# STRESS RESPONSIVE GENE BIOPROSPECTING STUDIES FROM EXTREMOPHILIC AND EXTREMOTOLERANT MICROALGAE: CHARACTERIZATION AND FUNCTIONAL VALIDATION OF GENES INVOLVED IN ACID, SALT AND THERMAL STRESS

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# *Certificate*

*This is to certify that the thesis entitled "**Stress responsive gene bioprospecting studies from extremophilic and extremotolerant microalgae: characterization and functional validation of genes involved in acid, salt and thermal stress**", is an authentic record of research work carried out by **Mr.Subin C S (Reg. No. 3781)** under my supervision and guidance in the Marine Biotechnology Division, Central Marine Fisheries Research Institute, in partial fulfillment of the requirements for the degree of **Doctor of Philosophy** in **Marine Sciences**, Cochin University of Science and Technology, Kochi and no part thereof has been presented before for the award of any degree, diploma or associateship in any University.*

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# Declaration

*I hereby declare that the thesis entitled "**Stress responsive gene bioprospecting studies from extremophilic and extremotolerant microalgae: characterization and functional validation of genes involved in acid, salt and thermal stress**", is a genuine record of research work done by me under the supervision of **Dr. K. K Vijayan**, Director, Central Institute of Brackish water Aquaculture, Chennai and that no part of this work, has previously formed the basis for the award of any degree, diploma associateship, fellowship or any other similar title of any University or Institution.*

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# List of Abbreviations

| | | |
|---|---|---|
| 3` | : | three prime DNA end |
| 5` | : | five prime DNA end |
| °C | : | degrees Celsius |
| β | : | Beta |
| % | : | Percentage |
| µE | : | Micro Einstein |
| µg | : | Microgram(s) |
| µl | : | Microlitre (s) |
| µM | : | Micro molar |
| AA | : | Amino acid(s) |
| ADP | : | Adinosine diphosphate |
| ATP | : | Adenosine Triphosphate |
| BLAST | : | Basic local alignment search tool |
| bp | : | Base pairs |
| cDNA | : | Complementary DNA |
| DHA | : | Docosahexaenoic Acid |
| DNA | : | Deoxyribonucleic acid |
| dNTP | : | Deoxyribonucleotide |
| *E. coli* | : | *Escherichia coli* |
| EDTA | : | Ethylene diamine tetra acetic acid |
| EPA | : | Eicosapentaenoic Acid |
| *et.al.* | : | And others |
| GLA | : | Gamma-linolenic Acid |
| g | : | Gram(s) |
| IPTG | : | Isopropyl β-D-1-thiogalactopyranoside |
| KDa | : | Kilo Dalton(s) |
| LB | : | Luria Bertani |
| MCS | : | Multiple cloning site |
| min | : | Minute(s) |
| mg | : | Milligram(s) |

| | | |
|---|---|---|
| ml | : | Milli litre(s) |
| mm | : | Milli metre(s) |
| mM | : | Milli molar |
| mRNA | : | Messenger RNA |
| NaCl | : | Sodium Chloride |
| NCBI | : | National Center for Biotechnology Information |
| ng | : | Nanogram(s) |
| NGS | : | Next Generation Sequencing |
| ORF | : | Open reading frame |
| OD | : | Optical Density |
| PAGE | : | Polyacrylamide gel electrophoresis |
| PCR | : | Polymerase chain reaction |
| pH | : | Hydrogen Ion Concentration |
| ppt | : | parts per thousands |
| PUFA | : | Poly Unsaturated Fatty Acids |
| RACE | : | Random Amplification of cDNA Ends |
| ROS | : | Reactive Oxygen Species |
| rDNA | : | Ribosomal DNA |
| RNA | : | Ribonucleic acid |
| RNAi | | RNA interference |
| rpm | : | Revolutions per minute |
| RT-PCR | : | Reverse transcription Polymerase chain reaction |
| SDS | : | Sodium dodecyl sulfate |
| sec | : | Second(s) |
| SSH | : | Suppressive Subtractive Hybridization |
| TAP | : | Tris-Acetic acid Phosphate |
| TEMED | : | Tetramethyl ethylene diamine |
| UTR | : | untranslated region |

# General Introduction

Micro algae are diverse group of microscopic photo-autotrophic non-vascular plants with photosynthesizing pigments. They are unicellular and sometimes form extended chains with simple reproductive structures. These unicellular primary producers are dispersed throughout the photic zones of the ocean and accomplish major share of the primary production in the marine environment and account half of the primary production in the earth. They belong to both prokaryotes (Blue Green algae, Cyanobacteria) and eukaryotes (True algae). The phylogeny of microalgae basically depends on the traditional morphological identification. Morphological identification, based on the structure and arrangement of cell organelles, has limited application when environmental condition like salinity, pH, light, temperature, nutrient condition can change the structure of the cell. Recently more research has been carried out in the field of algal taxonomy, wherein many exciting molecular and ultrastuctural evidences has emerged. Due to its diverse distribution, only about 40,000 to 60,000 species of microalgae have been described. There are many species yet to be described including the extremophiles (Norton *et al.*, 1996; Sastre and Posten, 2010).

As primary producers, micro algae play a vital role in the Earth's carbon cycle and it accounts for about 40-50% of the total global primary productions (Harlin and Darley, 1988; Van den Hoek *et al*., 1995; Graham and Wilcox., 2000). Ocean covers about 70% of the earth's surface. Marine microalgae contribute to most of the primary production by fixing carbon dioxide to organic matter hence they are the main regulators of global climatic

conditions (Raven and Falkowski, 1999). Micro algal culture was first started as live feed for early larval stages of shrimp, molluscs and fin fishes used in aquaculture. More than 40 species of microalgae are used as live feed and many of them like *Chlorella, Scenedesmus, Spirulina* etc. are also used in formulated animal feed (Becker, 2007; Cadoret *et al*., 2012). Since the early 1950's microalgae were studied for their nutritional and industrial application, relevance of micro algae in bioactive compounds, waste treatment, carbon sequestration, genetic engineering, agriculture and bio fuel production has recent origin.

Blue green algae or Cyanobacteria are primitive groups which forms the transition stage between prokaryotes and eukaryotes. They form the descendent of the present day eukaryotic photosynthetic organisms including land plants (Yoon *et al.,* 2004). The main photosynthetic pigments are chlorophyll a, carotenoids and phycobilins and starch form the main storage product. They have typical prokaryotic cell structure with only few membrane bound cell organelles so obvious in eukaryotes (Amos Richmond, 2008). Several species of cyanobacteria are capable of fixing atmospheric nitrogen to nitrates, ammonia and other reactive forms available for their metabolic needs. Nitrogen fixation takes place through the specialized cells called heterocyst. Cyanobacteria exhibit symbiotic association with other organisms and also live as epiphytes. Photosynthetic Blue green algae are important organism due to their vast application in the field of food, feeds, bio fuels, fertilizers etc. One of the important cynobacteria is spirulina which is extensively cultivated for nutrient supplement and which is known as "super food" because of its high protein content and other nutritional values such as high gamma linolenic acid and vitamin B12 levels. The natural colouring agent, phycocyanin is high

in spirulina so is used in cosmetic and food industry. Cynobacteria, *Aphanizomenon flosaquae* is used as dietary supplement.

Among the eukaryotic microalgae, green algae or chlorophycean algae has a significant role in algal biotechnology. Green algae are fast growing and the photosynthetic efficiency is high when compared to plants. Microalgae have the ability to synthesize long chain polyunsaturated fatty acids like gamma-linolenic acid (GLA), arachidonic acid (AA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). Terrestrial plants and animals lack the enzymes to synthesize these long chain fatty acids. Dietary supplement of PUFA has great beneficial effect on human health. The DHA content of marine stramenophile, *Schizochytrium* is very high up to 35- 45% when compared to the conventional omega 3 rich oils like walnut oil and canola oil which contain only 10% omega 3 fatty acids (Cadoret *et al.*, 2012).

Microalgae are rich source of natural pigment with antioxidant potential. The widely cultivated green algae such as *Dunaliella* and *Haematococcus* are rich source of β carotene and astaxanthin respectively. Besides β carotene and astaxanthin there are some other pigments such as leutein, alpha-carotene, lycopene and zeaxanthin which are also extracted from microalgae. The red microalga *Porphyridium purpureum* is a rich source of polysaccharide, which is used in industrial and health field (Huheihel *et al.*, 2002; Matsui *et al.*, 2003; Gourdon *et al.*, 2008).

## Micro algal Genomics

Advancement of technologies like Next Generation Sequencing (NGS) with reduced cost revealed the genomic data of many of the important microalgae. As a model organism *Chlamydomonas* acquired great attention and its complete genome sequencing was completed in 2007 (Merchant *et al.*,

2007). This study exposed the evolution of land plants, characterized the genes behind photosynthesis and flagellar function. Later on the applications of advanced genetic tools like microarrays, RNAi, genetic transformation, etc. helped to unravel the metabolic pathways and biological processes such as responses to stress, the circadian clock (Matsuo and Ishiura, 2011), photosynthetic electron transport chains (Hermsmeier *et al.*, 1991), mechanisms of carbon concentration (Yamano and Fukuzawa, 2009) and flagellar assembly (Iomini *et al.*, 2009). Along with *Chlamydomonas* some other microalgae with phylogenetic distribution, ecological importance and biotechnological applications were studied and these studies helped to identify specific metabolic pathways and associated genes. Further these sequence data may provide insight to post-genomic investigations and would reveal signal transduction pathways, adaptation related to environmental changes, cell physiology, life cycle and metabolisms.

The genome structure of microalgae is complex and it size range from 12.6 Mbp like in the smallest eukaryote *Ostreococcus tauri* to an estimated 10,000 Mbp as seen in the Dinophyta, *Karenia brevis.* Due to complexity in genome structure full genome sequencing is difficult, so transcriptome approach has been adopted to make gene catalogue. There are around 39 microalgal transcriptome which has been sequenced and some of the important algae with complete genome data available are green microalgae *Chlorella vulgaris* UTEX395 (Guarnieri *et al.*, 2011), Ochrophyta *Pseudochattonella farcimen*, which is associated with fish mortalities (Dittami *et al.*, 2011), *Dunaliella salina* (Zhao *et al.*, 2011), *D. tertiolecta* (Rismani-Yazdi *et al.*, 2011) and the coccolithophore *Emiliania huxleyi* (Von Dassow *et al.*, 2009). Genomic approach has significant impact on microalgal biotechnology. The molecular mechanisms behind the synthesis of valuable metabolites provide an

insight of genes and with the modification of these would enable the better production of many bioactive compounds. Genomic data unveiled many of the new bioactive compounds from microalgae, the Heterokonta *Aureococcus anophagefferens* genome revealed the enzymes involved in the synthesis of toxic isoquinoline alkaloid which was not reported from these harmful algae (Gobler *et al.*, 2011). Molecular farming is a sustainable approach to produce pharmaceutical molecules. Microalgae have several advantages as potential expression system for the production of recombinant proteins.

## Extremophilic microalgae and their importance

Extremophiles are organisms which thrive and flourish in extreme conditions like hypersaline (2-5M NaCl), high temperature (50ºC-120ºC) or lower temperature (-2 to -20ºC), either alkalinity (pH>8) or acidity (pH<4).The term extremophile was first described by MacElroy in 1974. Extremophiles have the ability to tolerate many stressful environments which would be detrimental to normal life. Many of the extremophile identified belongs to the domain archaea and recently works has been carried out to characterize eukaryotic Kingdoms and among these microalgae are significant because of their diverse distribution on the earth. Among the photosynthetic eukaryote Cyanidiales (red algae) and Chlorophyte are the predominant groups. The red algae have a higher tolerance level. But the ecologically important diatoms and prymnesiophytes (members of the Heterokont algae) and the dinoflagellates do not form extremophiles (Varshney., *et al*. 2014) with few exceptions such as diatom *Pinnularia sp*. found in low pH fresh water (Aguilera *et al*., 2006) and some psychrophilic diatoms (Seckbach, 2007). Extremophiles are under-exploited resources and characterization of these organisms has potent application in the field of biotechnology. The enzymes produced by mesophiles have limited application at extreme

condition whereas the extremozyme produced by extremophile shows a greater stability under different harsh conditions. So they have better potential applications. Extremophilic microalgae have the ability to grow in fluctuating environmental conditions and also the extreme conditions prevent the growth of undesired organisms, so extremophilic microalgae have potential application in bio-resource engineering. Extremophiles are good source of desired genes that would enable recombinant production of active molecules and these genes can be used to produce stress tolerant plants through genetic engineering.

Among Extremophiles, ample evidences are available for thermophiles because of the potent source of thermostable enzymes. Thermphilic algae can tolerate extreme conditions because of the accumulation of certain bioactive compounds like α- tocopherol, carotenoids, astaxanthin, etc. Thermophilic algae like *Galdieria sulphuraria* (a red alga) and *Desmodesmus* (a green alga) are used for the production of pigments. *G. sulphuraria* has potential application in waste water treatment. Thermostability under extreme condition is acquired through the physiological adaptation supported by molecular mechanisms by differential expression of certain genes. Thus gives stability to DNA and also show protein folding under extreme temperature. Application of thermophiles includes the characterization of these temperature tolerant genes for the genetic modification of economically important plants for survival under changing climatic conditions.

Halophilic microalgae inhabit the hypersaline lakes and solar salt evaporation ponds. Hypersaline conditions are also subjected to high temperature and radiation. So microalgae which thrive in such conditions develop fascinating mechanism to adapt to these environments. The adaptive mechanisms to overcome this hyper osmotic pressure are through the production of some

osmotolerant compounds like glycerol, glycine betaine etc. inside the cell. The excess production of β- carotene prevents oxidative damage caused by high iridescence. These stress tolerance mechanism is advantageous for the culturing of one of the most halotolerant eukaryote, *Dunaliella salina* for the commercial production of β- carotene and glycerol. Global warming seriously affects the soil salinity through sea water intrusion which negatively affects the land productivity. Due to adverse climatic condition and increased population density there will be insufficient food supply to the growing population. To circumvent this problem and to acclimatize the plant to these changing climatic conditions, the immediate and advanced solution is the genetic modification of the candidate plant genomes. This can be achieved through desired genes that are actively involved in salinity tolerance. As a eukaryote, halophilic microalgae are good source of desired genes for salinity tolerance. Presently few microalgae have been selected for genetic characterization, so as to explore their potential biotechnological applications.

Acidophiles are organisms which survive under high acidic conditions (usually at pH 2 or below). Large number of acidic environment covers the earth but the survivability of organisms to this extreme is limited. Low pH increases the solubility of metals which causes metal toxicity. Acidophilic microalgae have the ability to tolerate metal toxicity through genetic or physiological modifications. So they are potential candidate for bioremediation. Among eukaryotes, photosynthetic microalgae have the ability to tolerate acidic conditions and the group Cyanidiaceae has a better tolerance level. Complete genome sequencing of the most acid tolerant cyanide, *Galdieria sulphuraria* has been carried out (Weber *et al*., 2004; Barbier *et al*., 2005). In the present scenario global warming has significant impact on the ocean pH. Increased carbon dioxide concentration reduces the ocean pH which has serious impact on marine biodiversity. Genetic characterization of marine acidophile offers an

insight to the molecular mechanism behind the acidic stress. Characterization of genes which give acid tolerance can be used as quantitative trait for the isolation of stress tolerant organisms for future applications.

Abiotic stress tolerance mechanisms linked to different biological pathways are controlled by multiple genes. These stress tolerant gene products are mainly classified in to metabolic products which protect plant from stress, help in signal cascading and transcriptional controlling systems and also transport of ions and water. Engineering of genes involved in these pathways would enable plants to develop better tolerance under abiotic stresses. Success of the genetic transformation depends on the selection of suitable genes and their source. As a eukaryotic photosynthetic organism, extremophilic microalgae are a better source for the desired genes which would enable homologous expression in plant genome.

There are wide ranges of techniques available for gene discovery. Expressed sequence tag (EST) is a common approach with an advantage of functional characterization if full length cDNA are cloned. Microarrays can be used for the expression profile of cloned cDNA. Differential Display Reverse Transcription (DDRT-PCR) is another technique which helps to analyze and compare changes in gene expression at mRNA levels. Advancement in Next generation sequencing (NGS) has been employed for sequencing cDNA libraries. Even though they are efficient for quantitative analysis, they are labour intensive and highly expensive (Sahebi *et al*., 2015). Present study used the advantage of Suppression Subtractive Hybridization (SSH) technique for the identification of differentially expressed genes under various abiotic stresses. SSH is an effective molecular technique to identify genes with differential expression level under various biological processes including abiotic stresses. SSH is an effective method for enriching rare transcripts

(Neill and Sinclair, 1997). The principle of SSH is that selective amplification of target cDNA fragments and suppression of non-targeted cDNA fragment through the long inverted terminal repeats ligated to the 5' ends of single-strand cDNA fragments. The main distinguishing characteristics of SSH are the low false positives and target cDNA fragments are amplified selectively, whereas non-target cDNA fragments are suppressed from amplification (Diatchenko *et al*., 1996, Morissette *et al*., 2008, Coetzer *et al*., 2010, Zhang *et al*., 2012, Sahebi *et al*., 2015).

## Objectives of the study

1. Isolate, identify and culture extremophilic and extremotolerant microalgae.
2. Transcriptomic profiling of these algae for differentially expressed genes under various abiotic stresses such as acidity, salinity and temperature.
3. Complete characterization of important genes which tolerate various abiotic stresses.
4. Functional validation of identified genes through acquired stress tolerance in *E. coli* by recombinant expressions.

Chapter 1

# Differential gene expression under acidic stress from an acid tolerant euryhaline microalgae, *Dictyosphaerium ehrenbergianum*

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1.2 Materials and Methods
1.3 Results
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## 1.1 Introduction

Decreasing ocean pH is one of the major consequences of global warming which seriously damage marine organisms. Oceanic pH decreased 0.1 U due to the industrial revolution in the eighteenth century and it is further estimated to decrease 0.5 U and reaches around a pH of 7.7 by 2100. The main reasons for ocean acidification are anthropogenic activities which lead to uncontrolled emission of $CO_2$ causing the formation of carbonic acid ($H_2CO_3$). The dissociation of carbonic acid produces bicarbonate ($HCO_3^-$) and $H^+$ ions, which reduces oceanic pH. The changing global climate will affect the distribution of organisms in the ocean (Poloczanska *et al*., 2013) and also affect the community structure and physiology of organisms (Perry *et al*., 2005; Somero, 2010). The tolerance of these extreme limits is through physiological adaptation supported by

modified gene expression. Recently, more attention has been focused on the evolutionary adaptation of organisms towards climate change in the marine environment. The immediate effect of ocean acidification is reflected in calcareous algae, coral reefs and other ocean life with calcium carbonate exoskeleton because low pH reduces the carbonate absorption (Feely *et al*., 2004). Among eukaryotic photosynthetic organisms, limited studies has been conducted in coccolithophores to find out the molecular mechanism involved under increased $CO_2$ (Lefebvre *et al*., 2009; Richier *et al*., 2011; Rokitta *et al*., 2012 ).

This study focused on the adaptive mechanism of organism by differential gene expression under experimental acidic stress. The tolerance level for acidic environment is varied from organism to organism and some algae have the ability to tolerate pH 3 or even below. Microalgae are diverse group of photosynthetic eukaryotic organism distributed almost everywhere on earth. We can find them even in extreme environment such as acidic thermal springs, hypersaline lakes, acidic lakes etc. They are responsible for the major primary production in the ocean and it accounts for half of the earth's primary productivity. As a primary producer it is important to predict vulnerability of microalgae to varying climatic change by understanding the molecular mechanisms to compensate the effect of reduced ocean pH. A decreased pH reduces the photosynthetic efficiency of phytoplankton and thereby reduction in the ocean productivity is anticipated. Nutrition to coral reefs is mainly contributed by the symbiotically associated microalgae. Decreased ocean pH seriously affects associated algae which could be one of the reasons for phenomena such as coral bleaching. In the present study we have isolated euryhaline (wide range of salinity tolerance) microalgae, *Dictyosphaerium ehrenbergianum* (CMFRI-MBTD-S129) which grown under various salinity from 0‰ to 40‰ and also the saline acclimatized cells have the

ability to tolerate acidic environment up to pH 3. Multiple stresses enhance the expression of various genes to overcome these stressful environments. Earlier works mainly focused on the impact of ocean acidification on calcifying marine life because of the reduced absorption of carbonate under low pH. This study focused on the capability of organism to adjust with the changing climatic condition through the altered expression of functional genes to maintain homeostasis. Evolutionary adaptation is essential for the organism to persist in the changing environmental conditions. Among the evolutionary adaptations genetic adaptation has significant importance but the studies are scarce at molecular level.

Molecular approach provides better understanding of organism's response to changing climate. Suppression subtractive hybridization is a reliable molecular technique for the characterization of differentially expressed genes (Zhang *et al.*, 2012, Sahebi *et al.*, 2015). A combination of SSH and quantitative validation using Real-Time PCR revealed many genes which have active role in acidic stress tolerance. These studies throw light on whether the genetic adaptations are sufficient for the organisms physiological functioning to withstand changing climate and other abiotic stresses. An organism's adaptability depends on the functioning of protein because proteins control all cellular processes. Genetic modification is attained through the modification of proteins and these are achieved through gene mutation which gives tolerance to changing climate.

## 1.2 Materials and Methods

### 1.2.1 Isolation and Identification of algal strain

Algal strain was isolated from the Cochin estuary (9°59.321'N and 76°16.225'E) during monsoon season (July, 2010), where the salinity was very low (2 ppt). Water samples collected were enriched with f/2 medium and kept

at 25ºC with continuous illumination for a period of one week. Algal cells were isolated from the enriched sample by serial dilution. Further purification was carried out by streaking cells on agar plates enriched with f/2 media and supplemented with antibiotic mix to eliminate bacterial growth. It was then incubated at 25ºC with continuous illumination until visible colonies appeared. Single colony was picked and inoculated to 100 ml freshwater with f/2 media under sterile condition and kept at 25ºC with 30 $\mu E\ m^{-2}\ s^{-1}$white fluorescent light.

Identification of the isolated algae was carried out by analyzing morphological features like size, shape and colour of the cells, arrangement of chloroplast and other cellular organelles under phase contrast microscope. Morphological isolation was further confirmed with molecular technique by sequencing 18S rRNA gene and BLAST analyzed in NCBI gen bank.

### 1.2.2 Culture Maintenance and Stress Treatment

The observed growth in f/2 media was stagnant so the cells were inoculated to double strength of f/2 media and maintained under continuous illumination at 25ºC. The algal culture flourished in the f2 media were further screened to their tolerance levels under different salinities (0 ppt, 10 ppt, 20 ppt and 40 ppt) and pH (pH 3, pH 4, pH 6 and pH 8). The cells acclimatized under different salinities were also screened for pH tolerance.

### 1.2.3 RNA Isolation and Suppressive Subtractive Hybridization

Total RNA was isolated from the algal culture grown at normal (pH 8) and stressed condition (pH 4) during the exponential growth phase by using TRI reagent (Sigma, USA). Total RNA was also isolated from short term acid shocked cells (6 hrs, 12 hrs, 24 hrs and 48 hrs) and pooled to fore mentioned

RNA. Isolated total RNA was quantified by Bio photometer plus (Eppendroff, Germany) and integrity was checked in 1.5% agarose gel electrophoresis. Then the mRNA was purified with GenElute™ Direct mRNA Miniprep Kit (Sigma, USA). A total of 2 µg purified mRNA was used for synthesis of complementary DNA (cDNA) and subtractive hybridization was done using PCR Select cDNA subtraction kit (Clone tech, USA). Experiment started with the synthesis of first strand cDNA of both tester and driver using 2 µg purified mRNA, cDNA synthesis primer containing Rsa1 restriction site, dNTP mix (10mM each) smarts scribe reverse transcriptase at 42ºC. Both tester and driver first strand cDNA synthesized were immediately transferred to double strand DNA with a second strand enzyme cocktail (DNA polymerase I, RNase H and *Escherichia coli* DNA ligase and T4 DNA polymerase) followed by incubation at 16ºC. Double stranded DNA synthesized was further subjected to Rsa1 restriction digestion which created blunt end cDNA fragments. Rsa1 digested tester DNA was taken into two separate tubes and labeled as T1 and T2 and each tester cDNA was ligated with two different adapters (Oligonucleotides supplied with kit) at 16ºC for 12 hours and driver cDNA was not ligated with adapters.

