Full Length Research Article

CLONING AND EXPRESSION OF TWO GENES (*NHAS2* AND *NHAS4*) ISOLATED FROM *SYNECHOCYSTIS* SP. PCC 6803 IN YEAST

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ARTICLE INFO

ABSTRACT

Two genes encoding Na^+/H^+ antiporter isolated from Cyanobacterial strain *Synechocystis* sp. PCC 6803 were cloned and sequenced. These genes, namely, *nhaS2* and *nhaS4* were cloned into the pYES2 vector for expression in *Saccharomyces cerevisiae*. Transgenic yeast was examined for their ability to survive under salt- stress conditions and their growth response to increasing levels of NaCl (0, 1.7, 1.8, 1.9, 2.0, 2.1, 2.2 and 2.3M) for 7days, then the yeast growth was measured at OD_{600}nm in spectrophotometer. The results revealed significant differences in growth between the transgenic yeast and control. The salt concentration of 1.8M NaCl was critical in determining the ability of yeast to tolerate salt stress. At this concentration, the mean growth values for the transgenic yeast harboring the genes *nhaS2* or *nhaS4* were 1.2250_{cA} and 1.2180_{cA}, respectively, compared to 0.2620_{cB} for the control. Moreover, Increasing the salt concentration to 1.9 M NaCl, inhibited the growth of the control cells significantly. While, the transgenic yeast cells for the 2 genes maintained good growth and the growth continued until the 2.3 M NaCl, but with a lower level. RT–PCR confirmed the expression of the two genes in response to salt stress in transgenic yeast. The specific *nhaS2* and *nhaS4* primers successfully amplified the genes at 1623 bp and 1233bp, respectively in this transgenic yeast. In addition to the growth response of the transgenic yeast to salt stress, the results revealed that the intracellular proline accumulation in the transgenic yeast was significantly increased with raising salinization level.

INTRODUCTION

Salinity is a major abiotic stress limiting growth and productivity of plants in many areas of the world (Gupta and Huang, 2014).Salinity stress involves changes in different physiological and metabolic processes, depending on the severity and duration of the stress, and ultimately inhibits crop production (Rozema and Flowers, 2008; James *et al*., 2011and Gupta and Huang, 2014). In the initial phases of salinity stress, water absorption capacity of root systems decreases and water loss from the leaves is accelerated due to osmotic stress of high salt accumulation in soil and plants. Therefore, salinity stress is also considered as hyperosmotic stress. Osmotic stress causes various physiological changes (Gupta and Huang, 2014). Salinity stress is also considered as a hyperionic stress. One of the most harmful effects of salinity stress is the accumulation of Na^+ and Cl^- ions in tissues of plants exposed to soils with high NaCl concentrations. Entry of both Na^+ and Cl^- into the cells causes severe ion imbalance and excess uptake might cause significant physiological disorder(s).

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High concentration of Na^+ ions inhibits uptake of K^+ ions. These ions are essential for growth and development. In addition, the lack of these ions leads to lower productivity and may even lead to death (Gupta and Huang, 2014) .In order to avoid Na^+ toxicity, the plant cell may either transport the ions outside the cell or store them in the vacuole, processes mediated by specialized proteins. Some of these proteins belong to the family of Na^+/H^+ exchangers, which can be located in the plasma membrane or vacuole.

Na^+/H^+ antiporters have been identified in several mammals, bacteria and plants. These transporters play roles in pumping out Na^+ from the cytoplasm by exchanging it for H^+ at the expense of the proton gradient generated by specialized pumps in the cell and vacuolar membrane. This mechanism allows plant cells accumulate Na^+ in the vacuole and therefore, maintain the appropriate ion concentration in the cytoplasm (Baltierra *et al*., 2013). Intracellular NHX proteins are Na^+, K^+/H^+ antiporters involved in K^+ homeostasis, endosomal pH regulation, and salt tolerance (Gupta and Huang, 2014). Na^+/H^+ antiporters are integral membrane proteins that transport Na^+ and H^+ in opposite directions across the membrane and that

occur in virtually all cell types. These transporters play an important role in the regulation of cytosolic pH and Na^+ concentrations and influence proton or sodium motive force across the membrane (Hamada *et al*., 2001 and Tsunekawa *et al*., 2009). Proline has been recognized as a multi-functional molecule, accumulating in high concentrations in response to a variety of abiotic stresses (Kishor and Sreenivasulu, 2014). Proline is an important amino acid in terms of its biological functions and biotechnological applications. In response to osmotic stress, proline is accumulated in many bacteria and plant cells as an osmoprotectant (Takagi, 2008). It plays a highly beneficial role in plants exposed to various stress conditions. Besides acting as an excellent osmolyte, proline plays three major roles during stress, i.e., as a metal chelator, an antioxidative defense molecule and a signaling molecule (Hayat *et al*., 2012).

Cyanobacteria, the only known prokaryotes capable of oxygenic photosynthesis, are attractive organisms for solving environmental problems, due to their simple nutritional requirements, effective metabolism and the relatively simple genetic background of some species (Liu *et al*., 2012 and Sakr *et al.*, 2013). In addition, they have played a significant role in the Earth's history as primary producers of atmospheric oxygen (Nakao *et al*., 2010). According to the endosymbiotic theory, chloroplasts in plants and eukaryotic algae have evolved from cyanobacterial ancestors *via* endosymbiosis (Ragini and Pankaj, 2012). Cyanobacteria became dominant organisms with major impacts on biogeochemical processes of our planet, it was estimated that they account for about 30% of global primary production, especially in the central oceans, which make them an important sink for CO_2. Additionally, because of their N_2-fixing ability, many cyanobacterial strains are regarded as the major sources of combined nitrogen in the marine system. During their long evolution, cyanobacteria have adapted to aquatic habitats with various salt concentrations (Hagemann, 2011).

Synechocystis sp. PCC 6803 was the first cyanobacteria to have its genome sequenced in 1996 (Nakao *et al*., 2010) and is considered as halophytic, hypersaline strain or halotolerant strain. *Synechocystis* sp. PCC 6803 has revealed a comprehensive picture of the dynamic process of salt acclimation involving the differential expression of hundreds of genes. Therefore cyanobacterial stress genes were employed to improve the salt tolerance of sensitive organisms (Hagemann, 2011). *Synechocystis* sp. PCC 6803 contains six circular genomic molecules (chromosome and plasmids), with a total of 3725 genes (Nakao *et al*., 2010). The genome contains six genes encoding Na^+/H^+ antiporters (*nhaS1*–5 and *sll0556*) (Tsunekawa *et al*., 2009). Yeast species provide excellent models for fundamental biological research. Yeast (*Saccharomyces cerevisiae*) is a model organism for the expression of eukaryotic genes. The expression vector pYES2 is the most commonly used for the expression of genes cloned into yeast (Forsburg, 2005). In the present investigation, we report the isolation of two abiotic stress-related genes(Na^+/H^+ antiporter) *nhaS2* and *nhaS4* from cyanobacterium (*Synechocystis* sp. PCC 6803) and the expression of the cloned genes into yeast as a eukaryotic model system under salt stress conditions.

