Gene Expression Analysis in *Dunaliella salina* Under Salt Stress

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Received: 2019-02-29 Revised and accepted: 2019-08-14

Abstract

Different salt concentrations are found in the large portion of water resources and agriculture lands in which most of the plant species cannot be grown. Some microalgae species can be grown either in seawater or in brackish water which is not usable for normal agriculture. The present study analyzed the gene expressions associated with salt stress in *Dunaliella Salina*. The expression of three coding genes namely acetyl-CoA carboxylase (ACC), channelrhodopsin-1 DChR1 protein (DC), and sodium-coupled phosphate transporter protein (DSSPT) were evaluated at three salinity levels: 3 gl^{-1} (low), 32 gl^{-1} (control), 100 gl^{-1} (high) . The analysis were performed on the 5th, 10th and 17th day of culture. The results revealed that for low and high salt treatments, the lowest gene expressions were observed on 5th day of growth, however, the gene expressions were increased with the growth of algae cells until the 10th day. It should be noted that, under low salinity conditions (3 gl^{-1}), gene expressions were lower than high salinity concentration during the growth phase.

Keywords: Microalgae, Saltwater, *Dunaliella salina*, Gene expression, Salt stress.

Introduction

Freshwater resources are limited and it is expected to threaten the world in coming decades. Dry lands are usually located near coastal areas and use of seawater, even those with low salt concentration for agriculture, leads to the formation of saline crusts on the surface and damages the soil. Microalgaecan grow in various habitats due to their unique features. Using saltwater for marine microalgae cultivation may decrease the production cost of these organisms. Currently, large scale microalgae cultivation is globally brought to spotlight which is possible by high biomass productivity, no need for farmlands and freshwater. Microalgae are chiefly characterized by their ability to produce value-added compounds such as pigments, vitamins, pharmaceutical products and biofuels (Ben-Amotz, 2004; Chang et al., 2001; Spolaore et al., 2006). Different groups of algae are found in various climates and different aquatic ecosystems and habitants. Among algae groups, green algae are largely found in freshwater and marine water with different salinity. Some algae species exist in unbelievable environments such as salt lakes, hot springs, and glaciers (Baweja and Sahoo 2015).

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Dunaliella species are characterized by the lack of eukaryotic cell wall, but due to their haploid genome, they can grow in saline concentration in the range of 0.5-5 M. Due to-producing glycerol through photosynthesis and its accumulation, these algae can adjust intracellular osmotic potential(Ben-Amotz et al., 2009). *Dunaliella* species are viable in a great pH range. For example *D. acidophila* and *D. Salina* can survive between 1 to 11 pH ranges, respectively (Gimmler et al., 1989). However, they can grow in temperatures between 0-40°C, and depending on the strains, optimum growth temperatures are usually between 21-40°C (Werman et al. 2002). Carotenoids, glycerol, proteins and vitamins are the basic intracellular compounds in algae with autoxidation and anticancer properties (Gomez et al., 2003). *Dunaliella* produce glycerol as the osmoprotectant in order to cope with salt stress (Shariati and Hadi, 2011).

The process of gene expression allows the cells to control their structure and performance; in fact, this process is the basis of cell differentiation, evolution and the ability to adapt organisms to new conditions. Gene transcripts analysis is necessary for developing an inclusive profile of mRNA for key enzymes which are involved in environmental stresses (Zheng et al., 2014). Diagnostic methods are currently very popular for qualitative assessment. Real-Time Polymerase Chain Reaction amplify DNA chain and providing the possibility to measure nucleic acid production in every cycle. Microalgae species are widely used as a simple model organism for researches. In fact, microalgae are characterized by a rapid growth rate and low cost of production compared with other gene expression systems, and provide valuable chemicals and recombinant products.Unlike the extensive studies on *Chlamydomonas reinhardtii*, relatively few studies have been done on *Dunaliella Salina* in spite of its large scale cultivation. Some *Dunaliella* species are highly halophilic and used as model organisms in investigations of cellular adaptations and molecular mechanisms in response to salinity variations. (Ben-Amotz and Avron, 1990). Genome expression analysis is a primary step in diagnosis cellular responses to abiotic stresses. Molecular methods such as databases of Expressed Sequence Tag (EST), microarray analysis, transcriptome or genome sequencing, can be used for gene expression analysis. Due to extracellular osmotic pressure, intracellular glycerol concentration may reach to 50% of the intracellular content (Zhang et al., 2003). It has been proved that changing osmotic conditions results to synthesis or degradation of glycerol.under photosynthesis inhibition, glycerol can be produced from stored amides improving resistance to osmotic pressure. On the other hand, when extracellular salinity decreased, glycerol may be converted into amides or exported (Araújo et al., 2009). Some studies investigated relationship between sodium transporters and plasma membrane electron transporter (oxidation system) in *Dunaliella* and show that greater concentrations of intracellular sodium can help to reduce extracellular electron acceptor ferricyanide (Katz and Pick, 2001). Studies on various biochemical methods such as signal transduction, redox energy, protein synthesis, and sta-