Subtractive hybridization was carried out in two steps. During first hybridization an excess of Rsa1 digested driver cDNA was added to each tester cDNA and then incubated at 98ºC for 1.5 minutes. This was followed by annealing at 68ºC for 8 hours, leading to the enrichment of differentially expressed genes. Second hybridization was carried out by mixing both primary hybridized sample and fresh denatured driver cDNA which leads to further enrichment of differentially expressed genes. Polymerase chain reaction was carried out for selective amplification of differentially expressed genes under

acidic stress with adapter specific primers. Subtracted cDNA fragments amplified were cloned into pJET vector. cDNA clones obtained were screened with vector specific primers and insert size was analyzed by agarose gel electrophoresis. Positive clones were cultured, plasmids were isolated using GeneJET Plasmid Miniprep Kit (Thermoscientific, USA) and sequenced. The sequences were analyzed using both BLASTN and BLASTX for its homology with the available sequences (http://www.ncbi.nlm.nih.gov/BLAST/)

### 1.2.4 Quantitative Validation of Selected Genes using Real-Time PCR

Selected 11 genes of both functional and unknown genes were quantitatively validated using Real-Time PCR. Specific primers for both known and unknown genes were designed using Beacon Designer™software and synthesized. Total RNA was isolated from the normal and stressed cells as followed in the SSH procedure. Total RNAs isolated were quantified spectrophotometrically by using biophotometer (Eppendroff, Germany). DNA contamination was eliminated by treating with RNase free DNase (1U/µg). First strand cDNA was synthesized using Revert Aid Premium First Strand cDNA synthesis Kit (Thermoscientific, USA). Real-Time PCR was carried in iQ5 thermal cycler (Biorad, USA) using SYBR green master mix (Biorad, USA). Normalization of expression was carried out with 18S rRNA reference gene (Kuchipudi *et al.*, 2012). All the reactions were carried out in triplicate with a standardized procedure. Details of the primers used for the validation experiment is given in the Table.1.1

**Table 1.1** List of primers used for the quantitative validation of acidic stress induced genes

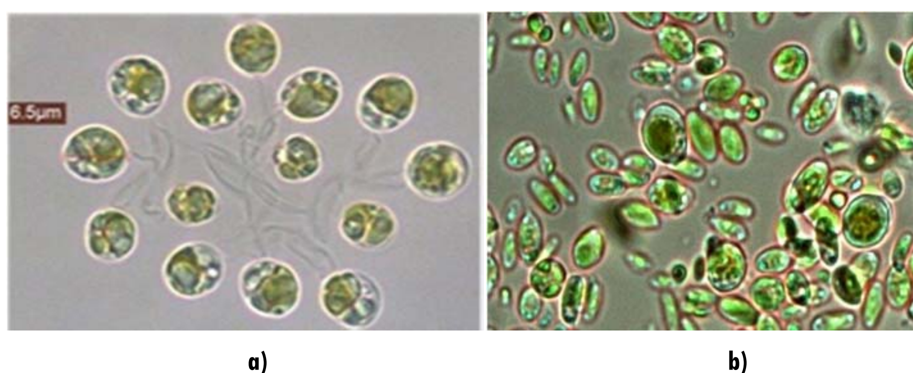
| SL. No. | Gene | Primer | Sequence | Product size (bp) |
|---|---|---|---|---|
| 1 | Ubiquitin | S129_ubiqtn_QPF | CTGACTACAACATCCAGAAGGAG | 138 |
| | | S129_ubiqtn_QPR | TACAGGGCTAGGTGGGATAC | |
| 2 | Cinnamyl alcohol dehydrogenase | S129CAD_QPF | GCAATGTGCCTACACAATGTT | 113 |
| | | S129CAD_QPR | GGCTTGCACGTTCACAATG | |
| 3 | Thioredoxin | S129thiordxn_QPF | GCCGCCACCAATTCAATAC | 124 |
| | | S129thiordxn_QPR | TGAACGACAAGCAGGAGTT | |
| 4 | Sugar epimerase | S129Sugrnucltdepimrse_QPF | AGCTTCTCCAGGCCGTAT | 120 |
| | | S129Sugrnucltdepimrse_QPR | CATCCTCCGCTTGCATCTAC | |
| 5 | ATP synthase | S129ATPsynthse_QPF | TTTGATGGCGAGCTTCCT | 102 |
| | | S129ATPsynthse_QPR | GAAGAGTGTTCTCTCCCAGATG | |
| 6 | Major Facilitator Super family protein | S129MFIT_QPF | AGGAACCCAACTCCATACTTTATC | 115 |
| | | S129MFIT_QPR | ACGGTATCCAAGAAGCAGAATAC | |
| 7 | Multi copper ferroxidase | S129MCF_QPF | CCGTTACACTCTTTCAAACTTCTC | 81 |
| | | S129MCF_QPR | TCGGGATTCATGTAGTAAGGTATT | |
| 8 | Unknown 328 | S129_UN_328_QPF | GGCAACAAGGCCTACTACAA | 141 |
| | | S129_UN_328_QPR | GGCATTAAACAGTGGCTTGTG | |
| 9 | Calmodulin | S129calmdn_QPF | CAACGGCACCATCGACTT | 110 |
| | | S129calmdn_QPR | CCGTCCTTGTCAAACACCTT | |
| 10 | Osmotically Induced protein | S129OsMc_QPF | CACAGTGGTTGTGGACAGAG | 109 |
| | | S129OsMc_QPR | CAATCAGGGCTCCTAAGAAGTG | |
| 11 | Unknown 541 | S129un_541_QPF | GGAAGGCTTGTTGGGACAAT | 149 |
| | | S129un_541_QPR | AGACGCCGAAGCATGAAAC | |
| 12 | 18S rRNA | Univ18SRT1F | GGGCTCGAAGACGATTAGATAC | 121 |
| | | Univ18SRT1R | GTGCTGGTGGAGTCATCAA | |

## 1.3 Results

### 1.3.1 Identification and Stress tolerance of *Dictyosphaerium ehrenbergianum*

Isolated strain of microalgae was identified as *Dictyosphaerium ehrenbergianum* (MBTD-CMFRI-S129) using morphological features combined with molecular identification by sequencing 18 S ribosomal rRNA gene (NCBI Acc. No. JF708180). *D. ehrenbergianum* cells have the ability to

tolerate wide range of salinity from 0 ppt to 40 ppt and the saline acclimatized cells are also able to withstand low pH even at pH 3. There was a drastic change in the morphology when the cells were acclimatized to stress conditions. In the optimum conditions cells are round in shape and form colonies with extracellular mucilage. Under stressed conditions cells become single, oval in shape and rigid. These morphological changes may be due to the physiological adaptation to overcome stressful environment.




a) b)

**Fig1.1.** *Dictyosphaerium ehrenbergianum* a) Normal cells b) Stressed cells

### 1.3.2 Assembly and analysis of differentially expressed genes under acidic stress

Genes differentially expressed under acidic stress from euryhaline acid tolerant micro algae, *D. ehrenbergianum* were characterized using Suppressive Subtractive Hybridization. A total of 200 clones with an insert size range of 0.2kb - 1kb were sequenced. All the sequence were edited and made in to contigs with overlapping sequence using Seqman sequence editor. A total of 78 contigs were formed from the differentially expressed gene sequence and these contigs were analyzed in NCBI gen bank using BLASTX and BLASTN programme.

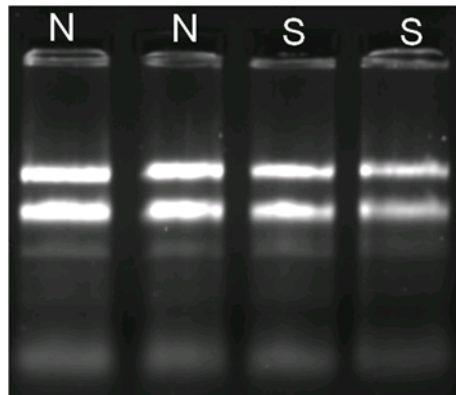



**Fig 1.2.** Total RNA isolated from *D.ehrenbergianum*on 1.5% AGE, N- Normal RNA (pH 8), S- Stressed RNA (pH4)

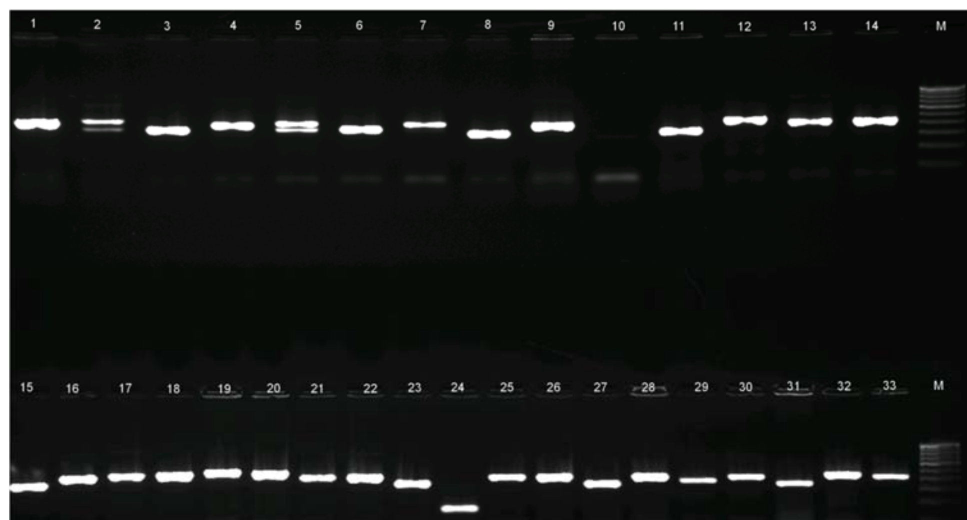



**Fig 1.3** Colony PCR with pJET primers on 1.5% AGE, 1- 33 differentially expressed gene fragments, M- 100bp Marker

BLAST analysis revealed the identification of differentially expressed genes under acidic stress. Among the differentially expressed genes 55% showed sequence similarity with the functional genes which are actively involved in stress tolerance mechanisms as well as metabolic processes, 21% showed no significant similarity with the reported nucleotide and this may be treated as unknown genes with functional role under acidic stress and the remaining 24% showed a sequence similarity with ribosomal genes (Fig1.4). Details of the functional genes differentially expressed under acidic stress were

given in the table 1.2. All the identified genes directly or indirectly involved in the acid or abiotic stress tolerance mechanisms are actively involved in biological process of the cell. Functional genes are classified based on their cellular function such as photosynthesis, cell proliferation and DNA repair, metabolic processes, stress response and cellular transport of ions (Fig 1.5). All these genes are directly or indirectly involved in acidic stress and also other abiotic stresses such as salinity, drought, temperature stress and oxidative stress etc.

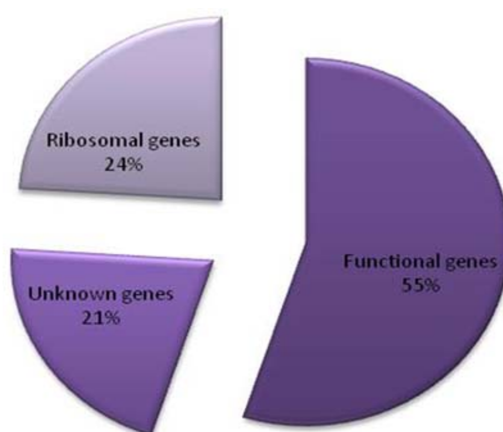



**Fig 1.4** Classification of differentially expressed genes under acidic stress

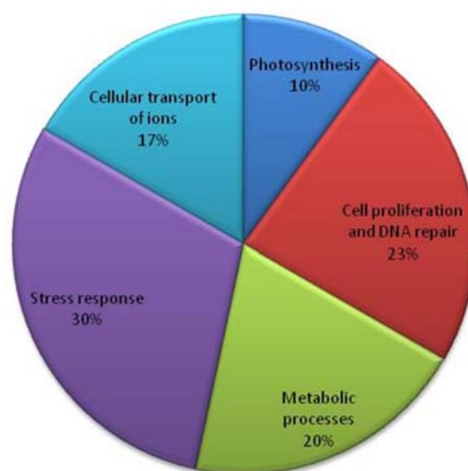



**Fig1.5.**Functional classification of identified acid tolerant genes

**Table 1.2** List of acidic stress induced genes showing significant similarity to known sequence in the public database

| Putative gene | Annotation e-Value | Functions | Reference |
|---|---|---|---|
| Multicopperferroxidase | 3e-04 | Cellular uptake of iron | Lang *et al.*,2012; Crysten and Sabeeha, 2012 |
| Major Facilitator Super family protein | 1e-57 | Acid stress, Drought stress | Remy *et al.*,2013; Xu *et al.*, 2014 |
| Cinnamyl alcohol dehydrogenase | 1e-10 | Abiotic and biotic stresses | Vidal *et al.*, 2009; Jin *et al.*, 2014 |
| ATP synthase beta chain | 1e-44 | ATP synthesis and hydrolysis, intracellular pH homeostasis drought, salt, cold and oxidative stress | Harold *et al.*, 1970, Legendre *et al.*, 2000;Breton *et al.* 2003; Tamura *et al.*, 2003; Cotter and Hill, 2003 |
| tRNA-splicing ligase RtcB | 2e-14 | stress-induced splicing of mRNA | Popow*et al.*, 2014 |
| 20S proteasome alpha subunit A1 | 3e-45 | Misfolded protein stresses and defenses | Sahana *et al.*,2012 |
| ATP-dependent RNA helicase eIF4A | 1e-164 | Salt and cold stress | Nakamura *et al.*, 2004 |
| Vacuolar ATP synthase subunit D | 2e-72 | ATP synthesis , ion transport, Salt stress | Golldack and Dietz,2001 |
| Oxygen evolving protein | 6e-21 | Photosynthesis, abiotic stress | Koichi *et al.*, 2000 |
| TBC-domain-containing protein | 6e-10 | protein-binding, GTPase activating | Ishibashi *et al.*, 2009 |
| Carbohydrate-binding module family 48 protein | 5e-11 | Carbohydrate metabolism | Camilla *et al.*, 2009 |
| RNI-like protein | 2e-43 | Protein binding, abiotic stress tolerance | Jensen *et al.*, 2013; Saeidm *et al.*, 2014 |
| Elongation factor alpha | 2e-130 | Protein synthesis, heat stress | Bhadula *et al.*, 2001 |
| Arginasedeacetylase | 7e-27 | Abiotic stress tolerance | Shi *et al.*, 2013 |
| NADP-dependent malic enzyme MaeA | 5e-63 | Abiotic stress tolerance | Laporte *et al.*, 2002, Zeng-Hui *et al.*, 2010 |
| Ubiquitin | 7e-38 | Regulatory pathway and abiotic stress | Lyzenga and Stone, 2011, Cui *et al.*, 2012 |
| Photosystem II 44 kDa protein | 4e-73 | Photosynthesis | Kristen *et al.*,2009 |
| Glycine-rich RNA-binding protein | 3e-13 | Environmental stress and metabolism of mRNA | Kim *et al.*, 2007, Singh *et al.*, 2011 |
| Phosphoglyceratemutase | 3e-35 | Glycolysis, Abiotic stress, oxidative stress | Zhao and Assmann, 2011, Y *et al.*, 2014 |
| 2-methyl citrate synthase | 4e-37 | Krebs cycle and plant stress tolerance | Tong *et al.*, 2009 |
| Sugar nucleotide epimerase | 2e-83 | Photosynthesis, growth regulation and osmotic stress | Seifert *et al.*, 2004; Li *et al.*, 2011 |
| Thioredoxin domain 2 | 3e-30 | Oxidative stress and other abiotic stresses | Nancy A. Eckardt, 2006; Santos and Rey, 2006; Zagorchev*et al.,* 2013, |
| Calmodulin | 6e-41 | Stress signaling pathway | Pardo *et al.*, 1998, Reddy *et al.*, 2010 |
| Glutathione peroxidase | 4e-23 | Abiotic stress | Faltin *et al.*, 2010; Gaber*et al.*, 2012 |
| Osmotically inducible protein C | 2e-10 | Oxidative stress and osmotic stress | Park *et al.*, 2008 |
| Elongation factor EF-3 | 7e-09 | Protein synthesis, heat stress | Ristica *et al.*, 2007; Fu *et al.*, 2012, |
| Light-harvesting complex II protein | 5e-39 | Photosynthesis, photo protection and energy dissipation | Siffel and Vacha, 1998; Barros and Kuhlbrandt, 2009; Dittami *et al.,*2010 |
| Transposase | 7e-67 | Biotic and abiotic stress | Marie-Angele Grandbastien,1998; Hidetaka Ito, 2013; Makarevitch et al., 2015 |
| DNA helicase | 7e-77 | Plant growth and development, Salinity stress and oxidative stress | Vashisht, 2005; NarendraTuteja, 2010; Tuteja *et al.*,2014 |

### 1.3.3 Expression Pattern of Differentially Expressed Genes using Real–Time PCR

Quantitative validation of the expression pattern of differentially expressed genes was carried out using Real–Time PCR. Selected 11 gene fragments from both novel as well as functional genes were validated under acidic stress. All genes showed an upward regulation under acidic stress and the expression level varied from 1 to 2.5 fold greater than the control (pH 8). This expression profile also strengthens the efficiency of SSH for the identification of differentially expressed genes. Among the highly expressed genes $H^+$ ATP synthase and major facilitator like ions transporter has significant roles in cell homeostasis.

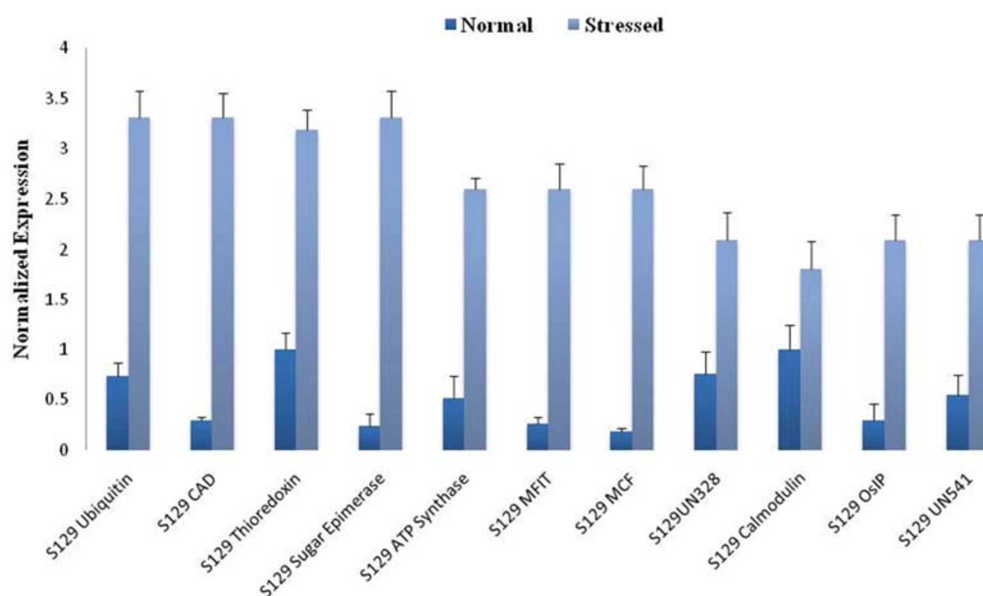



**Fig 1.6.** Expression profile of selected genes differentially expressed under acidic stress using Real-Time PCR. Error bar indicates ± standard deviation (SD), Number of replicate (n)= 3.

## 1.4 Discussion

This study has tried to elucidate the gene expression profile of euryhaline microalgae *Dictyosphaerium ehrenbergianum* under acidic stress and thereby analyze the effect of reduced ocean pH on marine life at molecular level. Microalgae are distributed almost everywhere on earth because of their inherent ability to tolerate extreme environment. These survival mechanisms under harsh conditions are attained through adaptive mutation. The adverse effect of ocean acidification is more pronounced in calcifying marine organisms where elevated $CO_2$ is more sensitive. This study highlights the effect of reduced pH on marine life. Here we focused on the effect of acidic pH on vulnerability of marine life through physiological adaptation achieved by genetic modifications. Genetic adaptability is acquired through the combined action of number of genes to mitigate the climatic change, but still there have been limited studies in genetic effect of climatic change (Franks and Hoffmann, 2012). In the present scenario global climatic change reduce the ocean surface pH in a predicted rate of 0.1 to 0.5 units in the next century (IPCC, 2007). It is essential to analyze effect of ocean acidification on the physiological adaptation of marine living organisms.

This study also revealed many genes which are actively involved in the tolerance mechanism under various stresses. The physiological adaptations are achieved through these differentially expressed genes. These functional genes were classified based on their functions to photosynthesis, cell proliferation and DNA repair, metabolic processes, stress response, cellular transport of ions.The homeostasis of the cell is achieved through the transport of ions across the membranes. During elevated pH acid-base balance is maintained by specific genes which control the ion regulatory pathways. Among the ions transporter proteins Major Facilitator Superfamily (MFS) has significant role

in homeostasis and provide tolerance to extreme acidic environment (Xu *et al.*, 2014). MFS are secondary or electro chemically gradient transporters functioning by the gradient generated through co-transported molecules (Petrasek *et al.*, 2006; Wisniewska *et al.*, 2006; Pfeil *et al.*, 2012). The MFS proteins are coded by multiple genes and the *Arabidopsis* genome contain more than 120 genes to encode MFS superfamily (Ren *et al.*, 2004). In plants only few MFS were characterized and these proteins are meant for the transport of sugar, oligonucleotides and nitrate (Buttner, 2007; Tsay *et al.*, 2007; Hamid and Parviz, 2014). The zinc induced facilitator transporter from *Arabidopsis* is involved in homeostasis and drought tolerance evidenced by the heterologous expression in yeast (Cabrito *et al.*, 2009; Remy *et al.*, 2013). In citrus plant induction of MFS was observed under salinity stress (Brumos *et al.*, 2009) which indicates the response of this protein family under multiple stresses. The energy expenditure for tolerance mechanisms under extreme condition is acquired through increased ATP synthesis. In the present study, increased expression of $H^+$ ATP synthase has helped to maintain homeostasis of cell as it works under proton motive force. Earlier works in bacteria and other micro organisms has proved the role of F1F0 ATPase which acts as a proton exporter under acidic conditions. (Harold *et al.*, 1970; Cotter and Hill, 2003). Along with ATP synthesis it has specific role in abiotic stresses like drought, salinity, temperature stress etc. (Legendre *et al.*, 2000; Breton *et al.*, 2003; Tamura *et al.*, 2003; Komatsu, 2010). The resistance of *Streptococcus mutans* under acidic condition is due to the over expression of ATP synthase as it extrude the $H^+$ ions (Len *et al.*, 2004).

Iron is an essential element for the normal physiological functioning of the cell. In plants, algae and other photosynthetic organism's iron function as co-factor for photosynthetic electron transport chain. Acidic condition reduces the

absorption and transportation of iron and hence there should be an effective iron uptake mechanism under acidic stress. Redox mediated iron uptake is a mechanism of iron uptake found in plants, yeast and algae and is under the control of multicopper feroxidase (Paz *et al*., 2007). During iron depreciation, an elevated expression of this gene enhances the iron absorption. In *Chlamydomonas reinhardtii* the regulation of iron transportation is by ferroxi reductase gene which is induced under iron starvation (Liping *et al*., 2013).

The cellular stress response is a well developed and generalized mechanism under various stresses. During acidic stress large number of proteins involved in cellular stress response is over expressed. All stress finally leads to oxidative damage to the cells. The over expression of antioxidative protein is essential to compensate all type of stress. The present study also showed differential expression of antioxidant genes such as thioredoxin, glutathione peroxidase, arginase deacetylase, etc. which were over expressed. In plants, thioredoxin (Trx) gene over expression is related to oxidative stress, which leads to increased ROS level (Nishinaka *et al*., 2001, Koharyova and Kollarova, 2008). Other than antioxidant function, thioredoxin is actively involved in DNA synthesis, sulfur assimilation, cell growth, inflammation reactions, apoptosis and transcription regulation. The trx gene is conserved in almost all organisms and their main function is oxidative stress response (Laloi *et al*., 2004). In plants numerous types of Trx has been identified compared to animals (Santos and Rey, 2006). *Arabidopsis* genome revealed 42 Trx gene and functional role of most of the Trx gene is not yet finalized. The over expression of Osmotically inducible protein C (OsmC) is also under defense mechanism and is actively involved in oxidative stress damage. The first demonstration of this gene was carried out in bacteria under osmotic stress (Gutierrez and Devedjian, 1991). *Mycoplasma genitalium*

which showed an effective resistance against oxidative stress caused by host ROS is due to the expression of a novel OsmC gene which possesses hydroperoxide reductase activity (Zhang and Baseman, 2013). The over expression of arginase deacetylase exerts resistance to multiple abiotic stresses. Arginase deacetylase is actively involved in arginine metabolism which finally produces metabolite such as putrescine, which scavenges reactive oxygen species produced during oxidative stress (Wimalasekera *et al*., 2011; Brauc *et al*., 2012). Argininine metabolism also leads to proline biosynthesis which is an important secondary metabolite to regulate cell membrane stability and osmotic compatibility under abiotic stress tolerance (Kishor *et al*., 2005). Over expression of this gene would enable resistance to oxidative stress caused by acidic stress and also confers tolerance to osmotic stress and membrane stability. The over expression of ubiquitin control regulatory proteins which are involved in adaptive response to various environmental stresses (Sophia L. Stone, 2014). During stress condition role of ubiquitin is to alter protein conformation in order to withstand the extreme condition. In *Arabidopsis* ubiquitin has active role in the regulation of salinity, cold, heat and drought stress tolerance (Lyzenga and Stone, 2011 and 2012; Cui *et al.*, 2012).

The genes which control the normal physiological function of the cell also showed an elevated expression. This may be due to the increased requirement of energy to adjust to the stressful conditions. The genes involved in photosynthesis like oxygen evolving protein, photosystem II protein and light harvesting complex II differentially expressed to meet the energy requirements. The enhanced expression of cinnamyl alcohol dehydrogenase (CAD) showed the stressed cells adaptability to overcome acidic stress by providing integrity to the cells through the synthesis of lignin. Cinnamyl

alcohol dehydrogenase is actively involved in the lignin biosynthesis in vascular plants. Even though true lignin was not observed in non-vascular plants some lignin like compound was reported in some primitive green algae and red algae (Gunnison *et al*., 1975; Delwiche *et al*., 1989; Martone *et al*., 2009). During this study the stressed cells acquired a higher rigidity compared to normal cells (observed during RNA isolation) and this may be due to the over expression of CAD gene and the resultant formation of lignified cell wall. Stress induced lignifications and over expression of CAD gene was observed in melon and sweet potato (Kim *et al*., 2010; Jin *et al*., 2014). A study on the transcript profile of *Arabidopsis thaliana* under different abiotic stresses revealed the upward regulation of CAD gene (Ma and Bohnert, 2007).

## 1.5 Conclusion

In conclusion these study insights to various genes which are differentially expressed under acidic stress from acid tolerant microalgae, *D. ehrenbergianum.* Most of the genes differentially expressed have active role in normal physiological functioning as well as the tolerant mechanisms under various abiotic stresses. This differential gene expression profile gives an idea about the molecular mechanism lying behind the organism's adaptability under decreased ocean pH. This genetic information can be used as molecular markers for the selection of potential candidate species for sustainable cultivation under changing global climate. The basic data generated in the present study, one of the first attempts using acid tolerant microalgal isolate from our sea water ecosystem would be useful for further generating plants with resistant to abiotic stresses. This would be a realistic possibility with coming future where climatic changes are increasingly visible.