MATERIALS AND METHODS

Bacterial strains and growth conditions

Cyanobacterial strain *Synechocystis* sp. PCC 6803 was grown photoautotrophically at 27ºC in Allen's medium (Allen, 1968) under illumination at 50 µE m^{-2} s^{-1} from fluorescent lamps with shaking at 130 rpm.

Isolation of the *nhaS2* (*sll0273*) and *nhaS4* (*slr1595*) genes from cyanobacteria

Chromosomal DNA was isolated from *Synechocystis* PCC 6803 according to Williams (1988). A DNA fragment containing the open reading frame of each of the *nhaS2* and the *nhaS4* genes was amplified using PCR. The primers used for amplification of the *nhaS2* gene were F(5'-CCAAGCTTATGATTAAGCTCCCTGTGCT-3') and R(5'-TTAGAATTCTCAGTCATCCTGCAGGGCTA-3'). While, for the *nhaS4* gene, the primers were F(5'-ATCAAGCTTATGGACACCAATACTTTACT-3') and R(5'-TAAGAATTCTTAATGGGCTGGGGCAGGAT-3').

These primers were designed according to the sequences in the database of the National Center for Biotechnology Information (NCBI). Moreover, the recognition sites of the two restriction enzymes *Hind III* and *EcoR I* were added to the primers sequences to facilitate the cloning of the amplified genes. Primers were synthesized at Bio Basic Inc. The PCR reaction was carried out in 20µl volume containing 1µl DNA, 2µl for each primer (10pmol/1ml primer), 10µl master mix of DNA polymerase (Promega) and 5µl d.dH$_2$O. The PCR was carried out using a DNA thermal cycler (Biometra). The PCR temperature profile was as follows: 95ºC/1min, followed by 30 cycles of (95ºC/ 30 sec. (60ºC/1 min. or 65ºC/1min. for the *nhaS2* gene and *nhaS4* gene, respectively) and 72ºC/ 1 min) and a final extension of 72ºC/7 min. The PCR products were resolved on 1% (w/v) agarose gel. The PCR products were cut from the gel and purified using the Qiagen band elution kit according to the manufacturer's instructions.

Cloning and Sequencing

The purified PCR products were cloned into pGEM®-T Easy cloning vector System I according to the manufacturer's protocol (Promega). The recombinant vectors were transformed into competent *E. coli*, strain xL1-blue bacterial cells. Transformed *E. coli* were grown on LB medium supplemented with 100 µg/ml ampicillin, 0.1M IPTG and 50mg/ml X-Gal.The recombinant clones were identified by blue-white selection and PCR colony screening. Recombinant pGEM®-T Easy vectors were isolated from white colonies and sequenced with an automated DNA sequencer (Macrogen Company, Germany). The nucleotide sequence and the deduced amino acid sequence of each cloned gene were compared and verified with the DNA and protein sequences available in the databases by means of the Basic Local Alignment Search Tool (BLAST).

Characterization and evaluation of the physiological roles of the selected genes in yeast

Recombinant plasmids were isolated from *E. coli* xL1-blue cells. These recombinant vectors were transferred to INVSc1

yeast cells by the Li-acetate method (Gietz *et al.*, 1992). The recombinant plasmids were digested with *HindIII* and *EcoRI* to release the cloned genes. These DNA fragments were cloned into the pYES2 expression vector (Fig.1) digested with the same restriction enzymes. The resulting constructs were propagated into *E. coli* and then transformed into competent yeast cells as a model eukaryotic system.
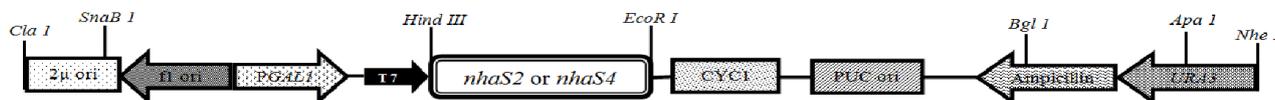
amplified by PCR using gene-specific primers for the *nhaS2* and *nhaS4* genes.

Estimation of intracellular proline content

Proline content was estimated according to Sasano *et al.* (2012).

Fig. 1. Schematic representation of the transformation vector pYES2 containing the *nhaS2* or *nhaS4* under the genetic control of T7 promoter

Transformation of yeast was verified by Colony Pick PCR as a rapid and sensitive method for DNA amplification in yeast (Mirhendi *et al.*,2007). Fresh (<2 days old) single colonies of approximately 1 x 1 mm were suspended in 20µl sterile water and boiled for 15 min., then added to the PCR reaction mixture in a volume of 50 µl containing (1 µM primer, 200 µM dNTPs, 0.5 units of *Taq* polymerase enzyme and 1X *Taq* polymerase buffer). The PCR temperature profile was as previously described.

Expression of the *nhaS2* (*sll0273*) and *nhaS4* (*slr1595*) genes in yeast

Measurement of yeast growth

Transgenic yeasts were grown on synthetic minimal defined medium (SC) (Sasano *et al.,* 2012) containing 2% glucose, 0.67% yeast nitrogen base without amino acids. This medium was supplemented with 0.01% (adenine, arginine, cysteine, leucine, lysine, threonine, and tryptophan), 0.005% (aspartic acid, histidine, isoleucine, methionine, phenylalanine, proline, serine, tyrosine and valine) with no uracil to make selective plates for growing pYES2 transformants for 2 days. The cultured sample (300µl) was inoculated into 25 ml of SC medium with different concentrations of NaCl, i.e., 0, 1.7, 1.8, 1.9, 2.0, 2.1, 2.2 and 2.3M for 7days, then the yeast growth was measured at OD_{600}nm in spectrophotometer (Jenway 6300). In this experiment, two controls were included. The first control was INVSc1 yeast which is a diploid strain auxotrophic for histidine, leucine, tryptophan, and uracil. Therefore, it will not grow in SC medium. The second control was transformed yeast cells with empty pYES vector.