bilizing plasma in response to salt stress indicates more than one mechanism is involved in unique salinity tolerance of *Dunaliella Salina* (Liska et al., 2004). Among abiotic stresses, mainly high salt stress is investigated due to its direct effect on cell division and growth rate. It might lead to ionic and osmotic effects in photosynthetic organisms (Allahverdiyev et al., 2011). Other reports show the role of salinity stress in some metabolites production such as carotenoids, glycerol or lipids (Bahieldin et al. 2014). Molecular response at the gene level for *Dunaliella salina* was investigated using microarray method in low and high salinity growth conditions during a long time (Kim et al., 2010). To find out mechanism of salinity stress and identify the genes involved in ion transport pathway under salt stress conditions, study of some genes expressions was done.

Materials and Methods

The microalgae *Dunaliella Salina* was prepared from plant biotechnology laboratory of Ferdowsi university of Mashhad with accession number KF477384 and algae cells grown in 500 ml Erlenmeyer flasks containing 250 ml culture medium (Talebi et al., 2015). Culture media formulated using several trails and errors, contained the following compositions as in 1 L distilled water: 1.50 g $NaNO_3$, 1.00 g KNO_3, 3 g $NaHCO_3$, 0.080 g $CaCl_2$. $2H_2O$, 1.5 g $MgCl_2.6H_2O$, 0.080 g $MgSO_4$. $7H_2O$, 15-100 g NaCl, 0.20 g KCl, 0.15 g K_2HPO_4, 0.15 g K_2HPO_4, 0.20 g KH_2PO_4 and 1 ml of the Hutner's trace elements stock solution. The medium and flasks were sterilized in an autoclave for 15 min at 121^oC to prevent any contamination during the growth. Culture was illuminated with cool white fluorescent lamps at an intensity of 6000 lux (equivalent to 69 µmol photon/m2s) in 16:8 h light-dark cycles. The cultures were incubated at 21± 2^oC. Because of the necessity for fully sterile conditions, no aeration pump was used during the cultivation (Moheimani et al., 2013). Three water resources including Caspian Sea, Oman Sea and well water were considered in this study. According to water analysis, three levels of salinity was determined. Three salinity levels (3, 32, and 100 grams of NaCl per liter) were selected (Hanson et al., 1999). In order to adapt at microalgae to stress condition, *Dunaliella* Salina was cultivated in optimum and pre-stress medium for three days before transferring to stress condition. Microalgae sampling was performed at 5th, 10th, 17th day of growth and algal biomass was analyzed to determine gene expression. Here, total RNA contents of algal biomass under different salt concentration (high, low and control) treatments were extracted (Kim et al., 2010).

Four genes for gene expressions analysis under various salt stress conditions, based on literature were selected. Expressed Sequence Tags for these genes were obtained from NCBI database and designed by primer3, primer premier and primer quest (Table 1) then analyzed and approved by Blast Primer.

RNA was extracted using total extraction RNA kit (Pars Toos Co., Iran). RNA quantity and quality were determined by Thermo Nano Drop2000 and 1% agarose gel. Purification of RNA free of DNA was performed using DNAse I (Roche Co., Germany). In addition,

Table 1. Sequences of primers used for amplification of target areas.

