

ISTANBUL TECHNICAL UNIVERSITY ★ GRADUATE SCHOOL OF SCIENCE
ENGINEERING AND TECHNOLOGY

**MOLECULAR AND PHYSIOLOGICAL CHARACTERIZATION OF A SALT-
RESISTANT *Saccharomyces cerevisiae* MUTANT OBTAINED BY
EVOLUTIONARY ENGINEERING**

Ph.D. THESIS

Şeyma Hande TEKARSLAN

Department of Molecular Biology-Genetics and Biotechnology

Molecular Biology-Genetics and Biotechnology Programme

JULY 2015

ISTANBUL TECHNICAL UNIVERSITY ★ GRADUATE SCHOOL OF SCIENCE
ENGINEERING AND TECHNOLOGY

**MOLECULAR AND PHYSIOLOGICAL CHARACTERIZATION OF A SALT-
RESISTANT *Saccharomyces cerevisiae* MUTANT OBTAINED BY
EVOLUTIONARY ENGINEERING**

Ph.D. THESIS

**Şeyma Hande TEKARSLAN
(521092077)**

Department of Molecular Biology-Genetics and Biotechnology

Molecular Biology-Genetics and Biotechnology Programme

**Thesis Advisor: Prof. Dr. Zeynep Petek ÇAKAR
Thesis Co-advisor: Prof. Dr. Carola HUNTE**

JULY 2015

İSTANBUL TEKNİK ÜNİVERSİTESİ ★ FEN BİLİMLERİ ENSTİTÜSÜ

**EVİRİMSEL MÜHENDİSLİK YÖNTEMİYLE ELDE EDİLMİŞ TUZA
DİRENÇLİ BİR *Saccharomyces cerevisiae* MUTANTININ MOLEKÜLER VE
FİZYOLOJİK KARAKTERİZASYONU**

DOKTORA TEZİ

**Şeyma Hande TEKARSLAN
(521092077)**

Moleküler Biyoloji-Genetik ve Biyoteknoloji Anabilim Dalı

Moleküler Biyoloji-Genetik ve Biyoteknoloji Programı

**Tez Danışmanı: Prof. Dr. Zeynep Petek ÇAKAR
Eş Danışman: Prof. Dr. Carola HUNTE**

TEMMUZ 2015

Şeyma Hande Tekarslan, a Ph.D. student of ITU Graduate School of Science, Engineering and Technology student ID 521092077 successfully defended the thesis entitled “**MOLECULAR AND PHYSIOLOGICAL CHARACTERIZATION OF A SALT-RESISTANT *Saccharomyces cerevisiae* MUTANT OBTAINED BY EVOLUTIONARY ENGINEERING**”, which she prepared after fulfilling the requirements specified in the associated legislations, before the jury whose signatures are below.

Thesis Advisor : **Prof. Dr. Zeynep Petek ÇAKAR**
Istanbul Technical University

Co-advisor : **Prof. Dr. Carola HUNTE**
University of Freiburg

Jury Members : **Prof. Dr. Ayten Yazgan KARATAŞ**
Istanbul Technical University

Prof. Dr. Süleyman AKMAN
Istanbul Technical University

Prof. Dr. Kutlu ÜLGEN
Boğaziçi University

Assoc. Prof. Dr. Fatma Neşe KÖK
Istanbul Technical University

Prof. Dr. Nazlı ARDA
Istanbul University

Date of Submission : 29 June 2015

Date of Defense : 13 July 2015

To my family,

FOREWORD

I would like to thank my advisor Prof. Dr. Zeynep Petek Çakar and my co-advisor Prof. Dr. Carola Hunte for giving me support and courage during my thesis study. It was my dream to work with Prof. Çakar since I saw her CV in 2006. I got the opportunity to work with her starting in 2008. In these 7 years, it was a pleasure for me to be in her group.

I would like to thank to Assoc. Prof. Dr. Erdal Cevher for his support and help during difficult times in my PhD. He always gives me courage for my academic career. It is really a chance for me to work with him. I would like to thank to Prof. Dr. Ahmet Oğul Araman and Prof. Dr. Yıldız Özsoy-Erginer for their support. Prof. Dr. Ahmet Oğul Araman teaches me that being in a department means being in a family.

I want to thank members of the ITU yeast group; especially Dr. Ceren Alkım, B. Gülçin Balaban, Arman Akşit, Seçil Erbil, Can Holyavkin, Nazlı Kocaefe, Dr.Ülkü Yılmaz, Dr.Burcu Turanlı Yıldız, Mevlüt Arslan, Naciye Durmuş, Burcu Sönmez.

I have wonderful memories with yeast group. Their optimistic viewpoint of life and joy made me really happy during my studies. During my Ph.D studies I gained friendships that will last all my life.

And my dearest friend Ceren...She is more than a friend, she is like my sister...We laughed, had fun, cried and studied together.

The times that I shared with Can and Nazlı are unforgettable.

I had the opportunity to work in Prof. Hunte's group since 2011. I learned a lot from them. I look forward to continue working with them in future. I would like to thank Dr. Xenia Bogdanovic, Dr. Thomas Kleinschroth, Dr. Christopher Lentjes, Dr. Christoph Wirth, Dr. Wei-Chun Kao, Dr. Syed Mir, Dominic Birth, Anika Fippel, Simon Fuchs and Claudia Escher.

I would like to acknowledge financial support from Istanbul Technical University Research Funds (ITU-BAP graduate student project no:37730); European Cooperation in Science and Technology Action (COST) CM0902 and I thank the Scientific and Technological Research Council of Turkey (TÜBİTAK) -National Scholarship Programme for Ph.D Students.

Finally, I would like to thank my family, Erdal Tekarslan, Şükran Tekarslan and Efnan Elif Tekarslan for their love and support. I dedicate this study to them.

My grandmother, Hatice Yalım, thank you for your prayers.

And Refik, I am amazed by his composure and patience. Thank you for waiting all these PhD years.

June 2015

Şeyma Hande TEKARSLAN
(Molecular Biologist)

TABLE OF CONTENTS

	<u>Page</u>
1. INTRODUCTION.....	1
1.1 The Model Organism <i>Saccharomyces cerevisiae</i>	1
1.2 Sodium Mechanism in <i>Saccharomyces cerevisiae</i>	4
1.2.1 Importance of NaCl.....	4
1.2.2 Role of Na ⁺ and K ⁺	4
1.2.3 Na ⁺ entry into cells.....	5
1.2.4 Na ⁺ efflux.....	5
1.3 Osmotic and Salt Stress.....	6
1.3.1 Strategies of yeast for survival in osmotic and salt stress.....	7
1.3.2 Phases of the osmotic response.....	8
1.4 Salt Response Pathways.....	9
1.5 Evolutionary Engineering.....	10
1.6 Trehalose and Glycogen.....	12
1.7 Structural and Functional Properties of Na ⁺ /H ⁺ Antiporter.....	16
1.8 Diseases Related to Na ⁺ -ion.....	17
1.9 The Aim of The Study.....	17
2. MATERIALS AND METHODS.....	19
2.1 Materials.....	19
2.1.1 Strains and glycerol stocks.....	19
2.1.2 Media.....	20
2.1.3 Chemicals, solutions, buffers, kits, enzymes and equipment.....	21
2.2 Methods.....	25
2.2.1 Cultivation of the cells.....	25
2.2.2 Growth Analysis.....	26
2.2.3 Determination of stress resistances.....	26
2.2.4 Growth Analysis of Wild Type and Salt resistant Mutant Strain 'T8'.....	27
2.2.5 Proton efflux.....	27
2.2.6 Analytical procedures.....	28
2.2.7 Quantitative assessment of glycogen and trehalose contents.....	31
2.2.8 Metal content determination by Flame Atomic Absorption Spectrometer (FAAS).....	32
2.2.9 Microarray analysis.....	32
2.2.10 Real-Time PCR.....	33
2.2.11 Sequence analysis of <i>NHA1</i> , <i>NHX1</i> and <i>KHA1</i> genes.....	35
2.2.12 Transformation experiments.....	39
2.2.13 Site directed mutagenesis of <i>NHA1</i>	43
2.2.14 Proteome analysis via electrospray ionization quadrupole time-of-flight (TOF) mass spectrometry.....	45
3. RESULTS.....	47
3.1 Growth Characteristics of Mutant Strain 'T8' and comparison of growth physiology between wildtype and T8.....	47

3.1.1 Growth Characteristics of Mutant Strain ‘T8’ in the presence of 0.5 M NaCl, 0.7 M NaCl and 0.9 M NaCl	47
3.1.2 Growth Characteristics of Salt-Resistant Mutant‘T8’ in the presence of 0.5 M NaCl	49
3.1.3 Growth Characteristics of Salt-resistant Mutant ‘T8’ in the presence of 5 mM LiCl	52
3.2 Spot test upon different stress types	56
3.2.2 Spot Test of CEN.PK 113.7D(wildtype) and T8 with NaCl, KCl and LiCl (YMM).....	57
3.2.3 Spot Test of CEN.PK 113.7D (wild type) and T8 with different concentrations of LiCl (YPD).....	61
3.2.4 Spot Test of CEN.PK 113.7D (wild type) and T8 with KCl and Sorbitol (YMM).....	62
3.2.5 Spot Test of CEN.PK 113.7D (wild type) and T8 with combination of NaCl and KCl stress (YMM)	64
3.2.6 Spot Test of CEN.PK 113.7D (wild type) and T8 with pH and 0.7 M NaCl stress effect (YMM).....	65
3.2.7 Spot Test of CEN.PK 113.7D (wild type) and T8 with pH and 150 mM and 300 mM NaCl stress effect (YPD).....	67
3.2.8 Spot Test of CEN.PK 113.7D (wild type) and T8 with pH and LiCl stress effect (YPD).....	68
3.2.10 Spot tests of FGY217, AB11c, KTA40-2 at pH3.5, pH 7.1, pH 8.75 with 150 mM, 300 mM, 400 mM NaCl.....	69
3.2.11 Spot tests of FGY217, AB11c, KTA40-2 at pH 3.5, pH 7.1, pH 8.75 with 50% (v/v) sorbitol	74
3.3 pH Determination Experiment.....	75
3.4 Proton Efflux	77
3.5 Estimation Of Stress Resistance For CEN.PK 113.7D By Spot Test (Cross-resistance).....	77
3.5.1 YMM.....	77
3.5.2 YPD	78
3.6 Most Probable Number (MPN) Assay.....	79
3.7 Metal Content Determination by Flame Atomic Absorption Spectrometer (FAAS).....	83
3.7.1 Potassium and sodium content determination via Flame Atomic Absorption Spectrometer (FAAS)	84
3.8 Determination of Metabolite Contents by HPLC	89
3.8.1 HPLC (0.5 M NaCl)	89
3.8.2 HPLC (5mM LiCl)	93
3.9 Quantitative assessment of glycogen and trehalose content.....	98
3.9.1 0.5 M NaCl	98
3.9.2 5 mM LiCl.....	99
3.10 Whole genome transcriptomic analysis of salt resistant mutant.....	101
3.10.1 RNA Isolation and RIN Analysis	101
3.10.2 Upregulated genes	102
3.10.3 Downregulated genes	108
3.11 Expression Level Determination of <i>ENA6</i> and <i>NHA1</i> for both Wild Type and T8.....	111
3.12 Proteomics Results of T8.....	113
3.13 Sequence analysis of <i>NHA1</i> , <i>NHX1</i> and <i>KHA1</i>	117

3.13.1 Genomic DNA isolation.....	117
3.13.2 Amplification of target sequences by PCR	118
3.14 Site directed mutagenized sequences by PCR.....	119
4. DISCUSSION	123
5. CONCLUSION.....	145
REFERENCES.....	147
CURRICULUM VITAE.....	163

ABBREVIATIONS

AVG	: Average
bp	: Base Pair
cDNA	: Complementary Deoxyribo Nucleic Acid
CDW	: Cell Dry Weight
CoCl₂	: Cobalt Chloride
C_T	: Cycle Threshold
DNA	: Deoxyribo Nucleic Acid
ds	: Double Strand
EMS	: Ethyl Methane Sulfonate
h	: Hour
H₂O₂	: Hydrogen Peroxide
HPLC	: High Pressure Liquid Chromatography
HSPs	: Heat-Shock Proteins
LB	: Luria Broth
MAT	: Mating Type
µg	: Microgram
µL	: Microliter
µm	: Micrometer
mM	: Micromolar
mg	: Milligram
mL	: Milliliter
min	: Minute
MPN	: Most Probable Number
ng	: Nanogram
OD	: Optical Density
PCR	: Polymerase Chain Reaction
RNA	: Ribonucleic Acid
RIN	: RNA Integrity Number
rpm	: Revolution per minute
RT-PCR	: Real-Time Polymerase Chain Reaction
wt	: Wild Type
YMM	: Yeast Minimal Medium
YPD	: Yeast Extract- Peptone - Dextrose

LIST OF TABLES

	<u>Page</u>
Table 1.1 : Evolutionary engineering experiments with yeasts exposed to environmental stress	12
Table 2.1 : Yeast Minimal Medium (YMM) contents.....	20
Table 2.2 : YPD (YEPD, YP Dextrose) medium contents.....	20
Table 2.3 : Luria Bertani medium contents.....	20
Table 2.4 : Drop-out medium contents	21
Table 2.5 : Chemicals used in this study	21
Table 2.6 : Name and content of the solutions and buffers.....	22
Table 2.7 : Laboratory equipment used in this study	23
Table 2.8 : Software and websites used in this study.....	25
Table 2.9 : Kits and enzymes used in this study.	25
Table 2.10 : Stock Solutions of HPLC Standards	29
Table 2.11 : HPLC Standard Solution Dilutions (Std: Standard Solution)(Solution A&B).....	30
Table 2.12 : HPLC Standard Solution Dilutions (Std: Standard Solution) (Solution C).....	30
Table 2.13 : Metabolite Concentrations in HPLC Standard Solutions (Std: Standard Solution, RT: Retention Time, RI: Refractive Index).....	31
Table 2.14 : The list of primer sequences	34
Table 2.15 : Q-PCR protocol.....	35
Table 2.16 : Q-PCR program	35
Table 2.17 : Sequencing primers of <i>NHA1</i> , <i>NHX1</i> and <i>KHA1</i>	37
Table 2.18 : PCR mixture.....	38
Table 2.19 : PCR program for Colony PCR (XB t7t3-44 35) (preheated lid at 94 °C)	38
Table 2.20 : Yeast transformation mixture.....	40
Table 2.21 : PCR mixture for colonies.....	41
Table 2.22 : PCR program for Colony PCR (XB t7t3-44 35) (preheated lid at 94 °C).....	41
Table 2.23 : List of site-directed mutagenesis primers	43
Table 2.24 : PCR mixture.....	43
Table 2.25 : PCR program for site-directed mutagenesis PCR.....	44
Table 3.1 : Average OD ₆₀₀ values of wildtype and wild type in the presence of 0.5 M NaCl, 0.7 M NaCl, 0.9 M NaCl	46
Table 3.2 : Average OD ₆₀₀ values of T8 and T8 in the presence of 0.5 M NaCl, 0.7 M NaCl and 0.9 M NaCl	46
Table 3.3 : ln OD ₆₀₀ and $\mu_{\max}$ (h ⁻¹) values of wt, wt in the presence of 0.5 M NaCl, 0.7 M NaCl, 0.9M NaCl , T8, T8 in the presence of 0.5 M NaCl, T8+0.7 M NaCl, T8+0.9 M NaCl.....	47

Table 3.4 : Average OD ₆₀₀ Values of wt, wt in the presence of 0.5 M NaCl, T8 and T8 in the presence of 0.5 M NaCl	49
Table 3.5 : ln (OD ₆₀₀) and $\mu_{\max}$ (h ⁻¹) values of wt, wt+0.5 M NaCl, T8 and T8+0.5 M NaCl	49
Table 3.6 : Average cell dry weight (CDW) mg/mL values of wild type, wild type in the presence of 0.5 M NaCl, T8, T8 in the presence of 0.5 M NaCl.....	51
Table 3.7 : Average (OD ₆₀₀) values of wild type, wild type in the presence of 5mM LiCl, T8 and T8 in the presence of 5mM LiCl	52
Table 3.8 : ln (OD ₆₀₀) and $\mu_{\max}$ (h ⁻¹) values of wt, wt in the presence of 5mM LiCl, T8 and T8 in the presence of 5mM LiCl.	52
Table 3.9 : Average CDW mg/mL values of wild type, wild type in the presence of 5 mM LiCl, T8, T8 in the presence of 5 mM LiCl.....	54
Table 3.10 : MPN cell number of wild type and T8 at NaCl, LiCl and KCl stresses	75
Table 3.11 : MPN cell number of wild type and T8 at different stress conditions ...	75
Table 3.12 : Survival ratios of T8 as fold of wild type in all stress conditions (MPN results)	77
Table 3.13 : Cell dry weight (CDW) values.....	78
Table 3.14 : Total cellular potassium, sodium and lithium amount (µg/mg) in wild type and T8 mutant, as determined by flame atomic absorption spectrometry	79
Table 3.15 : Total cellular potassium and sodium content (mM) and Na ⁺ /K ⁺ ratio.	82
Table 3.16 : Average amounts of metabolites (g/L) obtained from HPLC analysis for wild type	84
Table 3.17 : Average amounts of metabolites (g/L) obtained from HPLC analysis for T8.....	84
Table 3.18 : Average amounts of metabolites (g/L) obtained from HPLC analysis for T8+0.5 M NaCl	84
Table 3.19 : Average amounts of metabolites (g/L) obtained from HPLC analysis for wild type	88
Table 3.20 : Average amounts of metabolites (g/L) obtained from HPLC analysis for the wild type.....	88
Table 3.21 : Average amounts of metabolites (g/L) obtained from HPLC analysis for the wild type in the presence of 5 mM LiCl	88
Table 3.22 : Average amounts of metabolites (g/L) obtained from HPLC analysis for T8.....	89
Table 3.23 : Average amounts of metabolites (g/L) obtained from HPLC analysis for T8 in the presence of 5 mM LiCl.....	89
Table 3.24 : Relative trehalose and glycogen content of sodium-treated and non-treated samples of wild type and T8.....	92
Table 3.25 : Relative trehalose and glycogen contents of lithium-treated and non-treated samples of wild type and T8.....	94
Table 3.26 : Initial OD ₆₀₀ of the cultures and their OD ₆₀₀ values just before the RNA isolation.....	95
Table 3.27 : RIN values for the parallel sets of cultures	96
Table 3.28 : RNA Concentration (ng/µl) of the samples.....	96
Table 3.29 : Upregulated genes (about five-fold and higher) in T8 based on microarray results	98
Table 3.30 : Downregulated genes (about two-fold and higher) in T8 based on microarray results	103

Table 3.31 : Efficiency and error values for qPCR analysis	105
Table 3.32 : Relative changes in protein expression levels between <i>S. cerevisiae</i> wild type and T8	105
Table 3.33 : Important interactions according to the proteomic analysis results....	110

LIST OF FIGURES

	<u>Page</u>
Figure 1.1 : Haploid cells of <i>Saccharomyces cerevisiae</i>	1
Figure 1.2 : Phylogenetic tree of <i>S.cerevisiae</i> strains.....	3
Figure 1.3 : The metabolic pathways for glycogen and trehalose biosynthesis and biodegradation and for trehalose assimilation in the yeast <i>S. cerevisiae</i> . Abbreviation of the enzyme name: Hxk2p, hexokinase II; Hxk1p, hexokinase I; Glk1p, glucokinase; Pgm1,2p, phosphoglucose mutase isoform 1 and 2; Ymr278p, homologous to phosphoglucomutase; Ugp1p, uridylylglucose pyrophosphorylase; Glg1,2p, glycogenin isoform 1 & 2; Gsy1,2p, glycogen synthase isoform 1 & 2; Glc3p, glycogen branching enzyme; Gph1p, glycogen phosphorylase; Gdb1p, glycogen debranching enzyme; Sga1p, amylo (1 → 4), (1 → 6) glucosidase; Ath1p, acid trehalase or extracellular trehalase; Agt1p, a-methylglucose transporter; Nth1, 2p, neutral trehalase isoform 1 & 2; Sgp1p, putative sugar permease; Tps1p, trehalose-6-phosphate synthase; Tps2p; trehalose- 6-P phosphatase; Tps3p & Tsl1p, regulatory subunit of the trehalose synthase complex.....	13
Figure 3.1 : Growth curves of wild type and T8 strain in the absence and presence of 0.5 M, 0.7M,0.9 M NaCl.....	47
Figure 3.2 : Growth curves of wild type and T8 strain in the absence and presence of 0.5 M NaCl	50
Figure 3.3 : Average CDW (mg/ml) values of wild type and T8 in the absence and presence of 0.5 M NaCl.....	51
Figure 3.4 : Growth curves of wild type and T8 strain in the absence and presence of 5 mM LiCl	53
Figure 3.5 : Average CDW (mg/ml) values of wild type and T8 in the absence and presence of LiCl	54
Figure 3.6 : Spot test results of wild type and T8 in YPD, containing different amounts of TMA.....	55
Figure 3.7 : Spot test results of wild type and T8 in YPD containing different amounts of spermine	56
Figure 3.8 : Spot test results of wild type and T8 in YMM containing different amounts of NaCl and LiCl (24 th hour).....	57
Figure 3.9 : Spot test results of wild type and T8 in YMM containing different amounts of NaCl and LiCl (48 th hour).....	58
Figure 3.10 : Spot test results of wild type and T8 in YMM containing different amounts of KCl (24 th hour).	58
Figure 3.11 : Spot test results of wild type and T8 in YMM containing different concentrations of KCl (48 th hour).....	59
Figure 3.12 : Spot test results of wild type and T8 in YPD containing different amounts of LiCl.....	59