RT-PCR expression analysis

Transgenic yeasts were cultured in YPD medium (Morita *et al.,* 2003) for 2 days and 300µl of the cultured sample was inoculated into 25 ml of YPD medium with different concentrations of NaCl, i.e., 1.8, 2.0 and 2.3M for 7days. Total RNA was extracted from transgenic and non-transgenic yeast using the Vivantis (GF-TR-025) kit. First-strand cDNA was synthesized using reverse transcription reaction. Reverse transcription reaction was carried out in a 20µL reaction volume containing 11µl RNA, 1µl oligo(dT), 2µl dNTPs, 4 µl 5× reverse transcription buffer,1µl reverse transcriptase and 1µl RNase inhibitor. The reverse transcription reaction was incubated at 42°C/60 min. and then the reverse transcriptase was inactivated at 70°C/5 min. The cDNA template was

Transgenic yeasts were cultured in YPD medium for 2 days. Then, 300µl of the cultured sample was inoculated into 25 ml of YPD medium containing 1% yeast extract, 2% Bacto Peptone and 2% glucose with the eight concentrations of NaCl for 7days. Yeast cells were harvested, washed twice with water and resuspended in 1 ml of distilled water. The suspension was transferred to boiling water and intracellular amino acids were extracted for 20 min. The proline content was measured at OD_{520}nm in spectrophotometer .

Statistical Analysis

Yeast growth and proline estimates were statistically analyzed using the ASSISTAT software, version 7.7 according to Silva and Azevedo (2009). Experiments were designed as factorial experiments in a completely randomized design with three repetitions. Means were compared by t-test at a level of 5% probability

RESULTS AND DISCUSSION

Isolation and characterization of *nhaS2* and *nhaS4* genes

Na^+/H^+ antiporters were characterized in the hypersaline strain *Synechocystis* PCC 6803. This cyanobacterium harbours multiple Na^+/H^+ antiporter genes (Waditee *et al.,* 2001; Wutipraditkul *et al.*, 2005 and Hagemann, 2011). The genomic DNA isolated from *Synechocystis* PCC 6803 was PCR amplified with gene-specific primers for the two different Na^+/H^+ antiporter genes (*nhaS2* and *nhaS4*) (Fig .2 A and B). In this respect, Tsunekawa *et al.* (2009) isolated the *nhaS3* (sll0689) gene from chromosomal DNA of *Synechocystis* sp. PCC 6803 by PCR using *KpnI* site-containing forward primer and *SalI* site-containing reverse primer. Hamada *et al.* (2010) isolated the *synnhaP* gene from *Synechocystis* sp. PCC 6803 using the PCR technique. Jha *et al.* (2011) isolated the Na^+/H^+ antiporter *NHX1*from a halophytic plant *Salicornia brachiata* using PCR. In addition, Razzaque *et al.* (2014) amplified the coding regions of two Arabidopsis antiporters (*AtNHX*1 and *AtNHX*2) and one rice antiporter (*OsNHX*1) by target specific PCR. PCR amplicons were first cloned into *pENTR/D-TOPO* and later recombined with a destination vector (*pK*7*WG*2.0) by LR reaction. Positive clones were selected by PCR.

Cloning of the isolated genes in *E. coli*

The positive clones were selected using blue-white and PCR colony screening**.** The purified PCR products of 1623 bp and 1233 bp for *nhaS2* and *nhaS4* genes, respectively, were used
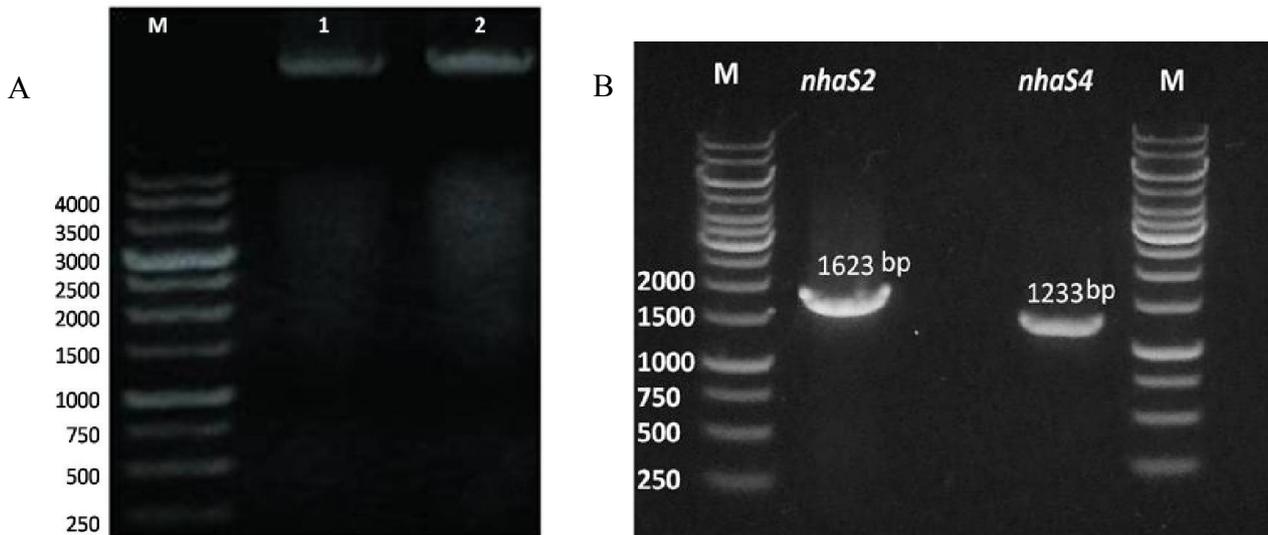
Fig. 2. (A) Genomic DNA of *Synechocystis* sp. PCC 6803. (B) The PCR amplification of the *nhaS2* and *nhaS4* genes revealing the amplified band at the expected size for each gene (1623bp and 1233 bp, respectively). M: 1kb ladder as a marker
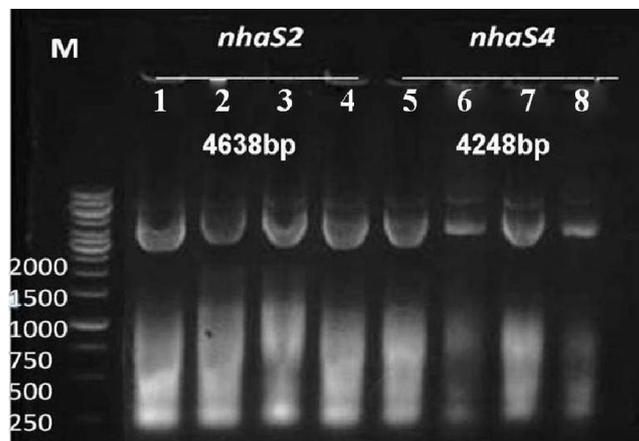