Gene name	Primer sequence	Length of multiplied section (bp)
DSSPT	GCCGTCCGAACTCCTATC (F) CACGAGCACCTACAACAACT (R)	165
DChR1	TTGGGATCAACAACAACGAAAC (F) GCACGAACACACACAGATAAAC (R)	111
Acetyl-CoA	AGTAGCCAGGAACGGATAG (F) CCGTGTAGTTCTTCATGTCG (R)	112
18S rRNA	TCGATGGTAGGATAGAGG (F) CTTCCTTGGATGTGGTAG (R)	103

cDNA was synthesized using Kit (Pars Toos Co., Iran). Gene expressions were evaluated in three biological replicates by Bio-Rad CFX manager iCycler real-time PCR and SYBR Green master mix kit (Pars Toos Co., Iran). Thermo cycles for amplification of the samples were as follows: first, 94°C for 5 minutes for initial denaturing of strands, followed by 40 PCR cycles (each cycle included 94°C for 30 s, 57°C for 30 s and 72°C for 30 s). After the end of these 40 cycles, the vials were stored for 8 minutes at 72°C and final extension and completion of strands performed. To explore the cellular reaction at gene level, effect of salt stress and growth phase on gene expression of three proposed genes acetyl-CoA carboxylase (ACC), sodium-coupled phosphate transporter protein (DSSPT), and channelrhodopsin-1 DChR1 protein (DC) of rhodopsin family were compared. At this stage, the PCR reaction was performed using a synthesized cDNA as a template and specific primers for DSS, DC and Acetyl-CoA carboxylase genes. Additionally, primers of 18S rRNA were used in amplifying of a 103-base-pair region of 18S rRNA as housekeeping gene. For *statistical analysis*, biologic cycles and 3 technical cycles were considered. However, before performing the main test for each primer, cDNA was diluted to determine the efficiency of the primer. After optimization of PCR reaction, the final reaction was performed with negative control and triple repetitions for the samples using Pars Toos Syber Green Supermix. To draw the DNA melting curve, the temperature changed from 50°C to 95°C during 90 cycles of 10 seconds So that in each cycle it increased 0.5 °C and the amount of light emitted from the samples was recorded at the end of 10 seconds. All melting curves and Ct values were analyzed by thermo-cycler and using $2^{-\Delta\Delta Ct}$ method (Wan et al., 2011). Data are normalized with respect to 18S ribosomal RNA gene and then variations in gene expression during different stress conditions wereanalyzed or measured in comparison with control (no stress applied) (Kim et al. 2010).

The experiments included factorial design with

three primers and 3 salt concentrations (low, high and no-salinity (control)) at three different growth phases (5th, 10th, 17th days after the main stress) in a Completely Randomized Design (CRD) with three replicates. Evaluation of data variability was performed by one-way analysis of variance (ANOVA) with Duncan's post hoc test utilizing the SAS 9.4 software. The P-valueless than or equal to 0.01 was considered statistically significant.

Results

Sudden salt stresses (either low or high) can lead to microalgal death, in this study algal cells were gradually transferred to the stress culture media (in three steps) and therefore, the samples were ready for main stress conditions. Proper performance of PCR and exclusive amplification of gene regions were approved for different samples using melting curve (Fig. 1). As shown in Figure 1, the curve includes similar peaks for all samples, indicating that no primer dimer or non-exclusive sections exists. Figure 2 shows the effect of different frequency of investigated gene transcripts in series of cDNA.

According to Figure 3, the activity of Acetyl-CoA carboxylase decreases in response to 3g/l and 100 g/l of salt on the 5th day apply stress. In addition, these two treatments led to 2.6 times greater gene transcripts during algal growth after 10 days. At the end of logarithmic phase and the beginning of the stationary phase, gene expression becomes stable. It is notable that during low salt stress, the expression of this gene is knocked out to near zero but with growth, gene expression increases and reaches the condition of control samples. This indicates the adaptation of cells with stress conditions as they grow.