Figure 3.13 : Spot test results of wild type and T8 in YMM containing different amounts of KCl (48 th hour).	60
Figure 3.14 : Spot test results of wild type and T8 in YMM containing different amounts of KCl and sorbitol (72 nd hour).....	60
Figure 3.15 : Spot test results of wild type and T8 in YMM containing different amounts of KCl and NaCl (48 th hour).	61
Figure 3.16 : Spot test results of wild type and T8 in YMM containing different amounts of KCl and NaCl (72 nd hour)..	62
Figure 3.17 : Spot test results of wild type and T8 in YPD containing 0.7 M NaCl and at different pH range (36 th hour).....	63
Figure 3.18 : Spot test results of wild type and T8 in YPD containing 0.7 M NaCl and at different pH range (48 th hour).....	63
Figure 3.19 : Spot test results of wild type and T8 in YPD containing 0.7M NaCl and at different pH range (72 nd hour).	64
Figure 3.20 : Spot test of wild type and T8 with different concentrations of NaCl at different pH values	65
Figure 3.21 : Spot test of wild type and T8 with 20 mM LiCl at different pH values.....	65
Figure 3.22 : Spot test of wild type and T8 with 50%(v/v) sorbitol at different pH values.....	66
Figure 3.23 : Spot test of FGY217, AB11c, KTA40-2 at pH 3.5, pH 7.1, pH 8.75 with 150 mM, 300 mM, 400 mM NaCl	66
Figure 3.24 : Spot test of scNha1p in FGY217, AB11c, KTA40-2 at pH 3.5, pH 7.1, pH 8.75 with 150 mM, 300 mM, 400 mM NaCl	67
Figure 3.25 : Spot test of PDD empty in FGY217, AB11c, KTA40-2 at pH 3.5, pH 7.1, pH 8.75 with 150 mM, 300 mM, 400 mM NaCl	68
Figure 3.26 : Spot test of GFP in FGY217 at pH 3.5, pH 7.1, pH 8.75 with 150 mM, 300 mM NaCl.	69
Figure 3.26 (cont) : Spot test of GFP in AB11c, KTA40-2 at pH 3.5, pH 7.1, pH 8.75 with 150 mM, 300 mM, 400 mM NaCl.	69
Figure 3.27 : Spot test of scNha in AB11c, KTA40-2 at pH 3.5, pH7.1, pH 8.75 with 50%(v/v) sorbitol	70
Figure 3.28 : Spot test of GFP in AB11c, KTA40-2 at pH 3.5, pH 7.1, pH 8.75 with 50%(v/v) sorbitol	70
Figure 3.29 : OD ₆₀₀ values of wild type, T8, wild type+0.7 M NaCl and T8+0.7 M NaCl	71
Figure 3.30 : pH values of wild type, T8, wild type+0.7 M NaCl and T8+0.7 M NaCl	72
Figure 3.31 : Proton efflux of wild type and T8	72
Figure 3.32 : Spot test results of wild type and T8 in YMM in the presence of different stress types.....	73
Figure 3.33 : Spot test results of wild type and T8 in YPD in the presence of different stress types.....	74
Figure 3.34 : Determination of survival ratio of T8 normalized to wild type at 4 mM CoCl ₂ , 1.5 M KCl, 6% (v/v) ethanol, 15 mM MnCl ₂ , 2m M CrCl ₃ , 10 mM AlCl ₃	76
Figure 3.35 : Determination of survival ratio of T8 normalized to wild type at 0.7 M NaCl, 10 mM ZnCl ₂ , 0.8 mM H ₂ O ₂	76
Figure 3.36 : Determination of survival ratio of T8 normalized to wild type at 5 mM LiCl.....	77

Figure 3.37 : Cell dry weight measurement results	79
Figure 3.38 : Total cellular potassium amount in wild type and T8 mutant	80
Figure 3.39 : Total cellular sodium amount in wild type and T8 mutant	81
Figure 3.40 : Total cellular lithium amount in wild type and T8 mutant.....	82
Figure 3.41 : HPLC standards for glucose, glycerol, acetate, ethanol	83
Figure 3.42 : Glucose utilization levels by wild type and T8 strain without and with 0.5 M NaCl stress conditions during 24 h incubation at 30°C, 150 rpm.....	85
Figure 3.43 : Ethanol production levels by wild type and T8 strain at control and stress conditions during 24 h incubation at 30°C, 150 rpm.....	86
Figure 3.44 : Glycerol production by wild type and T8 strain at control and stress conditions during 24 h incubation at 30 °C, 150 rpm.....	86
Figure 3.45 : Acetate production by wild type and T8 strain at control and stress conditions during 24 h incubation at 30°C, 150 rpm.....	87
Figure 3.46 : HPLC standards for glucose, glycerol, acetate, ethanol.....	87
Figure 3.47 : HPLC standards for maltose, citric acid, succinic acid.....	88
Figure 3.48 : Glucose utilization levels by wild type and T8 strain at control and stress conditions during 24 h incubation at 30°C, 150 rpm.....	90
Figure 3.49 : Ethanol production levels by wild type and T8 strain at control and stress conditions during 24 h incubation at 30°C, 150 rpm.....	90
Figure 3.50 : Glycerol production by wild type and T8 strain at control and stress conditions during 24 h incubation at 30°C, 150 rpm.....	91
Figure 3.51 : Acetate production by wild type and T8 strain at control and stress conditions during 24 h incubation at 30°C, 150 rpm.	91
Figure 3.52 : Trehalose levels of 0.5 M sodium-treated wild type, T8 and non- treated T8 samples as fold of wild type.....	93
Figure 3.53 : Glycogen levels of 0.5 M sodium-treated wild type, T8 and non- treated T8 samples as fold of wild type.....	93
Figure 3.54 : Trehalose levels of 5 mM lithium-treated wild type, T8 and non- treated T8 samples as fold of wild type.....	94
Figure 3.55 : Glycogen levels of 5 mM lithium-treated wild type, T8 and non- treated T8 samples as fold of wild type.....	95
Figure 3.56 : The percentage of upregulated genes to the total genes in each category (cut off value is ≥ 2 fold).....	97
Figure 3.57 : The distribution of transcriptional response of stress-related and cell cycle-related genes .The red line shows induced genes, and the green line shows repressed genes under environmental stress conditions.Bar chart shows the genes that are expressed under normal growth conditions in cell cycle. The black line shows the upregulated genes of T8 under normal growth conditions in cell cycle.....	102
Figure 3.58 : The percentage of downregulated genes to total genes in each category (cut-off value is ≥ 2 fold).....	103
Figure 3.59 : The distribution of transcriptional response of stress-related and cell cycle-related genes.The red line shows induced genes, and the green line shows repressed genes under environmental stress conditions. Bar chart shows the genes that are expressed under normal growth conditions in cell cycle. The black line shows the downregulated genes of T8 under normal growth conditions in cell cycle	104

Figure 3.60 : Expression level of <i>ENA6</i> gene in control, 0.7 M NaCl and 5 mM LiCl stress conditions.....	105
Figure 3.61 : Expression level of <i>NHA1</i> gene in control, 0.7 M NaCl and 5 mM LiCl stress conditions.....	106
Figure 3.62 : Expression of proteins related with metabolic pathways	108
Figure 3.63 : Proteins whose expressions are changed in the glycolytic pathway of <i>S. cerevisiae</i>	109
Figure 3.64 : Gel image of DNA isolated from the wild type and T8	111
Figure 3.65 : 1% (w/v) agarose gel image of amplification products of <i>NHA1</i> , <i>NHX1</i> and <i>KHA1</i>	112
Figure 4.1 : High osmolarity pathway in <i>S.cerevisiae</i>	122
Figure 4.2 : Metabolic pathways for glycogen and trehalose	124
Figure 4.3 : <i>HOG-1</i> Pathway related to <i>CTT1</i>	128
Figure 4.4 : Regulation of <i>ENA1</i> in <i>S.cerevisiae</i>	129

MOLECULAR AND PHYSIOLOGICAL CHARACTERIZATION OF A SALT-RESISTANT *Saccharomyces cerevisiae* MUTANT OBTAINED BY EVOLUTIONARY ENGINEERING

SUMMARY

Yeast is an excellent model system for molecular biology, cell morphogenesis, chromosome stability, and even aging. *Saccharomyces cerevisiae* CEN.PK 113-7D is widely used for metabolic engineering and systems biology research in industry and academia. A consortium of German yeast researchers developed the isogenic family of CEN.PK strains by crossing different laboratory strains of *S.cerevisiae* in the 1990s. The haploid MATa strain CEN.PK113-7D, has been extremely popular for studies in systems biology and is a preferred laboratory strain for industrial biotechnology research.

Maintenance of alkali cation homeostasis is a key process in most types of cells, including yeasts. Sodium chloride is very important for human biology. Na^+ and K^+ are essential for human nutrition and also Li^+ is an essential nutrient in rodents and goats. The budding yeast *S. cerevisiae* is able to grow in the presence of a broad range of external concentrations of K^+ (10 mM–2.5 M) and Na^+ (1.5 M).

The aim of this study was to investigate the effects of NaCl stress at physiological and transcriptomic level. *S.cerevisiae* was first mutagenized by ethyl methanesulfonate (EMS) and then exposed to salt stress (NaCl) by Sezgin T. (2010). An individual was chosen from the population and compared to wild type to investigate how it gained resistance to salt stress.

Specific growth curves of wild type and "T8" in YMM were calculated by growing these strains in 5 mM LiCl, 0.5 M NaCl, 0.7 M NaCl and 0.9 M NaCl. In all of these conditions, T8 grew better than the wild type.

Spot test results of T8 and wild type (CENPK-7D) showed that resistance to sodium and lithium are obvious, however potassium resistance was not observed. NaCl resistant mutant "T8" was resistant to sodium and lithium, while the wild type as well as T8 grew in the presence of potassium. It could be inferred that "T8" had resistance to cation toxicity, but not to osmotic stress.

Flame Atomic Absorption Spectrometry (FAAS) results showed that high extracellular Na^+ and Li^+ concentrations increase intracellular Na^+ and decrease intracellular K^+ , in both wild type and T8. Apparently, cells maintain the total monovalent cation content low after K^+ , Na^+ and Li^+ stress.

NaCl resistant mutant "T8" did not exhibit significantly increased lithium accumulation, compared to the wild type strain. The lithium resistance of the wild type was not caused by increased lithium efflux. "T8" accumulated slightly more Li^+ than the wild type in double stress conditions.

FAAS results showed that T8 has higher K^+/Na^+ ratio at every stress condition compared to the wild type. Difference of K^+/Na^+ ratio between T8 and the wildtype is obvious at 5 mM LiCl. K^+/Na^+ ratio of T8 was 4.21 and of wildtype was 1.97 at 5 mM LiCl stress. *ENA6* and *NHA1* genes' expression were lower at LiCl stress, compared to NaCl stress. The reason why K^+/Na^+ ratio is higher at LiCl stress can be the lower expression of these efflux genes so that T8 may not have active efflux at LiCl stress as at NaCl stress.

Glycogen and trehalose are two important glucose stores of the yeast *S. cerevisiae*, and production of them change in response to a number of environmental stress conditions. The disaccharide trehalose accumulates during salt adaptation. Assessment of glycogen and trehalose content showed that amount of trehalose and glycogen in T8 are prominently high compared to the wild type without stress conditions. Lithium triggers the amount of glycogen and trehalose more than sodium. It could be explained by higher toxicity of lithium. Trehalose accumulation is higher in T8 than glycogen without and with sodium and lithium stress.

In our NaCl-resistant evolved *S. cerevisiae* strain "T8", production of glycerol is higher and production of ethanol is reduced compared to the wildtype. The metabolism of the evolved strain is redirected toward glycerol and away from ethanol production.

The cross-resistance of T8 was assessed by growth on LiCl, ZnCl₂, H₂O₂, CoCl₂, KCl, ethanol, CuCl₂, MnCl₂, CrCl₃, AlCl₃YPD and YMM plates. T8 has significantly high cross resistance to lithium. T8 has slight resistance to Co⁺², Mn⁺², Ni⁺², Zn⁺² and is sensitive to aluminum.

The *S. cerevisiae* genome contains three genes encoding alkali metal cation/H antiporters (Nha1p, Nhx1p, Kha1p) that differ in cell localization, substrate specificity and physiological function. Sequencing of *NHX1*, *NHA1*, and *KHA1* were checked to be compared with mutant strain "T8". Sequencing results showed that EMS mutagenesis and NaCl stress did not affect the sequences of *NHX1*, *NHA1*, and *KHA1* genes.

The transcriptional response to high NaCl concentration stress in the yeast *S. cerevisiae* was analyzed by using DNA microarray to understand yeast strains showing tolerance to high salt stress. In microarray results, several hundred genes were identified associated with NaCl tolerance involving a broad range of functional categories including energy; cell rescue, defense and virulence; metabolism; cellular transport, transport facilities and transport routes; cellular communication/signal transduction mechanism. *FLO11* gene encoding GPI-anchored cell surface glycoprotein(flocculin) which is required for flocculation and biofilms was the most highly expressed gene in T8. In "T8" microarray results, *HOG* pathway is not induced. "T8" had slower glucose uptake and did not accumulate high levels of succinate and acetate.

In microarray results, T8 has induced *ENA6* expression, however *NHA1* is not induced. In Real-time PCR results, both *ENA6* and *NHA1* genes are induced. This may result from the differences in sensitivities of microarray and real-time PCR, the latter being more sensitive.

Microarray results indicated that "T8" has many genes overexpressed compared to the wild type even in the absence of NaCl stress. These upregulated and downregulated genes adapted this mutant to high sodium and lithium stresses.

Nha1 was cloned to be overproduced and it is checked by complementation assays. Overexpression of Nha1p showed that this protein complements better at pH 3.5. Specific single site mutations [G372S, D266H, D267A, D266H/D267A, D266N/D267N, N176, D177N] on Nha1 were applied to understand these influence of this mutations on pH.

In this study it was shown that under *S. cerevisiae* strains with enhanced glycerol and reduced ethanol yields could be obtained by evolutionary engineering for NaCl-stress resistance. This study is the first one that uses EMS and increasing NaCl stress levels for the selection of *S. cerevisiae* strains by evolutionary engineering. Additionally, the results of this thesis work suggest that *FLO11* may have an important role in high NaCl-resistance in *S. cerevisiae*.

EVRİMSEL MÜHENDİSLİK YÖNTEMİYLE ELDE EDİLMİŞ TUZA DİRENÇLİ BİR *Saccharomyces cerevisiae* MUTANTININ MOLEKÜLER VE FİZYOLOJİK KARAKTERİZASYONU

ÖZET

Maya; moleküler biyoloji, hücre morfojenezi, kromozom stabilitesi ve hatta yaşlanma için mükemmel bir model oluşturmaktadır. *Saccharomyces cerevisiae* CEN.PK 113-7D endüstri ve akademideki metabolik mühendislik ve sistem biyolojisi çalışmalarında geniş kullanım alanına sahiptir. 1990'larda Alman maya araştırmacılarından oluşan bir konsorsiyum *S.cerevisiae*'ın, farklı laboratuvar suşlarını çaprazlayarak CEN.PK suşlarının izogenik ailesini geliştirmişlerdir.

Alkali katyon homeostazinin sağlanması maya da dahil olmak üzere çoğu hücre tipinde anahtar prostestir. Sodyum klorür insan biyolojisi için çok önemlidir. Na^+ ve K^+ insanda beslenme için gereklidir ve Li^+ ise kemirgen ve keçilerde gerekli bir besin bileşenidir. Tomurcuklu bir maya olan *S. cerevisiae* harici K^+ konsantrasyonu (10 mM–2.5 M) ve Na^+ (1.5 M) durumlarında büyüebilmektedir.

Bu çalışmanın amacı fizyolojik ve transkriptomik seviyede NaCl stresinin prensiplerini araştırmaktır. *S.cerevisiae* daha inceleki bir çalışmada (Sezgin T., 2010) etil metansulfonat (EMS) ile muamele edilerek mutajenize edildi ve sonra tuz stresine maruz bırakıldı. Mutant bireyler seçilerek tuza karşı nasıl direnç kazandığı yabanıl tip ile karşılaştırılarak araştırıldı.

5 mM LiCl, 0.5 M NaCl, 0.7 M NaCl and 0.9 M NaCl varlığında YMM'de büyütülen yabanıl tip ve T8'in spesifik büyüme eğrileri oluşturuldu. Tüm bu şartlar altında T8 yabanıl tipe göre daha iyi üretti.

T8 ve yabanıl tip'e ait olan fenotipik sonuçlar T8'in sodyum ve lityuma karşı olan direncinin belirgin olduğunu ve T8'in potasyum varlığında yabanıl tip ile aynı düzeyde ürettiğini göstermiştir. Bu, T8 katyonik toksisiteye karşı dirençliyen osmotik strese karşı direnç göstermemektedir.

%25, %30, %35 sorbitol varlığında YMM'de büyütülen T8 ve yabanıl tip karşılaştırıldığında sorbitol varlığında yabanıl tip ve T8'in aynı düzeyde ürettiği görülmüştür. T8'in potasyum varlığında da aynı etkiyi gösterdiği görülmüş olup T8'in osmotik strese karşı direnç göstermediğinin kanıtıdır.

pH'ın NaCl direncine olan etkisini incelemek üzere T8 ve yabanıl tip değişen NaCl miktarlarında ve farklı pH aralığına sahip YPD'de büyütülmüş. T8'in NaCl olan direncinin pH değişikliklerinden etkilenmediği gözlenmiştir.

T8 ve yabanıl tip katyonik ilaçlara olan direnci test edilmiştir. Katyonik ilaç olarak Tetrametilammonyum (TMA) ve spermin seçilmiştir. Katyonik ilaçlar ile direnç testinin sebebi, eğer dirençte bir farklılık var ise, mutantta elektrokimyasal gradient değişikliği oluşmuş olma ihtimalidir. T8 ve yabanıl tip farklı spermin ve TMA

miktarlarına sahip olan YPD'de büyütülmüş. T8 ve yabancı tip'in TMA ve spermin dirençlerinin farklı olmadığı gözlenmiştir.

Alevli Atomik Absorpsiyon Spektrometri (AAAS) sonuçları göstermiştir ki, yüksek oranda harici Na^+ ve Li^+ konsantrasyonları hem T8 hem de yabancı tipteki hücre içi Na^+ miktarını yükseltirken hücre içi K^+ oranı düşmektedir. Hücreler toplam tek değerlikli katyon miktarını K^+ , Na^+ and Li^+ streslerinden sonra düşük tutmaktadır.

NaCl dirençli mutant T8 yabancı tip ile karşılaştırıldığında belirgin bir şekilde lityum birikmesi görülmemektedir. T8'in lityum direncinin sebebi artmış lityum efflux olmayabilir. Çift stres koşullarında T8 yabancı tipe göre çok az miktarda Li^+ biriktirmiştir. 1 M KCl varlığında yabancı tip ve T8 karşılaştırıldığında, T8 en yüksek potasyum ve sodyum miktarına sahiptir. T8 LiCl ve NaCl varlığında en yüksek LiCl miktarına sahiptir.

AAAS sonuçlarına göre, her stres koşulu altında T8 yabancı tipe göre yüksek K^+/Na^+ oranına sahiptir. T8 ve yabancı tipin K^+/Na^+ oranları arasındaki fark 5 mM LiCl stresi altında çok belirgindir. LiCl stresinde bu oranın daha fazla olmasının sebebi lityum efflux genlerinin az eksprese olmasından kaynaklı olabilir. Bu durumda T8, NaCl stresindeki gibi LiCl stresinde etkin efflux sistemine sahip olmayabilir.