Fig. 3. Recombinant plasmids (pGEM®-T Easy vector with *nhaS2 or nhaS4*). Lanes 1-4 plasmids with *nhaS2* gene, lanes 5-8 plasmids with *nhaS4* gene and M: DNA molecular marker 1kb ladder

for ligation into the pGEM-T Easy vector and transformation in *E. coli* xL1-blue cells. The blue- white screening is one of the most common molecular techniques that allow detecting the successful ligation of the gene of interest in vector. White colonies indicate insertion of foreign DNA and loss of the cells ability to hydrolyze the marker (Sherwood, 2003 and Padmanabhan *et al.*, 2011) .A recombinant colony for each gene was picked and grown in LB broth containing ampicillin and the recombinant plasmid DNA was extracted and electrophoresed on a 1% agarose gel (Fig. 3). The restriction enzyme analysis of recombinant plasmid was carried out using the *EcoRI* restriction enzyme to release the *nhaS2* (1623bp) and *nhaS4* (1233bp) insert DNA from the 3.015 kbp vector DNA (Fig. 4). Moreover, the presence of *nhaS2* (1623bp) and *nhaS4* (1233bp) in a recombinant plasmid was verified by the polymerase chain reaction technique (Fig. 5).

Sequencing of the cloned genes

The amplified genes were purified from the gel, cloned into pGEM-T Easy vector and sequenced. Sequence analysis was performed by Blasting with the available sequences in the National Centre for Biotechnology Information (NCBI). The results revealed 99% homology between the amplified genes and the corresponding sequences in the database, as shown in Figures (6 and 7). Translation of the nucleotide sequence of the *nhaS2* and *nhaS4* genes to a protein sequence using ExPASy (translate tool) is shown in Figure (8 A and B). The alignment of the amino acids was performed using the BLAST which revealed a homology of 100% for *nhaS2* and 99% for *nhaS4* homologues, as shown in Figure (9 A and B, respectively). These results revealed that the target genes were successfully isolated. Li *et al.* (2008) cloned a novel vacuolar Na^+/H^+ exchanger, *CgNHX1*, from a halophytic species *Chenopodium glaucum* by using reverse transcriptase-polymerase chain reaction (RT-PCR) and rapid amplification of cDNA ends (RACE) technique. Sequence alignment and phylogenetic analysis of 22 *NHX* genes from GenBank as well as the new *CgNHX1* gene indicated that *NHX* genes shared a great degree of similarity, regardless of their glycophytic or halophytic origin. Wang *et al.* (2011) isolated a vacuolar Na^+/H^+ antiporter gene (*CmNHX1*) from melon using the rapid amplification of cDNA ends approach.
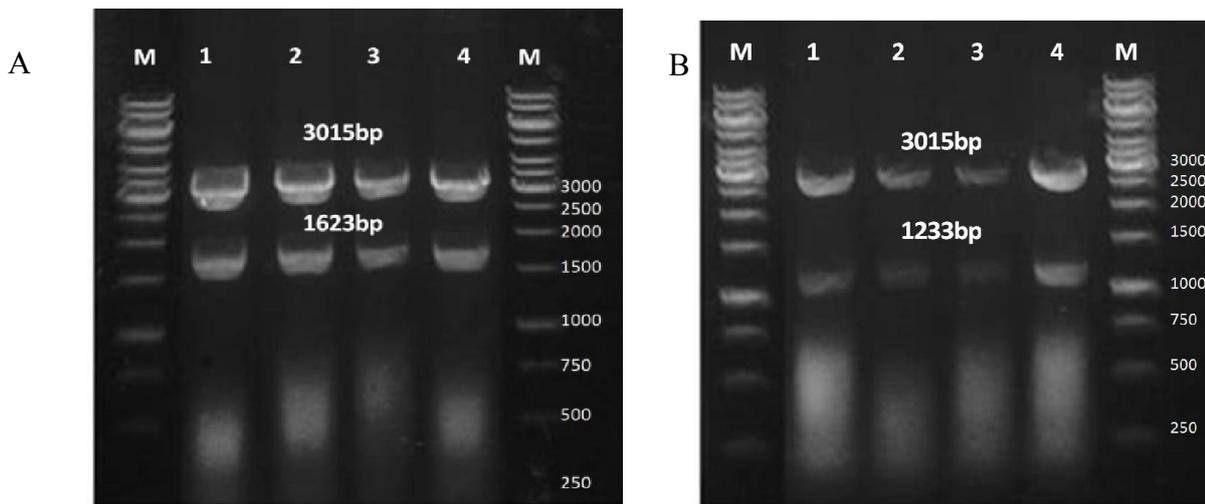
Fig. 4. Agarose gel showing release of inserts (*nhaS2* and *nhaS4* genes, 1623bp (A) and 1233 bp (B), respectively) from recombinant plasmids after digestion with *EcoRI*. M: 1kb ladder as a marker
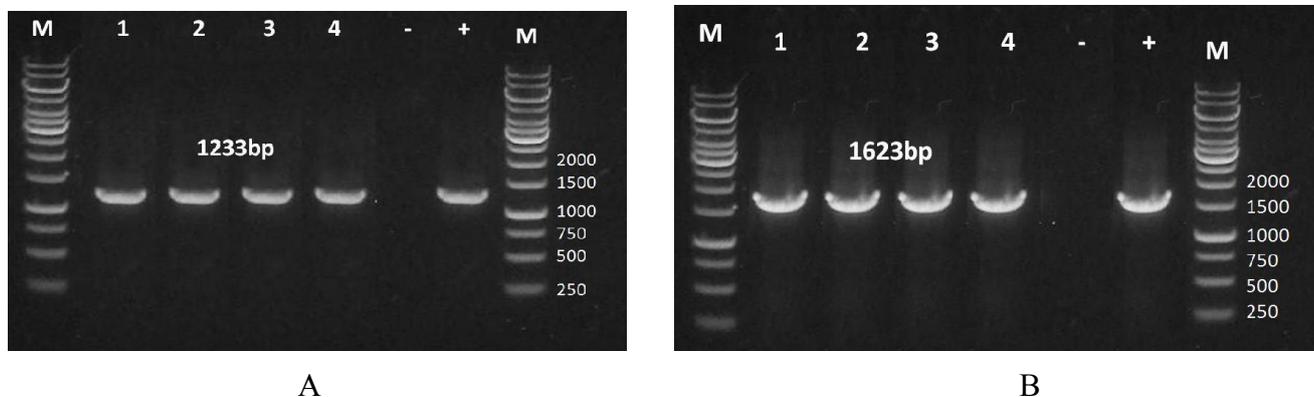

Fig. 5. The PCR amplification of the *nhaS2* and *nhaS4* genes (1623bp (A) and 1233 bp(B), respectively). M: 1kb ladder as a marker

Sequence analysis indicated that the full-length cDNA of *CmNHX1* was 2534 bp, including an open reading frame (ORF) of 1659 bp, which encoded a deduced polypeptide of 553 amino acids. The deduced protein contained conserved structural domains and shared a high degree of homology with putative vacuolar Na^+/H^+ antiporters from other higher plants. Subcellular localization revealed that this protein was located in the vacuolar membrane. Sun *et al.* (2014) characterized a heavy metal-associated protein, AcHMA1, from the halophyte, *Atriplex canescens*. Sequence analysis revealed that AcHMA1 contains two heavy metal binding domains.