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Chapter 2

# Molecular and functional characterization of proton donating $H^+$ ATP synthase differentially expressed under acidic stress

Contents

2.1 Introduction
2.2 Materials and Methods
2.3 Results
2.4 Discussion
2.5 Conclusion

## 2.1 Introduction

Changing climate develop adaptation mechanisms in living organisms to resist the determinant effect. These adaptation mechanisms alter normal physiological and biochemical processes through enhanced expression of functional genes involved in the complex stress tolerance mechanisms. Microalgae being unicellular eukaryote with wide distributional ranges are better candidates to study environmental stress tolerance mechanisms. They have the ability to tolerate wide range of extremities next to prokaryotes and these multiple stresses enhance expression of stress tolerant genes. ATP synthase is one of the functional genes with active roles in various abiotic stress tolerances. All living organisms posses ATP synthase which is a membrane protein located in mitochondria, chloroplast, and some other cell types like endothelial cells, keratinocytes and adipocytes (Hong and Pedersen, 2008). ATP synthase is a reversible multi subunit membrane associated enzyme which is

actively involved in synthesis of energy currency of the cell, ATP through phosphorylation of ADP by proton motive force generated through electron transport chain. In addition to ATP synthesis, the respiratory chain also regulates intracellular pH (Sun *et al*., 2012). It can also reversibly function by hydrolysis of ATP to ADP and thereby generation of proton gradient across cell membrane which assist the ion movements. The intracellular proton pump is mainly controlled by V-type ATPase and these are structurally similar to ATP synthase and working in the same principle. ATP synthase also called F0F1 ATPase has complicated protein structure with a soluble catalytic F1 sector and F0 sector which is membrane bound and involved in the proton translocation.

Plants possess a plant-specific subunit named $F_AD$ which has been extensively studied instead of F0 subunit (Heazlewood *et al*., 2003). ATP synthase is conserved from prokaryotes to eukaryotes and is subdivided in to different subunits (α, β, γ, δ, e, a, b2, c10-14) and these subunits vary from organisms to organisms (Cross, 1981, Senior and Wise, 1983; Walker *et al*., 1984). More complex ATP synthase enzymes are found in bacteria which posses 15 different proteins. The membrane ATP synthase posses some supernumerary subunits and their functions are not clear or poorly defined. These supernumerary subunits are involved in cellular processes beyond ATP synthesis. The expression level of the sub unit 'e' is more during stress and other physiological changes. Living cells require energy for metabolic processes which include cellular biosynthesis, ion transport, cell division, DNA repair and mobility of cells. This energy is mainly acquired through synthesis of ATP. ATP synthesis increased during acidic stress in acid tolerant *E.coli* cells and ATP synthase plays an important role in acid tolerance (Fortier *et al*., 2003; Sun *et al*., 2012). The main reason for increased expression of ATP synthase gene may be due to membrane potential and pH gradient that drive ATP synthesis. As a substrate for many metabolic processes ATP requirement will be higher under

stressful conditions to tide over unfavorable conditions. Besides intracellular pH homeostasis ATP synthase has significant role in various abiotic stresses such as osmotic stress, drought, oxidative and cold stress. The over expression of ATP synthase gene in *Arabdopsis* and Yeast showed an increased tolerance to abiotic stresses (Zhang *et al*., 2008; Ghosh and Xu, 2014).

The global climatic changes induce the organism's adaptive mechanisms to habituate to the altered environment. The immediate reflection is by differential gene expression to cope with these stressful conditions. The present work focused on the role of microalgal $H^+$ ATP synthase gene in acidic stress responses using candidate microalgae with proven acidic stress tolerance. Most of the studies on ATP synthase are focused on plants and higher organisms. Owing to eukaryotic and acidophilic origin De.$H^+$ ATP synthase gene can be a better allele for development of stress tolerant plant for enhanced production in varying agro- climatic conditions.

## 2.2 Materials and Methods

### 2.2.1 Isolation and Identification of $H^+$ ATP Synthase Gene using Suppressive Subtractive Hybridization

A partial sequence of *D.eherenbergianum* $H^+$ATP synthase (De.$H^+$ATPase) gene was identified using SSH. Total RNA was isolated from algal cells grown under normal pH 8 (driver) and an acidic pH 4 (tester) during exponential growth phase. RNA was also isolated and pooled to the fore mentioned total RNA from acidic shocked (6 hr, 12 hr, 24 hr, and 48 hr) cells. A total of 2µg mRNA was used for synthesis of first strand cDNA. Differentially expressed gene fragments amplified using suppression PCR were ligated to pJET vector and cloned to Top 10 competent cells. PCR screened positive clones were sequenced. Sequences were edited and made

into contigs with overlapping sequences using Seqman sequence analyzing software and BLAST analyzed in NCBI gen bank.

### 2.2.2 Quantitative Validation of Expression Profile of $H^+$ ATP Synthase under Acidic Stress by Real Time PCR

Differentially expressed De.$H^+$ATP synthase gene was validated for its expression level under acidic stress using Real-Time PCR. Gene specific primers were designed by Beacon Designer™software and synthesized. Primers were also designed and synthesized for 18S rRNA gene that serves as housekeeping gene. Details of primers used are given in Table 2.1

Expression level of De.$H^+$ATP synthase mRNA was estimated quantitatively by giving an acidic shock for a specific period of time intervals viz 0hrs, 6hrs, 12hrs, 24hrs and 48hrs. Total RNA was isolated using TRI reagent and quantified using Bio photometer plus (Eppenroff, Germany). DNA contamination was eliminated by treating total RNA with RNase free DNase (1U/µg RNA) and incubated at 37ºC for 30 minutes. Then EDTA was added and heated at 65ºC for 10 minutes to inactivate the DNase. The integrity of RNA was analyzed in 1.5% agarose gel electrophoresis. First strand cDNA was synthesized from 1µg of total RNA by using RevertAid Premium First Strand cDNA synthesis Kit (Fermentas, Germany). Real time PCR was carried out with 1X SYBR Green master mix (Biorad, USA), 10µM concentration of both forward and reverse primers and 2.5 µl cDNA in a 25µl reaction mixture. Quantitative PCR was carried out in IQ5 thermal cycler (Biorad, USA) under the following cycling conditions: Initial denaturation at 95ºC for 3min followed by 45 cycles of 95ºC for 30sec, 60ºC for 30sec. and 72ºC for 20sec. Finally a 91 cycles of melt curve with an interval of 30sec. was set from 50ºC to 90ºC. All the reactions were carried out in triplicates to reduce the standard error.

### 2.2.3 Full gene Amplification of $H^+$ ATP Synthase by RACE PCR

After validating expression level of De.$H^+$ ATP synthase mRNA under acidic stress, the partial sequence was used for Rapid Amplification of cDNA Ends (RACE) PCR. Gene specific primers for both 5' and 3'RACE was designed by using primer 3 plus software and synthesized. Details of the RACE primers are given in the table 2.1

Total RNA was isolated from stressed algal culture using TRI reagent and mRNA was purified by using GenElute mRNA purification Kit (Sigma, USA). RACE was carried out using SMARTer RACE cDNA Amplification Kit (Clonetech, USA). Both 5' and 3' RACE ready cDNA were synthesized and RACE PCR was carried out under the following programme for touchdown PCR: 5 cycles: 94°C 30 sec, 72°C 3 min. 5 cycles: 94°C 30 sec, 70°C 30 sec, 72°C 3 min. and finally 27 cycles: 94°C 30 sec, 68°C 30 sec, 72°C 3 min. Amplified products were ligated to pJET cloning vector and transformed to Top 10 competent cells. Transformed cells were grown over LB agar plates containing Ampicillin (100µg/ml) at 37ºC. PCR screening was carried out with vector specific primers. Positive clones with insert was isolated and cultured in LB broth supplemented with Ampicillin (100µg/ml). Plasmids were isolated from overnight grown cultures and sequenced with pJET specific primers.

**Table 2.1** List of primers used for the RACE PCR and recombinant expression of $H^+$ ATP synthase

| SL. No. | Gene | Primer | Sequence | Product size (bp) |
|---|---|---|---|---|
| 1 | H+ ATP Synthase | S129_ATP_synthase_5'GSP | GTGATCTCCTGCTCAGTGCTCTGCT | 548 |
| | | S129_ATP_synthase_3'GSP | GGGATACGTCAAGCAGGTGATTGGT | 1554 |
| 2 | H+ ATP Synthase | ATP.Syn_Nco1_F | TACTTACCATGGCGATGCGGAGGGCAGCGG | 1617 |
| | | ATP.Syn _Xho1_R | TAATACCTCGAGCTGGTTGGCCATCTCACGGGCC | |

### 2.2.4 Sequence Analysis and Phylogenetic Tree Construction

Sequences obtained from both 5’ and 3’ RACE were aligned and vector sequence was removed using Seqman software. Then the trimmed sequences were assembled to create contig with overlapping region. A single contig formed from both 5’ and 3’ regions were analyzed in NCBI using BLASTN and BLASTX programme to find the similarity with available sequences in the database. De.$H^+$ ATP synthase gene sequence was translated to an amino acid sequence using the Expert Protein Analysis System (EXPASY) (http://www.expasy.org/) translate tool. Homologous $H^+$ATP synthase gene sequences in other species were obtained from NCBI by BLAST analysis. Multiple sequence alignments were generated using Bio-Edit multiple alignment tool. Phylogenetic tree was constructed using Molecular Evolutionary Genetics Analysis (MEGA version 6) software with Neighbor-Joining method (Tamura *et al.*, 2013).

### 2.2.5 Recombinant Expression of De.$H^+$ ATP Synthase in *E.coli*

#### 2.2.5.1 Plasmid Construction and Transformation

pET28b expression vector was used for recombinant expression of De.$H^+$ATP synthase gene and BL21 *E.coli* cells were used as expression host. The ORF region of De.$H^+$ATP synthase gene was amplified using specific primers with Nco1 and Xho1 restriction site to insert in multiple cloning region of the vector. Details of primers are given in table 2.1. The PCR product was analyzed in 1.5% agarose gel and amplification of gene was confirmed. Then the PCR product was purified using Quiaquick PCR purification Kit (Quiagen, Germany) and quantified spectrophotometrically using Biophotometer (Eppendroff, Germany). One microgram of purified PCR product was double digested with Nco1 and Xho1 restriction enzyme at 37ºC

and purified. The expression vector pET28b also double digested with the same enzymes and purified. Then ORF region of the gene was ligated to the vector. The resultant pET28De.$H^+$ATPase construct was transformed to BL21 *E.coli* cells. The transformed cells were plated on agar plates containing Kanamycin (50 µg/ml) and kept at 37ºC overnight.

#### 2.2.5.2 Analysis of the Expressed Protein using SDS-PAGE

Transformed cells with pET28De.$H^+$ATPase was isolated from the agar plate and inoculated to 2ml LB media (stock culture) containing antibiotic Kanamycin (50µg/ml) and kept overnight at 37ºC in a shaking incubator at 225 rpm. About 2% of stock culture was inoculated to 2ml LB media containing Kanamycin (50µg/ml). To induce De.$H^+$ ATP synthase gene expression a final concentration of 0.5 mM of isopropyl-β-D-thiogalacto pyranoside (IPTG) was added after 2 hours of growth when the cells reached an $OD_{600} \sim 0.6$. It was kept further for 4 hours at 37ºC in a shaking incubator at 225 rpm. Uninduced cells were also kept in same conditions to verify the protein expression. 1ml of both induced and uninduced cultures were harvested by centrifugation at 12000 rpm for 5 min. Harvested cells were suspended in resuspension buffer and lysed with Cell LyticB solution (Sigma, USA). Protein expression was analyzed in 8% glycine SDS-PAGE.

### 2.2.6 Expression of De.$H^+$ ATP Synthase in *E.coli* improves Tolerance under Acidic pH

Recombinant *E.coli* cells with *Dictyosphaerium* $H^+$ ATP synthase gene and pET28b vector alone (control) was used to study pH tolerance. A starter culture was obtained by inoculating single colony of transformed cells to 1ml of LB media containing 50µg/ml Kanamycin and kept at 37ºC in a shaking incubator at 225 rpm. 2% of the starter culture was inoculated to 10 ml of LB

media tubes with different pH (pH 4, pH 5, pH 6,) containing 50 µg/ml Kanamycin and 0.5 mM IPTG. All experiments were carried out in triplicates for both pET28De.$H^+$ATPse and pET28b vector. The growth rate was monitored by taking $OD_{600}$ with an interval of 2 hours.

## 2.3 Results

### 2.3.1 Isolation and Identification of De.$H^+$ ATP Synthase Gene

$H^+$ATP Synthase gene partial sequence was isolated and identified from *D.eherenbergianum* under acidic stress using Suppressive subtractive hybridization. The nucleotide information of De.$H^+$ATP synthase gene partial sequence was used for full length amplification using RACE PCR method. Both 5' and 3' RACE was carried out to generate complete gene sequence. The 5' RACE product was 548 bp and 3' RACE product was 1554 bp. Both sequences were aligned and created single contig of De.$H^+$ ATP Synthase complete gene (NCBI Acc. No. KT875171). The isolated gene was identified using BLAST analysis in NCBI. De.$H^+$ATP Synthase gene has an ORF of 1617bp encoding a protein with 538 deduced amino acids. The predicted molecular mass is 57.6 KDa. The 5' UTR consists of 94 nucleotides and 3' UTR contain 151 nucleotides. The protein contains 53 Strongly Basic(+) Amino Acids (K,R), 70 Strongly Acidic(-) Amino Acids (D,E), 208 Hydrophobic Amino Acids (A,I,L,F,W,V) and 107 Polar Amino Acids (N,C,Q,S,T,Y).

### 2.3.2 Sequence Comparison and Phylogenetic Analysis

The results of multiple sequence alignment of De.$H^+$ATP Synthase gene showed homology with the photosynthetic organisms. $H^+$ATP Synthase gene is highly conserved from fungi to higher plants with variation only in C-terminal region. In this study De.$H^+$ATP Synthase showed the highest similarity with closely related green algae, *Coccomyxa subellipsoidea* (Acc. No.XP_005643804).

**Fig 2.1** cDNA and deduced amino acid sequences of De.$H^+$ATP Synthase

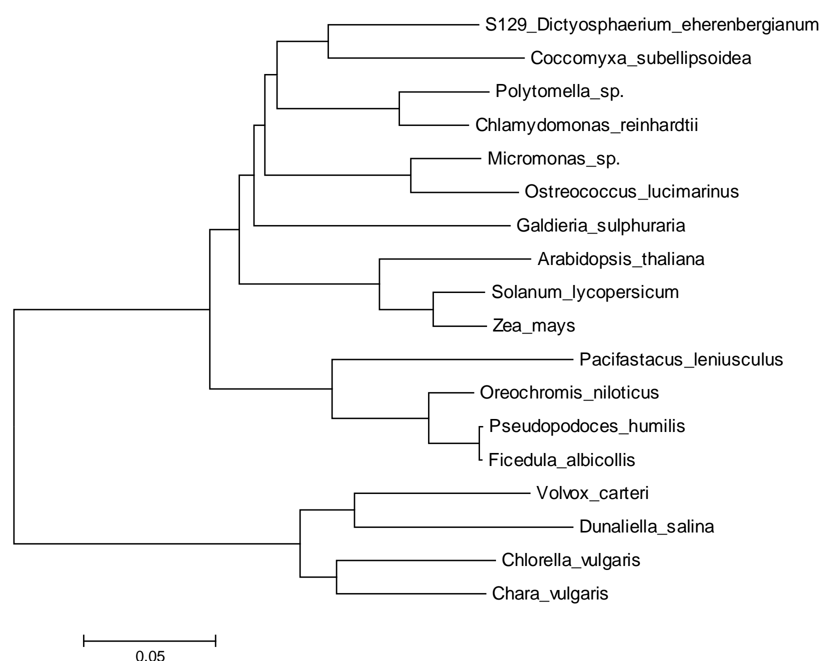



**Fig 2.2** Phylogenetic tree of De.H+ATP Synthase. The amino acid sequences were subjected to Bootstrap test of phylogeny by the MEGA 6.0 program, using neighbour-joining method with 1000 replicates

### 2.3.3 Quantitative Validation of De.H+ ATP Synthase Gene under Acidic Stress

Quantitative validation of gene expression of De.H+ATP synthase was carried out by Real-Time PCR. The expression of De.H+ATP synthase under acidic stress was analyzed by subjecting cells grown under normal pH (8.2) to acidic stress of pH 4. At specific time intervals, 0 hrs, 6 hrs, 12 hrs, 24hrs and 48 hrs, cells were harvested and analyzed for the gene expression. De.H+ATP synthase gene showed an upward regulation under acidic stress and which varied with exposure to acidic stress. The expression was maximum during initial shock after which it decreased till 24 hours. The expression reached more or less a stable condition afterward.

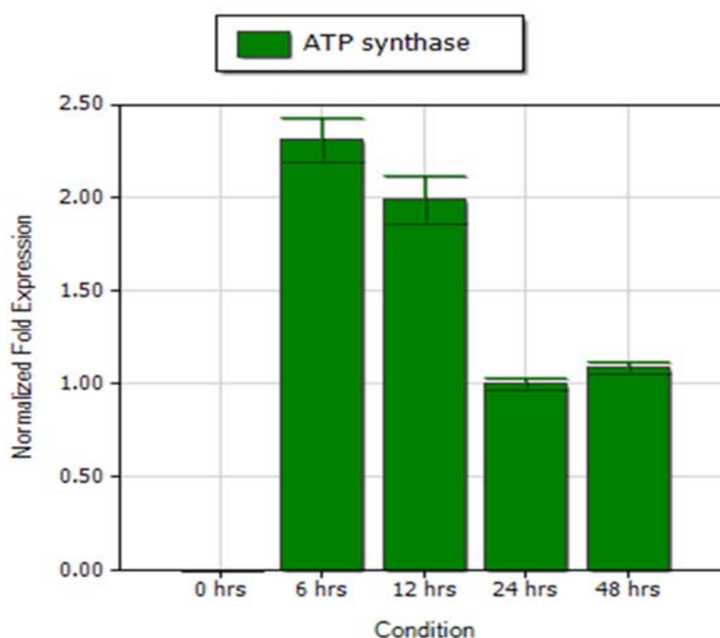
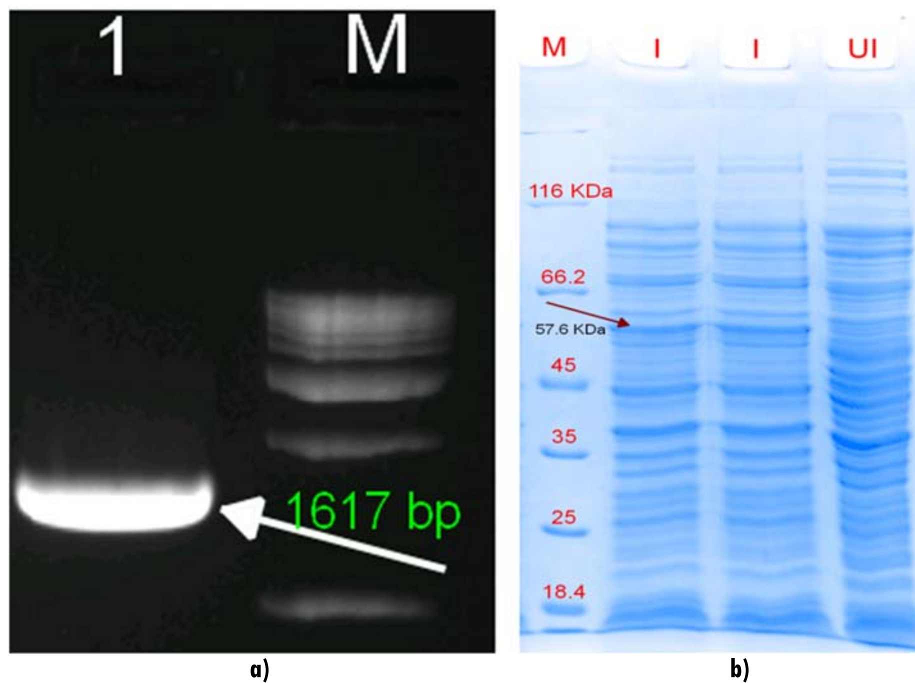



**Fig 2.3** Expression pattern of De.H+ATP synthase under acidic shock at different time intervals. Error bar indicates ± standard deviation (SD), Number of replicate (n)= 3.

### 2.3.4 Recombinant Expression of De.H+ ATP Synthase Gene in *E.coli*

The recombinant expression of De.H+ATP synthase gene was carried out in BL21 cell with pET28b expression vector. The coding region of De.H+ATP synthase gene was amplified using gene specific primers with restriction sites. The amplified product of size 1617bp was ligated to pET28b expression vector. The resultant pET28De.ATPase construct was transformed to BL21 expression host. The expressed pET28De.H+ATPase protein had a molecular weight of 57.6KDa visualized in 8% glycine SDS-PAGE.




a) b)

**Fig 2.4** a) 1.cDNA amplification of De.H+ATPase gene (ORF), M-1kb marker b) Expression profile of H+ ATPase on 8% Glycine SDS PAGE, M-Marker U-Uninduced I- Induced

### 2.3.5 Validation of Acid Tolerance Acquired by Recombinant *E.coli* with De.H+ ATP Synthase Gene

The *E.coli* cell with pET28De.ATPase acquired acidic tolerance when compared to cells with pET28b plasmid alone. This tolerance mechanism achieved may be due to presence of De.$H^+$ATP synthase genes which showed acidic tolerance even at pH 3. BL21 cells were tolerated up to pH6 below that cell growth was arrested. So the cells grown at pH6 were used for the validation of acidic stress tolerance. During initial growth phase (2-6hrs) the cells with $H^+$ATPse genes showed increased growth rate compared to control cells. The acidic stress may not affect growth of recombinant *E.coli* due to the presence of De.$H^+$ATPse gene which regulated internal homeostasis of the cell. The control cells took time to acclimatize to low pH medium. The expression level of De.$H^+$ATPse gene in stressed algae also showed a similar

pattern during Real-Time validation. These results indicate that $H^+$ATP synthase gene has instantaneous role in the stress tolerance mechanism by maintaining the homeostasis of the cells.

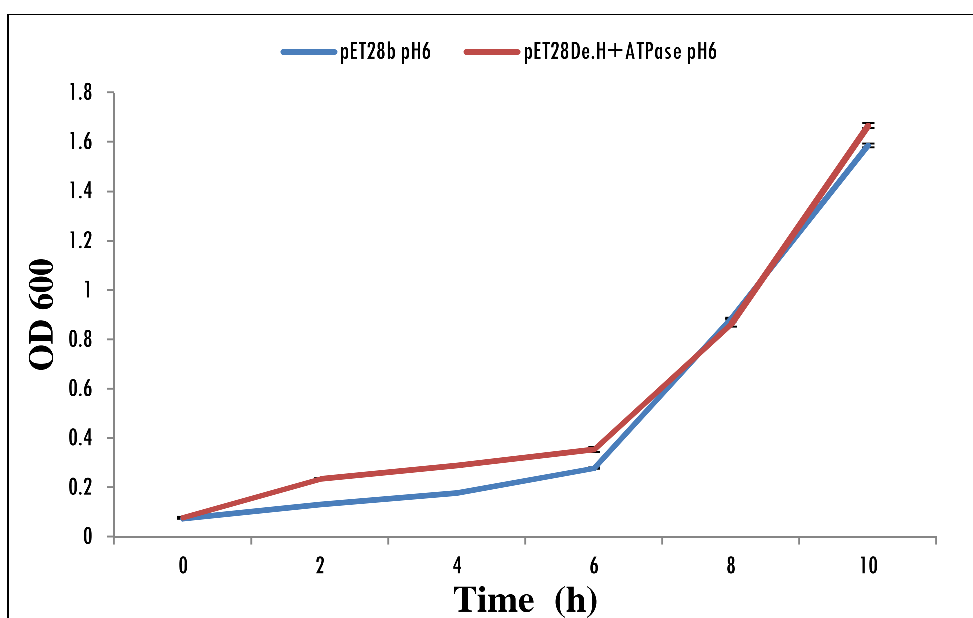



**Fig 2.5** Growth pattern of BL21 cells transformed with pET28De.H+ATPase and empty pET28b under acidic condition. Error bar indicates ± standard deviation (SD), Number of replicate (n) = 3.

## 2.4 Discussion

The determinant effect of reduced ocean pH has to be mitigated through the regulation of intracellular proton gradient. $H^+$ ATPase is actively involved in various physiological processes meant for the maintenances of intracellular ion homeostasis. In the present study a proton donating $H^+$ATP synthase gene from an acid tolerant microalgae was characterized and its expression validated under acidic stress. Sustainability of organisms under decreased pH is due to the physiological adaptation which is achieved through modification of gene expression. Homeostasis of cells are acquired through extracellular or intracellular ion transport. Various metabolic processes are

involved in the mechanism of acidic resistance (AR) like amino acid-dependent systems, (Richard *et al*., 2004) nucleotide biosynthesis induced AR, various genes etc. (Foster *et al*., 2004). F0 F1 ATPase is actively involved in both synthesis and hydrolysis of ATP through proton gradient, (Cross and Muller, 2004; Rappas *et al*., 2004) involved in acid tolerance (Len *et al*., 2004) and other abiotic stress maintenance. Studies on *E.coli* showed that ATP is essential for maintenance of acidic homeostasis under reduced pH. Acidic stress decreased growth of the mutant cells deficient in purA, purB and adk gene that are essential for ATP synthesis. This indicates that many processes require increased ATP to overcome acidic stress. For example ATP-dependent DNA repair system is one among these (Sun *et al*., 2012). The gram positive organisms like *Lactobacillus rhamnosus* showed an up regulation of F0F1 ATPase and also many ATP synthesis-coupled proton transport genes were up-regulated under acidic conditions. The survival of *Streptococcus mutans* under acidic conditions through up regulated expression of F0F1 ATPase extrude $H^+$ to exterior and maintain internal homeostasis (Belli and Marquis, 1991; Hamilton & Buckley, 1991; Dashper and Reynolds, 1992; Quivey *et al*., 2001; Len *et al*., 2004). The role of ATP synthase is not restricted to acidic stress alone but is actively involved in different environmental stresses like osmotic, cold, drought and oxidation stresses (Zhang *et al*., 2006; Ghosh and Jian Xu, 2014). Limited studies have been conducted to investigate role of F0F1 ATP synthase subunits under environmental stresses. Oxidative stress decreased the expression of *Arabidopsis* ATP synthase gene expression (Sweetlove *et al*., 2002). However rice ATP synthase showed an enhanced expression during osmotic and salinity stresses. Similarly transgenic tobacco with rice ATP synthase acquired a better tolerance under salt and osmotic stress (Zhang *et al*., 2006). Increased NaCl induce expression of subunits A, B,

E and C of vascular ATPase. The expression level of these subunits varied with plant system and stress regimes (Dietz *et al*., 1996). The expression of ATP synthase is also induced by metal toxicity; in aluminium (Al) tolerant wheat (*Triticum aestivum*) variety, increased Al level enhanced the expression of ATP synthase (Christie *et al*., 2001). Zhang *et al*., (2008) investigated the role *Arabidopsis* ATP synthase small sub unit gene under various abiotic stresses and confirmed the enhanced expression through recombinant expression in *Saccharomyces cerevisiae* and *Arabidopsis thaliana.* Studies on algal ATPase and its functional roles in abiotic stress tolerance are inadequate. Investigation on diatoms V-type ATPase exposed multiple genes encode for its subunits which are actively involved in acidic and salt stress (Bussard and Lopez, 2014). Studies by Lis *et al.,* (2007) investigated structural similarity of Chlamydomonad algae *Polytomella sp*. and *Chlamydomonas reinhardtii* and they found that both algae have structurally similar ATP synthase with an extension at their N- and C- terminal ends. The C-terminal region regulates ATPase activity which is induced by the physiological signals (Kinoshita and Shimazaki, 2002, Okumura *et al*., 2012). Investigation on plants, animals, bacteria and fungi showed significant structural similarity in the subunits of vascular ATPase (Binzel, 1995; Kluge *et al*., 1999). In the present study also *D.ehrenbergianum* showed conserved sequence with other chlorophytes and maximum similarity was observed with *Cocomyces subsiloides,* which also has the ability to tolerate acidic conditions.

