Uncharacterized protein	9PHYC	0	0	NA
M1I536	REVERSED Uncharacterized protein	9PHYC	0	0	NA
E1ZU40	Putative uncharacterized protein (Fragment)	CHLVA	0	0	ATP binding
E1Z287	Expressed protein	CHLVA	0	0	NA

Accession	**Protein Name**	**Species**	**% Coverage (95%)**	**Peptides (95%)**	**Function**
U5U5I3	Photosystem II D2 protein	9CHLO	0	0	photosynthetic electron transport in photosystem II
F2YGQ0	Photosystem II D2 protein	CHLVA	0	0	photosynthetic electron transport in photosystem II
C7BEZ1	Photosystem II D2 protein	PARKE	0	0	photosynthetic electron transport in photosystem II
E1ZRV0	Expressed protein	CHLVA	0	0	NA
E1ZT46	Putative uncharacterized protein	CHLVA	0	0	prolyl-tRNAaminoacylation
E1Z8A5	REVERSED	CHLVA	0	0	microtubule-based

	Putative uncharacterized protein				movement
E1ZQF5	REVERSED Putative uncharacterized protein	CHLVA	0	0	phosphatase activitY
E1ZIW3	Putative uncharacterized protein	CHLVA	0	0	NA
E1Z789	REVERSED Putative uncharacterized protein	CHLVA	0	0	NA
E1ZC08	REVERSED Putative uncharacterized protein	CHLVA	0	0	NA

Publications

1) Arvind M. Korwar, B. Santhakumari, Mahesh J Kulkarni. Identification of ripening specific proteins in tomato by intact tissue MALDI-TOF-MS. *Electronic Journal of Food and Plants Chemistry* 3 (1) 2008, 10-13.

2) Sunil R. Dhaneshwar, Vidhyak. Bhusari, B. Santhakumari, Mahadeo V. Mahadik. Application of a Stability-Indicating Thin-Layer Chromatographic Method to the Determination of Tenatoprazole in Pharmaceutical Dosage Forms.*Journal of AOAC International*.2009, 92(2) 387.

3) Sreekanth, D., A. Syed, S. Sarkar, D. Sarkar, B. Santhakumari, A. Ahmad, M. I. Khan. Production, Purification, and Characterization of Taxol and 10-DABIII from a newEndophytic Fungus Gliocladium sp. Isolated from the Indian Yew Tree, Taxusbaccata. *J. Microbiol. Biotechnol.*2009, 19(11), 1342–1347.

4)A. B. Pharne, A. S. Ghemud, H. K. Jain, M. J. Kulkarni, B. Santhakumari. Bioanalytical Method Development and Validation of Vildagliptin a Novel Dipeptidyl Peptidase IV Inhibitor by RP-HPLC Method.*Int J Pharm PharmSci,* 2012, 4(3), 119-123.

5) A. S. Ghemud, A. B. Pharne, M. M. Jadhav, K. S. Jain, M. J. Kulkarni, B. Santhakumari.Bioanalytical Method Development and Validation of Levalbuterol A β_2 -Adrenergic Agonist by RP-HPLC Method.*Int J Pharm Pharm Sci.*, 2012, 4(1), 249-253.

6) ShrikantWarkad, A. V. Chandewar, B. Santhakumari. Development and Validation of a Simple and Sensitive RP-HPLC Method for Simultneous Estimation of

Drospirenone and Ethinylestradiol in Combined Tablet Dosage Form.*Int J Pharm Pharm Sci*., 2013, 4(5), 452-457.

7) Amruta B. Kumbhar, UpendraC.Galgatte, ShrikantWarkad and B. Santhakumari. Development and validation of a sensitive bioanalytical method for the determination of Sumatriptan in Rat Plasma by UPLC-MS. *Int J Pharm PharmSci*, 2013, 5(3), 78-82.

8) MitalNakrani, DeepikaBairagee, PradeepGoyal and B. Santhakumari. Analytical and bioanalytical UHPLC-MS method validation for determination of metformin, a bigunaide and sitagliptin, a DPP-4 inhibitor.Int J Pharm Sci Res., 2015, 6(5), 1000-08.

9) Yogesh M. Kolekar, GarikapatiVannuruswamy, Sneha B. Bansode, Santhakumari B,Hirekodathakallu V. Thulasiram and Mahesh J. Kulkarni. Investigationof antiglycation activity of isoprenaline. RSC Adv., 2015, 5, 25051–25058.

Under communication

1) B. Santhakumari1, SandhyaSudage, ShwetaBhat, Suresh K Kesavan, Sandeep B Golegaonkar, Yogesh M Kolekar, Mahesh J Kulkarni ‘’Comparative proteomic profiling of *Dunaliella* and *Chlorella* under salt stress’’.

Conference Attended:

1) 5th Asia Oceania Human Proteome Organization (AOHUPO), 14th Association for the Promotion of DNA Finger Printing and Associated DNA Technologies (ADNAT) and 1st Proteomics Society India (PSI) on 21-25 February 2010 held at CCMB, Hyderabad, India.

2) Member of the local organization committee, International Symposium on Proteomics Beyond Ids…and Fourth Annual Meeting of Proteomics Society (India). November 22-24, 2012, held at CSIR-National Chemical Laboratory, Pune.

3) Member of the local Organization committee of the symposium of The Wonderland of Molecular Structures through the Looking Glass of X-ray Crystallography on 23rd September 2013 held at CSIR-National Chemical Laboratory, Pune.

Educational Attainment(s)

Qualification	Specialization/ Subject(s)	Year	Division	University/ Institute	Additional Information
M. Phil.,	Physical Chemistry	1987	First	S. V. University, Tirupati	Electrode processes are studied using dc Polarography and Cyclic Voltametry
M. Sc.,	Physical Chemistry	1985	First	S. V. University, Tirupati	_

Employment details

Grade/Post	Estt./Lab/Instt.	Duration From	Duration To	Remarks
Principal Scientist	NCL, Pune	29-06-2009	Till to date	_
Sr. Scientist	NCL, Pune	29-06-2003	28-06-2009	_
Scientist	NCL, Pune	29-06-1998	28-06-2003	_
Jr. Scientist	NCL, Pune	29-06-1993	28-06-1998	_
Lecturer	SKP Govt. Degree college, Guntakal, AP	Aug' 1988	April1993	Part-time lecturer