Sub-cloning into yeast expression vector

Recombinant plasmids pGEM-T/*nhaS2* and pGEM-T/*nhaS4* and the yeast expression vector pYES2 were restricted with *EcoRI* and *HindIII* enzymes to facilitate directional cloning. The inserts were ligated into linearised pYES2 vector and transformed into *E. coli* x1-blue cells . Plasmids DNA were further isolated and confirmed by PCR and restriction digestion using *EcoRI* and *HindIII* enzymes (Fig.10 A and B). In this regards, Song *et al.* (2005) cloned the chitinase gene (ech42) from the *Trichoderma aureoviride*. The gene was cut from apMD18-T vector with *EcoR I* and *BamH I* and then ligated into *EcoR I* and *BamH I* sites downstream of the GAL1 promoter of pYES2.

The resulting plasmid (pYES2/ech42) was characterized by restriction analysis. The pYES2/ech42 plasmid was transformed into *S. cerevisiae* H158 by the lithium acetate method. These yeast transformants were grown in the minimal medium containing raffinose as the sole carbon source for growth of yeast and induced by supplying the galactose as a carbon source. Further, chitinase activity of the recombinant yeast was assayed.

The maximum activity of the chitinase in yeast was found at 48 hr and 0.5 U/ml. Diao *et al.* (2010) cloned a GST gene (*LbGST1*) from *Limonium bicolor* into pYES2 under the control of the inducible GAL1 promoter, and this vector was designated as pYES2-LbGST1. The pYES2-LbGST1 and empty pYES2 vectors were transformed into yeast *S. cerevisiae* INVSc1 (His-, Leu-, Trp-, Ura-) using a lithium acetate method. Huang *et al.* (2014) isolated three cytochrome P450 monooxygenase *CYP52* gene family members from the sophorolipid-producing yeast *Starmerella bombicola* (former *Candida bombicola*), namely, *CYP52E3*, *CYP52M1*, and *CYP52N1*, and their open reading frames were cloned into the pYES2 vector for expression in *Saccharomyces cerevisiae*. The functions of the recombinant proteins were analyzed with a variety of alkane and fatty acid substrates using microsome proteins or a whole-cell system.

Fig. 6. The alignment of the amplified *nhaS2* (A) and *nhaS4* (B) genes and the corresponding sequences in the database

A

Description	Max score	Total score	Query cover	E value	Ident	Accession
Synechocystis sp. PCC 6803, complete genome	2835	2835	99%	0.0	99%	CP003265.1
Bacillus subtilis BEST7613 DNA, complete genome	2835	2835	99%	0.0	99%	AP012495.1
Synechocystis sp. PCC 6803 substr. PCC-P DNA, complete genome	2835	2835	99%	0.0	99%	AP012278.1
Synechocystis sp. PCC 6803 substr. PCC-N DNA, complete genome	2835	2835	99%	0.0	99%	AP012277.1

B

Description	Max score	Total score	Query cover	E value	Ident	Accession
Synechocystis sp. PCC 6803, complete genome	1925	1925	89%	0.0	99%	CP003265.1
Bacillus subtilis BEST7613 DNA, complete genome	1925	1925	89%	0.0	99%	AP012495.1
Synechocystis sp. PCC 6803 substr. PCC-P DNA, complete genome	1925	1925	89%	0.0	99%	AP012278.1
Synechocystis sp. PCC 6803 substr. PCC-N DNA, complete genome	1925	1925	89%	0.0	99%	AP012277.1

Fig. 7. Homology between the full nucleotide sequence of the *nhaS2* gene (A) and *nhaS4* gene (B) and the corresponding sequence in different organisms using BLAST

A

Met L N D G V A Met V L L S V I T T I H I Q G G F S A G E G I R Q I F V A F V G G G L V G L G L G Y L C V G L F R Q L N D D L S D I L L T V S V S L G T F Q I G Q Met L G V S S A I A V V V A G L V I G N L A L K Q T S A S I K V T L L S F W E Y A G F G V N T L I F L L V G I E V Y P S I L L S T I P A A L I A I V A Y Q I G R V F S I Y P L L Y L L S F F D R P L P L R W Q H V L I A G N V K G S L S Met A L A L A L P L T L P G R D Q V V T L V F S T V Met V S L I G Q G L S L P W V V K K L Q L S K P S P L A Q K I A Met L Q L N L V T A K A A Q G E L K Y L L E A G S L P K F L Y E E L F A D Y Q A R I A N S E Q E L R E F Y N Q R N L I F S E G E V E K K Y I D G L Y R R L Y I A E K S A I N D A L A K G I L A D D I S D E S H R C E

B

Met L G P S L L G L L S P A L E K S F F P A T T Q P F L Y L L S E I G L I F Y Met F L V G L E L N P Q Y L R Q K L K V A I L T S N V S I F F P F V L G I V L S F F V L Y S L N Q P N K T S F I P F A L F I G A A Met S I T A F P V L A R I L K D T G L D K T P L G T L G L T C A S V D D I S A W C L L A I A I A V T R T D N I F G A F P T L L G I I V Y T V F Met V T L G R K F F K Y I L R N Y G Q K N Y L S Q G L L T F I Y I Met V I L S A Met L T E W I G I D V I F G G F I L G A I L P K N T N L S T E L A T K T E D F V S T F L L P I F F A Y S G L S T D L G L L N K P Y L W A V C A L V V A A A I A G K Y C G V Y V T T R A L G V E K Q E A K A L G W L Met T R A D D I S H V V R A S V L P V I P

Fig. 8. Translation of the nucleotide sequence of the *nhaS2* gene (A) and *nhaS4* gene (B) to protein sequence using ExPASy (Translate tool)