## 2.5Conclusion

Previous works were less focused on role of algal ATP synthase in the environmental stress tolerance. Present study on ATP synthase mined from acid tolerant microalgae showed that it has active role in the mitigation of abiotic stresses. Significance of De.$H^+$ATP synthase is even higher owing to

its extreme tolerance under acidic environment and eukaryotic origin. Acidification of land and water is a major constraint in primary productivity. Development of new improved plant varieties with high adaptability to changing climate is the key to mitigate this problem. Successful mining, transfer and expression of De.$H^+$ATP synthase gene in the present study demonstrate the possibility of exploiting this versatile stress gene in developing crop varieties which can be grown in acidic or saline inhabited soils.

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Chapter 3

# Differential Transcriptomic Profile of Halophilic Microalgae, *Tetraselmis indica* Under Hypersaline Condition

Contents

*3.1 Introduction*
*3.2 Materials and Methods*
*3.3 Results*
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## 3.1 Introduction

Salinity is one of the severe abiotic stresses affecting plant productivity all over the world (Zhu *et al*., 2001; Shabala *et al*., 2007; Jamil *et al*., 2011; Pooja *et al*., 2014). Salinity is a major constraint in productivity both in terrestrial and aquatic habitat including anthropically modified environments (Fodorpataki and Bartha, 2004). Physiological functioning and metabolism require optimum salt concentration and any fluctuation leads to stress. Microalgae are organisms with diverse distribution and have the ability to tolerate wide range of extreme condition. Based on extent of salinity tolerance they are classified to halophilic and halotolerant. Halophilic algae require salt for optimum growth, where as halotolerant can tolerate salinity stress (Rao *et al*., 2007). Salinity stress affect normal physiological functioning which lead to alteration in the metabolisms like photosynthesis, ionic balance,

photorespiration, osmotic adjustment, etc. (Kawasaki *et al.,* 2001; Ozturk *et al.*, 2002). The mechanism of salinity tolerance is diverse and depends on organisms. The salinity tolerance level of halophilic organisms is extremely high and this achieved through the morphological and physiological adaptations (Galvan and Testerink, 2011), biochemical modification (Chatzissavvidis *et al.*, 2008), maintenance of ion homeostasis (Zhu, 2003) and all these functions are controlled by differentially expressed genes (Gong *et al.*, 2005). As a single celled eukaryote, microalgae serve as a model organism to investigate the complex physiological, biochemical and molecular processes under salt stress in higher plants (Fogg, 2001).

Sustainable agriculture requires development of stress tolerant plant varieties. Due to long generation time and complex trait, conventional breeding programme has limited success in the development of stress tolerant plants. The viable solution for this problem is the development of crop varieties with high adaptability to this changing environment through genetic engineering. Though presently transgenic food crops are not permitted or raised in India. It's going to be an unavoidable requirement in the near future. Successful generation of transgenic plant is requiring suitable genes with high abiotic stress tolerance. This can be achieved by the understanding of molecular mechanisms behind stress tolerance and characterization of potential salt tolerant genes. Many studies have been carried out to characterize gene response to salinity stress using different molecular approaches like cDNA libraries, T-DNA insertion mutation, miRNA microarrays, suppressive subtractive hybridization, homologous cloning etc, (Coetzer *et al.*, 2010, Zhang *et al.*,2012, Sahebi *et al.*, 2015). Advancement of molecular techniques created better understanding of stress tolerance in plants.

Most of these studies have been focused on glycophytes which shows limited adaptability to salinity stress. Marine and halophilic organisms have better adaptability to changing environment. As life originated in ocean, during evolution, organisms that shifted to land and fresh water lost their ability to tolerate fluctuating salinity. In this context microalgae and cyanobacteria have significant importance to study the mechanism of salinity tolerance since they inhabit diverse habitat including extreme environment (Kirroliaa *et al*., 2011). Among eukaryotes, hyperhaline microalgae show the extremity for salinity tolerance. Most of the molecular studies of stress tolerance in microalgae is concentrated on the most halotolerant eukaryote, *Dunaliella salina* (Chen and Jiang, 2009; Ramos *et al*., 2011; Gong *et al*., 2014). Apart from *Dunaliella*, hyperhaline environments are inhabited by diverse microalgae which show potential applications in biotechnology. Molecular mechanisms underlying stress tolerance in other hyperhaline microalgae will help to identify better alleles for development of stress tolerant plants. *Tetraselmis* is a dominant chlorophyte in the hyperhaline environment with substantial tolerance to hyperosmotic environment. Few studies have been carried out in different species of *Tetraselmis* to investigate the adaptation mechanism to various environmental stresses. In *Tetraselmis viridis* hyperosmotic stress induce the $Na^+$ ATPase protein which is involved in cytosolic homeostasis during salinity stress (Pagis *et al*., 2003; Strizh *et al*., 2004). Due to the euryhaline and eurythermal nature of *Tetraselmis* it is widely used in the aquaculture field. However, its exploration in tropical region is very limited, especially molecular characterization. In the present study, characterization of differentially expressed genes in hyper osmotic stress from halophilic chlorophycean algae *Tetraselmis indica,* newly described from Indian salt pan (Arora *et al*., 2011) was carried out.

## 3.2 Materials and Methods

### 3.2.1 Isolation Identification and Culturing of Algal Strain

The candidate halophilic microalga was isolated from the hypersaline water of Pulicat Lake, Chennai, Tamil Nadu, India. Isolation was done by serial dilution followed by agar plating to obtain pure culture. Isolated cells were maintained in f/2 media with 1.5M NaCl concentration. The culture room temperature was set to $25^0$ C and well illuminated by cool white fluorescent lamp. Salinity tolerance levels of the isolates up to 3M NaCl concentrations were analyzed. Identification of the isolate was carried out using traditional methods analyzing morphological characters such as size and shape of the cell, number and arrangement of flagella, chloroplasts and other cellular organelles. Identity of the species was confirmed using molecular methods by sequencing 18S rRNA and chloroplast genes.

### 3.2.2 RNA isolation and Suppressive Subtractive Hybridization

Total RNA was isolated from the cells grown under 1.5M NaCl and 3M NaCl during exponential phase of growth using TRI reagent. Algal cells grown under 1.5M NaCl salinity were subjected to shock by increasing salinity to 3M NaCl for varied durations of 6, 12, 24 and 48 hours. The total RNA was isolated and pooled as saline stressed RNA. Isolated RNA's were quantified and analyzed in 1.5% agarose gel electrophoresis (AGE). Equal quantities of both stressed and normal RNA were used for the purification of mRNA using GenElute mRNA purification kit (Sigma, USA). Suppressive Subtractive Hybridization (SSH) was carried out with PCR select cDNA subtraction kit (Clonetech, USA) using mRNA isolated from stressed (3M

NaCl) cells and normal (1.5M NaCl) cells as tester and driver respectively to isolate differentially expressed genes under hyperosmotic stress. Differentially expressed gene fragments were amplified using substraction PCR with adaptor specific primers. Amplified products were ligated to pJET vector and transformed. Transformed cells were screened using colony PCR and the size range of gene fragments were analyzed in 1% AGE. Positive clones were selected; plasmids were isolated and sequenced using vector specific primers. The generated sequences were edited using Seqman Sequence analyzing software and made into contigs with overlapping sequences. The contigs generated were analyzed using BLASTN and BLASTX softwares.

### 3.2.3 Quantitative validation of differentially expressed genes using Real-Time PCR

Expression profiles of the selected gene fragments under hyperosmotic stress were carried out using Real-Time PCR. Specific primers for both known and unknown genes were designed using Beacon Designer™software and synthesized. Real-Time PCR was carried out with both stressed and normal cells and 18S ribosomal gene used as internal control to normalize the expression level. Real- Time PCR was carried in iQ5 thermal cycler (Biorad, USA) using SYBR green master mix (Biorad, USA). PCR conditions for all the primes were standardized and all the reactions were carried out in triplicates. Details of primers used are given in table.3.1

**Table 3.1** List of primers used for the quantitative validation of hyperosmotically induced genes

| SL. No. | Gene | Primer | Sequence | Product size (bp) |
|---|---|---|---|---|
| 1 | Hydroxy pyruvate reductase | S093HPR_QP_F | TGCTCCAACGCCGTTATT | 118 |
| | | S093HPR_QP_R | TTCCAAACAGGGTATCCTTGG | |
| 2 | Guanine Nucleotide Exchange protein | S093GNEP_QPF | TCTGGAAGAGGGTGCTTAGT | 140 |
| | | S093GNEP_QPR | CCACCTCCACGAGTTCTTTC | |
| 3 | Glycine Serine hydroxymethyltransferase | S093GHMTransfrse_QPF | CGGGTGCAGACCATCTTG | 79 |
| | | S093GHMTransfrse_QPR | GGGTTCATCGCACTCTTGT | |
| 4 | ATP synthase | S093ATPsynthase_QPF | AACCACGATGGTGTGGTC | 95 |
| | | S093ATPsynthase_QPR | TGTATCTACGTTGCCGTTGG | |
| 5 | Inositol 3-phosphate synthase | S093Insostl3phphtesynthse_QPF | CATCGACCATCTACGCTCTTG | 85 |
| | | S093Insostl3phphtesynthse_QPR | CCTCTCGATAGCATACTCAATCAC | |
| 6 | Glyceraldehyde 3-phosphate dehydrogenase | S093GAPDH_QPF | GAATCCTTGGCTACACTGAGG | 123 |
| | | S093GAPDH_QPR | CAAGACACAAGCTTGACGAAAG | |
| 7 | MIF4G domain | S093MIF4G_QPF | TCCGAGTCCGATCGATGT | 115 |
| | | S093MIF4G_QPR | TGAACGAGCGGCTCAAC | |
| 8 | Fructose I,6 bisphosphatealdolase | S093FBA_QPF | TTCGCAAGATAGCGTGGTC | 117 |
| | | S093FBA_QPR | CCTGTTCGAGGAGACTCTGTA | |
| 9 | $Na^{+}K^{+}$ P-type ATPase | S093$Na^{+}/K^{+}$ATPase_QPF | CTTGTGGCGCATTGAAGATG | 106 |
| | | S093$Na^{+}/K^{+}$ATPase_QPR | TGAGGACAAGGAAGGAGGT | |
| 10 | Alkane hydroxylase | S093AHase_QPF | GAAGGACAAGAACGGCAAGTA | 116 |
| | | S093AHase_QPR | TGAGCGTGATGGTCTGAATG | |
| 11 | S93 Unknown 438 | S093un438_QPF | TCCTGAGGAGGAAGCTGAT | 99 |
| | | S093un438_QPR | CGGCTCATCGACCACAAA | |
| 12 | S93 Unknown 416 | S093un_416_QPF | ACAGGAGTAGACCGTCTGTATT | 97 |
| | | S093un_416_QPR | CCTGCATGAGCTTGACTTCT | |

## 3.3 Results

### 3.3.1Identification and Stress Tolerance of Isolated Halophilic Microalgae

Micro algal strain isolated was identified as *Tetraselmis indica*, a newly described species from India. BLAST analysis of both 18S and rbCL gene sequence showed 100% similarity with *T. indica* (NCBI Acc.No.KM087972).

The isolated strain has the capacity to tolerate wide range of salinity from 0.5 M NaCl to 3 M NaCl and optimum growth was observed at 1.5 M NaCl.

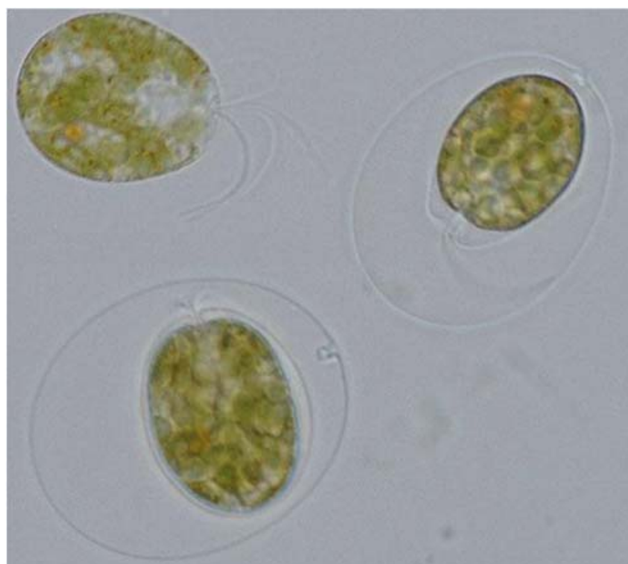


**Fig 3.1. Morphology of *T.indica* isolated from Pulicat Lake**

### 3.3.2 Assembly and Analysis of Differentially Expressed Genes under Hyperosmotic Stress

Transcriptome profile of the *T.indica* under hyperosmotic stress was generated using SSH technique. The subtractive PCR amplified differentially expressed genes with a size ranging from 0.15Kb to 1Kb. A total of 182 randomly picked colonies were sequenced. Seqman sequence editing software created 42 contigs from the overlapping regions of differentially expressed genes. All the contigs were analyzed using BLAST to identify the differentially expressed genes under hyperosmotic stress.

**Table 3.2** List of hyperosmotically induced genes showing significant similarity to known sequence in the public database

| Putative gene | Annotation e-Value | Functions | Reference |
|---|---|---|---|
| Hydroxy pyruvate reductase | 2e-55 | Photosynthesis and oxidative stress response | Wingler*et al.*, 1999 |
| Fructose-1,6-bisphosphate aldolase | 2e-75 | oxidative stress response | Lu *et al.*, 2012 |
| Fructose-1,6-bisphosphatase | 4e-63 | Osmotic, high temperature and desiccation stress | Kosova*et al.*, 2013, Haidong*et al.*, 2014 |
| Guanine nucleotide-exchange protein | 2e-66 | Signaling pathways including stress stimuli | Tuteja and Sopory, 2008 |
| TIM phosphate binding super family | 3e-07 | Amino acid biosynthesis, salt stress | Colaiacovo*et al.*, 2010, Fang *et al.*, 2014 |
| Alkane hydroxylase | 2e-64 | Photosynthesis and osmotic stress response, bioremediation | Bourdenx*et al.*, 2011, Nie*et al.*,2014 |
| F0F1 ATP synthase subunit alpha | 8e-33 | ATP synthesis, Oxidative stress, osmotic stress, drought stress etc | Yildirim*et al.*,2011, Lapaille*et al.*, 2011 |
| Glycine serine hydroxyl methyl transferase | 1e-05 | Temperature, cellular detoxification and oxidative stress responses | Moreno *et al.*, 2005, Zhou *et al.*,2012, Sirisattha*et al*, 2012 |
| MIF4G (middle portion of eIF4G) domain containing protein | 9e-05 | Protein protein and protein RNA interaction | Kmieciak*et al.*, 2002 |
| Isocitratelyase | 0.003 | Salt stress responses, Signal transduction, heat stress responses, descication | Cytryn*etal.*, 2007, Mizuno*etal.*, 2012 |
| Na+K+ P-type ATPase | 5e-64 | Photosynthesis, oxidative stress, osmotic stress | HartmutGimmler, 2000;Wiangnon*et al.*, 2007; Lind *et al.*, 2013 |
| Glyceraldehyde-3-phosphate dehydrogenase | 6e-12 | Salinity stress | Jeong*et al.*, 2001; Kosoava*et al.*, 2013; Cho *et al.*, 2014, |
| C2 super family/ protein kinase | 0.29/ 4.49e-04 | Hyperosmotic stress | Kobayashi *et al.*, 2004 |
| Inositol-3-phosphate synthase | 9e-41 | Abiotic stress | Wang *et al.*, 2011; Tan *et al.*, 2011; Astua*et al.*, 2007 |

Among the differentially expressed genes, 43% showed sequence similarity with functionally identified genes, 38% uncharacterized genes which may have functional roles in salinity stress tolerance and the rest 19% having sequence similarity with ribosomal genes (Fig.3.2). Details of the differentially expressed genes under hyperosmotic stress are given in the table.3.2. Functional

genes are classified (Fig.3.3) based on the cellular functions such as metabolic process, stress responses, cellular transport of ions, cell proliferation and DNA repair. All these functionally identified genes are actively involved in other abiotic stresses such as drought, temperature and oxidative stress.

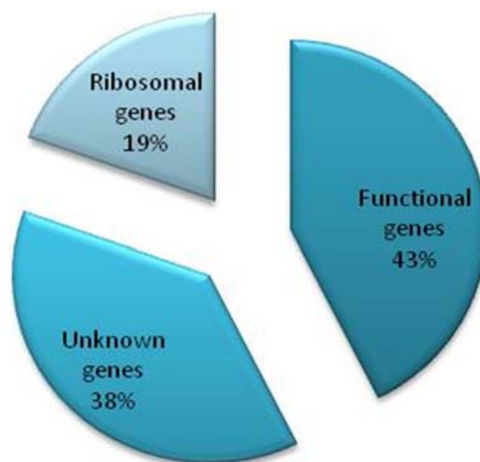



**Fig 3.2** Classification of differentially expressed genes under hyperosmotic stress

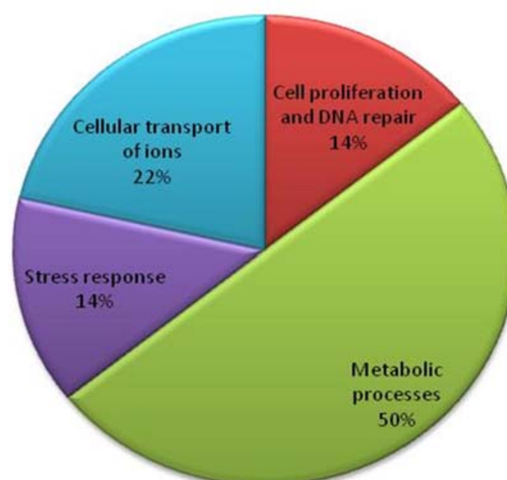



**Fig 3.3** Functional classification of identified genes under hyperosmotic stress

### 3.3.3 Validation of Differentially Expressed Genes under Hyperosmotic Stress using Real-Time PCR

The expression level of the differentially expressed genes under hyperosmotic stress was validated using Real-Time PCR. A representation of both novel and functional genes were quantitatively validated. Among the 12 gene selected, 10 genes showed a significant upward regulation with hyperosmotic stress (Fig 3.4) GAPDH and MIF4G domain protein did not show significant upward regulation under salinity stress. Highest expression under salinity stress was shown by hydroxyl pyruvate reductase (HPR) and fructose-1, 6 bisphosphate aldolase (FBA) genes.

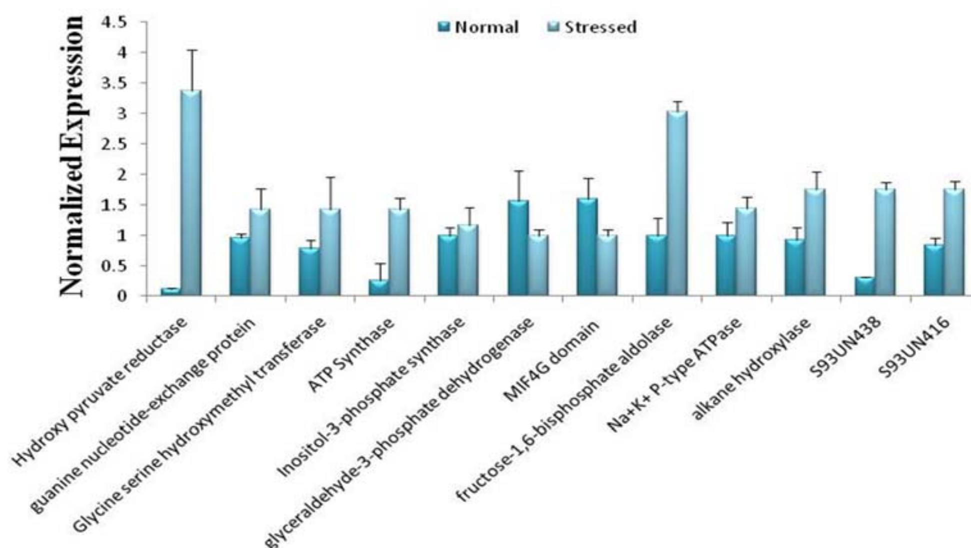



**Fig 3.4** Expression profile of differentially expressed genes in hyperosmotic stress using quantitative Real-Time PCR. Error bar indicates ± standard deviation (SD), Number of replicate (n)= 3.

## 3.4 Discussion

Salinity is one of the limiting factors for plant productivity worldwide. Climatic change has severe impact on ocean and land salinity. Halophilic organisms have fascinating mechanism to withstand high salinity. They have

adaptive mechanisms such as redox control, scavenging of radical oxygen species, metabolite accumulation and altered partitioning and ion homeostasis (Hasegawa *et al*., 2000). Earlier works have generated data regarding the physiological adaptations. Tolerance to abiotic stresses is a genetically determined trait and these catastrophic effects have mild or severe impact at any stage of plant development. Emergence of advanced technology in the field of molecular genetics and cell biology helps to reveal genetic determinants of salinity tolerance. Generation of EST and expression analysis strengthen the theory that the physiological mechanisms are under the control of genes. Transcriptomic profile under salinity stress provides useful information regarding the cell specific determinants of salinity tolerance. Present investigation focused on the analysis of gene expression of halophilic microalgae, *T. indica* under hyper saline conditions. This newly described strain has the ability to tolerate wide range of salinity from 0.5 M NaCl to 3 M NaCl. Most of the salinity tolerant studies have been carried out in the euryhaline microalgae, *Dunaliella salina* (Alkyal *et al*., 2010, Ramos *et al*., 2011, Zhao *et al*., 2011). The characterization of *T. indica* may provide an insight into better alleles responsible for salinity tolerance. SSH can be efficiently used for the characterization of functional genes without any sequence information. The EST generated in this work has active role in the process of biological stress management.

Photorespiration is a vital pathway essential for the normal physiological function of the plant cell (Bauwe *et al*., 2010). Hydroxypyruvate reductase (HPR) is an intermediate enzyme in the photorespiration pathway essential for the detoxification of 2-phosphoglycolate to glycerate, which enters the Calvin cycle. The increased expression of HPR was observed in barley (*Hordeum vulgare*) during drought stress which enhances the

photorespiration and thereby increased carbon fixation through photosynthesis (Wingler *et al*., 1999). Similar to drought, osmotic stress also leads to water deficit with interconnected protective mechanisms (Marcinska *et al*., 2013). This study revealed the enhanced expressions of the HPR gene in hyperosmotic stress which inturn enhance growth rate of *T.indica*. The fructose 1, 6 bisphosphate aldolase gene (FBA) which controls the glucose metabolism was found to be higher in the *T.indica* cDNA library. Calvin cycle is an initial pathway for the fixation of carbon through photosynthesis. Calvin cycle steps are under the control of different enzymes which control the carbon flux. FBA is one of the non-regulated enzymes involved in the glucose metabolism. Besides metabolic functions FBA actively involved in abiotic stress tolerance mechanisms (Uematsu *et al*., 2012). The expression patterns of eight FBA family genes in *Arabidopsis* and cotton showed an enhanced expression in various stresses like NaCl, Cadmium Abscisic acid, abnormal temperature and drought (Lu *et al*., 2012; Qaisar *et al*., 2015).Biochemical studies in maize showed an enhanced expression of FBA in salinity stress (Zorb *et al.,* 2004). These studies indicate the significance of FBA for the development of crop varieties with increased abiotic stress tolerance.

The intracellular ion homeostasis is maintained by P-type ATPase. Mostly plant cell posses $H^+$ ATPase for the ion transport but here we identified a $Na^+/K^+$ ATPase for homeostasis in hyperosmotic stress. During the evolution of vascular plants they loss $Na^+/K^+$ ATPase as they shifted to fresh water condition (Graciadeblas *et al*., 2001; Pedersen *et al*., 2012). Existences of $Na^+/K^+$ ATPase observed in organisms exposed to marine environment strengthen its role in osmotic stress tolerance. Animal type $Na^+/K^+$ ATPase was functionally characterized from a marine red seaweed *Porphyra yezoensis* which impart salinity tolerance in rice plant (Kishimoto *et al*., 2013). As a

most euryhaline eukaryote this gene was not reported from *Dunaliella salina* (Gimmler, 2000) but studies in other marine microalgae like *Tetraselmis, Heterosigma* and *Dunaliella maritima* revealed the existence of salinity induced animal type $Na^+/K^+$ ATPase (Balnokin and Popova, 1994; Shono *et al.*, 1996; Balnokin *et al.*, 1997; Gimmler, 2000; Popova *et al.*, 2005). Present study also revealed the occurrence of $Na^+/K^+$ ATPase in a new species of *Tetraselmis* which has the ability to tolerate hyperosmotic condition and further characterization of this gene would enable to understand the heterologous expression in other economically important agricultural crops.