Results have shown that gene expression of channelrhodopsin-1 DChR1 under low (3 g/l) and high (100 g/l) salt stress was dramatically decreased, while it was increased as soon as cells reached to the stationary phase. (10th day) and changed to stress tolerant strains. It is noticeable that huge increase in gene expression was observed with increasing salt stress, suggesting that salt stress can be controlled by increasing ion transportation and subsequently absorbing saline ions (Fig 4). The notable trend was the increase in gene expression in case of increasing salt stress, suggesting that salt stress can be controlled by increasing ion

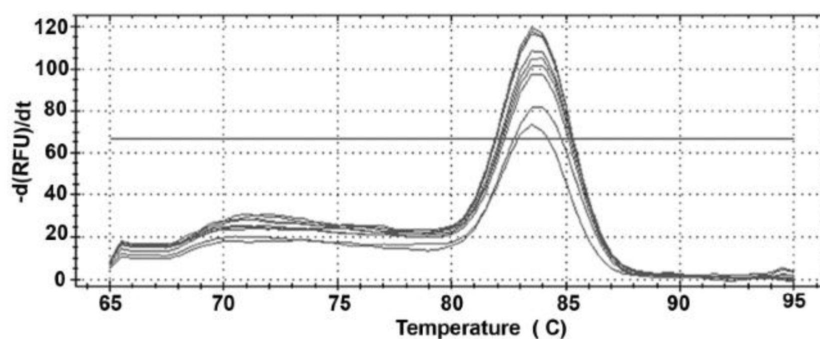

Fig. 1. Melting curve for multiplied samples with 18S rRNA primer after PCR amplification.

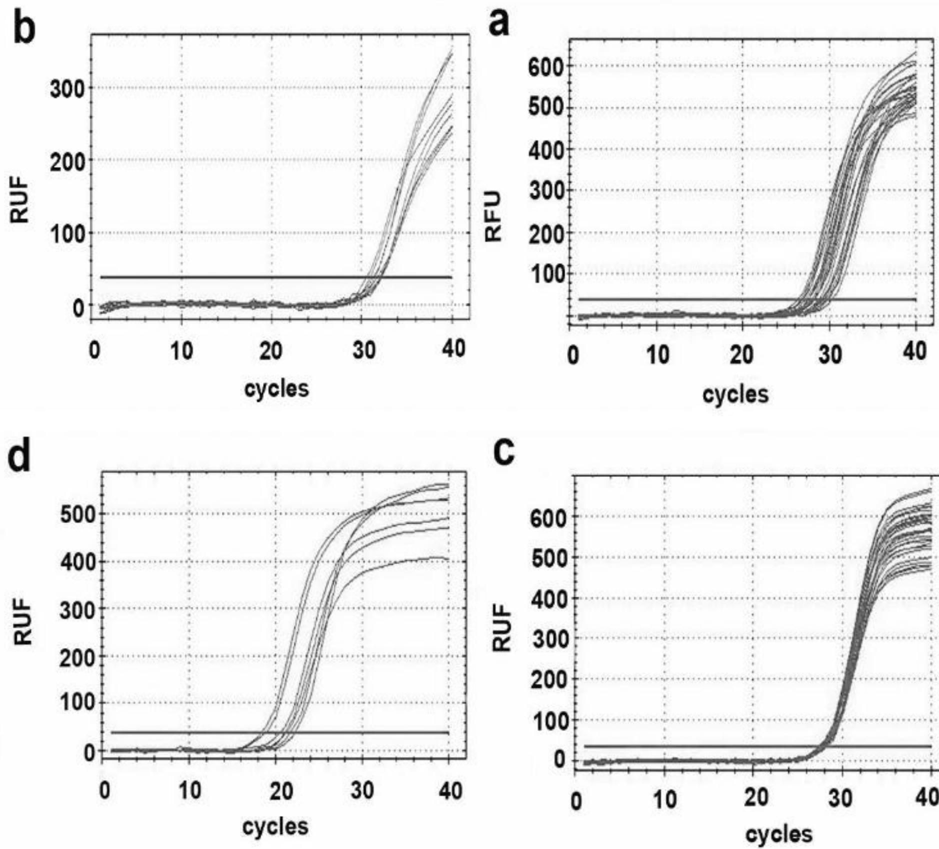

Fig. 2. Graphs for proposed genes: A- Channelrhodopsin-1, B- sodium coupled phosphate transporter protein, C- acetyl-CoA carboxylase, D-18S rRNA.