A

Description	Max score	Total score	Query cover	E value	Ident	Accession
sodium:proton antiporter [Synechocystis sp. PCC 6803]	695	695	98%	0.0	100%	WP_010873113.1
sodium:proton antiporter [Synechocystis sp. PCC 6714]	576	576	98%	0.0	92%	WP_028946316.1
sodium:proton antiporter [Myxosarcina sp. GI1]	487	487	98%	3e-166	74%	WP_036481945.1
MULTISPECIES: sodium:proton antiporter [Cyanothece]	486	486	98%	7e-166	69%	WP_009543862.1

B

Description	Max score	Total score	Query cover	E value	Ident	Accession
sodium:proton antiporter [Synechocystis sp. PCC 6803]	627	627	94%	0.0	99%	WP_010871619.1
sodium:proton antiporter [Synechocystis sp. PCC 6714]	558	558	91%	0.0	92%	WP_028947348.1
Kef-type K+ transport system membrane protein [Pleurocapsa minor]	380	380	94%	2e-122	60%	WP_015143332.1
sodium:proton antiporter [Chlorogloeopsis fritschii]	377	377	94%	2e-121	59%	WP_016877797.1

Fig. 9. Homology between the protein sequence of the *nhaS2* gene (A) and *nhaS4* gene (B) using BLAST
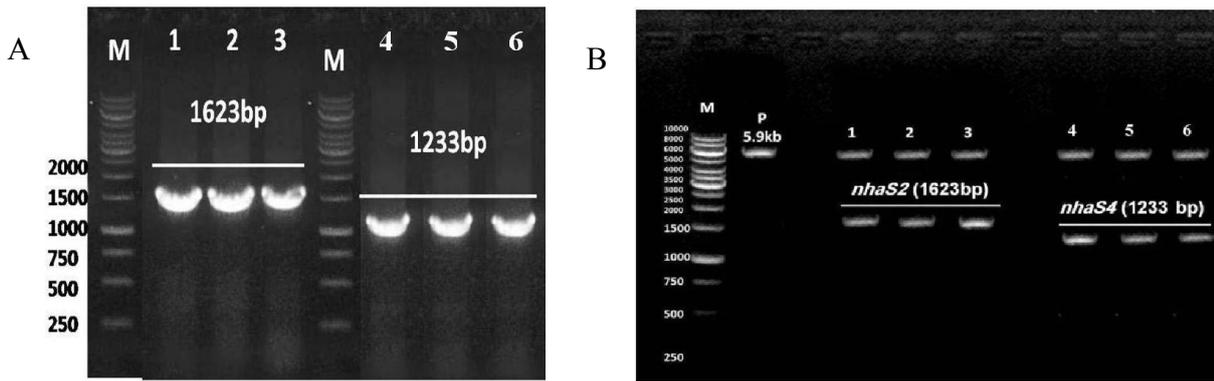
Fig. 10. (A) The PCR amplification of the *nhaS2* and *nhaS4* genes (lanes 1-3: *nhaS2* gene at the expected size (1623bp), lanes 4-6: *nhaS4* gene at the expected size (1233bp) and M. DNA molecular marker 1kb ladder). **(B)** Restriction digestion of the recombinant plasmids
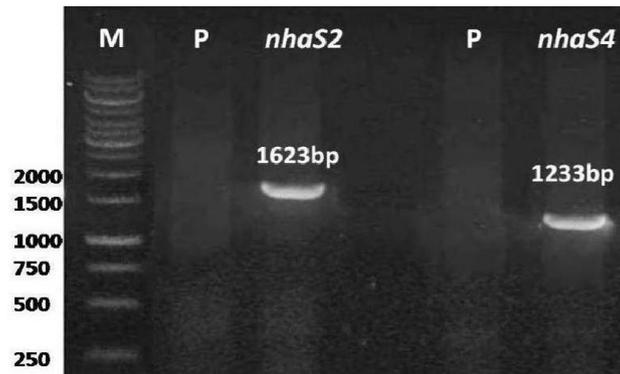

Fig. 11. The Colony PCR amplification of the *nhaS2* gene (1623bp) and *nhaS4* gene (1233bp). **(M)** 1kb ladder as a marker and **(P)** transformant yeast cells for empty pYES vector as negative control

Table 1. The mean values (OD$_{600}$)of the growth of transgenic yeast and controls (negative and positive) under salt stress

Salt conc. (M NaCl)	Negative control (-)	Positive control pYES2 (+)	*nhaS2*	*nhaS4*
0 M	0.0130$_{aB}$	1.9890$_{aA}$	1.9883$_{aA}$	1.9917$_{aA}$
1.7 M	0.0130$_{aC}$	1.1420$_{bB}$	1.3683$_{bA}$	1.3647$_{bA}$
1.8 M	0.0127$_{aC}$	0.2620$_{cB}$	1.2250$_{cA}$	1.2180$_{cA}$
1.9 M	0.0130$_{aB}$	0.0240$_{dB}$	0.9710$_{dA}$	0.9480$_{dA}$
2.0 M	0.0123$_{aB}$	0.0193$_{dB}$	0.8593$_{eA}$	0.8483$_{eA}$
2.1 M	0.0117$_{aB}$	0.0147$_{dB}$	0.6690$_{fA}$	0.6727$_{fA}$
2.2 M	0.0120$_{aB}$	0.0120$_{dB}$	0.3493$_{gA}$	0.3230$_{gA}$
2.3 M	0.017$_{aB}$	0.013$_{dB}$	0.1837$_{hA}$	0.1867$_{hA}$

Numbers with the same letters are not significantly different at 1%

Expression of the cloned genes in yeast

Yeast is a good model system for studying osmoadaptation in eukaryotes, as other fungi and plants share many of the stress response pathways and proteins involved in osmoadaptation in yeast (Dhar *et al.*, 2011 and Jin *et al.*, 2014). The transformants yeast were picked and streaked on SC medium devoid of uracil. Verification of the presence of the insert in these clones was performed by Colony Pick PCR (Fig. 11). In order to study the expression of the cloned genes in yeast, yeast growth under salt stress and intracellular proline accumulation were estimated.