Glikojen ve trehaloz *S. cerevisiae*'da iki önemli glikoz depolasudur ve bunların üretimi bir çok çevresel stres koşullarına bağlı olarak değişmektedir. Disakkarit trehaloz, tuz adaptasyonunda birikmektedir. Glikojen ve trehaloz miktarının ölçümü sonucunda T8'deki trehaloz ve glikojen miktarı stressiz koşulda göze çarpan bir şekilde yüksektir. Lityum glikojen ve trehaloz miktarını sodyuma göre daha fazla tetiklemektedir. Bu sonuç, lityumun yüksek toksisitesi ile açıklanabilir. Lityum ve sodyumlu stres ve stressiz koşullarda T8'deki trehaloz miktarı glikojene göre daha yüksektir.

NaCl ve LiCl varlığında ve yokluğunda T8 ve yabancı tipin metabolik ürün üretimi Yüksek Performanslı Sıvı Kromatografisi (HPLC) ile incelenmiştir. 0.5 M NaCl varlığında T8'in glukoz kullanımının ve etanol üretiminin yabancı tipe göre daha az olduğu ve en yüksek gliserol üretimine sahip olduğu gözlenmiştir. LiCl varlığında yabancı tipin glikoz kullanımının belirgin bir şekilde az olduğu ve en yüksek gliserol üretimine sahip olduğu gözlenmiştir.

NaCl dirençli evrilmiş *S. cerevisiae* suşu T8'de, yabancı tipe göre gliserol ve etanol üretimi daha azalmıştır. Evrilmiş mutantın metabolizması gliserola doğru ve etanol üretiminden uzağa doğru yönelmiştir.

Organizmalar strese karşı korunmak için birçok fizyolojik mekanizmalar kullanılmaktadır. Bu mekanizmalardan birisi çapraz direnç mekanizmasıdır. Bu mekanizmanın amacı bir çevresel faktöre kazanılan direnç sayesinde ikincil strese karşı direnç kazanılmasıdır. fakat, her strese başka bir strese karşı çapraz direnç sağlamamaktadır. YMM ve YPD katı besiyeri ortamında, T8'in LiCl, ZnCl_2 , H_2O_2 , CoCl_2 , KCl, etanol, CuCl_2 , MnCl_2 , CrCl_3 , AlCl_3 için çapraz direnci incelenmiştir. T8, lityum için yüksek çapraz dirence sahiptir. T8; Co^{+2} , Mn^{+2} , Ni^{+2} , Zn^{+2} ye az miktarda direnç gösterirken alüminyuma ise hassastır.

S. cerevisiae genomu hücre lokalizasyonu, sübstrat spesifikliğı ve fizyolojik fonksiyonları açısından birbirlerinden farklılaşan alkali metal katyon/ H^+ antiporterlarını (Nha1p, Nhx1p, Kha1p) kodlayan 3 gene sahiptir. *NHX1*, *NHA1*, ve *KHA1* genleri, mutant suşu T8 ile karşılaştırılmak için sekanslanmıştır. Sekanslama sonuçları, EMS

ve NaCl stresinin *NHX1*, *NHA1* ve *KHA1* gen sekanslarını etkilemediğini göstermiştir.

S. cerevisiae'nin yüksek NaCl stresine olan transkripsiyonal cevabı, DNA mikroarray kullanılarak analiz edilmiştir. Mikroarray sonuçlarında, enerji, hücre kurtarma, savunma ve virülans; metabolizma; hücresel transport, transport yolları; hücresel haberleşme/sinyal iletimi mekanizması dahil olmak üzere fonksiyonel kategorileri de içeren NaCl toleransı ile bağlantılı birkaç yüz gen tespit edilmiştir. Mikroarray sonucuna göre, 548 genin ekspresyonu artmış, 22 genin ise ekspresyonu azalmıştır. Hücresel transport ve transport yollarına ait genlerden ise 98 genin ekspresyonu artmışken 32 genin ekspresyonu azalmıştır. Flokülasyon ve biyofilm için gerekli olan GPI-bağlantılı hücre yüzeyi glikoproteini (flokulin) kodlayan *FLO11* geni T8'de en fazla eksprese olan genidir. T8'in mikroarray sonuçlarında, *HOG* yolizi indüklenmemiştir. T8 yavaş glukoz alımına sahip olup, yüksek seviyelerde süksinat ve asetat biriktirmemiştir.

FLO11 glukoz represyon yolizi ile de kontrol edilmektedir. Bu yolizinde, glukoz heksoz transporterları tarafından besiyerinden hücreye alınır ve hücre içinde glukoz heksokinazlar tarafından glukoz-6-fosfatta fosfatlanır. Bu fosfatlama Snf1 protein kinaz'ı ve Nrg1 inhibitör proteinini de inaktive eder. Bu inaktivasyon *FLO11*'in regulasyonunu baskılar. Glukoz miktarı az olduğunda Snf1 aktive olarak *FLO11* eksprese olur. *S.cerevisiae*'nin pH değişikliklerine cevabını kontrol eden transkripsiyon faktörü Rim101p hem *ENA6* hem de *FLO11* ekspresyonunu kontrol etmektedir. T8'de *HXT* genleri, *HXTK1*, *NRG1*, *FLO11* ve *ENA6* ekspresyonları artmıştır. Bu iki yolizi tarafında T8'in NaCl stresine direnci kontrol edilmesi muhtemeldir. Mikroarray sonucunda T8 indüklenmiş *ENA6* ekspresyonuna sahipken, *NHA1* indüklenmemiştir. Real-Time PCR sonuçlarında ise *ENA6* ve *NHA1* genleri indüklenmiştir. Bu sonuçların mikroarray ve real-time PCR arasındaki hassasiyet farklılıklarından kaynaklanması muhtemeldir, zira real-time PCR sonraki daha hassastır.

Mikroarray sonuçları T8'in pek çok geninin ortamda NaCl stresi yokken bile ekspresyonunun artmış olduğunu göstermektedir. Ekspresyonu artmış ve azalmış bu genler, T8'i yüksek sodyum ve lityum stresine adapte etmektedir.

Nha1 proteini daha fazla üretilmek için klonlanmış ve komplementasyon analizi ile kontrol edilmiştir. Nha1p'nin overekspresyonu göstermiştir ki protein en iyi pH 3.5'te komplement etmektedir. Nha1'e spesifik tek bölge mutasyonları [G372S, D266H, D267A, D266H/D267A, D266N/D267N, N176, D177N], bu mutasyonların pH üzerine olan etkisini anlamak için uygulanmıştır.

Bu çalışmada, gliserol üretimi artmış ve etanol üretimi azalmış *S.cerevisiae* suşlarının, tuz stresine direnç kazandırmaya yönelik evrimsel mühendislik yöntemiyle elde edilebileceği gösterilmiştir. Bu çalışma, EMS ve artan NaCl stres düzeyleri kullanılarak *S.cerevisiae* seleksiyonu için evrimsel mühendisliğin uygulandığı ilk çalışmadır. Ayrıca bu tez çalışmasının sonuçları, *FLO11*'in *S.cerevisiae*'nin yüksek tuz direncinde önemli bir rolü olabileceğine işaret etmektedir.

1. INTRODUCTION

1.1 The Model Organism *Saccharomyces cerevisiae*

Yeasts are in group of lower eukaryotic microorganisms, and are used in several industrial processes that include wine making, brewing, baking and the production of a variety of biochemical compounds (Boekhout and Robert, 2003).

Baker's yeast is one of the simplest eukaryotic organisms allowing simple cultivation, and many essential cellular processes are conserved between yeast and humans (Müller and Grossniklaus, 2010), (Figure 1.1).

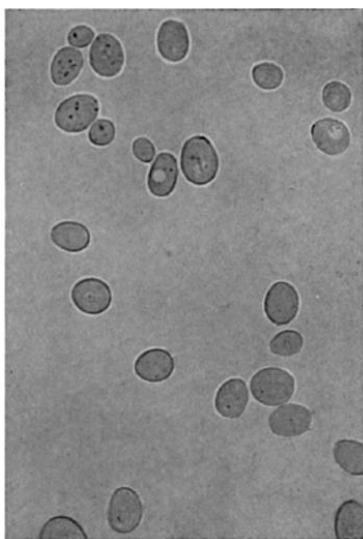


Figure 1.1 : Haploid cells of *Saccharomyces cerevisiae* (Barnett, 2007).

In the 1950s, Bob Mortimer set out yeast genome project. Genetic map of yeast could be at single nucleotide resolution and it could lead to the identification of all genes that comprise this relatively simple eukaryotic cell. Maynard Olson initiated its physical mapping in the late 1970s and DNA sequencing had begun in the late 1980s. The important thing is to understand the functions and inter-relationships of each of its ~6000 genes (Johnston, 2000). Øjvind Winge can be regarded as the founder of yeast genetics, as much of the pioneering and academic work was done in his group at the Carlsberg Laboratory in Copenhagen between 1935 and 1955 (Barnett, 2007). Many experimental techniques including four-strand crossing over, chromosome

mapping, the identification of lethal genes, and transformation of *Pneumococcus* made yeast a practical tool for genetics. Winge and many other scientists have improved knowledge of the genetics of the yeasts *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* and they made it possible to use these two yeasts for research on the cell cycle. The reasons for the budding yeast *S. cerevisiae* to be an excellent model system for eukaryotes are listed as follows: being a GRAS (Generally Regarded as Safe) organism, easy transformation, fast cultivation with few requirements, efficient expression of heterologous genes, cell morphogenesis, chromosome stability, and even aging and the many features shared with multicellular organisms (Yanagida, 2002; Müller and Grossniklaus, 2010).

S. cerevisiae CEN.PK 113-7D strain is widely used for metabolic engineering and systems biology research in industry and academia. A consortium of German yeast researchers developed the isogenic family of CEN.PK strains by crossing different laboratory strains of *S. cerevisiae* in the 1990s. Four *S. cerevisiae* strains compared in a subsequent multi-laboratory study confirmed that the CEN.PK strains combine good accessibility to classical and molecular genetics techniques with excellent growth characteristics under controlled, industrially relevant conditions. The haploid MATa strain CEN.PK113-7D, has been extremely popular for studies in systems biology (Nijkamp *et al.*, 2012). CEN.PK113-7D is a preferred laboratory strain for industrial biotechnology research (Otero *et al.*, 2010). The assembled sequence reveals that CEN.PK113-7D has a mosaic genome that combines characteristics of laboratory strains and wild-industrial strains (Nijkamp *et al.*, 2012).

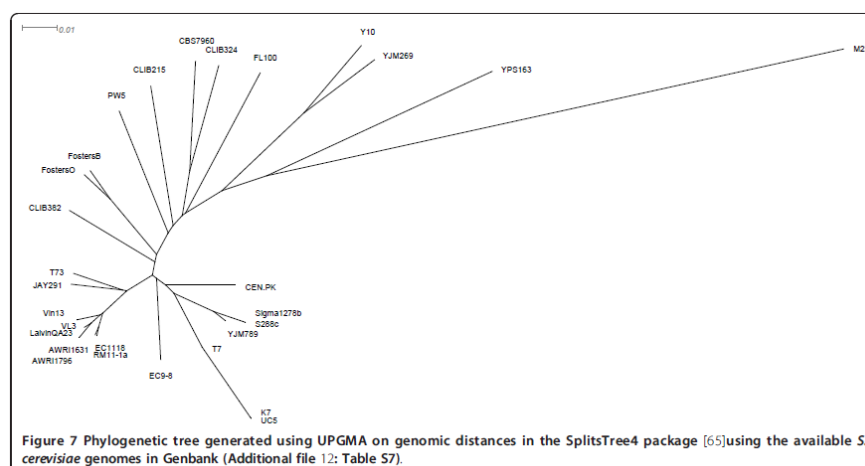


Figure 1.2 : Phylogenetic tree of *S.cerevisiae* strains (Nijkamp *et al.*, 2012).

Phylogenetic analysis aimed at comparing CEN.PK113-7D genome to the other *S. cerevisiae* genomes available in GenBank. Phylogenetic tree showed that the CEN.PK113-7D genome was located in between the laboratory strains (S288C and Sigma1278b) and industrial strains, such as the bioethanol (JAY291), wine (EC1118, RM11-1a, AWR11631, AWR11796, Vin13, VL3, T73 and Lalvin QA23) and beer (CLIB382, FostersO and FostersB) strains (Figure 1.2), (Nijkamp *et al.*, 2012).

The excellent growth characteristics of the CEN.PK strains have made it applicable for metabolic and evolutionary engineering studies, such as the fermentation of pentose sugars, production of ethanol and spirits, production of lactate and pyruvate, production of C4-dicarboxylic acids isoprenoids, and fungal polyketide (6-methylsalicylic acid) (Nijkamp *et al.*, 2012).

The major Na^+ - and Li^+ -exporting system in *S. cerevisiae* is the Na^+ -ATPase encoded by the *ENA/PMR2* locus (Kinclova *et al.*, 2002). The composition of the *PMR2* locus is strain-dependent and contains a cluster of one to five (*ENA1-5*) tandemly arranged and highly similar genes. The commonly used *S. cerevisiae* strains laboratory S288C, A364A and D273-10B contain five copies of *ENA* genes, DBY746 and W303.1A contain four copies and S1278b only contains a single *ENA* gene. CEN.PK's *PMR2* locus contains a single gene (*ENA6*) encoding a Na^+ and Li^+ pump similar to *ENA1*, 2 and 5 (Daran-Lapujade *et al.*, 2009).

The size of the *ENA* cluster varies according to strains. Na^+ and Li^+ tolerant strains contain more *ENA* genes (Arino *et al.*, 2010). CEN.PK strains, contain a single

atypical gene (*ENA6*) (Daran-Lapujade *et al.*, 2009). This could be the reason why CEN.PK derivatives are sensitive to Na^+ and Li^+ cations.

Osmoadaptation mechanisms have been studied in different yeast species such as *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, *Candida albicans*, *Zygosaccharomyces rouxii*, *Debaryomyces hansenii*. *Saccharomyces cerevisiae* is the most used yeast model. The reason why it is used mostly is the industrial interest, the large number of experimental tools, and the many laboratories worldwide studying it. Signal transduction pathways in osmoadaptation was also studied in fission yeast, *Schizosaccharomyces pombe*. Underlying mechanism of fission yeast and budding yeast is similar and claimed to be related to those of higher animals (Millar, 1999). *Candida albicans* is pathogenic to human health and osmoregulation mechanism of it is also studied. *Debaryomyces hansenii* is a highly sodium-tolerant marine yeast. *Zygosaccharomyces rouxii* is an important osmophilic food spoilage yeast (Blomberg and Adler, 1992).

1.2 Sodium Mechanism in *Saccharomyces cerevisiae*

1.2.1 Importance of NaCl

Sodium is an abundant cation in the environment, and sodium chloride is very important for human biology and the history of civilization. Several initial forms of word of economics were based on salt. Latin word of salt is *sal* and derivation of words of salary and soldier are *sal*. *Salus* means health in Latin. Although there are many elements in the earth crust, some of them are essential for human nutrition such as Na^+ and K^+ (Page and Di Cera, 2006).

1.2.2 Role of Na^+ and K^+

Family of alkali metals contain sodium and potassium, together with lithium, rubidium, cesium, and francium. They share similar properties and atomic structures and form monovalent cations. In cells, K^+ plays many important physiological roles such as negative charge compensation, activation of key metabolic processes, pyruvate synthesis and protein translation (Page and Di Cera, 2006). Maintenance of alkali cation homeostasis is a key process in most types of cells, including yeasts. The budding yeast *S. cerevisiae* is able to grow in the presence of a broad range of external concentrations of K^+ (10 mM–2.5 M) and Na^+ (1.5 M) (Marquina *et al.*, 2012). Na^+ is toxic at high levels and it substitutes for K^+ . Despite the much greater

abundance of Na^+ in the environment, yeast must maintain a high intracellular ratio of K^+/Na^+ . High intracellular ratio of K^+/Na^+ is achieved by selectively accumulating K^+ , while actively extruding Na^+ .

Role of K^+ is to balance charge across the plasma membrane, and to contribute to maintaining both intracellular pH and membrane potential. K^+ is continually taken up and extruded by cells, and both processes are important; membrane potential increases when potassium influx is crippled and decreases in cells defective for K^+ efflux (Madrid *et al*, 1998; Kinclova-Zimmermannova *et al*, 2006). Furthermore, K^+ efflux systems are regulated in response to changes in the membrane potential (Zahradka and Sychrová, 2012). Nha1 and Ena1 play primary roles in Na^+ transport, but also efflux K^+ (Kahm *et al*, 2012).

1.2.3 Na^+ entry into cells

Under standard growth conditions, cellular levels of Na^+ are low and Na^+ is not accumulated by yeast. However, when Na^+ levels are high in environment, cellular levels of Na^+ rise (Haro and Rodriguez-Navarro, 2002). To balance Na^+ levels, efflux mechanism of *S.cerevisiae* is triggered and uptake mechanism of *S. cerevisiae* is decreased. Efflux mechanism of *S.cerevisiae* is controlled by Pho89 and Pho84. Pho89 is highly specific for Na^+ , and is maximally active at alkaline pH (9.5) (Martinez and Persson, 1998). Pho84 mediates proton-coupled phosphate transport and is active at low pH (Pattison-Granberg and Persson, 2000).

1.2.4 Na^+ efflux

Two different mechanisms promote efflux of Na^+ , Li^+ , and to a lesser extent, K^+ ; 1) the Ena P-type ATPases and 2) the Nha1 antiporter. They both promote yeast growth in the presence of toxic cations. *S. cerevisiae* contains a group of tandemly repeated genes, *ENA1*–5 at chromosome IV. Products of *ENA* gene localize to the plasma membrane, and ATP is used to extrude Na^+ , Li^+ , and possibly K^+ (Cyert and Philpott, 2013).

Expression of *ENA1* is induced under alkaline conditions and controlled by signaling pathways including the osmotic stress-responsive HOG pathway, the Ca^{2+} -dependent calcineurin/Crz1 pathway and the Rim101 pathway. The Ca^{2+} -dependent calcineurin/Crz1 pathway promotes survival under a variety of stress conditions, and the Rim101 pathway modulates tolerance to NaCl and high pH as well as regulating

meiotic gene transcription, and nutrient-dependent pathways regulated by Snf1 and TOR. Ena-mediated Na⁺ and K⁺ efflux functions at high pH, Nha1 becomes essential in acidic pH (Arino *et al.*, 2010). *Nha1*, *Nhx1* and *Kha1* are also important for Na⁺ transport in *S.cerevisiae*.

Nha1 is a plasma membrane proton antiporter and extrudes Na⁺ and K⁺ with similar affinities and also provides alkali cations tolerance at acidic pH (Bañuelos *et al.*, 1998; Ohgaki *et al.*, 2005). Function of Nha1p is dimer and electrogenic. Nha1p exchanges multiple protons for each molecule of Na⁺ or K⁺ transported (Mitsui *et al.*, 2005; Ohgaki *et al.*, 2005). Nha1 is 985 amino acids in length; its overall structure is similar to other Na⁺/H⁺ exchangers (NHE) with a predicted short N-terminal cytosolic domain, followed by 12 membrane-spanning segments and a very long (551 amino acids) C-terminal cytosolic domain. Rapid adaptation to osmotic stress is controlled by Nha1 (Bañuelos *et al.*, 1998; Kinclova *et al.*, 2001; Proft and Struhl 2004), and it contributes to cell cycle regulation, and it is observed that Nha1 overexpression suppresses the G1 arrest of a phosphatase-deficient (*sit4 hal3*) strain (Simon *et al.*, 2003).

KHA1 encodes a Golgi-localized, 97-kDa protein with 12 predicted transmembrane domains that is related to bacterial K⁺ and Na⁺ transporters (Maresova and Sychrová, 2005). *Nhx1* encodes an endosomal Na⁺/H⁺ antiporter that is a member of the NHE superfamily and localizes mainly to the late endosome, as well as other secretory organelles (Nass and Rao 1998; Kojima *et al.*, 2012). *Nhx1* sequesters Na⁺ and K⁺ into secretory organelles and it utilizes the proton gradient established by the V-type ATPase for sequestration (Nass *et al.*, 1997; Brett *et al.*, 2005). *nhx1Δ* cells display growth defects in high salt or low pH conditions, and a decrease in intracellular pH (Nass *et al.*, 1997; Brett *et al.*, 2005). *Nhx1* was also identified in a screen for mutants with vacuolar protein sorting defects (Bowers *et al.*, 2000), and its role in determining the pH of endosomal compartments is critical to protein trafficking, where it may regulate the fusion of these compartments (Qiu and Fratti, 2010; Kallay *et al.*, 2011; Kojima *et al.*, 2012).