The high level expression of Glycine serine hydroxymethyl transferase during salt stress prevents cell death. It reduces the accumulation of reactive oxygen species through the correlated $Na^+/H^+$antiport activity (Moreno *et al.*, 2005; Zhou *et al.*, 2012). Most of the studies in this gene have been carried out in higher plants, animals, and prokaryote (Chang, *et al.*, 2007; Engel *et al.*, 2011; Jiang *et al.*, 2013). The over expression of this gene from halo tolerant cynobacteria *Aphanothece halophytica* in *E.coli* enhance the salinity tolerance (Sirisattha *et al*, 2012). The earlier works have not characterized this gene from microalgae. In the present study, we have identified a salinity induced Glycine serine hydroxymethyl transferase from halotolerant microalgae, *T. indica*. Quantitative validation also confirmed its enhanced expression in hyper osmotic stress. Isocitrate lyase (ICL), a glyoxylate pathway enzyme involved in oxidative stress and salinity stress was differentially expressed in hyperosmotic stress in *T.indica*. Its physiological function is the catalysis of conversion of isocitrate to succinate and glyoxylate.  Apart from this, it has active role in environmental stresses. Studies in *Shewanella sp* observed that an enhanced expression of ICL gene in salinity and acidic shock (Li *et al.*, 2006) and low temperature induce it in *Colwelliamaris* (Watanabe *et al.*, 2002). *Bradyrhizobium japonicum*, a symbiont

in soya bean showed an enhanced expression of ICL during desiccation stress (Jeon*et al*., 2015). Limited studies have been carried out to characterize algal ICL. Its role in carbon metabolism and oxidative stress was studied in *Chlamydomonas*. A mutant of this alga without ICL showed reduced growth in both dark (heterotrophic condition) and light (mixotrophic condition) and also susceptible to oxidative stress (Plancke *et al*., 2014). In this context further characterization of algal ICL gene has great significance.

The environmental stress stimuli in plants are transferred further downstream via network of signals. They are controlled by transcription factors and signal transducing GTPase, which is activated by guanine nucleotide exchange factors (GEF) (Schmidt and Hall, 2002). Osmotically induced *T.indica* cells also over expressed GEF, which activate the GTPase involved in salt stress mechanisms. Role of GTPase in salinity stress was investigated in *Arabidopsis thaliana* through the recombinant expression of *Medicago falcata* small GTPase gene. Transgenic *Arabdopsis* seedling showed a higher survival rate compared with wild types seedlings under salt stress (Wang *et al*., 2013). Other genes such as Glyceraldehyde 3-phosphae dehydogenase, alkane hydroxylase, inositol-3-phosphate synthase, phosphate binding superfamily, guanine nucleotide exchanger have direct or indirect role in various abiotic stresses including salinity stress. This study also revealed many novel genes having active role in osmotic stress which has to be further characterized.

## 3.5 Conclusion

In conclusion, the present work provides a detail understanding of differentially expressed genes essential for the survival of plants under osmotic stress. The EST generated in the present study seems to be either directly or indirectly involved in osmotic stress. Moreover these genes also

have active role in biological processes of cell apart from providing tolerance to osmotic stress. As the microalgae used in the study is a recently described halophilic species from tropical India, the differential gene expression profile threw light into the molecular mechanism of salinity tolerance. In depth studies on the complete characterization of novel/functional genes would unearth the physiological and molecular mechanisms involved in the abiotic stress tolerance. This would provide initial step in the development of economically important agriculture crops, which could grow in salinity inflated land area of India, in the near future.

Chapter 4

# Molecular and Functional Characterization of Fructose 1, 6 Bisphosphate Aldolase; a Potential Osmotolerant Gene

Contents

4.1 Introduction
4.2 Materials and Methods
4.3 Results
4.4 Discussion
4.5 Conclusion

## 4.1 Introduction

The osmotic tolerance mechanisms of plants varied from species to species some plants have the ability to tolerate wide range of salinity and some flourish in low salinity and some are very sensitive. Halophilic organisms have extraordinary mechanisms to withstand hyper osmotic stress. Among the halophiles, microalgae have special attention because of their eukaryotic origin, wide distribution and simplicity in cell structure. There are limited studies to find out molecular mechanisms behind osmotic adjustment and most of the studies have been done in euryhaline microalgae, *Dunaliella salina*. In the present study a potential osmotolerant gene, Fructose 1, 6 bisphosphate aldolase (FBA) differentially expressed under hyperosmotic stress from halophilic microalgae, *Tetraselmis indica* has been characterized.

Fructose 1, 6 bisphosphate aldolase is a key enzyme actively involved in the metabolism of glucose. In glycolysis FBA take part in the aldol cleavage of

fructose-1, 6-bisphosphate to dihydroxyacetone phosphate and glyceraldehyde 3-phosphate. FBA also participated in the anabolic process such as gluconeogenesis and photosynthesis. There are two closely related classes of FBA enzymes, class I and II but they differ in their action and also there is no sequence similarity (Sanchez *et al*., 2002). The class I enzyme posses lysine residue in the reaction site and form Shiffs base during reaction mechanism with substrate. The class II enzyme is a metallo enzyme which posses a divalent metal ion in the similar metabolic processes (Thomson *et al*., 1998). In animals the class I enzyme divided into three types, aldolase A found in muscle, alodolase B found in liver and aldolase C in brain (Rutter *et al*., 1965). The different enzymes also distributed in every organism with peculiar evolutionary lineage. Most of the animals, plants, and some protists contain class I enzyme and class II enzymes are characteristics of fungi (Marsh and Lebherz, 1992). Algae contain either class I or class II enzymes and both these genes found in *Euglena garacilis* (Nickol *et al*., 2000, Plaumann *et al*., 1997). These findings suggested that the same step of glycolysis and gluconeogenesis in different organisms are controlled by enzymes of different origin. Instead of glucose metabolism FBA has significant roles in different stress tolerance mechanisms (Lu *et al*., 2012). FBA take part in the normal growth and functioning and studies shown that reduced aldose activity reduce the photosynthetic activity and synthesis of starch and sugars there by inhibit growth in potato plant (Haake *et al*., 1998). During abiotic stress some metabolite disrupts their normal physiological function and accumulates to compete with the elevated condition. Some form compatible solutes and some form important signal molecules (Baier *et al*., 2004; Cho and Yoo, 2011). Among this glucose has vital role in cell processes like germination, development of seedlings and environmental stress tolerance. During salinity stress microbial cells faces decreased water activity because of the loss of intracellular water, modifying ionic fluxes and intracellular accumulation of osmolites. Long term osmotic tolerance is

achieved through the intracellular production of osmolyte, glycerol. As a key enzyme in the glucose metabolism FBA has significant role in abiotic stress tolerance. Studies on the Calvin cycle enzymes suggested that sedoheptulose 1,7-bisphosphatase (SBPase), transketolase (TK), and fructose 1,6-bisphosphate aldolase actively control the photosynthetic coefficient and thereby plant growth (Raines, 2003; Uematsu *et al*., 2012). As a non-regulated enzyme, over expression of aldolase increase the tolerance level of plants under abiotic stresses and enhance the growth through increased photosynthetic activity.

## 4.2 Materials and Methods

### 4.2.1 Identification and Quantitative Validation of Fructose 1, 6 Bisphosphate Aldolase Gene

A partial nucleotide sequence of *Tetrselmis indica* fructose 1, 6 bisphosphate aldolase gene (Ti.FBA) was isolated using SSH method under hyperosmotic stress. Algal culture stressed with salinity (1.5M NaCl to 3M NaCl) was used as tester and normal salinity (1.5 M NaCl) was used as driver. Isolated sequences were analyzed using BLAST in NCBI data base. The expression profile of the Ti.FBA was quantitatively validated using Biorad IQ5 Real-Time thermal cycler. Specific primers were designed from the partial sequence of FBA gene using Beacon Designer™software and synthesized. Algal cells grown under optimum salinity (1.5M NaCl) were subjected to salinity shock by increasing salinity to 3M NaCl. Total RNA was isolated after a specified period of time (0hr, 6hr, 12hr, 24hr and 48hr) using TRI reagent (Sigma, USA). Isolated RNA was quantified spectrophotometrically using Biophotometer (Eppendroff, Germany) and the quality was analyzed in 1.5% agarose gel electrophoresis. DNA contamination was eliminated by treating RNA samples with RNase free DNase enzyme (1U/µg RNA, Fermentas). 1µg RNA was used for the synthesis of complementary DNA using Revert Aid Premium cDNA synthesis Kit (Thermo scientific, USA). Real-Time PCR was

carried out in 25µl reaction with SYBR green Mastermix (Biorad, USA). 18S rRNA gene was used as internal reference gene and both the reactions were conducted in triplicate.

### 4.2.2 Full Length Amplification of *Tetraselmis* FBA gene using RACE PCR

After analyzing expressions under hyperosmotic stress, Ti.FBA gene was completely amplified using RACE PCR method. Gene specific primers for both 5' and 3' RACE were designed using Primer3 Plus software and synthesized. Total RNA was isolated from the hyperosmotically stressed cells using TRI reagent (Sigma, USA) and mRNA was purified using GenElute mRNA Purification Kit (Sigma, USA). Rapid Amplification of cDNA Ends were carried out using SMARTer RACE cDNA Amplification Kit (Clonetech, USA). Both 5' and 3' RACE ready cDNA were synthesized and RACE PCR was carried out. Amplified products were analyzed in 1% agarose gel electrophoresis and purified using Quiaquick PCR purification Kit (Quiagen, Germany). Purified products were ligated to pJETcloning vector and transformed to Top 10 chemically competent *E.coli* cells. Positive clones were screened using colony PCR. Plasmids were isolated from the positive clones and sequenced.

### 4.2.3 Recombinant Expression of Ti.FBA in *E.coli*

The ORF region of the Ti.FBA gene was amplified using specific primers with BbsI restriction site designed to produce cut ends compatible with NcoI digestion at the 5' end and XhoI digestion. The amplified product was purified and digested with BbsI and ligated to pET28b expression vector double digested with NcoI and XhoI restriction enzymes. The resultant pET28TiFBA vector construct was transformed to BL21 expression host. Transformed cells were plated on agar plate containing Kanamycin (50 µg/µl) and kept at 37$^{0}$C overnight.

**Table4.1** List of primers used for the RACE PCR and recombinant expression of Ti.FBA gene

| SL. No | Gene | Primer | Sequence | Product size (bp) |
|---|---|---|---|---|
| 1 | Fructose 1,6 bisphosphatealdolase | S93_FBA_5'GSP | GCTTAATGCCAGCCTCGGCCATCACAT | 466 |
| | | S93_FBA_3'GSP | CCTGTGAAGTCTGCTACGATGC | 1232 |
| 2 | Fructose 1,6 bisphosphatealdolase | >FBA_BbSI_F | ATCGAAGAAGACACCATGGCAGCCGCACTCAAGAGC | 1161 |
| | | >R_FBA_BbSI | GATGTCGAAGACACTCGAGATAAACATAGCCCTTCTCGAAC | |

### 4.2.4 Analysis of Recombinantly Expressed Ti.FBA in *E.coli* using SDS-PAGE

The stock culture of recombinant *E.coli* with *Tetraselmis* FBA gene was inoculated to LB media containing Kanamycin (50µg/µl) and kept at 37ºC over night. Two percent of the stock culture was inoculated to 2ml LB media containing Kanamycin (50µg/µl) and kept at 37ºC. After 2 hours of growth, cells were induced with 0.5mM concentration of isopropyl-b-D-thiogalacto pyranoside (IPTG) for the expression of recombinant protein and kept for further 4 hours at 37ºC. Control cells were also kept at the same condition without adding IPTG. After 4 hours of growth both induced and uninduced cells were harvested by centrifuging at 12000rpm for 5 minutes. Harvested cells were suspended in resuspension buffer and lysed with Cell LyticB solution (Sigma, USA). The over expressed recombinant protein were analyzed in 8% Glycine SDS-PAGE.

### 4.2.5 Validation of Acquired Salinity Tolerance of Recombinant *E.coli* with Ti.FBA Gene

Acquired thermal tolerance of recombinant *E.coli* with *Tetraselmis* FBA gene and control cells with pET28b alone was monitored. Transformed single colony was inoculated to 1ml LB media containing antibiotic Kanamycin (50µg/µl) and kept at 37ºC overnight. 2% of this stock culture was inoculated to 10ml LB containing 50µg/ml Kanamycin and 0.5mM IPTG with 0.5M NaCl concentration. The growth rate was monitored by taking $OD_{600}$ at intervals of 2 hours. All the experiments were carried out in triplicate.

## 4.3 Results

### 4.3.1 Identification and Expression Validation of *Tetraselmis* Fructose 1, 6 bisphosphate Aldolase Gene

A differentially expressed FBA gene fragment under hyperosmotic stress was isolated using suppressive subtractive hybridization technique. The partial sequence obtained with a size of 305 bp was analyzed using BLAST. The analyses showed high sequence homology with chlorophyte, Scherffelia dubia fructose 1, 6 bisphosphate aldolase, which is actively involved in the glucose metabolism. The expression of Ti.FBA of both stressed (3M NaCl) and normal cells (1.5M NaCl) was validated using Real-Time PCR. Hyper osmotic stress induced the over expression of FBA gene in T. indica and it depended on the duration of stress (Fig 4.1). During initial stress, both stressed and normal cells showed an optimum expression but after 48 hours of hyper osmotic shock, over expression of FBA gene was induced. This may lead to the increased production of glycerol, an osmoprotectant which may help to sustain algal cells under hyper osmotic stress.

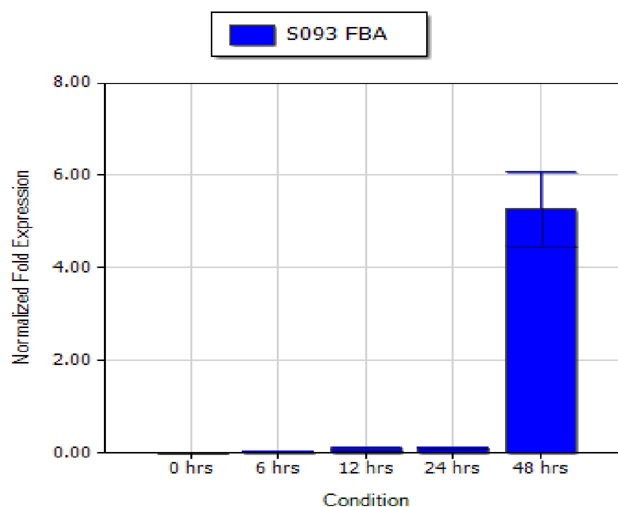



**Fig 4.1** Expression pattern of Ti.FBA gene under hyperosmotic stress at different time intervals. Error bar indicates ± standard deviation (SD), Number of replicate (n)= 3.

### 4.3.2 Full Gene Amplification of Ti.FBA using RACE PCR

Complete sequence of the Ti.FBA gene was obtained by RACE PCR. Both 5' and 3' RACE was carried out to find out the complete sequence. The 5' RACE product was 466 bp and 3' product was 1232bp and the overlapping regions of both the product were combined to create Ti.FBA gene sequence of 1383 bp nucleotides. The ORF region consist of 1161 bp nucleotide with 387 predicted amino acid translated to FBA protein with size of 41.4 KDa

### 4.3.3 Sequence Comparison and Phylogenetic Analysis

To analyze the evolutionary relationship of Ti.FBA, a phylogenetic tree was constructed using sequences of other species. The result indicated that *Tetraselmis* FBA has close similarity with various organisms and maximum was observed with chloroplast FBA of chlorophycean algae, *Scherffelia dubia* (similarity 77%, identity 79%). Based on the catalytic mechanisms FBA is classified in to Class I and Class II groups. Most of the plants contain Class I FBA with two isoforms namely plastid FBA and cytosol FBA. The sequence comparison of Ti.FBA showed maximum homology with plastidal Class I FBA than the cytoplasmic FBA which indicated that the *Tetraselmis indica* FBA belong to Class I plastidal isozyme.

**Fig 4.2** cDNA and deduced amino acid sequences of *Tetraselmis* FBA gene

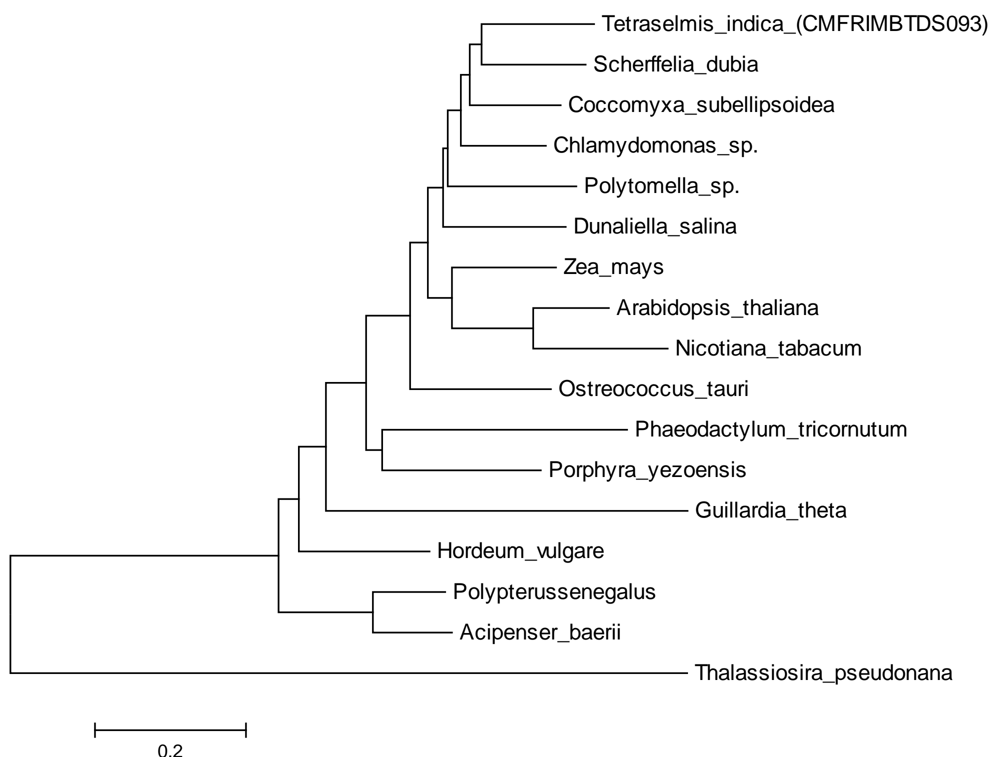
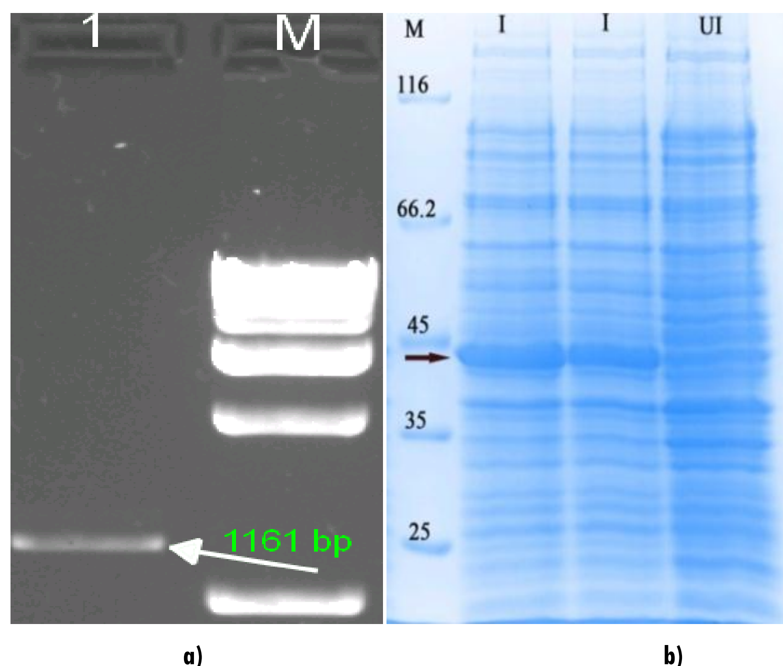



**Fig 4.3** Phylogenetic tree of Ti.FBA. The amino acid sequences were subjected to Bootstrap test of phylogeny by the MEGA 6.0 program, using neighbour-joining method with 1000 replicates

### 4.3.4 Recombinant Expression of Ti.FBA in *E.coli*

The differentially expressed Ti.FBA gene was recombinantly expressed in BL21 expression host through PET28b expression vector. The ORF region consisting of 1161 bp (Fig 4.4a) was amplified with specific primers and ligated to pET28b vector to create Ti.FBApET28 construct. The recombinant BL21 cell with Ti.FBApET28 construct over expressed the recombinant protein when induced with 0.5mM IPTG. The expressed protein with a molecular mass of 41KDa was visualized in 8% Glycine SDS-PAGE (Fig 4.4b)




a) b)

**Fig 4.4. a)** 1-cDNA amplification of Ti.FBA gene (ORF), M- 1 kb Marker b) Expression profile of Ti.FBA protein on 8% Glycine SDS PAGE M-Marker,U-Uninduced,I – Induced

### 4.3.5 Enhanced Growth of Recombinant *E.coli* with Ti.FBA Gene under Hyper Osmotic Shock

Recombinant *E.coli* with Ti.FBA gene acquired salinity tolerance under hyper osmotic shock when compared to *E.coli* cells with vector alone. Recombinant cells with Ti.FBApET28 and pET28 grown under normal conditions were subjected to a salinity shock (0.5M NaCl) and growth pattern monitored. Both the cells acclimatized to elevated salinity but the recombinant cells with Ti.FBA gene showed an enhanced growth rate compared to the control cells. The over expression of Ti.FBA gene in *E.coli* provide better tolerance thereby enhanced growth rate was observed in recombinant cells (Fig 4.5).

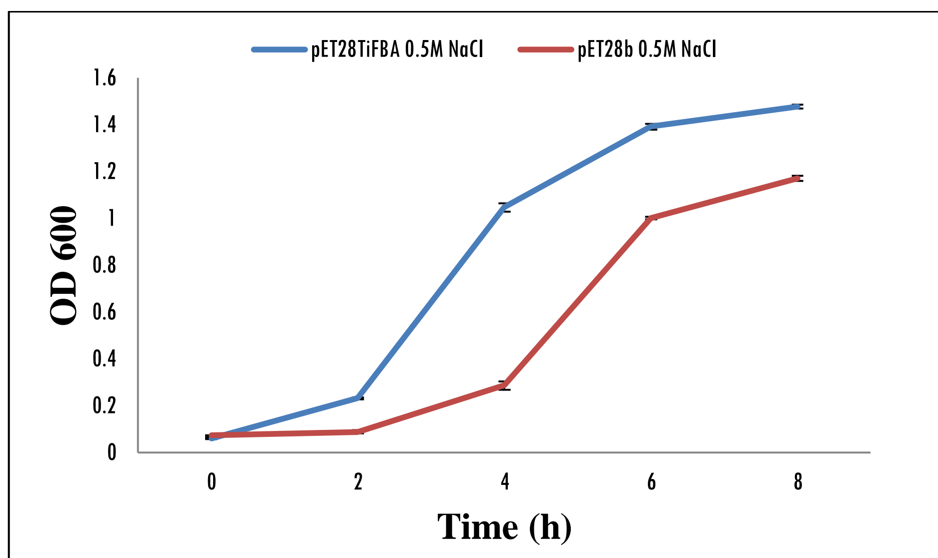



**Fig 4.5** The growth pattern of BL21 cells transformed with TiFBApET28 and empty pET28b under hyperosmotic shock. Error bar indicates ± standard deviation (SD), Number of replicate (n) = 3.

## 4.4 Discussion

Salinity is one of the major abiotic stress factors affecting worldwide plant productivity. Plants have developed many adaptive mechanisms to overcome these stressful environments. This is achieved through the physiological, biochemical and molecular changes (Bray 1997; Hasegawa *et al*. 2000). Salt stress induce the over expression of many genes which involved in the cellular pathways (Rock, 2000; Shinozaki and Yamaguchi-Shinozaki, 2000). In this study a hyperosmotically induced fructose 1, 6 bisphosphate aldolase (FBA) gene was characterized from a newly described halophilic microalgae, *Tetrasemis indica.* Most of the previous studies investigated the role of FBA in cellular metabolisms and abiotic stress tolerance in plants (Lu *et al*., 2012: Uematsu *et al*., 2012; Zeng *et al.,* 2015). FBA genes are highly conserved among eukaryotes and the Ti.FBA showed sequence similarity with other related algal groups and photosynthetic plants. Genomic analysis of rice, spinach and *chlamydomonas* revealed the existence of single isogene where as

presence of two isogenes in pea and tobacco plants (Razdan *et al*., 1992, Yamada *et al*., 2000). As a chlorophycean algae *T. indica* posses one isogene which is actively involved in osmotic stress tolerance mechanisms. Studies on the acidophilic red algae, *Galdieria sulphuraria* showed that the two isozymes of aldolases are homologous to the higher plants (Gross *et al*., 1999).