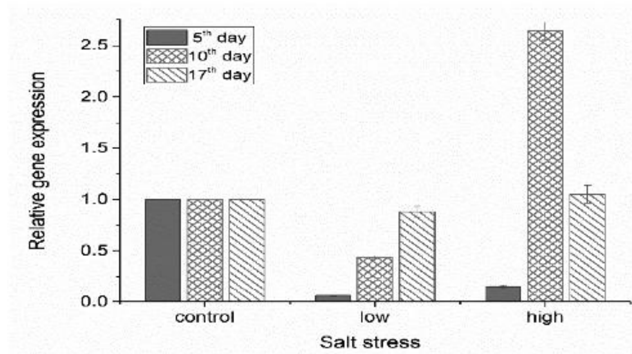

Fig. 3. Expression analysis of Acetyl-CoA carboxylase gene under low/high salt stresses in comparison with control sample during three samplings.

transportation and subsequently absorbing the saline ions (Fig 4).

The decrease in expression of genes involved in the pathway of photosynthesis and growth that are involved in regulating the absorption of light energy from the sun can lead to less growth. Given that *Dunaliella Salina* may grow in concentrations of 450 g/l NaCl or even on salt crystals, increasing salinity is unable to reduce its growth unless the changes occurs suddenly and the cell gets severe osmotic tension which prevents it from producing metabolites or controlling its channelrhodopsin-1 DChR1. In such a scenario, cells increase the gene expression responsible for producing channelrhodopsin-1 DChR1 and, thus promote ionic exchanges in order to simultaneously overcome high salinity and counterbalance the extra electrical conductivity brought about by salinity. Here, more solar energy is absorbed, leading to further photosynthesis.

The expression of the phosphate carrier gene

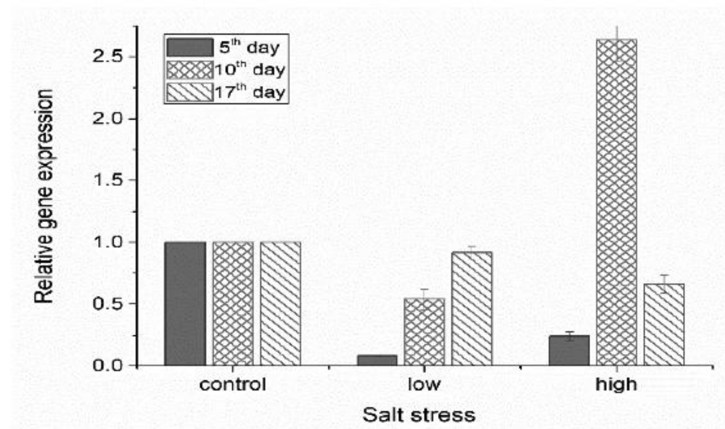

Fig. 4. Expression analysis of channelrhodopsin-1 DChR1 genes (in comparison with control samples) under high/low salt stress during three samplings.

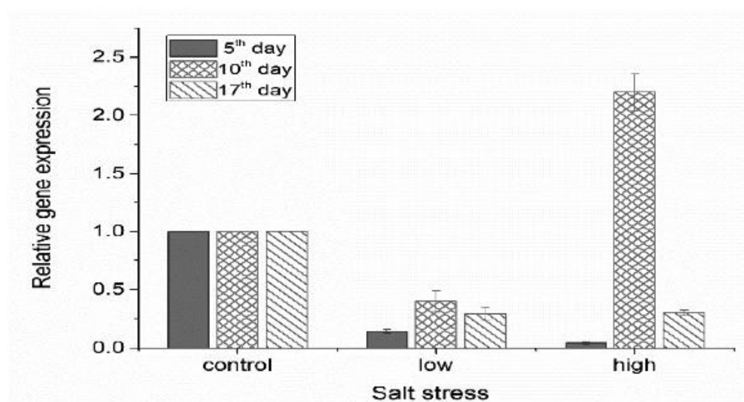

Fig. 5. Expression analysis of phosphate transporter genes under low/high salt stress in comparison with control samples during 3 samplings.