1.3 Osmotic and Salt Stress

Optimal growth is maintained by allowing the cell to maintain an optimal internal homeostasis. Stress means the lack of homeostasis. Yeasts are living in many variable environments and due to environmental changes yeasts develop mechanisms which

help them adapt and maintain their homeostasis. (Hohmann, 1997). Stress situation prevents or restricts growth of microorganisms. Since all biochemical reactions need water, survival of microorganisms stop when the water level is too low (Gervais *et al.*, 1996). When there is decrease of water in cell surrounding and increase of solute concentration, it means that osmotic stress occur. Increase of the concentration of various substances in the medium such as, sugars, polyols or ions generate osmotic stress. "Salt stress results from the presence of high salt concentrations and is distinguished from osmotic stress in the base of its effects on the cell, since it induces not only osmotic problems concerning the chemical species concentration, but also toxic effects related with the ion's properties" (Serrano, 1996).

Survival of sudden changes in the water activity and the acquisition of tolerance to low water activity are two important aspects of yeasts. The ability to survive a sudden change in water activity must be an intrinsic property of the cell. Survival mechanisms of yeasts need to respond within the first seconds after a sudden osmotic shift because fastness of passive water loss or uptake, and yeast cells have developed mechanisms to maintain or reestablish an inside-directed driving force for water (Somero *et al.*, 1997). NaCl is very commonly used in the laboratory to increase medium osmolality. NaCl also triggers osmotic responses as sugars or sugar alcohols at concentrations causing similar water activity (Rep *et al.*, 2000).

1.3.1 Strategies of yeast for survival in osmotic and salt stress

Microorganisms defend themselves from osmotic stress by two different strategies: 1) salt-in strategies 2) the compatible-solute strategy. In salt-in strategy, cells maintain a high intracellular salt concentration similar to the external concentration. In compatible-solute strategy, cells maintain a low salt concentration in their cytoplasm and accumulate low molecular weight solutes commonly termed osmolytes or compatible solutes (Oren, 1999). Salt-in strategy is used by aerobic extremely halophilic *Archaea* of the order *Halobacteriales* and, the anaerobic halophilic bacteria of the order *Haloanaerobiales* and the moderately halophilic aerobic bacteria (Ventosa *et al.*, 1998). All enzymes and cell components are adapted to high salt concentrations by using salt-in strategy (Oren, 1999).

Compatible-solute strategy has significant properties which are a minimal requirement for genetic change and a high degree of flexibility in allowing organisms to adapt to significant fluctuations in external osmolality. Yeasts are using

computible-solute strategy by accumulating osmolytes or compatible solutes (Jennings and Burke, 1990a). Compatible solutes work as osmotic balancers and protect enzyme function against stresses such as salinity, high temperature and freeze-thaw treatment. Osmolytes are amino acids, amino acid derivatives, potassium ions and polyalcohols, like glycerol, mannitol, arabinitol and erythritol. The compatible solute most used by yeasts is glycerol for salt stress. Glycerol is the osmolyte preferred for osmoregulation among eukaryotes. The reason why glycerol is the preferred one is structure and physical properties and its solubility in water is high (Oren, 1999).

Small molecules such as glycerol, sucrose, glycine betaine or ectoine, which is a novel cyclic amino acid, may be synthesized by microorganisms in the presence of high ionic stress in order to maintain osmotic balance with the extracellular medium (Yancey, 2005). In an alternative way, high levels of cytosolic K^+ are accumulated and higher levels of K^+ are allowed for adaptation of intracellular machinery in order to achieve osmotic balance (Vreeland, 1987). Growth of human pathogens such as *Vibrio cholerae* and *Candida albicans* may be inhibited when osmotolerance mechanisms are used as a therapeutic targets (Hase *et al.*, 2001).

1.3.2 Phases of the osmotic response

Three phases of yeast salt response are 1) dehydration, 2) triggering physiological and biochemical changes by regulation of gene expression 3) a state of balanced growth result (Mager and Siderius, 2002). The early (immediate) responses and the late (delayed) responses are responses of cells to stresses (Ruis and Schuller, 1995).

Two roles of the early (immediate) response is to provide minimal protection against sudden stress and initiate delayed response such as heat shock protein synthesis (Ruis and Schuller, 1995). Delayed responses are more effective on protecting cells and adapt cells to stress (Hohmann, 2002). Expression of many genes are induced to protect yeast from salt stress and these data showed that osmotic and salt response is a complex process with different mechanisms involved in determining the pattern of gene expression under conditions of hyperosmotic stress (Rep *et al.*, 2000; Posas *et al.*, 2000; Gasch *et al.*, 2000; Yale and Bohnert, 2001; Causton *et al.*, 2001).

1.4 Salt Response Pathways

Mitogen-Activated Protein (MAP) kinase cascades are highly conserved in all eukaryotes and these signaling pathways are also prominent in yeasts (Posas *et al.*, 1998). Five MAPKs are responsible in six pathways in *S.cerevisiae*. These six pathways are: the mating pathway, the filamentation-invasion pathway, the morphological switch pathway, the cell integrity pathway (or Protein Kinase C - PKC), the High- Osmolarity Glycerol pathway (HOG) and the STE vegetative growth pathway (Herskowitz, 1995; Gustin *et al*, 1998; Posas *et al.*, 1998; Lee and Elion, 1999; Elion, 2000). HOG pathway, PKC pathway and the calcineurin-mediated pathway are stimulated by osmotic and salt stress.

Osmotic shock triggers different pathways (Rep *et al.*, 1999; Rep *et al.*, 2000), but it seems that the HOG pathway is the dominant pathway for osmotic shock (Hohmann, 1997; Gustin *et al.*, 1998; Posas *et al.*, 1998; Hohmann, 2002). Sln1p (*Synthetic lethal of N-end rule*) and Sho1p (*Synthetic high osmolarity sensitive*) are two proteins in plasma membrane and are sensors of HOG pathway (Maeda *et al.*, 1994), and they are activated by a different range of solutes. Sln1 branch is known to be activated by a broad range of solute concentrations (O'Rourke *et al.*, 2002). When Sln1 branch is deleted, HOG pathway can be activated over 300 mM NaCl stress. Sln1 branch controls cell response to low NaCl stress. When Sho1 branch is deleted, Hog pathway activation starts at 100 mM NaCl (Hohmann, 2002). This HOG pathway is activated by NaCl, KCl, sorbitol or glucose (Brewster *et al.*, 1993; Davenport *et al.*, 1995). These data showed that increase in concentration of a specific solute activates HOG pathway (Tamas *et al.*, 2000). Na⁺, Li⁺ and Ca²⁺ activate HOG pathway in *S. cerevisiae* (Nakamura *et al.*, 1993; Aramburu *et al.*, 2000).

Calcineurin pathway mediates calcium ions by controlling P-type ATPases such as *ENA1* (*PMR2*) (Marquez and Serrano, 1996). Sodium tolerance in yeast is enhanced by continuous activation of calcineurin, which is required for modulation of the Na⁺ efflux mechanisms (Park *et al.*, 2001), and calcineurin pathway contributes to recovery from NaCl-induced osmotic stress (Hohmann, 2002).

General Stress Response is a global mechanism of yeasts for protecting cells from a number of different stress conditions (Hohmann, 1997; Causton *et al.*, 2001; Gasch *et al.*, 2000).

GSR is characterized by genes whose expression is induced by hyperosmotic stress, but also by other stress conditions such as heat stress, oxidative stress, nutrient starvation, weak acids and probably any condition that causes slow-down or arrest of cell proliferation. The expression of general responsive genes such *HSP12*, encoding a heat shock protein, and *CTT1*, encoding the Catalase T enzyme, are induced, suggesting that cytoskeleton alterations and oxidative species occur under osmotic stress (Rep *et al.*, 2000; Causton *et al.*, 2001; Yale and Bohnert, 2001).

1.5 Evolutionary Engineering

Metabolic engineering is the use of genetic engineering to manipulate cells with the objective to improve their properties for industrial applications (Bailey *et al.*, 1991; Stephanopoulos *et al.*, 1991). Evolutionary engineering is the application of a selection procedure to obtain a desired phenotype (Sauer *et al.*, 2001).

Evolutionary engineering is an inverse metabolic engineering approach for creating or improving desired microbial phenotypes (Patnaik, 2008). Strains with specific characteristics and/or complex phenotypes can be obtained by evolutionary engineering which is followed by high-throughput characterization and genome-wide microarray analysis of the desired phenotypes. Evolutionary engineering strategies have been successfully applied in biofuel research (Koppram *et al.*, 2012) or to obtain stress-resistant phenotypes and study their molecular mechanisms (Çakar *et al.*, 2005 and 2009; Küçükgöze *et al.*, 2013; Alkım *et al.*, 2013).

Yeasts are used for many biotechnological tasks such as production of biopharmaceutical proteins. Engineering microbial cells for biotechnological applications has been very important for about 25 years. Evolutionary engineering has major benefits compared to classical genetic engineering. Laboratory evolution experiments are based on early studies of Antonie van Leeuwenhoek, Louis Pasteur, Robert Koch and Charles Darwin. The first experimental study on microbial evolution was performed by Dr. William Dallinger, who investigated thermal adaptive change of protozoans with a short lifecycle. He cultured protozoans by gradually increasing their growth temperature for 7 years. Growth temperature was started at 16 °C and after 7 years protozoan cultures were able to grow at 70°C (Dallinger, 1887; Bennett and Hughes, 2009).

E.coli and *S.cerevisiae* were started to be used as a model organism in 1980s and 1990s (Bennett *et al.*, 1990; Paquin and Adams, 1983). The reasons why microbial cells were used were: 1) Simple nutrient requirements 2) easy cultivation in the lab 3) fast growth and cultivation for several generations within several weeks and months. For evolutionary engineering, time is an important criterion. Selection of phenotypes is affected by time, thus, selection experiments can be performed between 100 and 2000 generations (Dragosits and Mattanovich, 2013).

Cakar *et al.* (2012) reported that heterogenous populations are generally obtained during evolutionary engineering studies. Selection of the best phenotype depends on the highest fitness in certain conditions.

Table 1.1 : Evolutionary engineering experiments with yeasts exposed to environmental stress (Dragosits and Mattanovich, 2013).

Species	Environment	Selection time	Reference
<i>S.cerevisiae</i> CEN-PK EMS mutagenized	Chemostat and batch selection (multiple abiotic stresses)	Up to 68 generations	Cakar <i>et al.</i> , 2005)
<i>S.cerevisiae</i> CEN-PK EMS mutagenized	YMM, continuous and pulsed CoCl ₂ stress	25 transfers	Cakar <i>et al.</i> , 2009
<i>C. albicans</i>	fluconazole	330 generations	Selmecki <i>et al.</i> , 2009
<i>S.cerevisiae</i> BY4741	YP galactose medium, 0.5 M NaCl	300 generations	Dhar <i>et al.</i> , 2011
<i>S.cerevisiae</i> BL7	YP medium, 0-2.5 g L CuSO ₄	No data	Adamo <i>et al.</i> , 2012
<i>S.cerevisiae</i>	SD medium, 1.17% NaCl, 37 °C	25 generations	Gray and Goddard, 2012
<i>S.cerevisiae</i> W303	YP medium, 6-8% ethanol	141 generations	Avrahami-moyal <i>et al.</i> , 2012
<i>S.cerevisiae</i>	Salt and oxidative stress	300 generations	Dhar <i>et al.</i> , 2013

Improved phenotypes can be selected by mutations which are the basis of the genetic change. Also in evolutionary engineering experiments, various types of mutations were detected such as SNPs (single-nucleotide polymorphisms, small scale insertions and deletions (Conrad *et al.*, 2011). Additionally, chemical mutagens can be used to speed up the process of selection by increasing genetic diversity (Nielsen, 1998). Random mutagenesis is a common mechanism used for improved phenotypes in biotechnology. Jarosz *et al.* (2010) and Cowen *et al.* (2005) showed that protein

mutational robustness, protein folding and environmental stress also determine the evolution of new traits.

1.6 Trehalose and Glycogen

Glycogen and trehalose are two important glucose stores of the yeast *Saccharomyces cerevisiae*, and are accumulated in response to changing environmental conditions. Glycogen is a high molecular mass branched polysaccharide of linear α -(1,4)-glucosyl chains with α -(1,6)-linkages, whereas trehalose is a nonreducing disaccharide composed of two α -(1,1)-linked glucose molecules (François *et al.*, 2012).

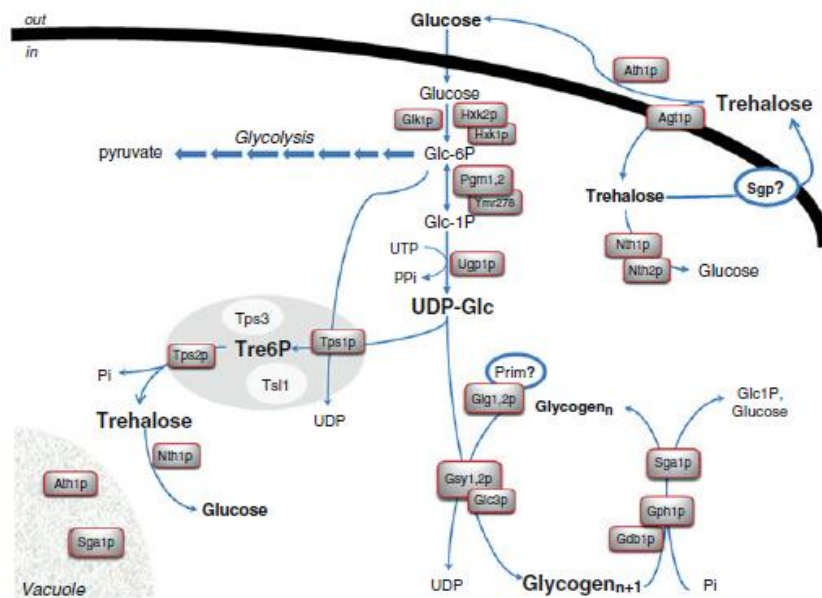


Figure 1.3 : The metabolic pathways for glycogen and trehalose biosynthesis and biodegradation and for trehalose assimilation in the yeast *S. cerevisiae*. Abbreviation of the enzyme name: Hxk2p, hexokinase II; Hxk1p, hexokinase I; Glk1p, glucokinase; Pgm1,2p, phosphoglucose mutase isoform 1 and 2; Ymr278p, homologous to phosphoglucomutase; Ugp1p, uridylylglucose pyrophosphorylase; Glg1,2p, glycogenin isoform 1 & 2; Gsy1,2p, glycogen synthase isoform 1 & 2; Glc3p, glycogen branching enzyme; Gph1p, glycogen phosphorylase; Gdb1p, glycogen debranching enzyme; Sga1p, amylo (1 → 4), (1 !→6) glucosidase; Ath1p, acid trehalase or extracellular trehalase; Agt1p, α -methylglucose transporter; Nth1, 2p, neutral trehalase isoform 1 & 2; Sgp1p, putative sugar permease; Tps1p, trehalose-6-phosphate synthase; Tps2p; trehalose- 6-P phosphatase; Tps3p & Tsl1p, regulatory subunit of the trehalose synthase complex (François *et al.*, 2012).

An initiator protein encoded by *GLGZ* and *GLG2* starts glycogen synthesis, and *GLG2* catalyses the formation of an $\alpha(1,4)$ glucosyl primer from UDP-glucose.

Elongation of this primer proceeds with glycogen synthase, which exists in two isoforms, encoded by *GSY1* and *GSY2*, and a branching enzyme encoded by *GLC3*, which introduces the $\alpha(1,6)$ branches on the mature glycogen. Gacp is a component of the protein-phosphatase complex and activates glycogen synthase by dephosphorylation. The breakdown of glycogen into Glc1P and free glucose occurs by the combined action of a glycogen phosphorylase encoded by *GPH* and a debranching enzyme carrying transferase/glucosidase activities (Parrou *et al.*, 1997).

Yeast glycogen metabolism responds to environmental stressors which is mediated by the regulation of the glycogen metabolic genes. Environmental stressors induce a number of glycogen metabolic genes, including *GPH1* which encodes glycogen phosphorylase. In addition to regulation by protein phosphorylation, yeast glycogen metabolism is regulated at the level of gene expression. The expression of genes involved in synthesis and degradation of glycogen, and the regulation of these pathways, is induced in early stationary phase.

GPH1 has TATA element in the upstream region. Mutation of the STREs showed that they were required for regulation of *GPH1* expression in early stationary phase, and in response to osmotic shock and heat shock. *GPH1* induction is related to HOG1 which encodes a MAP kinase. However, induction upon entry into stationary phase was only partially dependent on HOG1. Furthermore, the heat shock response, which can also be mediated by STREs, was independent of HOG1 (Sunnarborg *et al.*, 2001).

Examples of regulated genes include *GSY1* and *GSY2*, encoding glycogen synthase; *GPH1*, encoding glycogen phosphorylase *GLC3*, encoding the glycogen branching enzyme and *GAC1*, encoding the glycogen-binding subunit of protein phosphatase I. Stressors, such as nutrient limitation, induce the expression of a variety of yeast genes in addition to those involved in glycogen metabolism. Stress responsive genes are frequently those whose products exert a protective effect against damage to the cells by the stressor. Well-characterized examples include *CTT1*, encoding catalase T; *UBI4*, involved in ubiquitin-mediated protein degradation; *DDR2*, which is induced in response to DNA damage and *HSP12*, encoding a heat shock protein. We

have shown that one of the genes encoding glycogen synthase, GSY2, is also a member of this family. The response of these genes to stress requires the presence of a common stress response element (STRE) in their promoters. HOG1, which encodes a MAP kinase, participates in control mediated through STREs. Induction of *CTT1* and *HSP12* in response to osmotic shock was observed. The exact biochemical role of the HOG kinase in osmotic response has not been determined. The response of some STREs to osmotic shock has been shown to require a MAP kinase expressed from *HOG1* (Schüller *et al.*, 1994), although microarray analysis recently suggested that osmotic induction of the glycogen metabolic genes is *HOG*-independent (Posas *et al.*, 2000). Comparing osmotic shock induction of *GPH1*–lacZ in congenic *HOG1* wild-type and mutant strains, it is shown that the response of *GPH1* expression to osmotic shock does require a functional *HOG1* gene. *GPH1* expression responds to a variety of stressful conditions, such as growth into stationary phase, heat shock and osmotic shock. The HOG MAP kinase pathway is required for the STRE-mediated response of *CTT1*, *DDR2* and *HSP12* to osmotic shock. The response of *GPH1* to osmotic shock was also HOG-dependent, in contrast to conclusions from a microarray analysis study of saline-induced gene expression (Posas *et al.*, 2000).

At least two distinct pathways appear to control the response of *GPH1* to these stressors, one that is HOG-dependent and at least another one that is HOG-independent. Schüller *et al.* (1994) also found that *HOG1* was more stringently required for osmotic shock than for other stressors, for the regulation of *CTT1* expression. However, in contrast with *GPH1*, the *HOG* pathway did contribute to heat shock induction of *CTT1* (Schüller *et al.*, 1994; Sunnarborg *et al.*, 2001).

Glycogen metabolism is highly conserved from yeast to mammals. This conservation includes the protein sequences and the catalytic mechanisms for enzymes such as glycogen synthase and glycogen phosphorylase (Farkas *et al.*, 1990, 1991; Hwang and Fletterick, 1986). Regulation of enzyme activities is also conserved. For example, both yeast and mammalian glycogen metabolism are regulated by the phosphorylation of glycogen phosphorylase and glycogen synthase (Sunnarborg *et al.*, 2001). If there is a problem at the regulation of glycogen, glycogen storage diseases can be observed. Glycogen storage diseases are disorders which result from the deficiencies of glycogen synthesis or catabolism (Chen, 2011).

Wiggers discovered trehalose (alpha-D-glucopyranosyl-(1-1)-alpha-D-glucopyranoside), a nonreducing disaccharide of glucose, in 1832 in a mushroom crop and later in many other fungi, plants, and insects. Trehalose can accumulate up to 15% of the cell dry mass, depending on the growth conditions, the stage of the life cycle, or environmental stress in the yeast *S. cerevisiae*. Trehalose is immediately degraded by trehalase under normal conditions, so trehalose is not accumulated. This metabolic cycle, however, is important for glucose metabolism, because Tps1 controls the influx of glucose into the glycolytic pathway (Mulet *et al.*, 2004).

Moreover, the ability of this disaccharide to protect proteins and biological membranes against adverse conditions suggests that it also plays a stress-protectant role in this yeast and probably in other organisms that produce it (Jules *et al.*, 2004).

Intracellular levels of trehalose result from a well-controlled balance between enzymatic synthesis and degradation. In the yeast *S. cerevisiae*, the synthesis of trehalose is catalyzed by a UDP-glucose-dependent trehalose synthase (TPS) protein complex encoded by four genes. *TPS1* and *TPS2* encode trehalose-6-phosphate synthase and trehalose-6-phosphate phosphatase, respectively, while *TPS3* and *TSL1* code for two regulatory subunits of the TPS complex. Hydrolysis of trehalose into glucose can be carried out by at least two enzymes: a cytosolic or neutral trehalase encoded by *NTH1* and a vacuolar or acid trehalase encoded by *ATH1* (Jules *et al.*, 2004). The cytosolic neutral trehalase is subject to phosphorylation-dependent activation while *ATH1* is constitutively active. This differential regulation is necessary because trehalose lines the plasma membrane where it would be exposed to neutral trehalase; *ATH1* activity is sequestered within the vacuole. (Alizadeh and Klionsky, 1996)

The neutral trehalase Nth1p is responsible for the intracellular mobilization and/or recycling of trehalose. Lack of Ath1p does not alter intracellular levels of trehalose probably because the vacuolar localization of this enzyme prevents its action on cytosolic trehalose stores (Jules *et al.*, 2004).