Evaluation of transgenic yeast growth as an indicator of salt tolerance

Yeast strains proved to be suitable for gene engineering. Yeast can be used as a kind of vector to introduce foreign genes (Feng *et al.,* 2005). Transgenic yeasts were grown on SC medium devoid of uracil and supplemented with different concentrations of NaCl, i.e., 0, 1.7, 1.8, 1.9, 2.0, 2.1, 2.2 and 2.3M to evaluate their ability to tolerate salt stress. The results revealed significant differences in cells growth between the transgenic yeast and control. The data presented in Table (1) and the histogram in Fig. (12) Illustrate the effect of the different salt concentrations on the mean values of the growth at OD$_{600}$ in the transgenic yeast compared to the controls (negative and positive). In all salt treatments, the yeast cells in the negative control didn't grow due to the lack of uracil. At 0M NaCl the mean values of growth of the yeast cells were comparable in the second control (positive control) and transgenic yeast (1.9890$_{aA}$, 1.9883$_{aA}$ and 1.9917$_{aA,}$ respectively). The salt concentration of 1.8M NaCl was critical in determining the ability of yeast to tolerate salt stress. At this concentration, the mean growth values for the transgenic yeast harboring the genes *nhaS2* and *nhaS4* were 1.2250$_{cA}$ and 1.2180$_{cA}$, respectively compared to 0.2620$_{cB}$ for the control. Increasing the salt concentration to 1.9M NaCl, inhibited the growth of the control cells significantly.
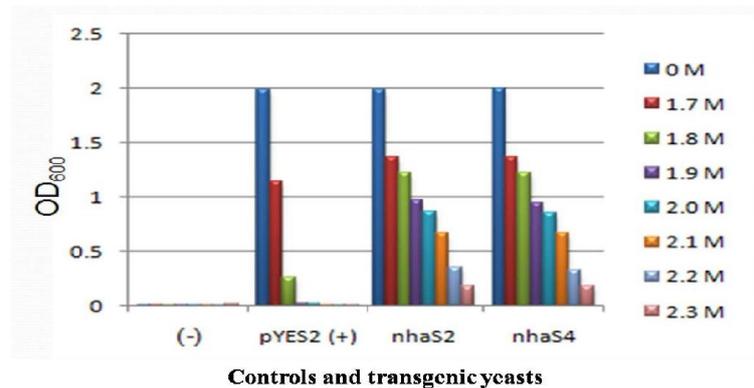
Fig. 12. Histogram illustrating means of measurements of the yeast growth at OD$_{600}$ under salt stress

While, the transgenic yeast cells for the 2 genes maintained good growth and the growth continued until the 2.3 M NaCl concentration but with a lower level. In this respect, Feng *et al.* (2005) cloned the thymosin α1 (Tα1) gene into pYES2 vector. Sequencing was performed to identify the recombinants. The sequence of Tα1 in recombinants coincided with the original one reported in Genbank. When pYES2- Tα1 plasmid was transformed into yeast, galactose instead of glucose was used to induce Tα1 expression. Western blot was performed to identify the quality of the expressed Tα1. Jin *et al.* (2014) transformed the rice *rgMT* gene in yeast (*Saccharomyces cerevisiae*) and investigated yeast growth under heavy metal ion, salt and oxidative stresses.

The results indicated that the *rgMT* gene was expressed in the cytoplasm of transgenic cells. Yeast cells transgenic for pYES2-rgMT showed vigorous growth compared to the nontransgenic controls when exposed to 7mM CuCl$_2$, 10mMFeCl$_2$, 1MNaCl, 24mM NaHCO$_s$ and 3.2mM H$_2$O$_2$, but there was no significant difference for other stresses tested. These results indicated that the expression of the rice *rgMT* gene in transgenic yeast is implicated in improving their tolerance for certain salt and peroxide stressors. Li *et al.* (2014) transformed full-length cDNAs of *P. tenuiflora* into *Saccharomyces cerevisiae* by using the full-length cDNA over-expressing gene-hunting system to identify novel salt-tolerance genes. In all, 32 yeast clones overexpressing *P. tenuiflora* cDNA was obtained by screening under NaCl stress conditions. Out of these, 31 clones showed stronger tolerance to NaCl, then they were amplified using PCR and sequenced.

RT-PCR expression analysis

To evaluate the expression of the *nhaS2* and the *nhaS4* genes in the transgenic yeast, RT-PCR analysis was performed. The total RNA was extracted and purified from transgenic yeast and control (transgenic yeast cells for empty pYES vector) grown on YPD medium supplemented with 1.8M, 2M and 2.3M NaCl. Reverse transcription reaction was performed using the first strand cDNA and the specific primers for the *nhaS2* and the *nhaS4* genes (Fig.13). The results indicated that the specific *nhaS2* and *nhaS4* primers successfully amplified the genes of 1623 bp and 1233bp, respectively, therefore, confirming the expression of the *nhaS2* and the *nhaS4* genes transcripts in the transgenic yeast.

Reverse transcriptase-PCR (RT-PCR) has been commonly employed to detect the presence of the transgene transcript in the transcriptome of transgenic yeast. In this regard, Aharon *et al.* (2003) used RT-PCR to determine the relative abundance of the *AtNHX* transcripts in *Arabidopsis thaliana*. They showed that while *AtNHX1* and *AtNHX2* transcripts were abundant and widely distributed in all tissues, *AtNHX3* and *AtNHX4* transcripts were almost exclusively detected in flower and root tissues, respectively. *AtNHX5* transcripts were observed at very low levels in all tissues.

Green *et al.* (2004) utilized the RT-PCR assay to analyze expression patterns of genes in the *Candida albicans* ALS (agglutinin-like sequence) family. Inoculation of a reconstituted human buccal epithelium (RHE) model of mucocutaneous candidiasis with strain SC5314 showed destruction of the epithelial layer by *C. albicans* and also formation of an upper fungal layer that had characteristics similar to a biofilm. RT-PCR analysis of total RNA samples extracted from *C. albicans*-inoculated buccal RHE showed that ALS1, ALS2, ALS3, ALS4, ALS5 and ALS9 were consistently detected over time as destruction of the RHE progressed. Li *et al.* (2008) cloned a novel vacuolar Na$^+$/H$^+$ exchanger, *CgNHX1*, from a halophytic species *Chenopodium glaucum* by using reverse transcriptase-polymerase chain reaction (RT-PCR). Wang *et al.* (2011)

detected the expression of *CmNHX1* in roots, stems and leaves of *Cucumis melo* using RT-PCR. They pointed out that the expression increased in the roots but decreased in the leaves with increasing NaCl concentration. Similarly, the expression increased with increasing time in transgenic plants treated with 100 mM NaCl. Expression of *CmNHX1* in ATX3 yeast Na$^+$/H$^+$ antiporter mutants showed functional complementation. No differences in yeast cell growth were detected in the presence or absence of *CmNHX1* on a NaCl-free medium. Control yeast growth was noticeably suppressed on medium containing NaCl, whereas, yeasts overexpressing *CmNHX1* showed increased population growth rates. These results indicate that the *CmNHX1* protein enhanced AXT3 salt tolerance.