Increased aldolase activity enhances the photosynthetic activity through the RuBP regeneration. In cyanobacteria increased photosynthetic activity through the regeneration of RuBP by the over expression of aldolase was observed (Kang *et al*., 2005; Ma *et al*., 2007 and 2008; Uematsu *et al*., 2012). The role of FBA in the model plant *Arabidopsis thaliana* was investigated by Moon *et al.,* 2012, and it was confirmed that this gene actively maintained the aldolase activity and NADPH level during abiotic stress. In mangrove plant *Sesuvium portulacastrum* salinity stress induce the over expression of FBA gene (Fan *et al*., 2009). The expression level of FBA in organisms inhabiting intertidal region was very high which indicate the active role of FBA in various environmental extremities (Tomanek, 2011). These studies reported the role of FBA in abiotic stress tolerance. In the present study we have investigated role of FBA in microalgae during salinity stress, and previous studies on *Dunaliella salina* fructose-1, 6-diphosphate (FDP) aldolase (DsALDP) proved its role in osmotic stress tolerance. Recombinant expression of DsALDP in *E.coli* and tobacco plant showed better survivability under osmotic stress through the elevated expression of DsALDP (Zang *et al*., 2002 and 2003). Our study in halophilic microalgae also confirmed the acquired salinity tolerance of *E.coli* cells through the recombinant expression of Ti.FBA gene (Fig.13). The rice FBA gene showed variation in expression with salt sensitive and salt tolerant rice varieties. In salt tolerant variety slight up regulation was observed after 24h and 7d of salinity stress. The expression

pattern was totally different in salt sensitive variety, the gene was down regulated during the initial stage of stress (24h) and then strongly expressed at 3 d and 7 d stress (Jankangram and Theerakulpisut, 2012). In the present study also Ti.FBA was not significantly expressed during initial stress but the expression was very high at 48h of stress. Transcriptomic study of the red algae *Gracilaria changii* under hyper and hypoosmotic stress showed that the expression of FBA actively up regulated during hyperosmotic stress and low salinity down regulated the expression (Teo *et al.*, 2009). Zeng *et al.* confirmed the role of CoFBA (*Camellia oleifera FBA*) gene in osmotic stress tolerance. The transgenic *Brassica napus* plant developed with CoFBA gene acquired better salinity tolerance than wild type (Zeng *et al.*, 2015). Transgenic potatoes with co-expression of FBA, triosephosphate isomerase (TBI) and fructose bisphosphatase (FBPase) achieved higher photosynthesis efficiency (Fan *et al.*, 2009). These studies depict the significance of FBA gene for the development of abiotic stress tolerant plant varieties with increased photosynthesizing efficiency as revealed in the present work.

## 4.5 Conclusion

In conclusion, Ti.FBA characterized in the present work is a better allele of FBA gene as it is characterized from halophilic chlorophycean algae, *T. indica*. Investigation of Ti.FBA gene revealed that they are actively involved in the hyper osmotic stress through elevated expression. Present study along with previous works on various FBA proved their role to impart hyperosmotic stress tolerance in other salt sensitive organisms through recombinant expression. As a eukaryotic extremophilic origin Ti.FBA has significant role in the development of stress tolerant plant varieties through homologous expression

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Chapter 5

# Analysis of Differentially Expressed Genes under Temperature Stress from Thermophilic Microalgae, Scenedesmus sp.

5.1 Introduction
5.2 Materials and Methods
5.3 Results
5.4 Discussion
5.5 Conclusion

## 5.1 Introduction

Evolution of organisms on earth primarily depends on climatic conditions of particular region (Bradshaw and Holzapfel, 2006). The constantly changing climate forcefully exposes organisms to new stressed environments (Parmesan and Yohe, 2003; Gienapp *et al.* 2008). Some species adapt themselves to withstand these changing environments while many others change their geographical distributions. Unicellular organisms can be found everywhere, in almost all stress conditions. Organisms which can withstand and flourish in harsh environments are called as extremophiles. Among extremophiles, thermophiles have great potential and are extensively studied (Valverde *et al*., 2012). Most organisms adapted to thrive under extreme conditions are identified to be either bacteria or archaea. However there are some eukaryotic micro algal species that successfully established in such conditions. Microalgae are diverse photosynthetic organisms adapted to thrive in highly diverse ecosystems. These unicellular

eukaryotes are distributed in a wide range of habitats including extreme environments such as thermal oceanic vents, acidic lakes, hyperhaline areas, hot springs, highly alkaline lakes etc., and are termed as extremophilic microalgae. These extremophilic algae have inherent ability to withstand extreme conditions by various physiological functioning and biochemical compositions regulated by differentially expressed genes. Among eukaryotes, unicellular red micro-alga *Galdieria sulphuraria* (Cyanidiales) was extensively studied by generating EST library (Weber *et al*., 2004). Gene expression analysis is an efficient tool for evaluation of organism's response to different abiotic stresses including temperature stress.

India has several hot springs but studies on utilization of its biotic potentials are limited. Microbial diversity in such habitats are employed in various biotechnological applications. Manikaran hot spring in H.P, India is identified as a unique environment with high water temperature of up to 96ºC and moderate salinity by the presence of $NaHCO_3Cl$ type and $NaCaHCO_3Cl$ type ions (Razdan *et al*., 2008). Studies on Manikaran quartzite fluids showed that salinity varies from 2.7 to 10.6 wt% NaCl equivalents (Sharma and Misra, 1998). The chloride content of Manikaran water ranges between 50 to 150 mg/l. Studies on hot spring micro biota were mainly restricted to archaea and prokaryotes (Murugan *et al*., 2014) however these habitats are also inhabited by thermophilic microalgae.

Heat stress leads to retarded growth, development and finally to cell death. Thermal tolerance is responsible for the emergence of various mechanisms involved in antioxidant production, membrane thermal stability, accumulation of osmolytes and most importantly activation of stress related genes. The adverse effect of high temperature in crop plants can be mitigated by various approaches including transgenic technology. There are successful

attempts to develop temperature tolerant plants through plant breeding programmes however transgenic technology applications are limited. This may be due to unavailability of desired genes. As a eukaryote, thermophilc microalgae are potent source for tolerant genes for extreme conditions.

Though mechanisms of stress tolerance in extremophilic prokaryotes are extensively studied, such studies on thermophilic eukaryotes are limited. In the present study we used an effective method termed as suppressive subtractive hybridization for isolation and characterization of differentially expressed genes under temperature stress. Here we used a combination of suppressive subtractive hybridization and quantitative Real-Time PCR for analysis of genes differentially expressed under temperature stress from thermophilic green algae, *Scenedesmus sp.,* an isolate from Manikaran thermal spring, Himachal Pradesh.

## 5.2 Materials and Methods

### 5.2.1 Isolation, Identification and Culture Optimization of Algal Strain

Temperature tolerant strain of green microalgae was isolated from Manikaran thermal springs at Himachal Pradesh, India. Sampling was done during December 2011 and water samples collected were enriched with 'D' medium (John *et al.*, 1975) and kept at 42ºC in an air incubator with 30 µE $m^{-2}$ $s^{-1}$white fluorescent light. Isolation of algal strain was done by serially diluting enriched sample with 10 ml 'D' medium in test tubes and kept at controlled conditions for growth. After one week of incubation, when growth was observed in all tubes and the tube with higher dilutions ($10^{-5}$) were taken for purifying the isolate as monoculture. The isolate was further streaked on 'D' medium agar plates supplemented with antibiotics to eliminate associated microorganisms and plates were incubated at 42ºC. Single independent

colonies on agar plates were isolated and inoculated in 10ml 'D' medium and further incubated at (42ºC). Identification of isolated strain was done by analyzing morphological features such as cell size, shape, arrangement of chloroplast and other organelles under phase contrast microscope and further confirmed by sequencing partial 18S rRNA gene.

**Fig 5.1** Sampling site, Thermal spring at Manikaran, Himachal Pradesh

### 5.2.2 Optimization of Culture Conditions and Temperature Stress Treatment

Isolated algal strain was maintained in 'D' medium at 42ºC and as growth observed was weak further standardization of culture medium was done and Tris Acetate Phosphate (TAP) medium was selected. Algae grown under optimum temperature (42ºC) was acclimatized to lower temperature (22ºC) to give efficient heat shock. A further increase from optimum temperature may affect stability of mRNA which interrupts growth. Acclimatized cells were further maintained at 42ºC. mRNA from algal cells grown at 22ºC and 42ºC were used as driver and tester respectively for the characterization of differentially expressed genes under temperature stress.

### 5.2.3 RNA Isolation and Suppressive Subtractive Hybridization

Total RNA was isolated from exponential phase of growth using TRI reagent (Sigma, USA). Isolated total RNA was quantified by Bio photometer plus (Eppendroff, Germany) and integrity was checked in 1.5% agarose gel electrophoresis. Then the mRNA was purified with GenElute™ Direct mRNA Miniprep Kit (Sigma, USA). A total of 2μg purified mRNA was used for synthesis of complementary DNA (cDNA) and subtractive hybridization was done using PCR Select cDNA subtraction kit (Clone tech, USA) according to the manufacturer's directions. Subtracted cDNA fragments amplified were cloned to cloning vector- pJET. cDNA clones obtained were screened with vector specific primers and insert size was analyzed by agarose gel electrophoresis. Positive clones were cultured, plasmids were isolated using GeneJET Plasmid Miniprep Kit (Thermoscientific, USA) and sequenced. The sequences were analyzed using both BLASTN and BLASTX for its homology with the available sequences (http://www.ncbi.nlm.nih.gov/BLAST/)

### 5.2.4 Quantitative Validation of the Expression Profile of Selected Genes under Heat Shock

Quantitative Real-Time PCR was carried out to validate genes differentially expressed under temperature stress. Real-Time PCR was performed with selected 17 genes in triplicate and reference gene used was 18S rRNA with a stable expression. Isolated RNA was treated with RNase free DNase (1U/μg RNA) to eliminate genomic DNA contamination. The qPCR was performed with the cDNA prepared from normal cells and heat stressed cells using Biorad SYBR green master mix in Biorad iQ5 thermal cycler. Primer annealing temperature for all selected genes and the reference gene were 60°C and the programme used was as follow: initial denaturation at 95°C for 3 minutes followed by 45 cycles of

denaturation at 95ºC for 20 seconds, primer annealing at 60ºC for 10 seconds and primer extension at 72ºC for 20 seconds, followed by 91 cycles of melt curve. Details of primer used for the PCR given in the table. 5.1

**Table 5.1** List of primers used for the quantitative validation of thermal stress induced genes

| SL. No. | Gene | Primer | Sequence | Product size (bp) |
|---|---|---|---|---|
| 1 | FKBP 12 | S154FKBP353_QPF | CCTCACATGCTCACCAGACT | 96 |
| | | S154FKBP429_QPR | GAGATCAGCTCCACGTCAAA | |
| 2 | S154UN463 | S154C3UN463_QPF | GCTCTTGCCAAGTATGTGGG | 103 |
| | | S154C3UN463_QPR | GTTTAGCCAACCCCGGAAAG | |
| 3 | S154UN343 | S154C13UN343_QPF | TGATTGGTTGGGCTGAGAGA | 89 |
| | | S154C13UN343_QPR | AGTTTTAGCAGAGCGTGGTC | |
| 4 | GlutamyltRNA synthase | S154SenGltmyl-tRNA synthtse_QP_F | CCCCCACTTCAGTCTTGGTA | 100 |
| | | S154SenGltmyl-tRNA synthtse_QP_R | ACGCTCACAGACTTGGACTA | |
| 5 | Aminotransferase | S154aminotrnsfrse_QPF | CTGGGTGTATAAGGTCTGCTAAG | 122 |
| | | S154aminotrnsfrse_QPR | GCTGCCAATGACTCAAGAAAG | |
| 6 | S154UN425 | S154C31UN425_QPF | CAACTGAGCTGTGACCATGA | 89 |
| | | S154C31UN425_QPR | CATGTAGGGTGTGGTGCAAT | |
| 7 | S154UN456 | S154C43UN456_QPF | TGTGCTGTGCAGAAGTCAAG | 149 |
| | | S154C43UN456_QPR | ATTTGCGCATGACAAGCAAC | |
| 8 | S154UN297 | S154C100UN297_QPF | GATCAATGGGCCTGGTAGGA | 70 |
| | | S154C100UN297_QPR | CCGAGGTACCGCTTCAGAT | |
| 9 | S154UN379 | S154C85UN379_QPF | AGGTGGCAGTAGTTGTCCG | 98 |
| | | S154C85UN379_QPR | GCCTGAGATATCACGCTGAC | |
| 10 | S154UN370 | S154C2UN370_QPF | AACGGAGAGTGACACCAACT | 76 |
| | | S154C2UN370_QPR | GCAGCTAACACTCAACCCTG | |
| 11 | S154UN196 | S154C122UN196_QPF | GCCTGCAGAGCTCACACA | 89 |
| | | S154C122UN196_QPR | ACGTGTGGGGAACTAGCG | |
| 12 | Ferredoxin-NADP$^+$reductase | S154ferxrdxn_QPF | GGTCTGATGTGGGTGTTCAT | 121 |
| | | S154ferxrdxn_QPR | TCACGGGATAGGGCATAGT | |
| 13 | Nucleoside diphosphate kinase | S154NDPK_QPF | CGACCTCAATGGCGTAGTC | 122 |
| | | S154NDPK_QPR | CAGTTGTTGCCATGGTGTG | |
| 14 | ATP synthase | S154ATPsynthse_QPF | TTTGATGGCGAGCTTCCT | 119 |
| | | S154ATPsynthse_QPR | TGGCAAAGATGACCGACTG | |
| 15 | Aldehyde dehydrognase | S154ADH_QPF | TGCGCATGCTCCTGAAA | 107 |
| | | S154ADH_QPR | CTACATGCAGGTGAAGGCTATT | |
| 16 | S154UN1082 | S154_UN1082_QPF | CCCTCAAAGGACAGTGGTATG | 121 |
| | | S154_UN1082_QPR | TGGAAGCCCGTGTTGTATATC | |
| 17 | S154UN370 | S154_UN_370_QPF | ATTCTGGCACCCACTCTTTC | 153 |
| | | S154_UN_370_QPR | TGGTTGGCGGAACTTCTTT | |

## 5.3 Results

### 5.3.1 Isolation, Identification and Culture Optimization of Algal Strain

Algal strain isolated from Manikaran thermal spring was identified as *Scenedesmus sp*. by observing morphology of cells. This was confirmed with molecular analyzes of 18S rRNA sequence (NCBI Acc.No.KM087971). BLAST analyzes showed its similarity with *Scenedesmus* sequence submitted in NCBI data bank. Growth observed in TAP medium was very high so it was used as standard medium for *Scenedesmus* culture**.**

### 5.3.2 Assembly and Analysis of *Scenedesmus* ESTs Generated by SSH.

Isolation and characterization of temperature tolerant genes from thermophilic strain of chlorophycean microalgae, *Scenedesmus sp*. was carried out by forward subtractive hybridization. A total of 325 clones were randomly picked and fragments with a size range of 0.1kb to 1.1kb were sequenced. All sequences were edited using Seqman sequence editor; vector and adapter sequences were removed. Further they were assembled to form contigs with overlapping sequences. A total of 148 contigs were formed from 325 clones sequenced and these sequences were aligned with gen bank data using BLASTN and BLASTX programmes.

BLAST analysis of differentially expressed gene contigs in NCBI gen bank revealed that out of 148, majority contigs (78%) has no significant similarity with reported sequences and it is kept as unknown genes with important functions in thermal tolerance. Thirteen clones showed sequence similarity with ribosomal gene and remaining contigs showed significant sequence homology with the functionally identified genes (Fig 5.2). Most of the differentially expressed gene fragments belonged to functional gene category that was directly or indirectly linked to temperature and other abiotic stresses. These functionally important genes have broad spectrum of cellular activity such as cell maintenance and development, signal transduction, energy metabolism, photosynthesis, transcription factors (Fig 5.3).

### 5.3.3 Expression Profile of Differentially Expressed Genes under Hyper Temperature Stress from *Scenedesmus sp.*

**Table 5.2** List of differentially expressed genes in hyper temperature stress showing significant similarity to known sequences in the pubic database

| Putative gene | Annotation e-Value | Functions | Reference |
|---|---|---|---|
| Light harvesting chlorophyll-ab binding protein | 2e-55 | Photosynthesis and oxidative stress response, | Andersson *et al.*, 2001; Dittami*et al.*, 2009; Xu *et al.*, 2012; Liu *et al.*, 2013, , |
| Rhodanase Homology Domain (RHOD), Gonidia specific protein | 2e-75 | oxidative stress response | Pantoja-uceda*et al,*, 2004; Cereda*et al.,* 2009, |
| chloroplast ribulose-1 5 bisphosphate carboxylase oxygenase | 4e-63 | Photosynthesis, water stress and oxidative stress response | Cunaseker and Berkowiz,1993, Chen *et al.*, 2011 |
| sedoheptulose-1,7-bisphosphatase | 2e-66 | Photosynthesis, salt stress responses | Feng *et al.*, 2007 |
| glutamyl-tRNAsynthetase | 3e-07 | Protein synthesis, heat stress and oxidative stress responses | Katz and Orellana, 2012, Linga and Solla, 2009, Tian*et al.*, 2009 |
| Rieske iron-sulphur protein | 2e-64 | Photosynthesis and stress response | Maiwald*et al.*, 2003 |
| aldehyde dehydrogenase | 8e-33 | Oxidative stress, osmotic stress, drought stress | Sunkar*et al.*, 2003; Kotchoni and Bartels, 2003;Gaoand Han, 2009; Xu, *et al*, 2013 |
| sulfotransferase domain protein | 1e-05 | Temperature stress, cellular detoxification and oxidative stress responses | Sebastia*et al.*, 2008; Wasternack and Hause, 2013 |
| aurora like protein kinase | 9e-05 | chromosome segregation and cytokinesis | Demidov*et al.*, 2005 |
| Serine threonine protein kinase | 0.003 | Salt stress responses, Signal transduction, heat stress responses | Hardie,1999 ; Diedhiou*et al.*, 2008 ; Kulik*et al.*, 2011 ; Pais *et al.*, 2009 |
| chloroplast ATP synthase | 7e-18 | Heat stress responses | Chen *et al.*, 2011 |
| oxygen evolving protein of photosystem II | 2e-08 | Oxidative stress, Heat stress | Kimura *et al.*, 2002 ; Murata*et al.*, 2007, |
| 2-oxoglutarate dehydrogenase | 1.5 | Heat stress resistance, oxidative stress, energy metabolism | Tretter*et al.*,2005; Li et al., 2013 |
| acyl carrier protein | 0.002 | Drought, hypoxia | Klinkenberg*et al*,. 2014 |
| nitrile hydrates | 2e-07 | Abiotic stress | Machingura and Stephen, 2014 |
| retrotransposon Ty3-gypsy | 2e-26 | Plant stress and defence | Grandbastien, 1998; Echenique*et al.*,2002; |
| Ubiquinol cytochrome c oxidoreductase biogenesis factor | 1e-17 | Electron transport and osmotic stress | Jia*et al.*, 2004 |
| Carboxy methylene butenolidase | 1e-26 | Hydrolase, carbohydrate metabolism, abiotic stress | Kim *et al.*, 2009; Yan Baglo, 2014 |
| nucleoside diphosphate kinase | 7e-57 | High temperature stress, salt stress, oxidative stress etc | Tang *et al.*, 2008; Li *et al.*, 2011 |
| FKBP 12 | 3e-35 | Refolding of denatured proteins; Ca2+ channel regulation, abiotic stress | Pemberton,2006; Geislerand Bailly,2007; Yu *et al.*, 2012 |
| DNA dependent RNA-polymerase | 5e-20 | Transcription | James *et al.*, 1979 |
| branched-chain amino acid aminotransferase II | 9e-10 | Drought and heat strss | Rizhsky*et al.*, 2004 |
| ferredoxin-NADP+ reductase | 2e-144 | oxidative stress | Rodriguez *et al.*, 2007; Musumeci*et al.*, 2012, |

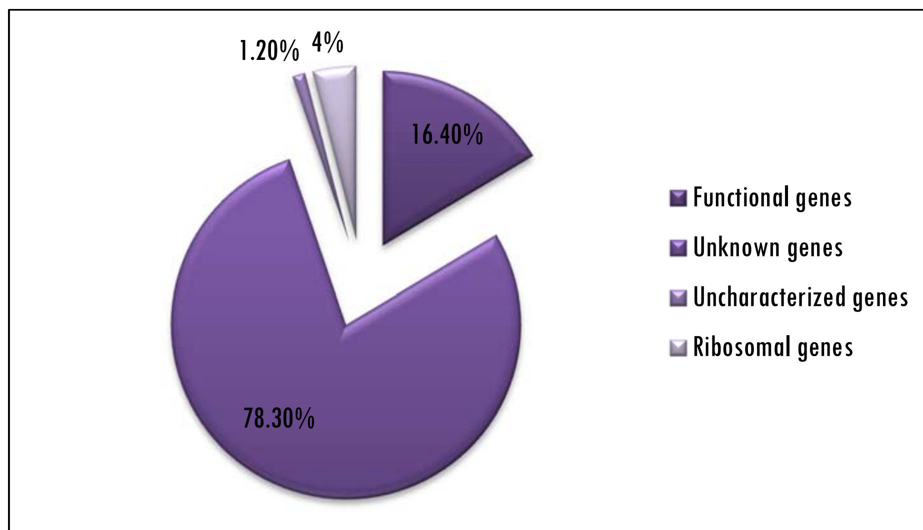


**Fig 5.2.** Classification of *Scenedesmus* genes differentially expressed under hyper temperature stress

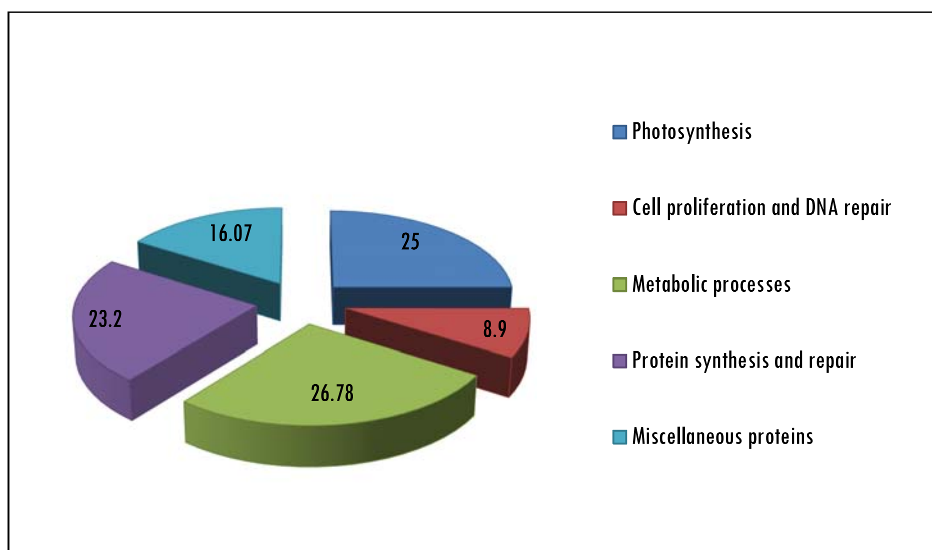


**Fig 5.3.** Functional classification of identified genes differentially expressed under hyper temperature stress

### 5.3.4 Quantitative Validation of Gene Expression by Real-Time PCR

To validate expression profile of the selected genes differentially expressed under temperature, we used qPCR method. A total of 19 genes were selected for the quantitative validation. Both known and unknown gene fragments

were selected for qPCR analysis. Among the selected gene fragments all showed an upward expression except one fragment (UN343), which did not show any significant expression under heat shock. As shown in figure 5.4 the mRNA transcript of the aldehyde dehydrogenase gene was highest in the stressed cells when compared to the normal cells. These results proved the reliability of SSH for isolation of differentially expressed genes under two different conditions.

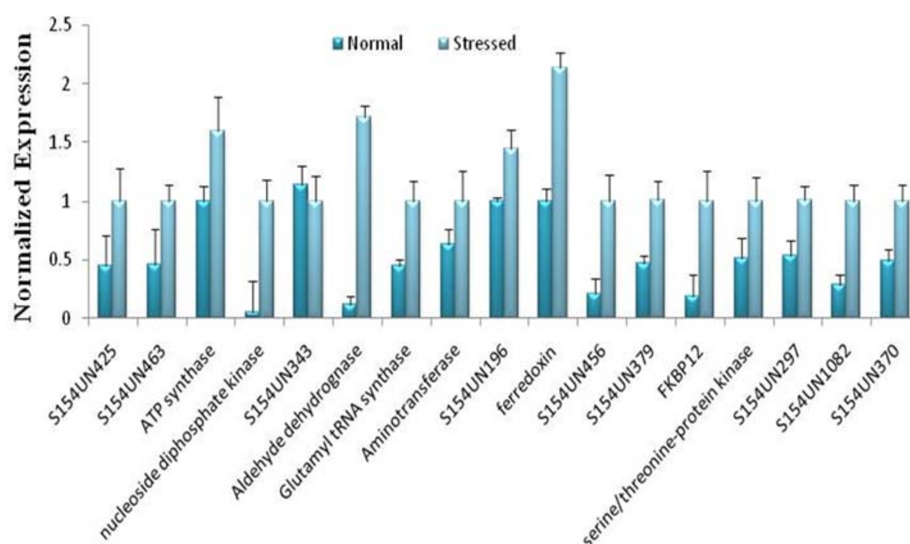


**Fig 5.4** The expression profile of selected ESTs generated from SSH library using Real-Time PCR. Error bar indicates ± standard deviation (SD), Number of replicate (n)= 3.

## 5.4 Discussion

Temperature stress is one of the important abiotic stresses in arid tropical countries. High temperature adversely affects normal functioning of cellular enzymes and thereby reduces its productivity. The present study tried to elucidate important genes differentially expressed under temperature stress from a thermophilic micro alga, *Scenedesmus sp.* The mechanism of stress tolerance is complex and controlled by a combination of genes expressed

under stress condition. The mechanism was extensively studied among microalgae but only few studies in thermophilic algae. In the present study we have isolated a thermophilic strain of green microalgae, *Scenedesmus sp*. and characterized genes differentially expressed under temperature stress using a powerful molecular method termed, Suppressive Subtractive Hybridization (Machida *et al*., 2008; Fan *et al*., 2012). The reliability of the SSH was further investigated by performing quantitative expression of selected genes by using Real-Time PCR.

High temperature adversely affects photosynthetic activity of plants by degrading photosystem II of the thylakoid region. Light-harvesting chlorophyll a/b-binding protein (LHCP) is an abundant protein and functionally involved in dissipating light energy to photosynthetic reaction centers in the chloroplast (Xia *et al*., 2012). There are various allelic forms of LHCP present in photosynthetic organisms and their level of expression varies under different abiotic stresses. The present study also identified different forms of LHCP gene fragments differentially expressed under elevated temperature. Dittami *et al*., (2009) studied the thirty light-harvesting chlorophyll a/b-binding proteins from brown algae and reported. Most of them showed a down regulation while three genes showed an up regulation under hyperhaline, hypohaline and oxidative stresses. In Antarctic diatom, *Chaetoceros neogracile*, temperature shock induced upward regulation of five and downward regulation of ten LHC proteins (Hwang *et al*., 2008). Similar result was observed in *Sueda salsa*, a halophytic plant which showed an up regulation of LHC protein during heat and salt shock (Li *et al*., 2011).