There are two pathways to be used for trehalose degradation: one of them, the Agt1p-Nth1p-dependent route, is dependent on the genetic background of the strains and can be almost completely inactivated. The other one, the acid trehalase-dependent pathway thus appears to be the most important system for trehalose assimilation. As

yeast cells have very efficient hexose transporters, the rate of extracellular trehalose hydrolysis into extracellular glucose, which can be easily monitored by the amount of Ath1p at the level of gene expression, limits glucose flux into yeast cells (Jules *et al.*, 2004).

Blazquez *et al.*(?) found that T6P competitively inhibits the hexokinases from *S. cerevisiae*. They suggested that T6P “could play in yeast a role as feed-back inhibitor similar to that played by glucose 6-P in higher organisms”. T6P is the only reported physiological inhibitor of yeast hexokinase (Gancedo and Flores, 2004)

The loss of *TPS1* not only abolishes the synthesis of trehalose, but also causes several other metabolic disorders (Jules *et al.*, 2004). Two major problems need to be solved. The first one is to identify the alternative priming system that allows glycogen to be synthesized without glycogenin that also includes the elucidation of the stochastic nature of this alternative mechanism. Answering this question can be expected to have a strong impact on human glycogen and its related metabolic disorders such as glycogen storage diseases (François *et al.*, 2012).

The trehalose biosynthetic pathway is an attractive potential specific target for the development of antifungal drugs due to its absence in vertebrates; therefore the effects of the *tps1* mutation have been studied in some pathogenic yeasts (Zaragoza *et al.*, 1998).

A strong relationship was found between intracellular trehalose levels and resistance to osmotic stress in yeast. The mutant strains unable to produce trehalose were more sensitive to severe osmotic stress than the isogenic wild-type strain, confirming a role for trehalose in survival (Hounsa *et al.*, 1998).

1.7 Structural and Functional Properties of Na^+/H^+ Antiporter

Na^+/H^+ antiporters were first described by West and Mitchell and are found in the cytoplasmic membranes of almost all cells and in many organellar membranes. The NhaA Na^+/H^+ antiporter is essential for adaptation of the enteric pathogen *E. coli* to alkaline pH in the presence of Na^+ but not in the absence of added Na^+ . The K^+/H^+ antiport activity of a $\text{K}^+(\text{Na}^+)(\text{Ca}^{2+})/\text{H}^+$ antiporter, ChaA, and of the MdfA antiporter, which has both multi-drug/ H^+ and $\text{K}^+(\text{Na}^+)/\text{H}^+$ antiport capacities, could support pH homeostasis under those conditions. Homologues and orthologues of NhaA with

related functions are present in other bacteria, as well as in humans. The human genome has NHE type antiporters of which hNHE1 defects lead to heart hypertrophy, ischemia, and reperfusion. hNHA2 may be involved in human essential hypertension (Krulwich *et al.*, 2011).

1.8 Diseases Related to Na⁺-ion

Ionic balance is regulated in humans by several mechanisms. The concentration of Na⁺ in the human cardiovascular system is regulated by control of water flux. Electrolyte balance is controlled by hormone signaling between the hypothalamus, adrenal cortex, heart, and kidney which is mediated through vasopressin (antidiuretic hormone), aldosterone, and atrial natriuretic peptide (ANP) (Page and Di Cera, 2006). Osmoreceptors sense changes in osmolarity of the extracellular fluid and they control vasopressin release from the hypothalamus. After V2 receptors are activated by vasopressin, the volume of circulating water is increased by acting on renal collecting ducts. When they are directed to cell membranes by aquaporin channels, cell permeability and reabsorption are increased (Page and Di Cera, 2006). Epithelial sodium channel (ENaC) has an important role in the regulation of sodium transport, sodium balance and maintenance of the proper level of Na⁺ in the kidney. Ion homeostasis is maintained by ENaC which transports Na⁺. In all vertebrates, the first step of active Na⁺ reabsorption, electrolyte and water homeostasis are mediated by ENaC. ENaC channel is activated in response to dietary Na⁺ intake (Garty and Palmer, 1997). Genes involved in ENaC trafficking are associated with diseases and linked with hypertension and hypotension (Lifton *et al.*, 2002). Excess sodium intake affects human health negatively and it may cause hypertension and other health problems. Hyponatremia is caused by low serum Na⁺ concentration and is the most common electrolyte disorder. It affects 1–5% of all hospital patients. Life-threatening hyponatremia may be caused by Vigorous exercise, such as marathon running (Page and Di Cera, 2006).

1.9 The Aim of The Study

The aim of the study was firstly to characterize a salt-resistant *S.cerevisiae* mutant which has been previously obtained by evolutionary engineering, and to clarify important pathways of salt resistance mechanism, and their interaction with the

environment. The second aim was to clone the Na^+/H^+ antiporter which is known to have an important function for salt tolerance and Na^+ -related diseases and to check its pH influences.

2. MATERIALS AND METHODS

2.1 Materials

2.1.1 Strains and glycerol stocks

Strains of *Saccharomyces cerevisiae* used in this study were: CEN.PK 113.7D (*Mata URA3 HIS3 LEU2 TRP1 MAL2-8c SUC2*), AB11c (*MATa ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 mall0 ena1Δ::HIS3::ena4Δnha1Δ::LEU2 nhx1Δ::TRP1*), KTA40-2 (*MATa ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 mall0 ena1Δ::HIS3::ena4 Δnha1 Δ::LEU2 nhx1Δ::TRP1 kha1Δ::kanMX*) (Maresova and Sychrova, 2005), FGY217 (*MATa ura3-52 lys2Δ201 pep4Δ*) (Kota *et al.*, 2007).

The AB11c strain resulted from a cross between AXT3 and W303-1A (Wallis *et al.*, 1989). The AXT3 strain (Quintero *et al.*, 2000) was white and grew better on standard media than its parental strain W303-1B, although both strains harbour the *ade2* mutation causing the accumulation of a toxic red pigment (Woods, 1969). Therefore, the AXT3 was crossed with W303-1A. Resulting diploids were sporulated and subjected to tetrad analysis in order to select a pink haploid strain with deletion of genes for Na⁺ transport (*ena1 Δ::HIS3::ena4 Δ nha1 Δ::LEU2 nhx1 Δ::TRP1*) and growth characteristics identical to that of W303-1A. The KTA40-2 resulted from a cross between AB11c and MA-KN (*ena1Δ::HIS3::ena4 Δnha1 Δ::LEU2 kha1Δ::KanMX*) (Maresova and Sychrova, 2005). PLY127 was transformed with a *EcoRI-XhoI* DNA fragment containing *pep4Δ::hisG-URA3-neo-hisG* from pAS173 and a Ura⁺ transformant was propagated on medium containing 5-fluoroorotic acid (FOA), resulting in the unmarked *pep4Δ::hisG* strain FGY217 (Kota J., 2007). *Escherichia coli* DH5α cells were used for transformation experiments.

Culture stocks of CEN.PK 113.7D were prepared from 1 mL of exponentially growing cells harvested in 1.5 mL microfuge tube at 10000 *g* for 5 min. The supernatant was discarded and the pellet was resuspended in 30% (v/v) glycerol. Stock cultures of AB11c, KTA40-2 and FGY217 were prepared by spreading

cultures on plate, removing the entire grown culture from plate and resuspending it in a 1.5 mL microfuge tube containing 750 μ L YPD, 250 μ L 80% (v/v) glycerol and 35 μ L dimethyl sulfoxide (DMSO). Cell stocks were stored at -80 °C.

2.1.2 Media

Table 2.1 : Yeast Minimal Medium (YMM) contents.

Content	Percentage in YMM (w/v)
Glucose	2.00 %
Yeast Nitrogen Base without amino acids	0.67 %
Agar (for solid media)	2.00 %

Table 2.2 : YPD (YEPD, YP Dextrose) medium contents.

Content	Percentage in YPD (w/v)
Glucose	2.00 %
Peptone	1.00 %
Yeast extract	1.00 %
Agar (for solid media)	2.00 %

Table 2.3: Luria Bertani (LB) medium contents.

Content	Percentage in LB Medium (w/v)
Tryptone	1.00 %
Yeast Extract	0.50 %
NaCl	0.50 %
Agar (for solid media)	2.00 %

Table 2.4 : Drop-out medium contents.

Content	Percentage in Drop-out Medium (w/v)
Glucose	2.00 %
Yeast Nitrogen Base without amino acids	0.67 %
Synthetic Complete Drop Out Mix	0.192 %
Agar (for solid media)	2.00 %

2.1.3 Chemicals, solutions, buffers, kits, enzymes and equipment

Table 2.5 : Chemicals used in this study.

Chemicals	Company	Country
5-fluoro-orotic acid	Euromedex	France
Acetic acid glacial	MERCK	Germany
Agarose	Applichem	Germany
Aluminium Chloride hexahydrate, $\text{AlCl}_3\cdot 6\text{H}_2\text{O}$	MERCK	Germany
Ammonium iron (II) sulfatehexahydrate, $(\text{NH}_4)_2\text{Fe}(\text{SO}_4)_2\cdot 6\text{H}_2\text{O}$	MERCK	Germany
Ammonium sulphate	VWR BDH PROLABO	UK
Ampicillin	Roche	Switzerland
Amyloglycosidase (3500 U)	Roche	Switzerland
Bacto Peptone	BD DifcoTM	USA
Calcium Chloride hexahydrate, $\text{CaCl}_2\cdot 6\text{H}_2\text{O}$	Sigma ALDRICH	USA
Cobalt chloride, CoCl_2	Fluka	USA
Copper (II)Sulfatepentahydrate, $\text{CuSO}_4\cdot 5\text{H}_2\text{O}$	Sigma ALDRICH	USA
DNA markers	Fermentas	Lithuania
Ethanol	J.T.Baker	Netherlands
Ethylenediaminetetraacetic acid disodium salt dihydrate, EDTA	MERCK	Germany
Glucose	VWR BDH PROLABO	UK
Glucose Standard Solution	Sigma	USA
Glycerol	Sigma ALDRICH	USA

Table 2.5 (continued) : Chemicals used in this study.

Chemicals	Company	Country
Glucose oxidase/peroxidase reagent	Sigma ALDRICH	USA
Hydrogen peroxide	MERCK	Germany
Lithium acetate	Sigma ALDRICH	USA
Lithium Chloride anhydrous, LiCl ₂	Sigma ALDRICH	USA
Magnesium Chloride hexahydrate, MgCl ₂ ·6H ₂ O	MERCK	Germany
Manganese (II) Sulfate monohydrate, MnSO ₄ ·H ₂ O	Sigma	USA
NaCl	Carlo Erba	Italy
NaOH	Carlo Erba	Italy
Nickel(II)Chloride hexahydrate, NiCl ₂ ·6H ₂ O	MERCK	Germany
o-Dianisidinedihydrochloride	Sigma	USA
PEG-4000	Sigma	USA
Potassium acetate	MERCK	Germany
Potassium acetate	Sigma ALDRICH	USA
RNase A	Roche	Switzerland
Salmon Sperm DNA	Sigma	USA
SDS	Sigma ALDRICH	USA
Sorbitol	MERCK	Germany
Trehalase (from porcine kidney)	Sigma	USA
Tris-HCl	MERCK	Germany
Tryptone	MERCK	Germany
X-gal	Fermentas	Lithuania
Yeast Extract	MERCK	Germany
Yeast nitrogen base without amino acid	BD Difco™	USA
Zinc Sulfateheptahydrate, ZnSO ₄ ·7H ₂ O	Sigma ALDRICH	USA

Table 2.6 : Name and content of the solutions and buffers.

Buffer/Solutions	
Potassium phosphate buffer	50 mM, pH 7
Sodium thiosulfate	10% 'w/v'
Glycerol	30% 'v/v'
Solution I	50 mMTrisHCl pH8, 10 mM EDTA, 100mg/L RNase A
Solution II	200 mMNaoH, 1 % SDS
Solution III	Potassium Acetate, Acetic acid glacial

Table 2.7 : Laboratory equipment used in this study.

Equipment	Company	Country
Autoclave	Tuttnauer Systec Autoclave 2540ml - 2870ELVC	Switzerland
Autoclave	Tomy SX 700E	China
Balance	Precisa BJ 610 C - 620 SCS	Switzerland
Benchtop Centrifuge	Beckman Coulter Allegra 25R	USA
Bioanalyser 2100	Agilent Technologies	USA
Deep Freezer	Sanyo (-80°C) Ultra Low MDT- U40865	Japan
Refrigerator	Arçelik 3011 NY	Turkey
Digital Color Camera	Leica, DFC 300FX	Germany
Electrophoresis Tank	Midicell PrimoTM EC 320 - 330	USA
Flame Atomic Absorption Spectrometer	Varian AA 280FS	USA
Glass slides for microarray analysis	Agilent	USA
Hybridization Chamber	Discovery from Ventana Medical System	USA
Incubator	Nüve EN400 - EN500	Turkey
Laminar Flow Hood	Biolab Faster BH-EN 2003	Italy
Light Microscope	Olympus CH30	USA
Magnetic Stirrer	Labworld	Germany
Microfuge	Eppendorf Centrifuge 5424	Germany
Micropipettes	Eppendorf	Germany
Microplate Reader	Biorad Model 3550 UV	USA
NanoDrop 1000 Spectrophotometer	Thermo SCIENTIFIC	USA

Table 2.7 (continued) : Laboratory equipment used in this study.

Equipments	Company	Country
Objective	Leica, 0.6 Oil HCX Plan-Apochromat	Germany
Orbital Shaker	Certomat S II	Germany
Incubators	Sartorius	
pH-meter	Mettler Toledo MP220	Switzerland
Power supply for Electrophoresis	EC Apparatus Corporation EC 250-90	
Real Time PCR equipment	Roche, Light Cycler 480 II	Switzerland
Rotor	Beckman Coulter JA-30.50i	USA
Spectrophotometer	Shimadzu UV-1700	Japan
Thermal cycler	Bio-Rad My cycler TM	USA
Thermal Cycler	TC-412 Techne	USA
Thermomixers	Eppendorf, Thermomixer Comfort	Germany
	Eppendorf, Thermomixer Compact	Germany
Ultracentrifuge	Beckman Coulter, Avanti J-30I	USA
Ultrapure Water System	USF-Elga UHQ	USA
Vortex mixer	Heidolph	Germany
Water Bath	Memmert wb-22	Switzerland

Software and websites used in this study are demonstrated in Table 2.8.

Table 2.8 : Software and websites used in this study.

Software and websites	
Microsoft Office 2007	
Primer3Plus	http://frodo.wi.mit.edu/primer3/
<i>Saccharomyces</i> genome database	http://www.yeastgenome.org/
YEASTRACT	http://www.yeasttract.com
FunSpec - Web-Based Cluster Interpreter of Yeast	http://funspec.med.utoronto.ca/
A five-tube MPN table	http://www.jlindquist.net/generalmicro/102dil3a.html

Table 2.9 : Kits and enzymes used in this study.

Kits and Enzymes	Company	Country
Fusion DNA polymerase	FINNZYMES, No. F-540S	Finland
High Pure RNA Isolation Kit	Roche	Switzerland
IQ™ SYBR® Green	BIO-RAD	USA
Supermix		
iScript™ cDNA Synthesis Kit	BIO-RAD	USA
Label Star Reverse Transcriptase	QIAGEN	Germany
Light Cycler 480 SYBR Green I Master	Roche	Switzerland
MinElute spin columns	QIAGEN	Germany
QIAquick Gel Extraction Kit	QIAGEN, No. 28706	Germany
SV Total RNA Isolation System	Promega	USA
T4 DNA ligase	Biolabs, No. M0202S	USA
TaqDNA polymerase	Biolabs, No. M0267S	USA
Transcriptor High Fidelity cDNA Synthesis Kit	Roche	Switzerland
Yeast DNA Purification Kit (MasterPure™)	EPICENTRE, MPY2-70921	USA

2.2 Methods

2.2.1 Cultivation of the cells

2.2.1.1 Regular growth of the yeast and bacteria cultures

-80°C stock cultures of CEN.PK 113.7D were inoculated into 50-mL culture tubes containing 10 mL YPD. After overnight incubation, cultures were inoculated in 100 mL YMM in 500 mL flasks at an initial OD₆₀₀ value of 0.25. Incubation of cultures was carried out at 30°C at a shaking speed of 150 rpm.

-80°C stock cultures of FGY217, KTA40-2 and AB11c were inoculated into 50-mL culture tubes containing 10 mL YPD. Incubation of cultures was carried out at 30°C at a shaking speed of 200 rpm. One-fifth of the flask volume was used for the medium. After overnight incubation, cultures were inoculated in 10 mL YPD or 200 mL YPD at an initial OD₆₀₀ value of 0.2.

Escherichia coli cells were grown in LB medium at 37°C at a shaking speed of 200 rpm.

2.2.2 Growth Analysis

2.2.2.1 Spectrophotometric measurements

OD₆₀₀ values were measured by using Shimadzu UV-1700 Spectrophotometer (Japan).

2.2.2.2 Maximum specific growth rate determination

Plotting OD₆₀₀ values versus time were used for drawing growth curves of the cultures. The maximum specific growth rate ($\mu_{\max}$), which corresponds to the maximum slope from this semi-logarithmic representation were calculated by the natural logarithm of the OD₆₀₀ values.

2.2.2.3 Cell dry weight determination

Firstly, 1.5 mL microfuge tubes were dried in an oven at 80 °C for 24 h. After two days of drying, they were placed in desiccators (Finemech Bola-Star Vitrium, USA) for 30 minutes to cool down to room temperature and then weighed. During the growth curve experiment, 1.5 mL of samples were taken for at 6 h and 1 mL of samples were taken at 7.5 h, 9 h, 10.5 h, and 24 h samples into dried microfuge tubes. Cells were centrifuged at 14000 rpm for 5 minutes and supernatants were discarded. The tubes containing cells were dried in an oven at 80°C to evaporate the remaining liquid on the cell pellet. After 24 h of drying, the tubes with dried cell extracts were again placed in a desiccator for 30 minutes and then weighted. The cell dry weight (CDW) was calculated by subtracting the weight of empty tubes from the weight of tubes containing cells.

2.2.3 Determination of stress resistances

2.2.3.1 Most probable number (MPN) assay

Resistance against various stress conditions was determined by using Most probable number (MPN) assay (Russek and Colwell, 1983). Viable cell counts were determined by serial dilutions in 96-well plates containing 180 μ L YMM and 20 μ L cells. Dilutions were made in the range of 10^{-1} to 10^{-8} for five biological repeats. The most probable number of survivors based on the ability of yeast cells to grow at higher dilutions were estimated by using published MPN tables (Lindquist, 2008). Survival rate was calculated by dividing the number of stress-treated viable-cells to that of non-treated cells to compare the resistance to various stress types.

2.2.3.2 Spot Test

2.2.3.2.1 Spot test of CEN.PK 113.7D

Spot test was used to determine the resistances or sensitivity of yeast strains to various stress conditions. Cells were cultivated in 10 mL liquid YMM placed in 50 mL culture tubes at 30°C and 150 rpm until logarithmic growth phase of growth. After growing them until OD₆₀₀ value of 1.2, equal numbers of yeast cells were harvested by centrifugation at 10000 rpm for 5 min and the supernatant was removed. The pellets were resuspended in 100 µL water and diluted serially as 10⁻¹, 10⁻², 10⁻³ and 10⁻⁴ in 96-well plates. Three µL from these diluted suspensions were dropped onto solid control plates and onto plates containing stress factors in their media. They were incubated at 30°C.

2.2.3.2.2 Spot test of FGY217, KTA40-2, AB11c

-80°C stock cultures of yeast cells were inoculated into 50-mL culture tubes containing 10 mL YPD. After overnight incubation, cultures were inoculated in 10 mL YPD in 50 mL flasks at initial OD₆₀₀ value of 0.2. After growing them until OD₆₀₀ value of 0.5-0.6, equal numbers of yeast cells were harvested by centrifugation at 2500 rpm for 10 minutes at room temperature. The pellets were resuspended in 200 µl medium. Cells were diluted serially between 10⁻¹ to 10⁻³ in 96-well plates. Three µL from these diluted suspensions were dropped onto solid control plates and onto plates containing NaCl in their media. Each strain was dropped in one plate four times. They were incubated at 30 °C.