Measurement of intracellular proline contents

Proline is an amino acid that plays an important role in plants. It protects the plants from the various stresses and also helps the plants to recover from stress more rapidly.

Table 2. The mean values (OD$_{520}$) of the proline in transgenic and control yeast under different concentrations of salt (NaCl)

| Salt conc. (M NaCl)	negative control (-)	Positive control pYES2 (+)	*nhaS2*	*nhaS4*
0 M	0.2537$_{bA}$	0.2550$_{bA}$	0.2600$_{hA}$	0.2613$_{hA}$
1.7 M	0.3577$_{aB}$	0.3573$_{aB}$	0.4673$_{fA}$	0.4697$_{fA}$
1.8 M	0.1157$_{cB}$	0.1207$_{cB}$	0.4950$_{eA}$	0.4973$_{eA}$
1.9 M	0.0427$_{dB}$	0.0427$_{dB}$	0.5217$_{dA}$	0.5240$_{dA}$
2.0 M	0.0360$_{dB}$	0.0370$_{dB}$	0.5420$_{cA}$	0.5433$_{cA}$
2.1 M	0.0263$_{eB}$	0.0273$_{eB}$	0.5550$_{bA}$	0.5547$_{bA}$
2.2 M	0.0213$_{efB}$	0.0210$_{efB}$	0.5647$_{aA}$	0.5650$_{aA}$
2.3 M	0.0147$_{fB}$	0.0143$_{fB}$	0.4450$_{gA}$	0.4467$_{gA}$
Mean	0.1085$_{b}$	0.1094$_{b}$	0.4813$_{a}$	0.4827$_{a}$
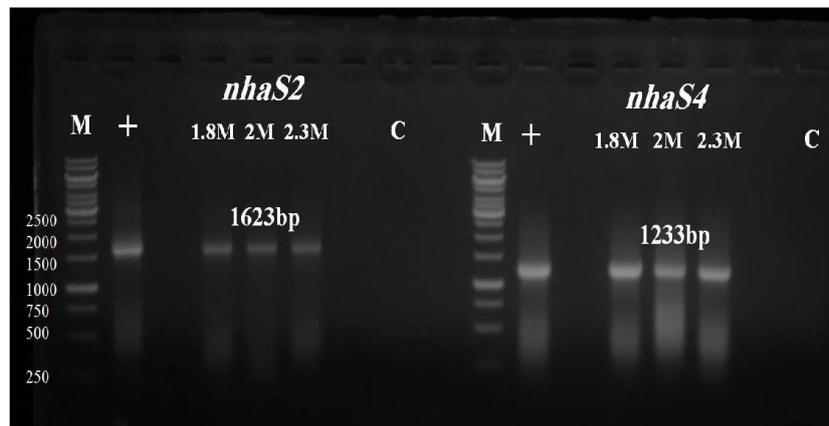
Fig. 13. The RT-PCR analysis of the *nhaS2* and *nhaS4* genes revealing the amplification of the bands at the expected size (1623bp and 1233 bp, respectively)
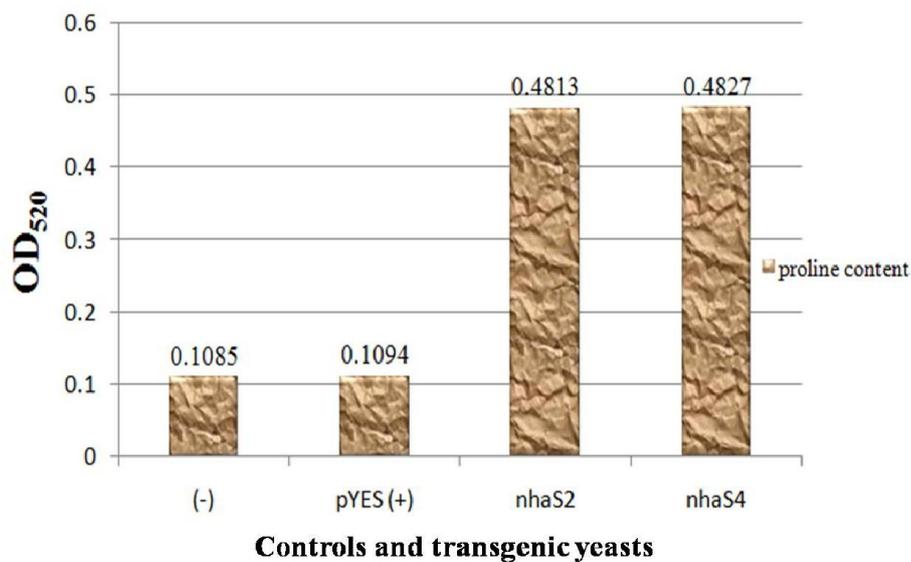

Fig. 14. Histogram illustrating general means of measurements of the proline at OD$_{520}$ from yeast under salt stress

Hayat *et.al.* (2012) reported that low concentrations of exogenous proline protect plants from salinity, drought and temperature stress. However, higher doses will impart toxic effects. In response to osmotic stress, proline is accumulated in many bacterial and plant cells as an osmoprotectant (Takagi, 2008). In the present study, to assess the response of the transgenic yeast to salt stress, intracellular proline accumulation has been determined. The results presented in Table (2) and Figure (14) showed that the mean values (OD_{520}) of the intracellular proline concentration of the controls was significantly decreased with raising salinization level. While, in the transgenic yeast the proline concentration was significantly increased with raising salinization level. Figure (14) illustrates the general mean values (OD_{520}) of the proline calculated from the different salt treatments in transgenic and controls yeast. The results confirmed that the proline content of the transgenic cells was significantly higher than that of the controls (0.4813_a for *nhaS2* and 0.4827_a for *nhaS4 vs.* 0.1094_b for the positive control and 0.1085_b for the negative control). Matsuura and Takagi (2005), Morita *et al.* (2002 and 2003) Takagi *et al.* (1997, 2000 and 2005) and Terao *et al.* (2003) constructed *S. cerevisiae* cells that accumulate proline, and the engineered strains successfully showed enhanced tolerance to many stresses, including freezing, desiccation, oxidation and ethanol. Huang *et al.* (2013) evaluated proline accumulation profiles in roots, stems and leaves of Jerusalem artichoke (*Helianthus tuberosus L.*) plantlets under NaCl stress. This plant is a moderately salt tolerant species. Their results revealed that the proline accumulated in roots, stems and leaves were three fold higher than each control.

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