was severely knocked out with initial stress. However, after passing the log phase, gene expression dramatically increased under salt stress in comparison with the control conditions and tended to decrease again after this phase. However expression of the gene after suppression in the first stages of growth, until the end of growth, did not reach the level of expression in the control sample under low salt stress (Fig. 5). The expression of the gene in the first stage of sampling after applying the stress (5th day) in both salinity stress conditions was decreased by 0.04 and 0.14, respectively, than the control (32 g/l). However, by increasing the salt stress in the second stage, a significant difference (0.04 to 2.2) was observed in comparison with a control sample.
In the third stage (17th day), dwindled to 0.3. Under low salt stress, gene expression was 0.14 during the first stage, 0.4 during the second stage and the third stage decreased to 0.29 in comparison with the control sample. Generally, the expression of membrane proteins under low salt stress conditions is reduced that is probably due to the diminished role of ion exchange inside the cell (Kim et al., 2010).

Discussion

The scarcity of freshwater resources is a constraint to the mass production of microalgae biomass. Thus identification and development of salinity resistant and tolerant micro algal strains is essential. Salt stress can cause accumulation of lipid in some microalgae but it also may inhibit the growth (Ben-Amotz and Avron, 1973). Therefore, it is critical to investigate the factors involved in the production of biomass as well as lipid accumulation under stress conditions (Ho et al., 2017). Acetyl-CoA carboxylase is a participant for 4% of enzyme reactions during mevalonate cycle and citric acid cycles. In the first cycle of citric acid, acetyl-CoA enzyme which is a 2-carbon substance reacts with oxaloacetate and loses its CoA enzyme activity while pyruvate is oxidized. The final product is called citrate CoA-SH. During the intracellular mevalonate cycle, HMG-CoA reductase acts as a catalyzer in converting HMG-CoA into mevalonate. Metabolism of mevalonate is an important metabolic pathway for eukaryotes and many bacteria that cholesterol, isoprenoids, terpene, lycopene, carotene, eucalyptol, pinene and other important compounds are their final products (Sayanova et al., 2017).
Cells may try to produce more glycerol to achieve maximum salinity tolerance by increasing the lipid production potential. At the end of their growth, they will achieve the maximum ability to accumulate lipid and then they are self-regulating to control and reduce more fat production by reducing the activity of the responsible genes, especially key fat-producing genes. Fat-related plastid proteins undergo increased gene expression and decreased gene expression during high and low salt stresses in their gene transcripts, respectively (Kim et al., 2010). Naturally, algae need to produce high amounts of lipid in order to counter high salt stress and to prevent cell plasmolysis, thus the expression of genes involved in the production pathway of glycerol and lipid would increase. Generally, microalgae culture under biological stress conditions such as ni-

trogen starvation or high salinity can lead to high lipid and starch synthesis. The pathway of lipid and starch production in *Chlamydomonas reinhardtii* through stabilizing carbon dioxide has shown that Acetyl-CoA carboxylase is a key gene in the biosynthesis of lipid and under no stress conditions, more lipid would be produced. However, under high salt stress, less lipid would be produced initially then would enhance to higher level than that of under non-stress conditions. Also, expression level of Acetyl-CoA carboxylase gene under high salt stress is initially increased and subsequently decreased during the log growth phase (Ho et al., 2017). Ben-Amotz and Avron (1973) showed that salinity stress to 4M NaCl would cause osmotic stress and considerable accumulation of glycerol in *Dunaliella* strains. The difference in gene expression of Acetyl-CoA carboxylase under poor nutrition stress showed that this gene is one of the important ones in the biosynthesis of the lipid, making it a candidate for genetic manipulation of algae (Fan et al., 2014). Similar to other plants, microalgae are capable of synthesizing and storing biomass and energy. The pathway for biosynthesis of triglycerides in microalgae includes three major steps: 1- formation of Acetyl-CoA carboxylase in cytoplasm; 2- elongation and saturation of carbon chain in fatty acids; and 3- biosynthesis of triglycerides. Totally, L-a-phosphoglycerol and Acetyl-CoA carboxylase are the main elements in the biosynthesis of triglycerides (Deng et al., 2014; Patil et al., 2011).