2.2.4 Growth Analysis of Wild Type and Salt resistant Mutant Strain ‘T8’

Precultures were grown overnight, at 30 °C and 150 rpm. These cultures were used to inoculate 100 mL YMM with and without NaCl and LiCl in 500 mL flasks starting with an OD₆₀₀ value of 0.25. Those flasks were incubated at 30 °C, 150 rpm. Growth was monitored by optical density measurements at 600 nm (OD₆₀₀) using Shimadzu UV-700 (Japan) spectrophotometer.

2.2.5 Proton efflux

After cells were grown to mid-log phase (OD₆₀₀= 0.6), they were washed three times with distilled water by centrifugation at 14000 rpm for 2 minutes. Then, they were stored on ice for at least 1 h. Cells were pelleted by centrifugation at 14000 rpm for 5 minutes and resuspended in 2 mL of a 10-mM glycylglycine solution (pH 4.5

adjusted with HCl) containing 50 mM KCl. Glycyl-glycine solution (6 mL) was added to 1.8 mL of cell suspension and pH was monitored with constant stirring until a stable baseline was reached. Glucose was then added to a final concentration of 20 mM. The pH was recorded every 10 s with a pH-meter GLP21 (CRISON) and the slope was calculated. All experiments were repeated at least three times and the standard deviations were calculated (Naverrete *et al.*, 2010)i

2.2.6 Analytical procedures

2.2.6.1 Determination of Metabolite Contents by HPLC

While collecting samples for the growth curve of the wild type and the mutant, simultaneously, 1 mL samples from all cultures were taken into microfuge tubes with known-weight (which were weighed for cell dry weight experiment) with 1.5 h time intervals and the flasks were replaced to 30 °C immediately. After centrifugation (13000 rpm, 5 min), the supernatants were taken with a syringe, filtered using a 0.22 µm pore filter and transferred to new microfuge tubes. Those supernatants were kept at -20 °C for further High Performance Liquid Chromatography analysis. HPLC analysis of metabolites was performed by using the Aminex[®] HPX-87H column which was eluted with H₂SO₄ (5 mM) at a flow rate of 0.6 mL/min and at 60 °C. Injection volume per sample was adjusted as 20 µl. Determination of metabolic compositions were done by comparison of 6 known standards. Calibration curve was obtained by analyzing standard solutions. By using that calibration curve, the concentrations of metabolites in each sample were determined as g/L.

Stock solutions components and amounts are listed in Table 2.10. 5 mM sulphuric acid (H₂SO₄) was used as the eluent.

Table 2.10 : Stock Solutions of HPLC Standards.

SOLUTION A	
Component	Amount
Glucose	120 g
in 1 liter of distilled water (ddH ₂ O)	
SOLUTION B	
Component	Amount
Acetate	4 g
Glycerol	2 g
Ethanol	30 g
in 1 liter of distilled water (ddH ₂ O)	
SOLUTION C	
Component	Amount
Maltose	20 g
Citric Acid	20 g
Succinic Acid	20 g
in 1 liter of distilled water (ddH ₂ O)	

Standard solutions for HPLC standard curve were prepared according to the following dilutions given in Table 2.11 and Table 2.12.

Table 2.11 : HPLC Standard Solution Dilutions (Std: Standard Solution) (Solution A&B).

Standard Solutions	Mixing Volumes		Final Volume
Standard 1	1 mL Solution A 3 mL Solution B	2 mL Eluent	6 mL
Standard 2	0.75 mL Std 1	0.25 mL Eluent	1 mL
Standard 3	0.50 mL Std 1	0.50 mL Eluent	1 mL
Standard 4	0.25 mL Std 1	0.75 mL Eluent	1 mL
Standard 5	0.125 mL Std 1	0.875 mL Eluent	1 mL
Standard 6	0.063 mL Std 1	0.937 mL Eluent	1 mL

Table 2.12 : HPLC Standard Solution Dilutions (Std: Standard Solution) (Solution C).

Standard Solutions	Mixing Volumes		Final Volume
Standard 1	1 mL Solution C	-	1 mL
Standard 2	0.75 mL Std 1	0.25 mL Eluent	1 mL
Standard 3	0.50 mL Std 1	0.50 mL Eluent	1 mL
Standard 4	0.25 mL Std 1	0.75 mL Eluent	1 mL
Standard 5	0.125 mL Std 1	0.875 mL Eluent	1 mL
Standard 6	0.063 mL Std 1	0.937 mL Eluent	1 mL

Concentrations of metabolites in the prepared standard solutions are shown in Table 2.13.

Table 2.13 : Metabolite Concentrations in HPLC Standard Solutions (Std: Standard Solution, RT: Retention Time, RI: Refractive Index).

Metabolite (g/L)	Std1	Std2	Std3	Std4	Std5	Std6	RT (min)	Detector
Glucose	20	15	10	5	2.5	1.25	9.43	RI
Glycerol	1	0.75	0.5	0.25	0.125	0.0625	13.52	RI
Acetate	2	1.5	1	0.5	0.25	0.125	14.83	RI
Ethanol	15	11.25	7.5	3.75	1.875	0.9375	19.65	RI
Maltose	20	15	10	5	2.5	1.25	6.95	RI
Citric Acid	20	15	10	5	2.5	1.25	7.54	RI
Succinic Acid	20	15	10	5	2.5	1.25	10.948	RI

2.2.7 Quantitative assessment of glycogen and trehalose contents

The procedure was performed as described previously (Parrou and François, 1997). Briefly, the cell pellet (collected from 25 OD₆₀₀ units of culture) was suspended in 250 µL 0.25 M Na₂CO₃ in screw-top microfuge tubes (Corning, USA). After vortexing, tubes were placed to a thermomixer tube to be heated at 95 °C for 4 h. Then, 150 µL 1 M acetic acid was added to the mixture, and subsequently 600 µL of 0.2 M sodium acetate buffer (pH 5.2) was added into the cell suspension. Half of this mixture (500 µL) was incubated overnight at 57 °C in the presence of 100 mg of amyloglucosidase (Roche, Switzerland). The second half of the mixture (500 µL) was incubated overnight at 37 °C in the presence of trehalase (Sigma). The next day, suspensions were centrifuged for 2 min at 3500 rpm, and 50 mg/ml glucose solution was prepared stock glucose solution. By using this stock solution, 0.5 mg/ml, 0.25 mg/ml, 0.125 mg/ml, 0.0625 mg/ml, 0.03125 mg/ml and 0.0156 mg/ml standard solutions were prepared. 200 µl of glucose oxidase/peroxidase reagent was delivered to the wells of the microplate (This reagent is prepared by solubilizing 1 tablet of glucose oxidase/peroxidase reagent in 39.2 ml water. Then 1 tablet of o-Dianisidine dihydrochloride was dissolved in 1 ml water and 400 µl of it was added to glucose oxidase/peroxidase reagent). 20 µl standards, water and all samples were delivered to the wells of the 96 well plate. Following 30 min incubation at 37°C, the absorbance of the samples was measured at 420/490 nm. The released glucose was determined using the glucose oxidase/peroxidase method (Cramp, 1967).

2.2.8 Metal content determination by Flame Atomic Absorption Spectrometer (FAAS)

Wild type (905) and salt resistant mutant (T8) were inoculated into 50 mL yeast minimal medium (YMM) in 250 mL flasks at an initial OD₆₀₀ value of 0.35 ($2-4 \times 10^6$ cells/mL). When their OD₆₀₀ was about 3 (3×10^7 cells/mL), they were transferred to 10 mL fresh YMM in 50 mL culture tubes.

Cultures were incubated for 24 hours at 30°C, 150 rpm in the absence of the stresses and in the presence of 0.7 M NaCl, 5 mM LiCl, 1M KCl, both 0.7 M NaCl and 5mM LiCl, both 0.7 M NaCl and 1M KCl, both 1M KCl and 5mM LiCl. Three tubes were prepared for each condition. After incubation, cells were centrifuged at 4000 rpm for 10 min. The supernatants were discarded and the cell pellets were washed twice by using autoclaved distilled water to separate cells from any residual NaCl, LiCl and KCl. 42 of 50 mL test tubes were labeled for each stress condition via three sets of each condition and were weighed out. Then, these empty test tubes were dried at 80°C for 16 h. The 24-hour incubated cells were transferred into these dry tubes and centrifuged for 10 minutes at 5000 rpm. Discarding the supernatants, the pellets of cells were washed with ddH₂O to remove NaCl, LiCl and KCl residues left on the cells. Then, washed cell pellets were dried for 16 hours at 80°C and cooled in a dessiccator for 30 minutes. The cells were then hydrolyzed with 10 M HNO₃ solution for two hours at 90°C.

After the lysis of the cells, 5 mL of dH₂O were put into each test tube and the potassium, sodium and lithium content of the cells were measured via flame atomic absorption spectrometer (FAAS) (Varian AA 280FS (USA)). Measurements were carried out at 766.5 nm, 589 nm and 670.8 nm wavelengths for potassium, sodium and lithium, respectively. Cell dry weights were determined as mg/mL. These experiments were repeated three times and the standard deviations were calculated. Cation values were expressed as millimoles per milligram of dry weight of cells.

2.2.9 Microarray analysis

2.2.9.1 RNA isolation for the microarray experiment

Cultures of wild-type and T8 were grown at 30 °C, 150 rpm in 100 mL of YMM in 500-mL flasks as three replicates. Initial OD₆₀₀ of cultures were 0.1 and they were grown until their OD₆₀₀ values reached ~1 (5×10^7 cells/ml). The total RNA were

isolated from cultures of wild-type and T8 by using “QiagenRNeasy Mini Kit” (Cat: 74104, Qiagen, USA). The total RNA of cultures were stored at -80°C.

2.2.9.2 cDNA synthesis and hybridisation to microarrays

Concentrations and quality of isolated RNA was checked by RNA 6000 Nano Assay Kit (Cat: 5067-1511, Agilent, USA). The samples were analyzed with Agilent BioAnalyzer 2100 and the RNA integrity numbers (RIN) were calculated.

2.2.9.3 DNA microarray

First, internal controls with known concentrations were obtained by “Agilent one-color RNA spike-in kit” (Cat: 5188-5282, Agilent, ABD). Then the total RNA samples were mixed with diluted RNA spike-in controls and labelled with Cy3 by using “Agilent low input quick amp labeling Kit” (Cat: 5190-2305, Agilent, USA). Absolutely RNA Nanoprep Kit” (Cat: 400753, Agilent, USA) was used to purify the Cy3-labelled samples. The concentrations of the samples were measured by NanoDrop, and the same volumes of each triplicate samples were placed on microarray slides (*RNA 6000 NanoLabChip® Kit*, Cat: 5065-4476, Agilent, USA). The microarray slides were transferred into Agilent Microarray Hybridization Chamber. After 17 h of hybridization process, the slides were washed and transferred into Agilent C scanner device.

2.2.9.4 Transcript data acquisition and analysis of microarray

The microarray data was based on three independent biological replicates for the wild type and salt resistant mutant T8. The mean expression profile of T8 was normalized to wild-type by GeneSpring GX 12.5 software. FunSpec and FunCat databases were used for the functional categorization of up- and down-regulated genes of T8. The gene expression and growth relation analysis were compared by using Princeton growth rate/gene expression relationship database.

2.2.10 Real-Time PCR

2.2.10.1 Primer design

The primer design of genes were analyzed by “Primer3 Plus Software” and “Amplify3 - 3.1.4 version - MacOS X” software. The specific primers are listed in Table 2.14. *ENA6* and *NHA1* genes were analyzed for their expression levels by Real-Time PCR.

Table 2.14 : The list of primer sequences.

Gene Name	Primer Sequence		Amplicon (bp)
	Forward (5'→3')	Reverse (5'→3')	
<i>ACT1</i>	CTTTCAACGTTCCAGCCTTC	TCACCGGAATCCAAA ACAAT	94
<i>ENA1</i>	GTCTGGTGAAGGTCGGTGAT	ACCCACGGAGGTTTCT TCTT	155
<i>NHA1</i>	ACATTTGCATTGAGCACAGC	TTCCATCTCGTCTTCG CTTT	237

2.2.10.2 Total RNA isolation from yeast cells (CEN.PK 113.7D)

RNA was obtained from untreated cells and cells treated with 0.7 M NaCl and 5mM LiCl, respectively. Wild type and T8 were grown in 10 mL YMM at 30 °C, 150 rpm, until they reached OD₆₀₀ of 1. Cells were centrifuged at 13000 rpm for 5 min and supernatants were discarded. Total RNA was isolated with RNA isolation kit (Roche - High Pure RNA Isolation Kit (Cat. No. 11 828 665 001). Purification was carried out following manufacturer's instructions.

2.2.10.3 Measurement of total RNA concentration

Total RNA concentrations were measured by NanoDrop.

2.2.10.4 cDNA synthesis

cDNA from total RNA samples was synthesized by Transcriptor High Fidelity cDNA Synthesis Kit (Cat. No. 05 081 955 001).

2.2.10.5 Real-time PCR applications

Roche-LightCycler® 480 DNA SYBR Green I Master Kit (Cat. No. 04 707 516 001) was used for real-timePCR applications. The components of the reaction mix are indicated in Table 2.15. Standard curves were drawn for each gene and the internal control housekeeping gene beta-actin (*ACT1*). The relative expression levels of each target gene were analysed using $2^{-\Delta\Delta C_t}$ method (Livak and Schmittgen, 2001). The efficiency, error and slope values of each standard curve and the melting curves of each Q-PCR was analyzed in detail (Dorak, 2006).

Table 2.15 : Q-PCR protocol.

PCR Components	Volume
Water	3 µl
PCR Primers (F+R)	2 µl
SYBR Green I Master	10 µl
Template	5 µl
Total Volume	20 µl

The Real-Time PCR program is indicated in Table 2.16.

Table 2.16 : Q-PCR program.

	Temperature (°C)	Time	Cycle
Pre-incubation	95	10 min	1
Denaturation	95	10 sec	
Amplification	56	18 sec	45
	72	20 sec	
	95	5 sec	
Melting	65	1 min	1
	97	-	
Cooling	40	10 sec	1

2.2.11 Sequence analysis of *NHA1*, *NHX1* and *KHA1* genes

2.2.11.1 Genomic DNA isolation

Genomic DNA isolation was carried out following procedures detailed in the protocol titled Small-Scale Preparations of Yeast DNA in "Molecular Cloning: A Laboratory Manual" (Green and Sambrook, 2001). Cultures of wild-type and T8 were grown overnight in 10 mL YPD at 30 °C, 150 rpm. 5 mL of cells was transferred to another culture tube and the other half of the cells were stored at 4 °C. 5 mL of cells were centrifuged at 2000g for 5 minutes. The pellet was resuspended in 500 µl sorbitol buffer and the cells were transferred into a 2 mL centrifuge tube. Zymolase 20T (2.5 mg/ml in sorbitol buffer) was added to the cells and they were incubated in a thermomixer for 1 hour at 37 °C. Then, the cells were centrifuged at 13000 rpm for 1 minute. The supernatant was removed and the cells were

resuspended in 500 µl yeast resuspension buffer. 50 µl 10% SDS was added to cells. After mixing the contents, the tube was incubated for 30 minutes at 65 °C. 200 µl 5 M potassium acetate was added and the tube was stored for 1 hour on ice. Cells were centrifuged at 13000 rpm for 5 minutes. The supernatant was removed and transferred to another centrifuge tube. DNA was precipitated by adding an equal volume of isopropanol. The tube was stored for 5 minutes at room temperature. Then, it was centrifuged at 13000 rpm for 10 seconds. After removing the supernatant, the pellet was dried for 10 minutes. 300 µl TE containing 20 µg/mL RNase was added to dissolve the pellet. The tube was incubated for 30 minutes at 37 °C. After adding 30 µl 3 M sodium acetate (pH 7.0) to the cells, 200 µl isopropanol was added. Precipitated DNA was recovered by centrifugation at 13000 rpm for 30 seconds. After removing the supernatant, the pellet was dried for 20 minutes. Then, DNA was dissolved in 150 µl TE (pH 7.4).

2.2.11.2 Amplification of target sequences by PCR

DNA sequences of *NHA1*, *NHX1* and *KHA1* in wild type and T8 were analysed as follows: genomic DNA was extracted by using Small-Scale Preparations of Yeast DNA protocol (Sambrook and Russell, 2001). The extracted DNA was used for the amplification of *NHA1*, *NHX1* and *KHA1* genes by using sequencing primers (Table 2.17). *Nha1*_Forward primer_1 was designed from the beginning of the gene, *Nha1*_forward primer_4 was designed from the end of the gene (Table 2.17). The PCR products were purified from the gel. QIAquick Gel Extraction Kit (QIAGEN, No. 28706) was used for both gel extractions.

Table 2.17 : Sequencing primers of *NHA1*, *NHX1* and *KHA1*.

Primer Name	Primer sequence 5'→3'	Tm
Nha1_Foward primer_1	ATGGCTATCTGGGAGCAACT	52 °C
Nha1_Reverse primer_1	TTACTTATTGAGACCAAGCG	48 °C
Nha1_Reverse primer_2	GCACGCAGATATCAACAAAC	50 °C
Nha1_Foward primer_2	GTCCTGGCGTTCATGTGTGC	56 °C
Nha1_Foward primer_3	GAAGATGAAGAAGGATTAAA	44 °C
Nha1_forward primer_4	ATAAAAGTAAAAACCGCCCA	46 °C
Nhx1_Foward primer_1	ATGCTATCCAAGGTATTGCT	48 °C
Nhx1_Reverse primer_1	CTAGTGGTTTTGGGAAGAGA	50 °C
Nhx1_Reverse primer_2	CTCGTACCCACTATTTAAAA	46 °C
Nhx1_Foward primer_2	TCGTCGGTTTTTGAAGGGGC	54 °C
Nhx1_Foward primer_3	GCTCGTTGGTGTGCTGTGTT	54 °C
Nhx1_forward primer_4	TTGTTTTAACAGTTATCATT	39 °C
Kha1_Foward primer_1	ATGGCAAACACTGTAGGAGG	52 °C
Kha1_Reverse primer_1	TTATTCAGACGAAAAATGGT	44 °C
Kha1_Reverse primer_2	AAATAAAGGAATCGCCAGCA	48 °C
Kha1_Foward primer_2	AGGACCCATGAATTGGACAG	52 °C
Kha1_Foward primer_3	CTGTCTTTAGGGGTATCTCC	52 °C
Kha1_forward primer_4	CAAATACCTATGGCAGATTA	46 °C

PCR experiment was performed by using Novagen KOD Hot Start DNAPolymerase (EMD Millipore, Germany) kit. The PCR mixture and the PCR program are indicated in Table 2.18 and Table 2.19.

Table 2.18 : PCR mixture.

Ingredients	Volume (μl)
dH ₂ O	2.4 μl
10X Buffer	2 μl
10 mM dNTP	2 μl
10 μM Nha1p/Nhx1/Kha1 F	1 μl
10 μM Nha1p/Nhx1/Kha1 R	1 μl
Template from T8 (mutant) or wt (50 ng)	-
MgSO ₄ (25 mM)	6.4 μl
DTT (100mM)	1.5 μl
BSA(4 mg/ml)	1 μl
DMSO (100% (v/v))	0.4 μl
KOD DNA polymerase	0.3 μl
Final volume	20 μl

Table 2.19 : PCR program for Colony PCR (XB t7t3-44 35) (preheated lid at 94 °C).

	Temperature	Time	Cycle number
Initial denaturation	94 °C	30 sec	1
Denaturation	94 °C	10 sec	
Annealing	55°C	30 sec	35
Extension	72 °C	60 sec	
Final extension	72 °C	5 min	1
Storage	16°C	∞	

2.2.11.3 Agarose Gel Electrophoresis of PCR Products

The DNA bands belonging to the PCR products were visualized on a 1% (w/v) agarose gel. 1% mini or midi gels were prepared. Midi gels were prepared with 0.5 g agarose and 5 μl SYBR Gold added into 50 mL of 1X TBE buffer, which was diluted from 10X stock TBE solution. Gels (1%) were prepared with 2 g agarose and 20 μL SYBR Gold added into 200 mL of 1X TBE buffer. 10 μL of PCR product was mixed

with 5 µL 1X loading dye and loaded into the wells. GeneRuler™ 100bp Plus marker (Fermentas) was used as the DNA ladder. The gels were run in 1X TBE buffer, at 100V for 30 min, and observed under UV light using a transilluminator. Gel images were obtained using UV PhotoMW software.

2.2.11.4 Data analysis of sequencing

Sequences of CEN.PK 113-7D wild type and T8 for *NHA1*, *NHX1*, *KHA1* were compared by program called SeqScape Software v.3.0.

2.2.12 Transformation experiments

2.2.12.1 Plasmid

PDD (3040 bp) was used for complementation of *Nha1* in the strain FGY217, AB11c, KTA40-2.