High temperature causes accumulation of reactive oxygen (ROS) species in algal cells which leads to the cellular damage and cell death. In the present study also we have identified many differentially expressed genes which are

directly or indirectly involved in oxidative stresses. Aldehyde dehydrogenase is one of the important enzymes actively involved in oxidative stress to detoxify aldehydes generated during the peroxidation of lipids. In the present study aldehyde dehydrogenase was over expressed in stressed cells when compared to normal cells. Besides temperature, other environmental stresses such as high salinity, drought and abscisic acid application also induce expression of aldehyde dehydrogenase (Gao *et al*., 2009). Glutamyl tRNAsynthatase gene which is differentially expressed under heat stress is also actively engaged in oxidative stress. The quantitative validation using Real-Time PCR also proved its elevated expression under temperature shock. It acts as key enzyme involved in protein synthesis by establishing genetic code through single aminoacylation reaction. Role of Glutamyl tRNAsynthatase in temperature stress was first reported among plants, in a thermal adapted grass *Agrostis scabra,* where elevated expression of Glutamyl tRNAsynthatase conferred long term tolerance under heat stress (Tian *et al*., 2009).

In the present study, we identified a temperature tolerant gene FKBP type peptidyl prolylcis-trans isomerase. The classification of FKBP is mainly based on molecular weight. In the present study differentially expressed FKBP have a molecular weight of 12 KDa and thus it belongs to FKBP12 category and this is the lowest form with only one substrate binding domain. FKBP is directly involved in protein folding by cis/trans isomerization of proline imidic peptide bonds (Lang *et al*.,1987; Wang *et al*., 2010). Sometimes they are not essential for normal physiological functioning of the cell but actively expressed under challenging environments (Pemberton; 2006). Study on some FKBPs reported their elevated expression pattern under different stress conditions (Geisler and Bailly, 2007) which include wounding stress, salt stress (Vucich and Gasser., 1996), heat and cold shock (Aviezer-Hagai *et*

*al*.,2007), water stress (Ahn*et al*., 2010), light stress (Luan *et al*., 1994) and malondialdehyde treatment (Weber *et al*., 2004).

Nucleoside diphosphate kinase (NDPK) has significant role in stress tolerance mechanisms of plants. This enzyme is actively involved in maintenance of intracellular dNTPs except ATP and also takes part in signal transduction pathways involved in oxidative stress (Otero, 2000), heat stress (Escobar *et al*., 2001) etc. In *Arabdopsis,* the over expression of NDPK2 enhanced the tolerance level under multiple environmental stresses (Moon *et al*., 2003). In our study we have identified a temperature tolerant form of NDPK gene differentially expressed under heat stress from thermophilic microalgae, *Scenedesmus sp*. The quantitative validation of the expressed NDPK gene fragment also showed an elevated expression under temperature shock. Among microalgae, identification and expression of NDPK was done in *Dunaliella tertiolecta* (Anderca *et al*., 2002) and there was no more functional characterization of micro algal NDPKs. So it is important to functionally characterize them from microalgae because of their high diversity and adaptability to thrive under extreme environmental conditions.

Ferredoxin-NADP(H) Reductase (FNR) is actively involved in the key step of electron transport during photosynthesis. Over expression of this enzyme during heat stress will reduce damage caused due to oxidation at higher temperatures. This was evidenced from enhanced tolerance to photo oxidative stress in transgenic tobacco with over expressed pea (*Pisums ativum*) FNR gene (Rodriguez *et al*., 2007). The thermophilic strain of *Scenedesmus sp*. also showed a differential expression of FNR gene under heat stress and it was further confirmed with the quantitative validation using Real-Time PCR. Other genes which are differentially expressed during this study were also directly or indirectly involved in enhancing the stress tolerance mechanisms.

The serine threonine protein kinase was highly expressed in plant during environmental stresses (Kulik *et al*., 2011).

## 5.5 Conclusion

Development of transgenic crops with desired capabilities will be the prime requirement in nearby future. This is especially important to feed the growing human population confronted with climate change issues. Transfer of extreme climate tolerant genes to crop plants will help to utilize and convert tropical arid waste land and flooded wet lands and marshes into fertile lands. This will help in global poverty alleviation also. In this context our present investigations of temperature tolerant genes in thermophilic *Scenedesmus sp*. offers scope and hope for transgenic crop plants.

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Chapter 6

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6.5 Conclusion

## 6.1 Introduction

The threat of global warming is real and imminent. Environmental consequences of climate changes are rising temperature, low pH, drought, sea level rise, increased salinity etc., and these changes will greatly affect the food production systems such as agriculture, animal husbandry and aquaculture (Adams *et al*., 1998; Smit *et al*., 1988; Sirohi and Michaelowa, 2007; Gornall *et al*., 2010; Yazdi and Shakouri, 2010; Naqvi and Sejian, 2011; Lobell and Gourdji, 2012). Development of transgenic crop varieties capable of growing and producing optimally under conditions of increased temperature and salinity would be a feasible approach to mitigate this threat, especially in coastal and estuarine areas where effects of climate change will be profound (Jewell *et al*., 2010; Hemantaranjan *et al*., 2014). Identification of a suitable gene or set of genes that can be used to impart the properties of salinity and

temperature tolerance to the crop would be one of the preliminary steps towards transgenic improvement of a crop for stress tolerance. Organisms that live and thrive in environments of high temperature and salinity would be the natural choice for screening for such genes.

Manikaran hot springs offers such a unique environment where the temperature ranges from 32ºC to 96ºC, and is also saline due to the presence of Na-$HCO_3$-Cl type ions (Chandrasekharam *et al.*, 2005 and 2008). A chlorophycean microalga *Scenedesmus sp.* was isolated from this dynamic environment where the pool formed by the hot spring mixes partially with the flowing cold water of the stream, the candidate algal isolate lives in an environment where sudden temperature changes in the range of 10ºC to 65ºC happens. The organism would have developed molecular adaptations to survive the sudden changes in temperature and salinity of its immediate environment. Suppressive subtractive hybridization technique was employed to identify and mine out genes that were differentially expressed by *Scenedesmus sp.* during high temperature. Among the many genes identified there was a chaperone known as peptidyl-prolylcis-trans isomerase.

Peptidyl-prolylcis-trans isomerases are a group of chaperones belonging to a family of unique proteins known as cyclophilins that are found in all classes of organisms. They forms one of the largest protein family, most of the higher forms occur in plants and are involved in several biochemical processes including signal transduction, protein folding and development. They are also known as FK506 binding protein (FKBP) due to their ability to bind immunosuppressive drugs like FK506, cyclosporine, rapamycin etc.

Recent studies revealed that they have specific role in stress tolerance mechanisms, protein transportation and apoptosis through their molecular

interactions with receptors or proteins. The interaction between Small Ubiquitin like Modifiers (SUMO) and PPIase have important function in protein folding and the process of aggregation of protein is minimized by molecular chaperons under stress conditions (Mueller *et al.,* 2006). PPIase accelerate the slow rate-limiting isomerisation step in refolding of proteins (Vierling E, 1991; Boston *et al*., 1996; Miernyk, 1999; Asadulghani *et al*., 2004). The sub cellular localization of FKBPs within the cell is diverse and are localized among cytoplasm, nucleus rough ER, plastid stroma and mitrochondrial matrix (He *et al*., 2004). FKBP are classified based on their molecular weight. There are various forms of FKBP comprising size range from 12 KDa (Faure *et al*., 1998) to 135 KDa in plants (Kurek *et al*., 2002b; Galat A, 2003). These different forms of FKBP are characterized by the presence of at least one FK506 binding domain (FKBd). Higher forms of the plant FKBP posses functional domain in addition to the obligatory FKB domain, and common among these are tetra tricopeptide repeat (TPR) units, Calmodulin (CaM)-binding domains (CaMBds). Plant FKBP posses different functions which depends on the functional domains. Isoforms of the multidomain FKBP62 (ROF 1) and FKBP65 (ROF 2) are actively involved in the long term tolerance of high temperature stress. This is achieved through the modulation and expression of several small heat shock proteins (sHSPs) involved in recovery from heat stress (Hagai *et al*., 2007).

FKBP 12, a canonical member of the FK506 binding protein is the basic domain of the FK506 binding proteins, which show PPIase activity. These canonical members function as molecular chaperones and are associated with the protein folding and modulation of oxidative stress (Kang *et al.*, 2008). FKBP 12 is also involved in regulation of cell cycle through interaction with various protein partners (Aghdasi *et al*., 2001; Vespa *et al*., 2004). FKBP12-

rapamycin-associated protein (FRAP) plays an important role in intracellular signaling network which ensure normal growth (Desai *et al.*, 2002). Among the FKBP protein family FKBP 12 has been extensively studied and it posses only a single FK506 binding domain (FKBD) comprised of 108 amino acid. FKBP complexes formed with ligands are more resistant to proteolytic cleavage and form an appropriate site binding to CaN and mTOR (mammalian target of rapamycin) (Harrar *et al.*, 2001; Kurek *et al.*, 2002b). FKBP 12 has ryanodine receptors (RyRs) and modulates it in the absence of FK506, which is one of the major $Ca^{2+}$ -releasing channels in the sarcoplasmic reticulum (Brillantes *et al.*, 1994; Breiman and Camus, 2002; Wang *et al.*, 2004). Together with the above properties of FKBP it is suggested that it has important role in the activities of cellular partners.

As consequences of global climatic change the temperature increases which indirectly affect soil salinity through the intrusion of saline water because of sea level rise. High temperature, salinity and other forms of stress results in misfolded proteins that tend to disrupt vital cellular functions. Organisms that live in harsh environments have developed distinct molecular mechanisms to adapt and thrive; they have proteins with altered structure to withstand and function in extreme environment, and also express stress responsive chaperones that enable proper folding of proteins and refolding of misfolded proteins. In this study we have completely characterized a stress responsive molecular chaperone, FKBP type peptidyl-prolylcis-transisomerase from thermophilic algae, isolated from the hot springs of India. This gene has exhibited tolerance to temperature and salinity. Hence is an ideal gene candidate for raising stress tolerant crop plant for the sustainable production in the changing global climate.

## 6.2 Materials and Methods

### 6.2.1 Sequence Analysis of Sce.FKBP 12

The Open Reading Frame (ORF) of *Scenedesmus*FKBP12 gene was identified and the amino acid sequence predicted using Editseq of Laser gene software. Homologous FKBP12 gene sequences in other species were obtained from NCBI by BLAST query. Multiple sequence alignments were generated using Bio-Edit multiple alignment tool. Phylogenetic trees were constructed using Molecular Evolutionary Genetics Analysis (MEGA version 6) software with the Neighbour-Joining method (Tamura *et al*., 2013).

### 6.2.2 FK506 Sensitivity Assay

Growth of *Scenedesmus* cells on TAP (Tris Acetate Phosphate) media containing different concentration of immunosuppressant drug, FK506 was assayed by spotting 5µl of normalized cultures in different dilution (100mM, 200mM, 500mM and 1000mM) during the exponential phase of growth. Spotted plates were incubated at 25ºC under a cool white fluorescent light.

### 6.2.3 Quantitative gene Expression of Sce.FKBP 12 under Temperature and Osmotic Stress

Gene expression profile of the isolated FKBP-type peptidyl-prolylcis–transisomerase gene was analyzed using Bio Rad IQ5 Real-time Thermal cycler. Quantitative validation of Sce.FKBP 12 gene under various temperatures and osmotic stresses were carried out. Algal cells grown at lower temperature was subjected to a heat shock (22ºC to 42 ºC) for specified time (0 Hrs, 6Hrs, 12Hrs, 24Hrs and 48Hrs). Osmotic stress was given by treating cells with 0.5M NaCl with same duration followed in the temperature stress treatment. All RNA samples were quantified spectrophotometrically

using Bio photometer plus (Eppendroff, Germany) and the integrity of the samples were analyzed in 1.5% agarose gel electrophoresis. Quantified RNA samples were treated with RNAse free DNAse 1(1u/µg RNA, Fermentas) to remove the genomic DNA contamination. First strand cDNA was synthesized using Revert Aid Premium cDNA synthesis Kit (Thermo scientific, USA). The resultant cDNA was used as template in 25µl PCR reaction mixture with Power SYBR Green Master Mix (Applied Biosystem, USA) and gene specific primers designed from the ORF of *Sce.*FKBP12 gene. The 18S rRNA gene was chosen as an internal reference gene for determining the RT-PCR amplification efficiency among different reactions. Details of primers used are given in table 5.1. PCR amplification was carried out using the following programme: 94 ºC for 3 min followed by fifty cycles at 94ºC for 10s, 62ºC for 30 s and 72ºC for 20s and final melt curve of 90 cycles 50-90ºC . The specificity of the reactions was verified by melting curve analysis. PCR conditions were standardized for both FKBP and 18S rRNA reference genes.

### 6.2.4 Recombinant Cloning and Expression of Sce.FKBP12

The coding sequence of the FKBP gene was amplified using the oligonucleotide forward primer 5' ACTGTAACCATGGGAGTCACCAAGG AGACTG (>FKBP_F) and oligonucleotide reverse primer CGTGGAGCTGATCTCAATCTCACTCGAGAGTTGTA (>R_FKBP) and cloned into NcoI and XhoI site of pET28b expression vector (Novagen, USA) and was termed pET28Sce.FKBP12. pET28Sce.FKBP12 construct was transformed to *E.coli* BL21 (DE3) competent cells. Transformed colonies were inoculated to LB broth supplemented with Kanamycin (50µg/ml) and grown at 37ºC with continuous shaking at 225rpm. The expression of the FKBP gene was induced at $OD_{600}$ ~ 0.6 using 0.5mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 4 hours. The induced and uninduced (control) cultures were

harvested by centrifugation at 8000rpm for 5 min and protein expression analyzed in 15% tricine gel SDS-PAGE.

### 6.2.5 Temperature and Salinity Tolerance of Recombinant *E. coli* cells with Sce.FKBP12 Gene

The *E.coli* cells (BL 21) transformed with pET28Sce.FKBP12 gene and with pET28b vector alone (control) were used for the tolerance study under temperature and salinity stress. A starter culture was prepared by inoculating single colony from both the transformed cells in LB broth with Kanamycin (50µg/ml) and kept overnight at 37 ºC in a shaking incubator. 2% stock culture was inoculated in 10ml LB broth containing 50µg/ml Kanamycin and 0.5mM IPTG. Initial $OD_{600}$ was taken after inoculation and the tubes kept in a shaking incubator with an rpm of 225 at 45ºC. For studying salinity tolerance cultures were inoculated into LB media containing different concentration of NaCl (0.5M, 1M, 1.5M and 2M NaCl) and 0.5mM of IPTG and kept at 37ºC with an rpm of 225. Both the cultures were made as triplicate to reduce the standard error. The growth rate was monitored by taking $OD_{600}$ with an interval of 2 hours.

## 6.3 Results

### 6.3.1 Isolation and Sequence Analysis of FKBP-type Peptidyl-prolyl cis–Trans Isomerase

Suppressive subtractive hybridization technique was adopted to reveal differentially expressed genes under temperature stress from thermophilic micro algae, *Scenedesmus sp.* The SSH clones were sequenced and contig aligned. The contigs were screened using NCBI blast query search (http://www.ncbi.nlm.nih.gov/BLAST/), one contig showed high similarity to FKBP type peptidyl-prolylcis–transisomerase. The Sce.FKBP12 contig (NCBI Acc. No. KR908645) contained the complete open reading frame (ORF) of

327bp for the gene and encoded a 108 amino acid protein with a predicted molecular mass of 12kDa. FKBP type protein families are mainly classified based on their molecular weight so the identified PPIase gene from *Scenedesmus* belongs to FKBP 12 family. FKBP 12 is the lowest form of the identified FKBPs, which have only one domain for substrate binding. To study the evolutionary relationship of FKBP 12 from different organisms a phylogenetic tree was constructed using neighbour joining method with 1000 replicates of bootstrap analysis for statistical reliability. As shown in the fig.6.3 the *Scenedesmus* FKBP 12 gene sequence shows highest phylogenetic similarity with closely related *Chlamydomonas* FKBP 12. The identity between the *Scenedesmus* FKBP12 and homologs in other organisms ranges from 69% to 79% at amino acid level. Analysis of the Sce.FKBP 12 amino acid sequence showed a single amino acid deletion of glutamic acid at 56$^{th}$ position as compared with its orthologs from other related organisms.

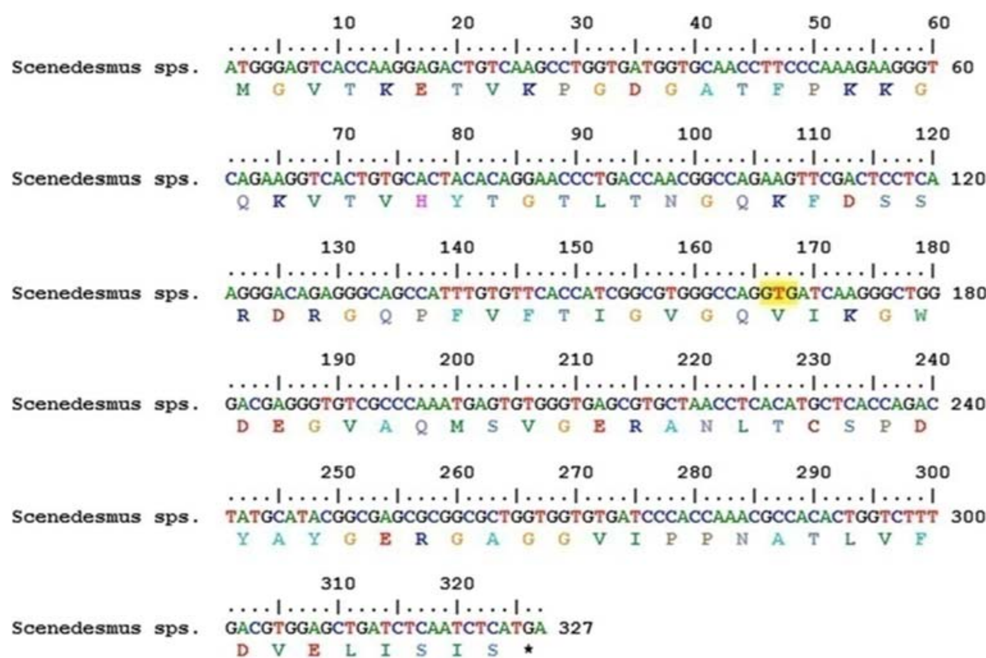


**Fig 6.1**cDNAand deduced amino acid sequences of FKBP 12 gene from *Scenedesmus sp.*

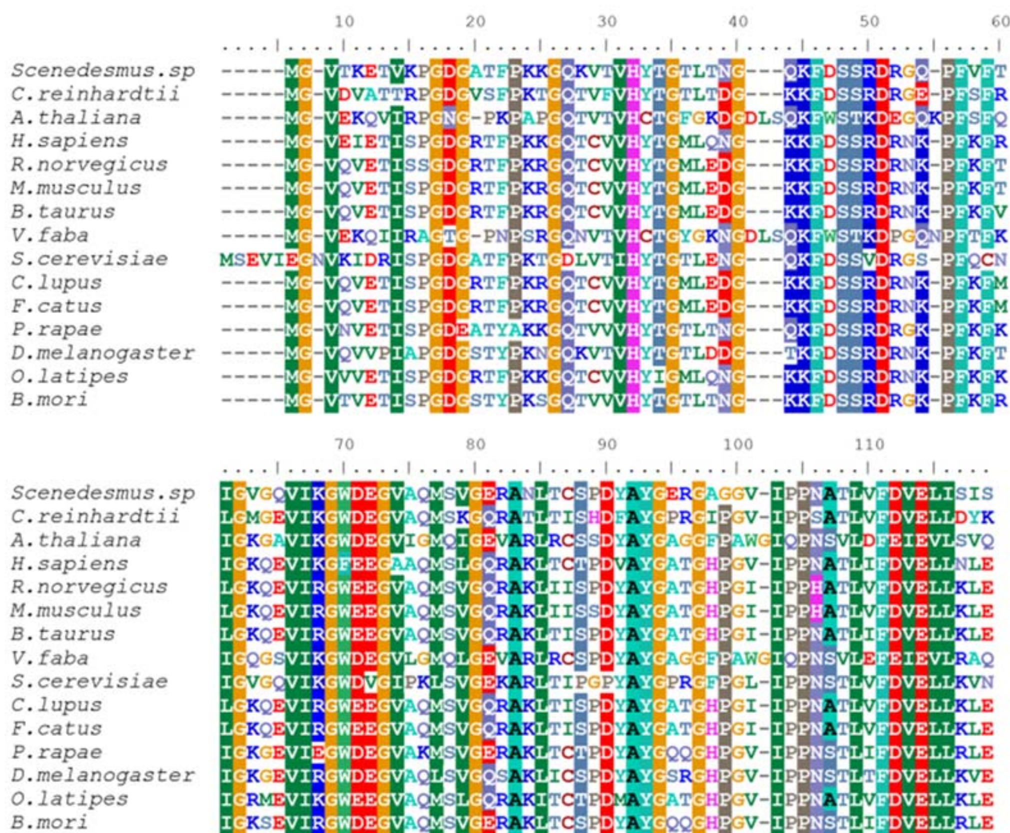


**Figure 6.2** Alignment of deduced amino acid sequences of FKBP12 from *Scenedesmus* sp. and orthologs of other eukaryotic organisms. Conservation of amino acid residues with similarity 90% and above are shown by colour shadings. Accession numbers of sequences for FKBP 12 proteins are: XP_001693615 (*Chlamydomonasreinhardtii*), NP_201240 (*Arabidopsis thaliana*), NP_004107 (*Homo sapiens*), NP_037234 (*Rattusnorvegicus*), NP_032045(*Musmusculus*), AAI02339 (*Bos Taurus*), NP_014264 (*Saccharomyces cerevisiae*), NP_001239119 (*Canis lupus*), AFN85815 (*Pierisrapae*), CAA88904(*Drosophila melanogaster*),XP_004083389 (*Oryziaslatipes*), ABK15648 (*Bombyxmori*), M3WX41 (*Feliscatus*) and O04287(*Viciafaba*)

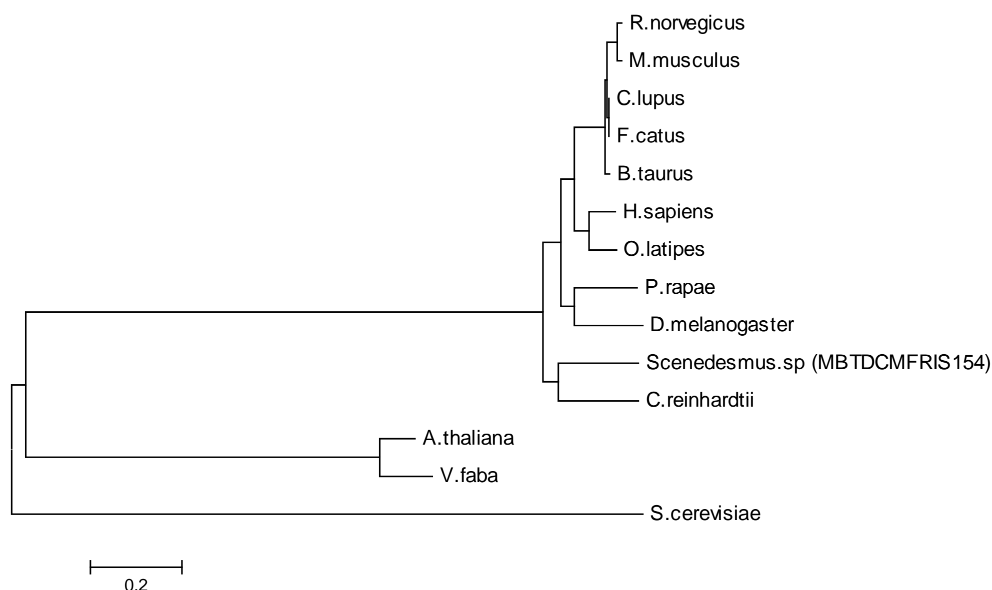


**Fig 6.3** Phylogenetic tree of FKBP 12. The amino acid sequences were subjected to Bootstrap test of phylogeny by the MEGA 6.0 program, using neighbour-joining method with 1000 replicates

## 6.3.2 Influence of FK506 on Growth of *Scenedesmus* Cells

The growth rate of *Scenedesmus* cells grown on TAP medium plate supplemented with different concentration of FK506 drug and also control plates without the drug was monitored. After 10 days of incubation plates were investigated for the growth and found that there was no significant difference in the growth of FK506 *Scenedesmus* cells in FK506 treated plates as compared to the control plates (Fig 6.4). The effect was negligible even at higher concentration (1000nM) which is about 10 times the concentration inhibiting yeast growth (Gollan and Bhave, 2010). This result shows that FK506 has no significant effect on the growth of *Scenedesmus* cells. The drug binding capacity of the FBKP depends on the hydrophobic pockets formed by several amino acid residues. Among this glutamic acid at position 54 or surrounding area is essential for high affinity towards the immunosuppressant drugs. The inability of FK506 to inhibit the cell growth may be due to the

deletion of glutamic acid at 56th amino acid position in *Scenedesmus* FKBP12 which may affect the affinity of FKBP12 towards the immunosuppressant drugs, or it may also be due to the high expression of duplicate forms under stress conditions (Gollan and Bhave, 2010).