Channelrhodopsin-1 DChR1 receive light directly and act as optical sensors in green flagellate algae, allowing it to choose the optimum light conditions for growth. In the case of poor lights, electric signals are often strengthened by secondary receptor channels like receptor channels of calcium. In case of intensive lights, channelrhodopsin-1 DChR1 directly conduct the electric current (Spudich et al., 2014), channels named CHR1 and CHR2 were found in *Chlamydomonas reinhardtii*. Later, other channels of the same type were found and the initial letter of the genus was added to its name. For example, the name DChR1 in *Dunaliella*, MChR1 in Mesostigma viride and VChR1 in Volvox (Ernst et al., 2008). ChRs are characterized by their ability to high cation selectivity and pass sodium, potassium, calcium and magnesium ions under various physiological conditions. Each cation can influence the channel's pores according to strong electric conductivity (Nagel et al., 2003). Two types of flagellate microalgae's ion channels and their responses to light conditions in some mutant's species of *Chlamydomonas reinhardtii* have been reported (Sineshchekov et al., 2009). These channels act selectively than different ions. Thus, studying ionic bonding and transportation is particularly important and many researchers have tried to find possible algorithms for enzyme activity under different conditions for microalgae cultivation and the correlation with the conductivity of sodium, calcium, magnesium and other ions (Schneider et al., 2013). Similar to our study, previous research has shown that genes involved in photosynthesis pathway and channelrhodopsin-1 DChR1 are knocked out under decreasing salt stress. The present study

showed that, in early stages of growth, low salt stress forced gene expression down to 0.08 compared with control samples while gene expression roughly reached the expression of control samples (0.91) until the third stage (Kim et al., 2010).

Proteins are vital to microalgal chemical composition and structure. Approximately, 20% of all proteins are coupled to the cell membrane, while 50% of proteins act within cells and 30 percent remain to move into or out of the cells (Berliner, 1986). Some intracellular phosphate transporters were identified in plants that facilitate selective phosphate transportation. These transporters are probably involved in N-glycosylation of proteins and cell wall biosynthesis, necessary features for creating tolerance to salt stress. (Cubero et al. 2009). Additionally, phosphate transporter proteins play a vital role in producing ATP in mitochondria and undergo overexpression in case of salt stress that reaches its maximum during log phase, a fact indicating a response mechanism to salt stress (Zhu et al., 2012).It has also been reported that sodium/hydrogen transporters in *Dunaliella Salina* increase their activities in the plasma membrane in different salinity levels or increased pH levels in order to help algae adapt to the immediate conditions. The increased activity concurred with increased amounts of sodium penetration and its concentration inside vesicles on the plasma membrane. This adaptation leads to further production of transporters in the plasma membrane of *Dunaliella Salina* (Katz et al., 1992).

Halophyte *Dunaliella Salina* shows acceptable tolerance in high salinity levels that for this adaptation, there is an efficient mechanism for regulating intracellular sodium. A report on the export of sodium ions by transporters on the plasma membrane has shown that the reducing electron acceptors like ferricyanide leads to more intracellular sodium (Katz and Pick, 2001). These results are consistent with the performance of an NADPH conductor coupled to exported sodium in the plasma membrane. Extrusion and recycling of Na in *Dunaliella* is an adaptation mechanism for hyper saline environments in the present study, the model for transcripts of proposed genes' level under stress matched with the models proposed by Kim et al. (2010) under salinity stress: in both attempts, gene expression increased with high salt stress and decreased with low salt stress. The light regulating genes and genes involved in lipid biosynthesis pathway have greater expressions under high salinity levels while coding genes in plasma membrane display decreased expression. With increasing sodium and chloride ions and increasing salinity, the osmotic pressure of the environment around the microalgae cells will also increase. Given the fact that cells need to balance the osmosis, they consume extra energy that affects metabolic production and cell division and growth Furthermore, during lag phase, the enzymes, and metabolism involved in cell division increase, leading to delay in the physiological and molecular adaptation of micro-algal cells to the surrounding environment.

As such, decreased expression of proposed genes during the early growth phase seems inevitable (Meseck et al., 2005).

Acknowledgements

This study was financially supported from the Ferdowsi University of Mashhad (Grant Number 3/32961).

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