2.2.12.2 Preparation of competent cells

Preculture of strains were precultured into 10 mL liquid YPD and incubated overnight at 30 °C, 150 rpm. Next day, stocks of 10X TE, 10X LiAc and 50% PEG 4000 were prepared freshly. During the experiment, autoclaved distilled water and stocks were kept on ice. Single-stranded DNA was heated for 5 min at 65 °C and distilled water was kept on ice. 200 ml cells that were grown overnight were diluted to OD₆₀₀ of 0.2 and incubated until they reached an OD₆₀₀ of 0.5-0.6. 200 ml culture was split into 4 centrifuge tubes and spun down at 4300 rpm for 9 min at 4 °C. Then, each pellet was resuspended in cold 10 ml ddH₂O and spun down at 4300 rpm for 9 min at 4 °C. Each pellet was resuspended in 1 ml ddH₂O and transferred into a 2 ml tube. The cells were harvested at 6000 rpm for 30 sec. The supernatant was poured off and 1 mL of 1X TE/LiAc solution was added to the cell pellet. Cells were spun down at 6000 rpm for 30 sec and resuspended in 1 ml 1X TE/LiAc solution.

2.2.12.3 Yeast transformation

After preparing competent cells, transformation mixture was added to competent cells (Table 2.20).

Table 2.20 : Yeast transformation mixture.

Component	Amount
Competent cells	50 μ l
Buffer (PEG-TE/LiAc pH 7.5)	300 μ l
SS Herings DNA	7 μ l
Transforming DNA(plasmid+template)	3-10 μ l (1-6 μ g)

Transformation mixture was mixed by vortexing. Then, the mixture was incubated at 30°C for 45 min followed by 15 min of incubation at 42 °C. Cells were washed by 1 ml YPD four times, repeatedly. This mixture was resuspended in 200 μ l YPD and spread on drop-out plates.

2.2.12.4 Transformation verification

Verification of transformation was done by colony PCR. Colonies were selected from selective media and masterplate of this colonies were struck to drop-out media plates. Colonies were picked and grown in liquid drop-out media. 20 μ l ddH₂O was put into microfuge tubes. These tubes were put into thermomixer and incubated at 30 °C for 30 min. 1 μ l zymolase (20T, Amsbio, UK) per tube was added and a colony from the masterplate was added to the water. The tubes were incubated at 30°C for 20 min; afterwards incubated at 95 °C for 35 min. The tubes were centrifuged at 13000 rpm for 1 min. 1.2 μ l supernatant was taken as template for PCR. KOD Hot Start DNA polymerase (Novagen, UK) was used for Colony PCR. PCR mixture for colonies and PCR program are shown in Table 2.21 and Table 2.22.

Table 2.21 : PCR mixture for colonies.

Ingredients	Volume (μl)
dH ₂ O	3.4 μ l
10X Buffer	1 μ l
10 mM dNTP	1 μ l
10 mM T7 primer	0.5 μ l
10 mM T3 primer	0.5 μ l
Template from colony	1.2 μ l
MgSO ₄ (25 mM)	3.2 μ l
DTT (100mM)	0.75 μ l
BSA(4 mg/ml)	0.5 μ l
DMSO(100%)	0.2 μ l
KOD DNA polymerase	0.15 μ l
Final volume	10 μ l

Table 2.22 : PCR program for Colony PCR (XB t7t3-44 35) (preheated lid at 94 °C).

	Temperature	Time	Cycle number
Initial denaturation	94 °C	30 sec	1
Denaturation	94 °C	10 sec	
Annealing	44 °C	30 sec	35
Extension	72 °C	60 sec	
Final extension	72 °C	5 min	1
Storage	16°C	∞	

Genomic integration of the gene was identified by gel electrophoresis of PCR products, based on their sizes.

2.2.12.5 Plasmid isolation from yeast

The plasmid was extracted using Qiagen Spin Mini Prep Kit (Qiagen, Germany). 10 mL overnight cultures in drop-out medium was harvested by centrifugation at 4700 rpm, 4 °C for 5 min. The supernatant was discarded and the pellets were resuspended

in 500 μ L Buffer P1 and split into two microfuge tubes containing glass beads of mm diameter. The tubes were vortexed for 15 min and centrifuged for 5 min at 13000 rpm. The supernatant was removed and 250 μ L Buffer P2 was added to the resuspended cell solution and the tube was inverted several times for mixing. 350 μ L Buffer N3 was added and the tubes were mixed by inverting several times. The tubes were centrifuged for 10 min at 13000 rpm. The supernatants were applied to the Qiaprep spin column and centrifuged for 1 min. After discarding the flow-through, 500 μ L Buffer PB was added to the spin column and centrifuged for 1 min at 13000 rpm. After once again discarding the flow-through, 750 μ L Buffer PE was added to the spin column and centrifuged for 1 min at 13000 rpm. The tubes were centrifuged for 1 min at 13000 rpm once more to remove residual wash buffer. 50 μ L Buffer EB was added at the center of the spin column. After waiting for 5 min, the tubes were centrifuged for 1 min at 13000 rpm. Concentration of plasmid was checked and the samples were kept at -20 °C.

2.2.12.6 Transformation to bacteria

Thermo-competent *E.coli* DH5 α cells were used for transformation experiments (Cohen *et al.*, 1972). Plasmid of true transformant which was from yeast was added to 100 μ L thermo-competent *E.coli* DH5 α cells. The tube was kept for 5 min on ice. The tube was then placed at 42 °C for 45 seconds. The tube was kept again on ice for 5 min. 800 μ L LB was added to the tubes and incubated at 37 °C for 1 hour, 600 rpm in thermomixer and spread on LB plates containing carbenicillin. The plates were incubated at 37°C overnight and the next day colonies were struck to masterplates and also inoculated to LB carbenicillin-containing liquid media separately.

2.2.12.7 Plasmid isolation from bacteria

The plasmid was extracted using Qiagen Spin Mini Prep Kit (Qiagen, Germany). Ten ml overnight cultures in LB medium with carbenicillin was harvested with centrifugation at 4700 rpm, 4 °C for 5 min. The supernatant was discarded and the pellet was resuspended in 500 μ L Buffer P1 and split into two microfuge tubes. 250 μ L Buffer P2 was added to the resuspended cell solution and the tube was inverted several times to mix. 350 μ L Buffer N3 was added and mixed by inverting several times. The tubes were centrifuged for 10 min at 13000 rpm. The supernatants were applied to the Qiaprep spin column and centrifuged for 1 min. After discarding the flow-through, 500 μ L Buffer PB was added to spin column and centrifuged for 1 min

at 13000 rpm. After discarding the flow-thorough, 750 μ L Buffer PE was added to the spin column and centrifuged for 1 min at 13000 rpm. The tubes were centrifuged for 1 min at 13000 rpm once more to remove residual wash buffer. 50 μ L Buffer EB was added at the center of the spin column. After 5 min, they were centrifuged for 1 min at 13000 rpm. Concentration of plasmid was checked and the samples were kept at -20 °C.

2.2.13 Site directed mutagenesis of *NHA1*

2.2.13.1 Site directed mutagenesis primers for *NHA1*

Primers for site directed mutagenesis were listed at Table 2.23. Primers were designed with high annealing temperatures.

Table 2.23 : List of site directed mutagenesis primers.

Primer Name		Primer sequence 5'→3'	Tm (°C)
G372S	ggt → agc	gtgggccattttgggtcctattagcgtaggtgcaattttt gccgct	70
D266H	gat → cat	gttcggctccattttgggtgtgcatgacctattggatc atttcagccg	72
D267A	gac → gcc	gttcggctccattttgggtgtggatgccctattggatc atttcagccg	73
D266H/D267A	gat → cat gac → gcc	gttcggctccattttgggtgtgcatgccctattggatc atttcagccg	73
D266N/D267N	gat → aat gac → aac	gttcggctccattttgggtgtgaataacctattggatc atttcagccg	70
N176D	aat → gat	ctgtctgcagagtcaggctgcgatgatggatggcct ttccttttta	71
D177N	gat → aat	ctgtctgcagagtcaggctgcaataatggatggcct ttccttttta	69

2.2.13.2 Amplification of site directed mutagenized sequences by PCR

Mutated versions of *NHA1* were generated by PCR. In all cases *Nha1*-GFP was used as the template, and amplified fragments were verified by DNA sequencing. To introduce codons at positions G372S (ggt → agc), D266H (gat → cat), D267A (gac → gcc), D266H/D267A (gat → cat, gac → gcc), D266N/D267N (gat → aat, gac → aac), N176 (aat → gat), D177N (gat → aat), site directed mutagenesis PCR was performed. PCR mixture and PCR program are shown in Table 2.24 and 2.25 (Fischer and Pei, 1997).

Table 2.24 : PCR mixture.

Ingredients	Volume (μl)
dH ₂ O	4.7 μl
10X Buffer	2μl
10 mM dNTP	2 μl
10 μM SDM primer (undiluted)	1 μl
Template (100 ng) (NHA1-GFP)	1 μl
MgSO ₄ (25 mM)	6.4 μl
DTT (100mM)	1.6 μl
BSA(4 mg/ml)	1 μl
DMSO(100%)	0.4 μl
KOD DNA polymerase	0.3 μl
Final volume	20 μl

Table 2.25 : PCR program for site-directed mutagenesis PCR.

	Temperature	Time	Cycle number
Initial denaturation	95°C	5 min	1
Denaturation	95°C	50 sec	
Annealing	60°C	50 sec	36
Extension	68 °C	10.5 min	
Final extension	68 °C	7 min	1
Storage	16°C	∞	

The PCR product was digested with 0.25 μL *DpnI* (20 U/μl, New England Biolabs). *DpnI* only cleaved at methylated sites, so it digested the template plasmid but not the PCR product. It was incubated at 37 °C for 2 hours. *DpnI*-digested product was transformed into *Escherichia coli* DH5α.

2.2.14 Proteome analysis via electrospray ionization quadrupole time-of-flight (TOF) mass spectrometry

The protein expression levels of NaCl- resistant mutant T8 was analyzed in reference to wild type by electrospray ionization quadrupole time-of-flight (TOF) mass spectrometry in TÜBİTAK Research Institute for Genetic Engineering and Biotechnology (RIGEB) Laboratories by A. Gökçe, Y. Öztürk and T. Baykal, according to the protocol mentioned by Callister *et al.*, 2006.

3. RESULTS

3.1 Growth Characteristics of Mutant Strain 'T8' and comparison of growth physiology between wildtype and T8

3.1.1 Growth Characteristics of Mutant Strain 'T8' in the presence of 0.5 M NaCl, 0.7 M NaCl and 0.9 M NaCl

3.1.1.1 Shake flask cultivation of wt and T8 in the presence of 0.5 M NaCl, 0.7 M NaCl and 0.9 M NaCl

-80 ° C stock cultures of wild type and mutant T8 were inoculated into 50-mL culture tubes containing 10 mL YPD. After overnight incubation, cultures were inoculated in 100 mL YMM in 500 mL flasks to an initial OD₆₀₀ value of 0.25. Incubation of cultures was carried out at 30 °C, 150 rpm. OD₆₀₀ values were measured and samples for later analyses were taken every 1.5 h. Growth curves of wild type, wild type in the presence of 0.5 M NaCl, 0.7 M NaCl, 0.9 M NaCl , T8 and T8 in the presence of 0.5 M NaCl, 0.7 M NaCl, 0.9 M NaCl were obtained. OD₆₀₀ values were measured by using Shimadzu UV-1700 Spectrophotometer.

Table 3.1 : Average OD₆₀₀ values of wild type and wild type in the presence of 0.5 M NaCl, 0.7 M NaCl, 0.9 M NaCl.

OD ₆₀₀	Wt-AVG	ST Dev	Wt	ST Dev	Wt	ST Dev	Wt	ST Dev
			+0.5 M NaCl -AVG		+0.7 M NaCl-AVG		+0.9 M NaCl AVG	
t=0 h	0.35	0.01	0.35	0.01	0.09	0.00	0.01	0.00
t=1 h	0.58	0.01	0.47	0.01	0.44	0.01	0.45	0.02
t=2 h	1.21	0.03	0.61	0.05	0.57	0.02	0.52	0.00
t=3 h	2.52	0.20	0.79	0.01	0.60	0.05	0.68	0.00
t=4 h	3.58	0.18	0.83	0.01	0.79	0.00	0.71	0.06
t=5 h	4.16	0.24	0.91	0.00	0.97	0.07	0.80	0.00
t=6 h	5.73	0.21	1.10	0.07	1.14	0.03	0.93	0.00

Table 3.2 : Average OD₆₀₀ values of T8 and T8 in the presence of 0.5 M NaCl, 0.7 M NaCl and 0.9 M NaCl.

OD ₆₀₀	T8-AVG	ST Dev	T8+0.5 M NaCl- AVG	ST Dev	T8+0.7 M NaCl- AVG	ST Dev	T8+0.9 M NaCl- AVG	ST Dev
t=0 h	0.35	0.02	0.32	0.02	0.29	0.00	0.32	0.02
t=1 h	0.52	0.03	0.42	0.01	0.47	0.02	0.37	0.00
t=2 h	0.92	0.03	0.58	0.01	0.60	0.02	0.47	0.00
t=3 h	1.64	0.01	0.89	0.05	0.83	0.00	0.66	0.01
t=4 h	2.56	0.03	1.23	0.02	0.79	0.04	1.05	0.11
t=5 h	3.34	0.06	1.73	0.10	1.40	0.16	1.06	0.01
t=6 h	5.00	0.03	2.20	0.01	1.89	0.06	1.43	0.01

According to Average OD₆₀₀ values, μ (h⁻¹) values were calculated (Table 3.1 and 3.2).

Table 3.3 : ln (OD₆₀₀) and $\mu_{\max}$ (h⁻¹) values of wt, wt in the presence of 0.5 M NaCl, 0.7 M NaCl, 0.9M NaCl , T8, T8 in the presence of 0.5 M NaCl, T8+0.7 M NaCl, T8+0.9 M NaCl.

ln (OD ₆₀₀)								
h	wt	wt+0.5 M NaCl	wt+0.7 M NaCl	wt+0.9 M NaCl	T8	T8+0.5 M NaCl	T8+0.7 M NaCl	T8+0.9 M NaCl
0	-1.06	-1.04	-2.43	-5.20	-1.05	-1.14	-1.22	-1.14
1.5	-0.54	-0.75	-0.82	-0.81	-0.65	-0.87	-0.77	-0.98
3	0.19	-0.50	-0.56	-0.65	-0.08	-0.55	-0.51	-0.76
4.5	0.93	-0.23	-0.50	-0.39	0.49	-0.11	-0.19	-0.42
6	1.28	-0.19	-0.24	-0.34	0.94	0.21	-0.24	0.05
7.5	1.43	-0.10	-0.03	-0.23	1.21	0.55	0.34	0.06
9	1.75	0.09	0.13	-0.08	1.61	0.79	0.63	0.36
μ	0.44	0.18	0.11	0.09	0.35	0.24	0.19	0.17

μ values of T8 were significantly higher than that of the wild type (Table 3.3). While 0.9 M NaCl inhibited wild type significantly, it did not affect T8's growth. It was obvious that T8 was resistant to 0.5 M NaCl, 0.7 M NaCl and 0.9 M NaCl (Figure 3.1).

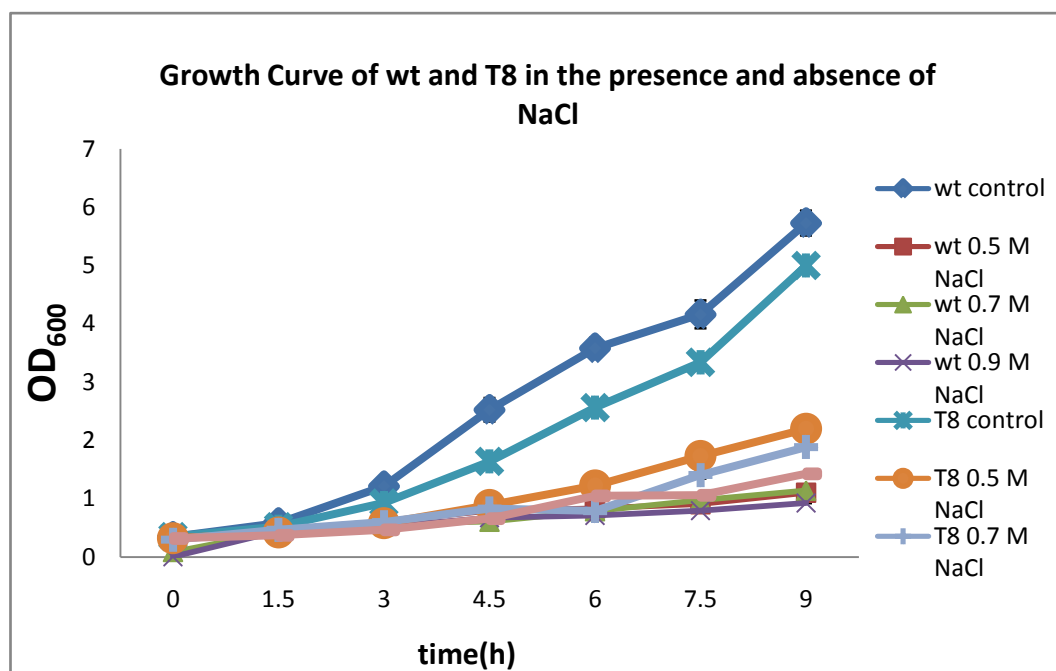


Figure 3.1 : Growth curves of wild type and T8 strain in the absence and presence of 0.5 M, 0.7M,0.9 M NaCl.

3.1.2 Growth Characteristics of Salt-Resistant Mutant‘T8’ in the presence of 0.5 M NaCl

3.1.2.1 Shake flask cultivation of wt and T8 in the presence of 0.5 M NaCl

-80 °C stock cultures of wild type and mutant T8 were inoculated into 50-mL culture tubes containing 10 mL YPD. After overnight incubation, cultures were inoculated in 100 mL YMM in 500 mL flasks to an initial OD₆₀₀ value of 0.25. Incubation of cultures was carried out at 30°C, 150 rpm. OD₆₀₀ values were measured and samples for later analyses were taken every 1.5 h. Growth curves of wild type, wild type in the presence of 0.5 M NaCl, T8 and T8 in the presence of 0.5 M NaCl were obtained. OD₆₀₀ values were measured by using Shimadzu UV-1700 Spectrophotometer.

Table 3.4 : Average OD₆₀₀ Values of wt, wt in the presence of 0.5 M NaCl, T8 and T8 in the presence of 0.5 M NaCl.

OD ₆₀₀	WT-AVG	ST Dev	WT+0.5 M NaCl-AVG	ST Dev	T8-AVG	ST Dev	T8+0.5 M NaCl-AVG	ST Dev
t=0 h	0.28	0.00	0.29	0.00	0.27	0.00	0.27	0.00
t=1 h	0.40	0.00	0.37	0.00	0.32	0.00	0.31	0.01
t=2 h	0.77	0.01	0.56	0.01	0.46	0.01	0.41	0.01
t=3 h	1.38	0.01	0.80	0.01	0.77	0.01	0.56	0.00
t=4 h	2.63	0.03	1.21	0.01	1.28	0.01	0.87	0.01
t=5 h	4.39	0.05	1.38	0.03	2.15	0.03	1.47	0.03
t=6 h	4.76	0.02	1.66	0.06	3.45	0.17	1.95	0.03
t=7 h	5.75	0.04	1.50	0.02	4.33	0.04	2.43	0.04
t=8 h	6.47	0.24	2.13	0.03	5.57	0.09	5.86	0.14

$\mu_{\max}$ values of wild type and T8 were not significantly different from each other (Table 3.5). However, 0.5 M NaCl was inhibiting wild type's growth and T8 was resistant to 0.5 M NaCl.

Table 3.5 : ln (OD₆₀₀) and $\mu_{\max}$ (h⁻¹) values of wt, wt+0.5 M NaCl, T8 and T8+0.5 M NaCl.