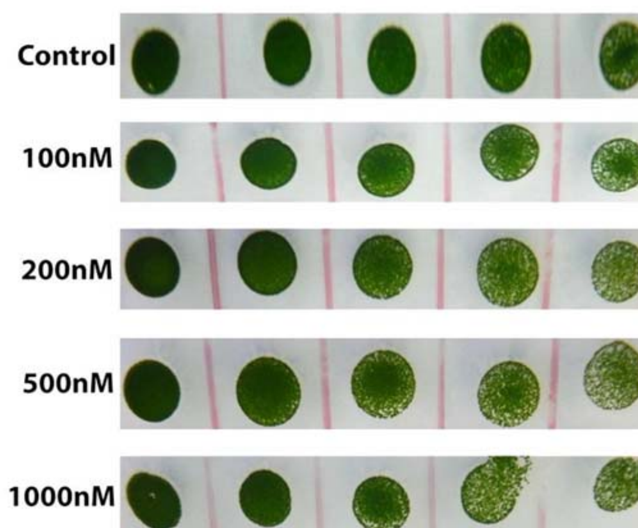


**Figure 6.4** Effect of FK 506 on growth of *Scenedesmus*sp. Thermophilic*Scenedesmus* cells at different dilutions were spotted onto TAP plates containing the indicated concentrations of FK 506. Plates were incubated at 25°C under continuous illumination.

### 6.3.3 Expression Profile of FKBP 12 under Temperature and Osmotic Stress

Expression pattern of the isolated FKBP 12 gene was analyzed using real time PCR. Specificity of the PCR reactions for both FKBP 12 and 18S rRNA genes were confirmed with real time dissociation curve. The expression of Sce.FKBP12 gene under temperature shock (22ºC to 42ºC) and a hyper osmotic stress at different time interval (0 hr, 6 hr, 12 hr, 24 hr and 48 h) were analyzed. Both the thermal and osmotic stress showed a significant variation in the level of expression of the FKBP 12 gene with the duration of time and there was an upward regulation of Sce.FKBP12 gene under temperature and salinity shock. The expression profile was highly significant

with duration of heat and osmotic shock that there was a rapid change of mRNA transcript level at 24 hours and it increased at 48 hours, after which the expression data was not taken (Fig 6.5 and 6. 6).

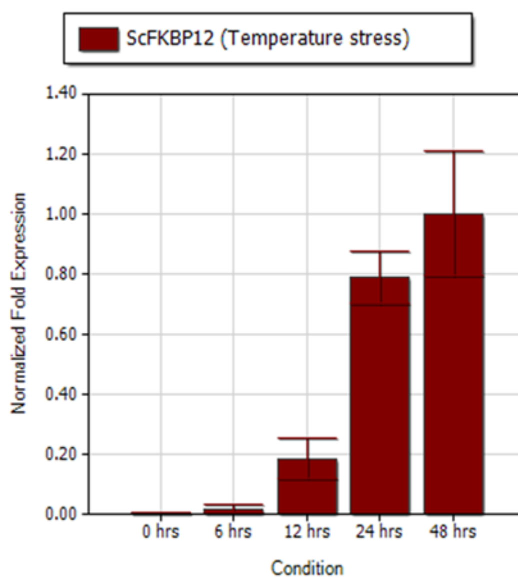


**Fig 6.5** Expression pattern of ScFKBP 12 gene under heat shock at different time intervals. Error bar indicates ± standard deviation (SD), Number of replicate (n)= 3.

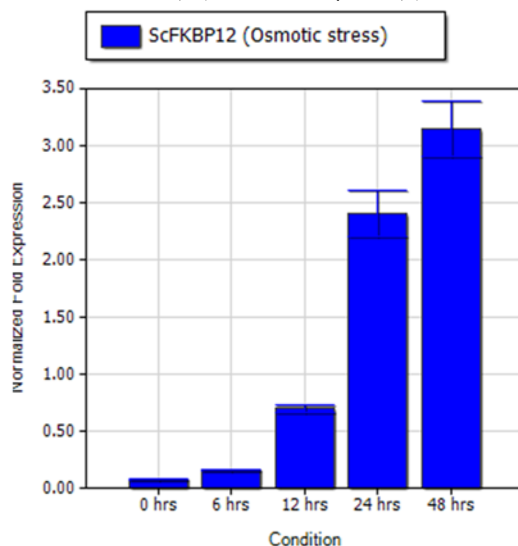


**Fig 6.6** Expression pattern of Sce.FKBP 12 gene under osmotic stress at different time interval. Error bar indicates ± standard deviation (SD), Number of replicate (n) = 3.

### 6.3.4 Recombinant Expression of Sce.FKBP 12 Gene in *E. coli*

The coding sequence of the Sce.FKBP12 gene was cloned to pET28b expression vector and was transformed to BL21 *E. coli* cells. The expressed recombinant Sce.FKBP12 (rSce.FKBP12) protein was visualized in 15% tricine gel SDS-PAGE (Fig.6.7b) with size near 14 kDa (including the C terminal His tag)

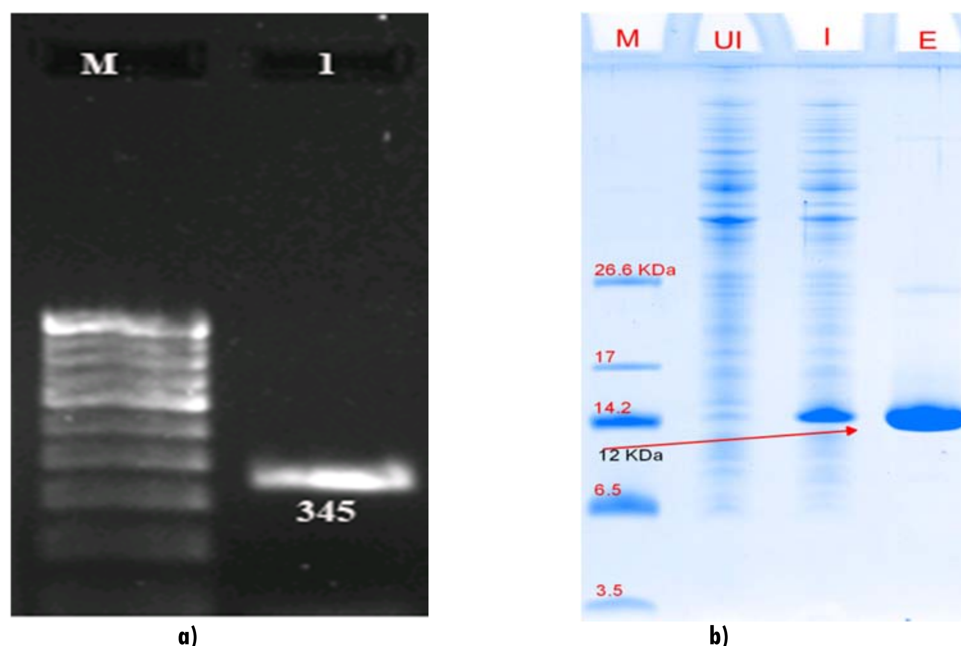


**Fig 6.7.** a) 1-cDNA amplification of FKBP 12 gene (complete CDS), M-100bp marker b) Expression profile of FKBP12 protein on 15% SDS-PAGE

### 6.3.5 Temperature and Salinity Tolerance of Recombinant *E. coli* cells with Sce.FKBP12 Gene

The recombinant *E.coli* cells were initially grown under normal temperature (37ºC) was subject to a temperature stress (42 ºC) and the growth rate was monitored by measuring $OD_{600}$ at 2 h. intervals. It was observed that both the transformed cells acclimatized to elevated temperature but the growth rate was higher in the *E.coli* transformed with pET28Sce.FKBP12 compared to the control *E.coli* cells transformed with empty pET28b vector (Fig 6.8).

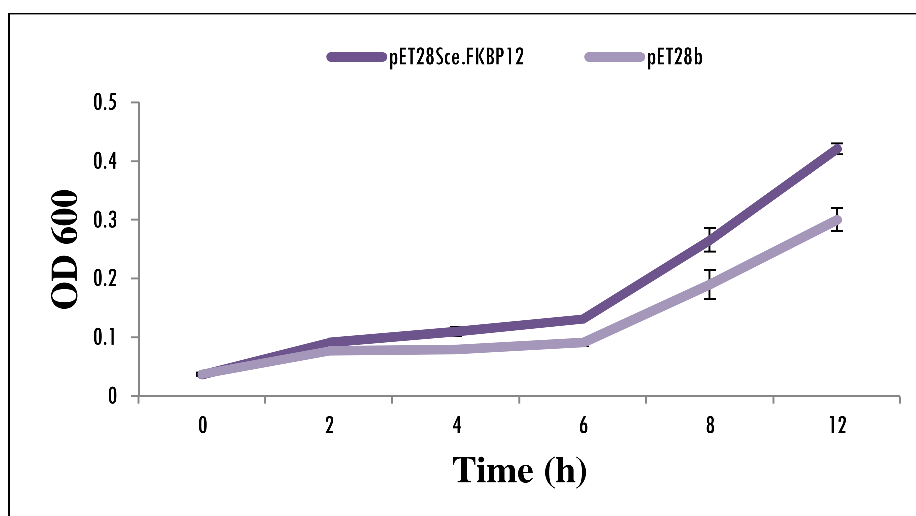


**Fig 6.8** The growth pattern of BL21 cells transformed with pET28FKBP12 and empty pET28b under hyper temperature. Error bar indicates ± standard deviation (SD), Number of replicate (n) = 3.

Hyperosmotic stress retarded the growth rate of pET28 (control), but the Sce.FKBP12 gene transformed BL21 cell acquired a better growth rate when compared to the control cells under elevated salinity. An enhanced growth rate was observed in the cells with Sce.FKBP12 gene when compared to the pET28 vector alone transformed cells at 0.5 M NaCl (Fig.6.9). pET28Sce.FKBP12 transformed *E.coli* cells acquired temperature and salinity tolerance when compared to the empty pET28b transformed cells.

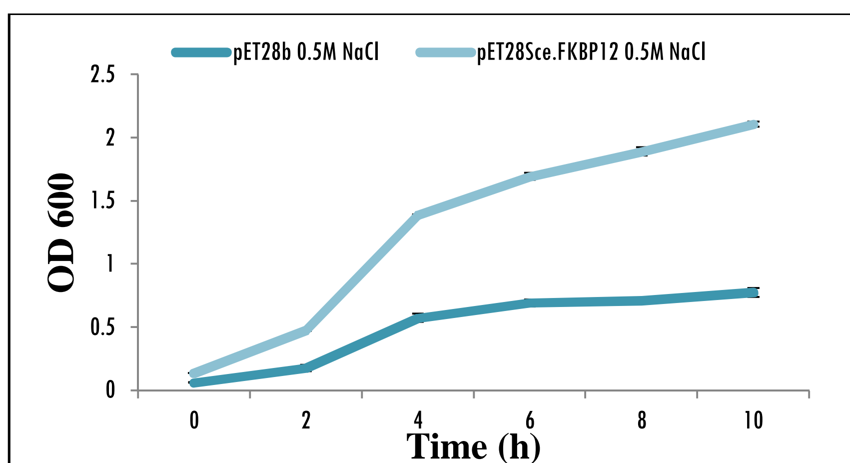


**Fig.6.9** Growth curve of recombinant BL 21 E.coli cells with pET28FKBP12 gene and pET28 (control) under hyper osmotic stress. Error bar indicates ± standard deviation (SD), Number of replicate (n)= 3.

## 6.4 Discussion

In the present research work temperature and salinity responsive form of FKBP 12 gene was isolated and characterized from thermophilic green algae, *Scenedesmus sp*. This is the first report of temperature and salinity tolerant FKBP 12 gene cloned and functionally characterized. Phylogenetic analysis of the *Scnedesmus* FKBP 12 gene showed a close relationship with *Chlamydomonas* FKBP 12 gene, both the algae belongs to same family chlorophyceae. In vitro study on the effect of immunosuppressant drug, FK506 on the growth of *Scenedesmus* showed that there is no significant effect on the growth as compared to *Chlamydomonas*, which is sensitive to immunosuppressant drugs (Crespo*et al*., 2005). Compared to other eukaryotes, plants are insensitive to immunosuppressant drug due to the inability of plant FKBP12 to bind immunosuppressant drugs (Xu *et al*., 1998). Multiple alignment of Sce.FKBP 12 protein with other FKBPs revealed a single amino acid mutation, absence of Glu at position 56, which would significantly reduce the binding of immunosuppressant drugs. This result is in agreement with the yeast complement assay done by Crespo *et al*. with mutant FKBP 12s where the presence of Glu either at position 54 or surrounding area significantly increased drug binding. The reason behind the high affinity of immunosuppressant drug on this Glu residue is the establishment of additional hydrogen bond with immunosuppressant drugs, as reported by Choi *et al*., (1996). This insensitivity may also be due to the high expression of duplicate forms under stress conditions (Gollan and Bhave, 2010). Stress conditions modulate the formation of CsA and FK506-insensitive FKBP with PPIase activity, thus suggesting that it may also be involved in stress adaptation (Sharma and Singh, 2003). We also investigated the reliability of subtractive hybridization by validating the

expression pattern of FKBP 12 gene under elevated temperature at different time intervals using Real Time PCR.

In plants, FKBPs are regulators of normal growth and development and provide a tool for enhancement of crop yield under stress conditions (Sharma and Singh, 2003). Study on some FKBPs reported their elevated expression pattern under different stress conditions which include wounding stress, salt stress (Vucich *et al*., 1996), heat and cold shock (Kang *et al*., 2008), water stress (Ahn*et al*., 2010) and light (Luan *et al*., 1994). In the present study Sce.FKBP12 gene was recombinantly expressed in *E.coli.* The recombinant cells over expressing ScFKBP12 acquired increased tolerance to temperature and salinity stress, the recombinant *E.coli* with Sce.FKBP 12 gene revealed a better growth when compared to *E.coli* transformed with empty pET28b vector. Trivedi *et al.* showed similar results in *E.coli* with PPIase gene from *Piriformos poraindica*, which enhance the growth rate of recombinant cells with PiCyPA under multiple abiotic stresses (Trivedi *et al*., 2013). This result strengthened the role of FKBP in temperature and salinity tolerance mechanisms. In higher plants multi- domain FKBPs are involved in heat stress responses (Yu *et al*., 2012), however here we report a single-domain ScFKBP12 which has significant up regulation under temperature and salinity stress. In higher eukaryotes even though the FKBP family is large but each member has its unique biological role and one member cannot completely complement the absence of another one (Breiman and Camus, 2002).There has been substantial progress in the past few years towards the characterization of FKBPs participating in stress responses in Arabidopsis (Vucich and Gasser, 1996; Hagai *et al*., 2007), rice (Ahn *et al*., 2010) and wheat (Blecher *et al*., 1996; Reddy *et al*., 1998; Kurek *et al*., 2002b; Dwivedi *et al*., 2003).

## 6.5 Conclusion

As a eukaryote, extremophilic microalgae are suitable source for "candidate genes", as they can survive unfavourable condition which cannot be sustained by normal life. Studies on algal FKBPs are rare and it is important to characterize each FKBP from microalgae because of their diverse distribution, abundance and simple cell structure. Our investigation on thermophilic green algae, *Scenedesmus sp*. isolated from thermal spring for identifying genes differentially expressed during temperature stress provided us with a temperature and salinity tolerant FKBP12 gene. It was seen that *Scenedesmus* FKBP12 showed up regulated expression in conditions of both increased temperature and salinity. When *Scenedesmus* FKBP12 was recombinantly expressed in *E. coli* the cells gained temperature and salinity tolerance, the gene now needs to be further validated in model plant systems before exploiting the gene for development of transgenic crop, which could be cultivated in higher saline/ and high temperature habitat.

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Chapter 7

# SUMMARY AND CONCLUSION

As a eukaryote, extremophilic and extremotolerant microalgae have potential application in the field of biotechnology and genomics. Microalgae can be used as model organism for the study of complex cell processes of plants because of their diverse distribution, simplicity in cell structure and convenience of raising and maintaining the culture in laboratory environments. Extremophilic organisms flourish in the adverse condition through special mechanisms like osmolite accumulation, physiological and biochemical adaptation, etc. Abiotic stress tolerance mechanism is a complex polygenic trait with allelic variations. Extremophilic microalgae have the inherent ability to with stand adverse climatic condition through the altered gene expression. Characterizations of these genes and their expression in suitable system have potential application for the development of stress tolerant plant varieties through genetic engineering. Apart from this extremophilic microalgae are good source of bioactive compounds which have potential applications in biotechnology. Global warming has serious impact on earth climate which greatly affect both land and ocean productivity. Among the various abiotic stresses salinity, temperature and pH has the maximum impact. To mitigate this problem it is necessary to develop food producing plants and other organisms which tolerate the changing climatic conditions. Previous approaches depend mainly on traditional selective breeding, but it is time consuming and has limited application. Advancement of biotechnological

methods like Next Generation Sequencing provides an insight to molecular mechanisms underlying stress tolerance. Transcriptomic profile of crop varieties has revealed the genes involved in various cascades of enzymatic action during abiotic stress tolerance. Alterations of specific pathways with a better allele of an extremophilic origin can enhance the tolerance level of sensitive species. In this context, characterization of functional genes differentially expressed under extreme condition from these algae have significant application for the development of crop varieties with better tolerance in varying agro-climatic conditions.

In India there are vast stretches of land unutilized due to the extreme abiotic conditions such as salinity temperature and pH. We need to explore ways to bring these unutilized areas for raising food crops. Hence we have to explore modern tools in biotechnology such as gene mining, expression and their use in the development of agricultural crops which could grow in extreme conditions. In the present research work, we have looked into the possibility of gene mining from extremophilic and extremotolerant microalgae from diverse Indian ecosystems with special reference to acidity, salinity and temperature.

## Major Achievements of Present study are as follows

- This study focuses on the extremophilic and extremotolerant microalgae which tolerates various extremities like hyperosmotic, acidic and thermal conditions. Isolation of the algal strain was carried out from various habitats like salt lakes, salt pans, thermal springs, coastal areas and estuaries.

## I. Acid tolerant gene mining, characterization and expression

- A euryhaline acid tolerant microalgae *D. Ehrenbergianum* was isolated and characterized from the Cochin estuary. Isolated strain with wide range salinity (0-40 ppt) and pH (3-8.5) tolerance was used for the characterization of differentially expressed genes in acidic stress.
- Suppressive subtractive hybridization (SSH) generated a differential trancriptome under acidic stress. A total of 200 transcripts of differentially expressed gene fragments were analysed. All the identified genes are directly or indirectly involved in the acid or abiotic stress tolerance mechanisms and are actively involved in biological process of the cell. Among the gene fragments analysed 55% showed sequence similarity with functional genes, 21% unknown genes which may have functional roles in abiotic stress and the remaining 24% contributed to ribosomal genes.
- Quantitative validation of selected gene fragments showed upward regulation in acidic condition. Some of the selected gene which showed substantial upward regulation include $H^+$ATP synthase, major-facilitator-like ion transporter, thioredoxin, calmodulin, osmotically inducible protein, glutathione peroxidase, cinnamyl alcohol dehydrogenase etc.
- Full gene amplification of a proton donating $H^+$ ATP synthase was carried out using RACE PCR. The amplified De.$H^+$ATPase gene has an ORF of 1617 bp encoding 538 amino acid protein with a predicted molecular mass of 57.6 KDa.
- De.$H^+$ATPase gene was recombinantly expressed in *E.coli* (BL21) with pET28b expression vector. Acquired tolerance of recombinant *E.coli* with De$H^+$ATPase in acidic pH was analysed.

**II. Saline tolerant gene mining characterization and expression**

- A halophilic microalga, *Tetraselmis indica* was isolated from Pulicat Lake which tolerates up to 3M NaCl concentration. Characterizations of saline responsive genes were carried out from this algae using SSH.
- A total of 182 differentially expressed transcripts were sequenced. Among the differentially expressed genes 43% showed sequence similarity with functionally identified genes, 38% unknown gene which may have functional roles in salinity stress tolerance and the remaining 19% showed sequence similarity with ribosomal genes.
- Expression profiles of selected 12 genes were carried out using Real-Time PCR. All the genes showed an upward regulation except GAPDH and MIF4G domain. Maximum expression was observed for the genes hydroxyl pyruvate reductase (HPR) and fructose-1,6bisphosphatealdolase (FBA).
- Among the highly expressed genes fructose-1,6 bisphosphate aldolase (FBA) was fully amplified by RACE PCR. The full gene with an ORF of 1161 bp nucleotide with 387 predicted amino acids was translated to FBA protein of size 41.4 KDa.
- Recombinant expression of Ti.FBA imparts salinity tolerance in *E.coli* under hyperosmotic stress.

**III. Thermotolerant gene mining characterization and expression**

- Isolation of thermophilic microalgae *Scenedesmus sp.* was carried out from Manikaran hot spring at Himachal Pradesh, India. Manikaran hot spring is unique for its high temperature and moderate salinity.

- A differential gene expression profile was created by analysing 325 gene fragments differentially expressed under hyper temperature stress. Among the gene fragments analyzed 78% has no significant similarity with the reported sequences and it is assigned as unknown genes with important functions in thermal tolerance. Thirteen clones showed sequence similarity with ribosomal gene and the remaining contigs showed significant sequence homology with the functionally identified genes.
- Quantitatively validated selected genes using Real-Time PCR showed an upward regulation under heat shock.
- Among the differentially expressed genes, a molecular chaperone called peptidyl-prolyl-cis-trans isomerase was obtained with complete ORF of 327 bp, encoding a 108 amino acid protein with a predicted molecular weight of 12 KDa.
- Molecular characterization and recombinant expression of PPlase gene was carried out. As a chaperon this gene has roles in various abiotic stresses. This study proved its role in temperature and salinity stress through recombinant expression and validation in *E.coli*

## Conclusion

Abiotic stress has severe impact on plant productivity worldwide. Among the various abiotic stresses salinity, temperature and acidity has significance because of the global warming. This study helps to understand the molecular mechanism of stress tolerant microalgae under acidic, hyperosmotic and hyper temperature condition through the differentially expressed genes. Most of the genes differentially expressed in these conditions have active role in various abiotic stresses. Some genes uniformly expressed in one or more stress conditions has proved their role in multiple stresses. In the present study all

the three stressors, namely salinity, low pH and temperature induced the over expression of ATP synthase gene in all the three different microalgae studied. One of the osmotic stress tolerance mechanisms is through the elevated synthesis of different osmolites. FBA is an important enzyme in the production pathway of glycerol, a vital osmolite produced during hyperosmotic stress. Apart from thermal stress PPlase has significant role in the various stresses because of its role in protein aggregation and refolding. All the abiotic stress finally leads to oxidative damage, so the expression of genes involved in antioxidant mechanisms were observed in all these stresses. Genes like thioredoxin, aldehyde dehydrogenase, peptidylprolyl-cis-trans isomerase, ferredoxin-$NADP^+$reductase, nucleoside diphosphate kinase, glutathione peroxidase etc. are actively involved in oxidative stress tolerance mechanisms. Recombinant expression of De.$H^+$ ATP synthase, Ti.FBA and Sce.FKBP12 in *E.coli* impart resistance to low pH, osmotic stress and temperature stress respectively. In addition to temperature stress Sce.FKBP12 recombinant cells acquires osmotic stress tolerance also. Bioprospecting of FKBP12 with versatile tolerance to temperature and salinity and their functional validation in cell system is a major achievement of present work. The gene constructs developed in the present study should be further characterised and validated using model eukaryotic organisms like Arabidopsis, Tobacco etc., for its suitability as a transgene for imparting stress tolerance in higher eukaryotes. After validation, if found suitable, it can be used for generating stress tolerant transgenics of agriculturally and economically important species for enhancing growth and productivity under various abiotic stresses.

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## RESEARCH PUBLICATION

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# FKBP-type peptidyl-prolyl *cis-trans* isomerase from thermophilic microalga, *Scenedesmus* sp.: molecular characterisation and demonstration of acquired salinity and thermotolerance in *E. coli* by recombinant expression

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**Abstract** A novel complementary DNA (cDNA) encoding temperature and salt stress-responsive FK506-binding protein 12 (FKBP12), a chaperone with peptidyl-prolyl *cis-trans* isomerase (PPIase) activity was isolated using suppression subtractive hybridization (SSH) technique from a thermophilic chlorophycean microalga, *Scenedesmus* sp., isolated from the Manikaran hot springs at Himachal Pradesh, India. To our best knowledge, this is the first report of temperature and salt-responsive FKBP12 isolated from a microalgae. The *Scenedesmus* FKBP12 (Sce.FKBP12) nucleotide sequence revealed the presence of an open reading frame of 327 bp, encoding 108 amino acid protein containing a single FKBP-like domain with a predicted molecular weight of 12 KDa. Amino acid sequence comparison of Sce.FKBP12 with its ortholog from other eukaryotic organisms showed structural conservation among each other. Expression profile of Sce.FKBP12 gene using quantitative real-time PCR showed significant up-regulated expression under temperature and salinity stress. The recombinant Sce.FKBP12 was over-expressed in *Escherichia coli* using pET28b expression vector. The recombinant expression of Sce.FKBP12 in *E. coli* cells imparted temperature and salinity tolerance to the host cells, implying a significant role of Sce.FKBP12 in stress tolerance mechanisms. Sce.FKBP12 could be a promising transgene candidate for imparting abiotic stress tolerance in crop plants, and they need to be further evaluated using plant models like *Arabidopsis* or Tobacco for confirming its potential as a transgene candidate in development of temperature and salinity-tolerant transgenic crops for improved and sustained productions in adverse agro-climatic conditions.

**Keywords** *Scenedesmus* · Thermophilic microalgae · FKBP · Peptidyl-prolyl *cis-trans* isomerase (PPIase) · Abiotic stress tolerance · Transgene · Recombinant expression

## Introduction

The threat of global warming is real and imminent. Environmental consequences of climate change are rising temperature, low pH, drought, sea level rise, increased salinity, etc., and these changes will greatly affect the food production systems such as agriculture, animal husbandry and aquaculture (Adams et al. 1998; Smit et al. 1988; Sirohi and Michaelowa 2007; Gornall et al. 2010; Yazdi and Shakouri 2010; Naqvi and Sejian 2011; Lobell and Gourdji 2012). Development of transgenic crop varieties capable of growing and producing optimally under conditions of increased temperature and salinity would be a feasible approach to mitigate this threat, especially in coastal and estuarine areas where effects of climate change will be profound (Jewell et al. 2010; Hemantaranjan et al. 2014). Identification of a suitable gene or set of genes that can be used to impart the properties of salinity and temperature tolerance to the crop would be one of the preliminary steps towards transgenic improvement of a crop for stress tolerance. Organisms that live and thrive in environments of high temperature and salinity would be the natural choice for screening for such genes.

Manikaran hot springs offers such a unique environment where the temperature ranges from 32 to 96 °C and is also

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