Time (h)	ln (OD ₆₀₀)			
	wt	wt+0.5 M NaCl	T8	T8+0.5 M NaCl
0	-1.28	-1.25	-1.32	-1.31
1.5	-0.91	-1.00	-1.13	-1.16
3	-0.26	-0.58	-0.78	-0.89
4.5	0.32	-0.22	-0.26	-0.58
6	0.97	0.19	0.25	-0.14
7.5	1.48	0.32	0.76	0.38
9	1.56	0.50	1.24	0.67
10.5	1.75	0.41	1.47	0.89
24	1.87	0.76	1.72	1.77
$\mu_{\max}$ (h ⁻¹)	0.41	0.18	0.36	0.28

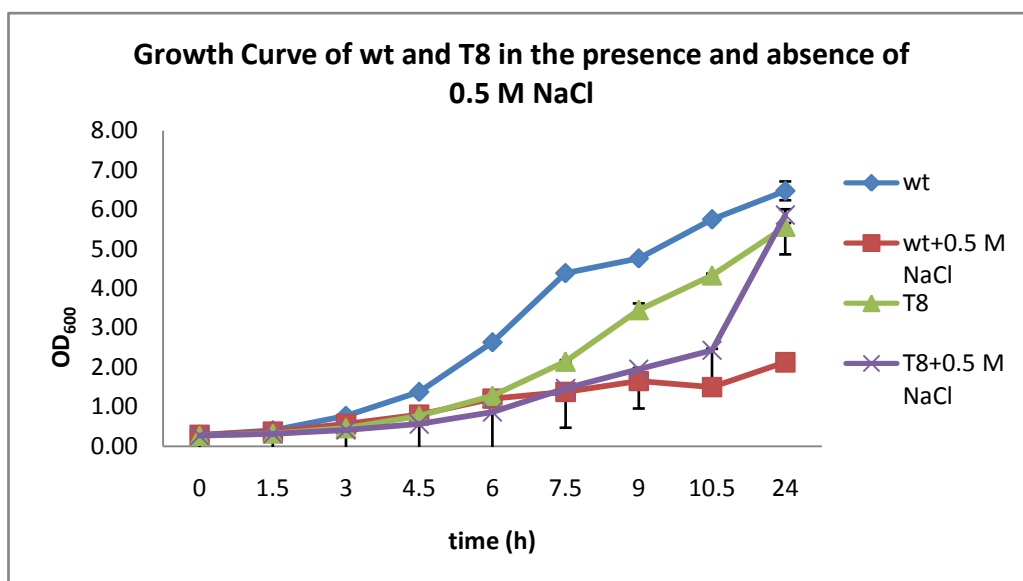


Figure 3.2 : Growth curves of wild type and T8 strain in the absence and presence of 0.5 M NaCl.

In Figure 3.2, better growth of T8 in the presence of 0.5 M NaCl was obvious after 10.5th hour.

3.1.2.2 Measurement of Cell Dry Weight in the presence of 0.5 M NaCl

Cell dry weight analysis was performed for each sample. Firstly, 1.5 mL microfuge tubes were dried in an oven at 80 °C for 24 h. After two days of drying, they were placed in desiccators for 30 minutes to cool down to room temperature, and then weighed. Throughout the growth experiment, 2 mL of samples were taken at t=7.5 h, t=9 h, t=10.5 h, and t=24 h and samples were placed into dried microfuge tubes via pipetting at particular time intervals. Cells were centrifuged at 14000 rpm for 5 minutes and supernatants were discarded. The tubes were dried in an oven at 80°C to evaporate the remaining liquid on the cell pellet. After 24 h of drying, the tubes with dried cell extracts were again placed in a desiccator for 30 minutes and then weighed. The cell dry weight (CDW) was calculated by subtracting the first weight from the second weight.

Table 3.6 : Average cell dry weight (CDW) mg/mL values of wild type, wild type in the presence of 0.5 M NaCl, T8, T8 in the presence of 0.5 M NaCl.

CDW (mg/mL)								
Time (h)	wt		Wt+0.5 M NaCl		T8		T8+ 0.5 M NaCl	
	avg	st dev	avg	st dev	avg	st dev	avg	st dev
7.5	1.12	0.15	0.75	0.08	0.9	0.28	0.7	0.13
9	1.33	0.11	1.13	0.07	1.05	0.09	1.23	0.07
10.5	1.37	0.14	1	0	1.45	0.1	1.27	0.11
24	1.75	0.07	1.25	0.07	2.25	0.07	1.63	0.13

CDW (mg/mL) results (Table 3.6) were in accordance with the OD₆₀₀ results (Table 3.4). It was observed that wild type and wild type samples treated with NaCl were different. The growth inhibition of sodium on wild type was obvious (Figure 3.3). Moreover, T8 was less affected by sodium stress.

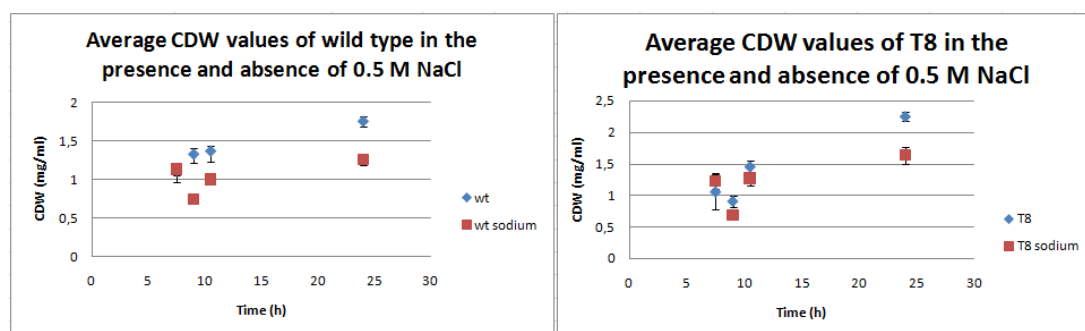


Figure 3.3 : Average CDW (mg/ml) values of wild type and T8 in the absence and presence of 0.5 M NaCl.

3.1.3 Growth Characteristics of Salt-resistant Mutant 'T8' in the presence of 5 mM LiCl

3.1.3.1 Shake flask cultivation of wt and T8 in the presence of 5 mM LiCl

NaCl-resistant mutant "T8" and the wild type *S.cerevisiae* strain were incubated to observe their physiological traits in aerobic-batch condition in the presence and in the absence of LiCl. Optical density and μ_{max} of salt-treated and non-treated samples were determined. -80°C stock cultures of wild type and mutant T8 were inoculated into 50mL culture tubes containing 10 mL YPD. After overnight incubation, cultures were inoculated in 100 mL YMM in 500 mL flasks with initial OD₆₀₀ value of 0.25. Incubation of cultures was carried out at 30°C, 150 rpm. OD₆₀₀ values were measured and samples for later analyses were taken every 1.5 h. Growth curves of wild type, wild type in the presence of 5mM LiCl, T8 and T8 in the presence of 5mM LiCl were obtained. OD₆₀₀ values were measured by using Shimadzu UV-1700

Spectrophotometer. Growth curves of the cultures were drawn by plotting OD₆₀₀ values versus time. The maximum specific growth rate ($\mu_{\max}$), which corresponded to the maximum slope from this semi-logarithmic representation were calculated by the natural logarithm of the OD₆₀₀ values.

Table 3.7 : Average OD₆₀₀ values of wildtype, wildtype in the presence of 5mM LiCl, T8 and T8 in the presence of 5mM LiCl.

Time (h)	OD ₆₀₀							
	WT- AVG	ST Dev	WT+5mM LiCl-AVG	ST Dev	T8- AVG	ST Dev	T8+5mM LiCl-AVG	ST Dev
0	0.32	0.00	0.30	0.01	0.32	0.00	0.33	0.01
1.5	0.45	0.00	0.40	0.00	0.37	0.01	0.41	0.01
3	0.78	0.01	0.63	0.01	0.55	0.02	0.64	0.02
4.5	1.33	0.03	0.81	0.02	0.98	0.05	1.15	0.02
6	2.87	0.02	1.01	0.01	1.83	0.00	1.72	0.03
7.5	3.61	0.11	1.15	0.03	2.43	0.07	2.28	0.03
9	4.80	0.12	1.34	0.03	3.48	0.03	3.50	0.00
10.5	5.73	0.07	1.50	0.04	3.68	0.01	3.88	0.03
24	6.47	0.24	2.13	0.03	5.57	0.09	5.86	0.14

Average OD₆₀₀ values at 24th hour showed that obviously T8 was growing better than wild type (Table 3.7). Until 4.5th hour, growth characteristics of T8 and wild type were the same. After 4.5th hour, LiCl diminished wild types's growth.

Table 3.8 : $\ln(\text{OD}_{600})$ and $\mu_{\max}$ (h^{-1}) values of wt, wt in the presence of 5mM LiCl, T8 and T8 in the presence of 5mM LiCl.

Time (h)	$\ln(\text{OD}_{600})$			
	wt	wt+5mM LiCl	T8	T8+5mM LiCl
0	-1.12	-1.21	-1.13	-1.11
1.5	-0.80	-0.91	-1.01	-0.90
3	-0.24	-0.47	-0.61	-0.45
4.5	0.28	-0.21	-0.02	0.14
6	1.06	0.01	0.60	0.54
7.5	1.28	0.14	0.89	0.82
9	1.57	0.29	1.25	1.25
10.5	1.75	0.41	1.30	1.35
24	1.87	0.76	1.72	1.77
$\mu_{\max}$ (h^{-1})	0.41	0.09	0.36	0.33

μ values of wild type and T8 were not significantly different from each other. Growth rate of wild type was slightly higher than that of T8 in the absence of LiCl stress (Table 3.8). Oppositely, the growth rate of T8 in the presence of LiCl was approximately three-fold higher than that of wild type (Table 3.8).

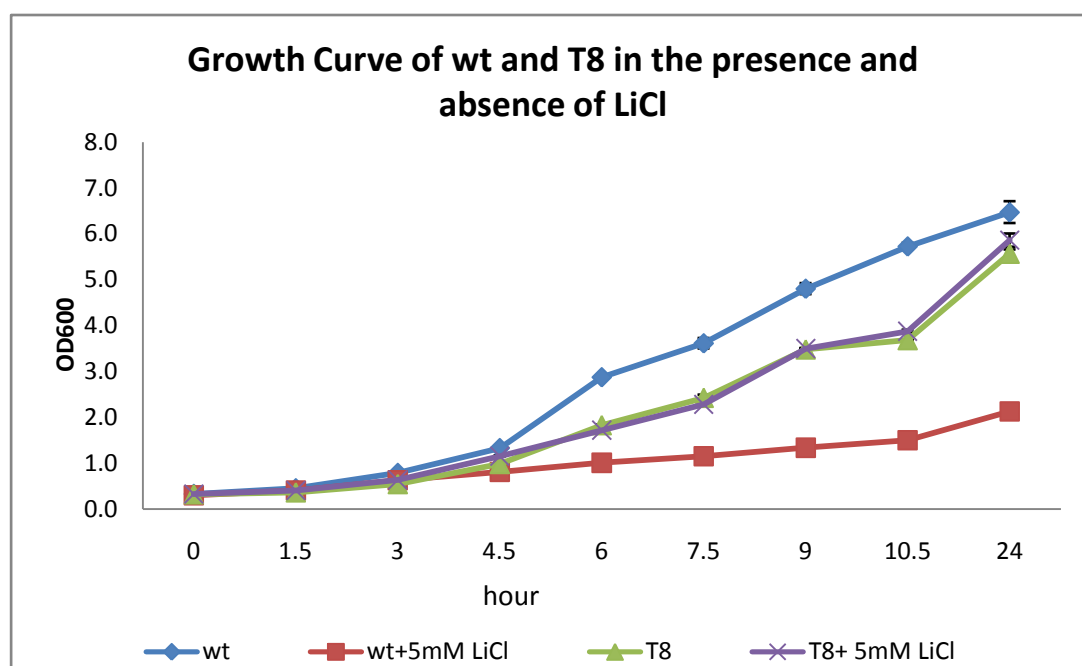


Figure 3.4 : Growth curves of wild type and T8 strain in the presence and absence of 5 mM LiCl.

Growth results clearly showed that 5 mM LiCl did not affect growth of T8; the growth curve was nearly the same for T8 in the presence and absence of 5 mM LiCl.

However, the presence of LiCl effectively reduced growth of the wild type (Figure 3.5).

3.1.3.2 Measurement of Cell Dry Weight in the presence of 5 mM LiCl

Cell dry weight analysis was performed for each sample. Firstly, 1.5 mL microfuge tubes were dried in an oven at 80°C for 24 h. After two days of drying, they were placed in a desiccator for 30 minutes to cool down to room temperature, and then weighed. Throughout the growth experiment, 1.5 mL of samples were taken for t=6 h samples and 1 mL of samples were taken at 7.5 h, 9 h, 10.5 h, and 24 h into dried microfuge tubes. Cells were centrifuged at 14000 rpm for 5 minutes and supernatants were discarded. The tubes containing cells were dried in an oven at 80°C to evaporate the remaining liquid on the cell pellet. After 24 h of drying, the tubes with dried cell extracts were again placed in a desiccator for 30 minutes and then weighed. The cell dry weight (CDW) was calculated by subtracting the weight of empty tubes from that of the tubes containing cells.

Table 3.9 : Average CDW mg/mL values of wild type, wild type in the presence of 5mM LiCl, T8, T8 in the presence of 5mM LiCl.

CDW (mg/mL)								
Time (h)	wt		Wt+ 5mM LiCl		T8		T8+ 5mM LiCl	
	avg	st dev	avg	st dev	avg	st dev	avg	st dev
6	1.18	0.17	0.83	0	1.27	0.29	0.62	0.23
7.5	1.17	0.32	0.6	0.38	1.13	0.3	1.5	0.34
9	1.4	0.21	0.77	0.32	1.5	0.25	1.3	0.23
10.5	1.73	0.18	0.77	0.15	1.77	0.22	1.43	0.06
24	1.73	0.31	1.27	0.32	2.13	0.32	2.23	0.12

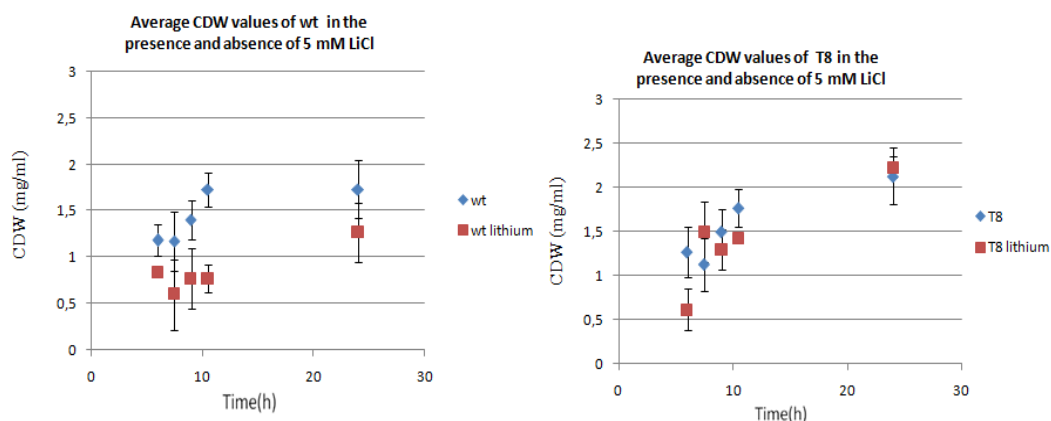


Figure 3.6 : Average CDW, mg/mL values of wild type and T8 in the presence and absence of LiCl.

CDW (mg/mL) results were in accordance with the OD₆₀₀ results (Table 3.9). CDW values of wild type and wild type samples treated with LiCl were significantly different, the growth inhibition of lithium on wild type was very obvious. Moreover, T8 (salt resistant mutant) seemed to be unaffected by high lithium stress (Figure 3.5).

3.2 Spot test upon different stress types

3.2.1 Tetramethylammonium (TMA) and spermine

Wild type and T8 were tested for their possible resistances to cationic drugs, TMA (Tetramethylammonium) and spermine (Sigma). For that reason, T8 and the wild type were grown in YPD until their exponential growth phase. Their diluted samples were then dropped into the media supplemented with TMA (0.2, 0.3, 0.4, 0.5 and 0.6 M), spermine (0.3, 0.4, 0.5, 0.6 and 0.7 mM).

If there was an alteration in the electrochemical gradient, mutants would be abnormally sensitive to toxic cations. Altered tolerance to diverse toxic cations, differing in the specific uptake transport system and toxicity mechanisms, would reflect a change in the electrochemical gradient (Barreto *et al.*, 2011).

Salt resistant strain (T8) exhibited slight resistance to spermine, but it did not exhibit any resistance to TMA. Slight resistance could be observed in 0.3 mM and 0.4 mM spermine plates (Figure 3.6 and 3.7).

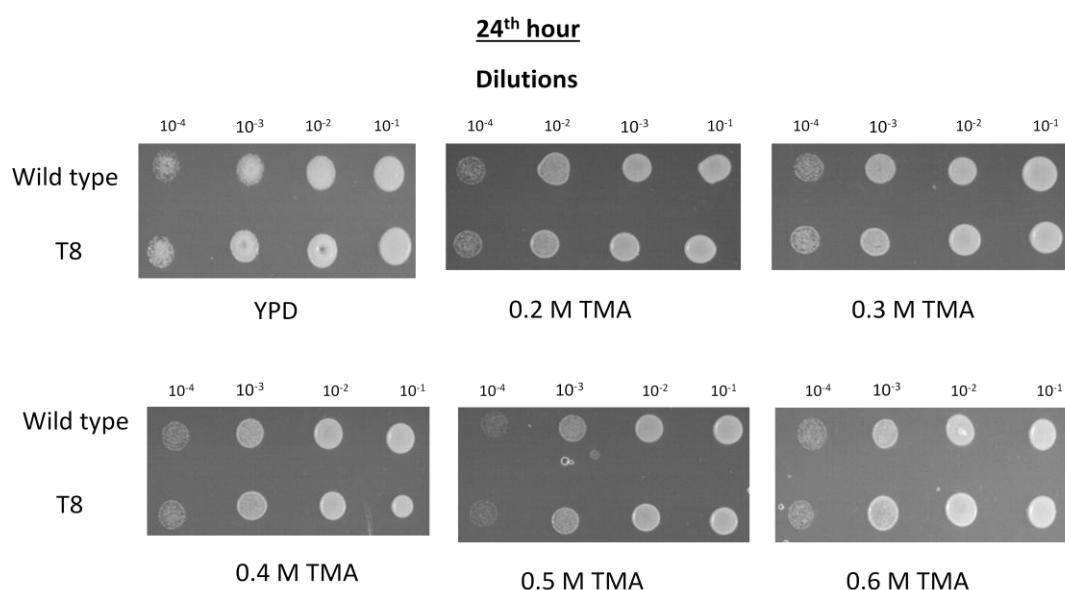


Figure 3.6: Spot test results of wild type and T8 in YPD containing different amounts of TMA.

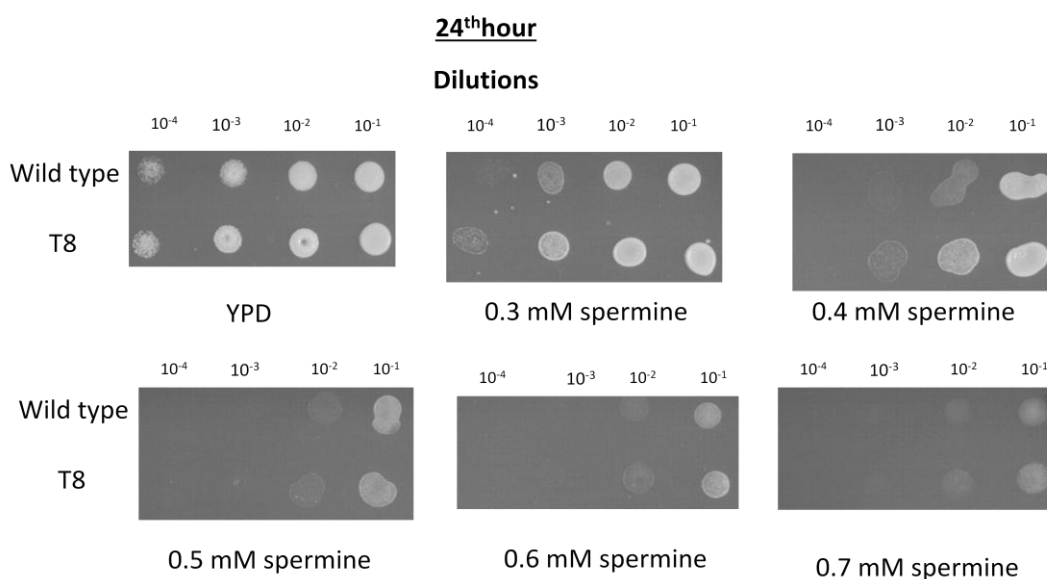


Figure 3.7 : Spot test results of wild type and T8 in YPD containing different amounts of spermine.

It seemed that there was no change in the plasma membrane potential of T8.

3.2.2 Spot Test of CEN.PK 113.7D(wildtype) and T8 with NaCl, KCl and LiCl (YMM)

Wild type and T8 were tested for their possible resistances to NaCl, LiCl and KCl. T8 and wild type were grown in YMM until their exponential growth phase. Their

diluted samples were then dropped into the media supplemented with NaCl (0.7, 1 and 1.3 M) ,KCl (500 mM and 1M) and LiCl (10 mM and 15mM).

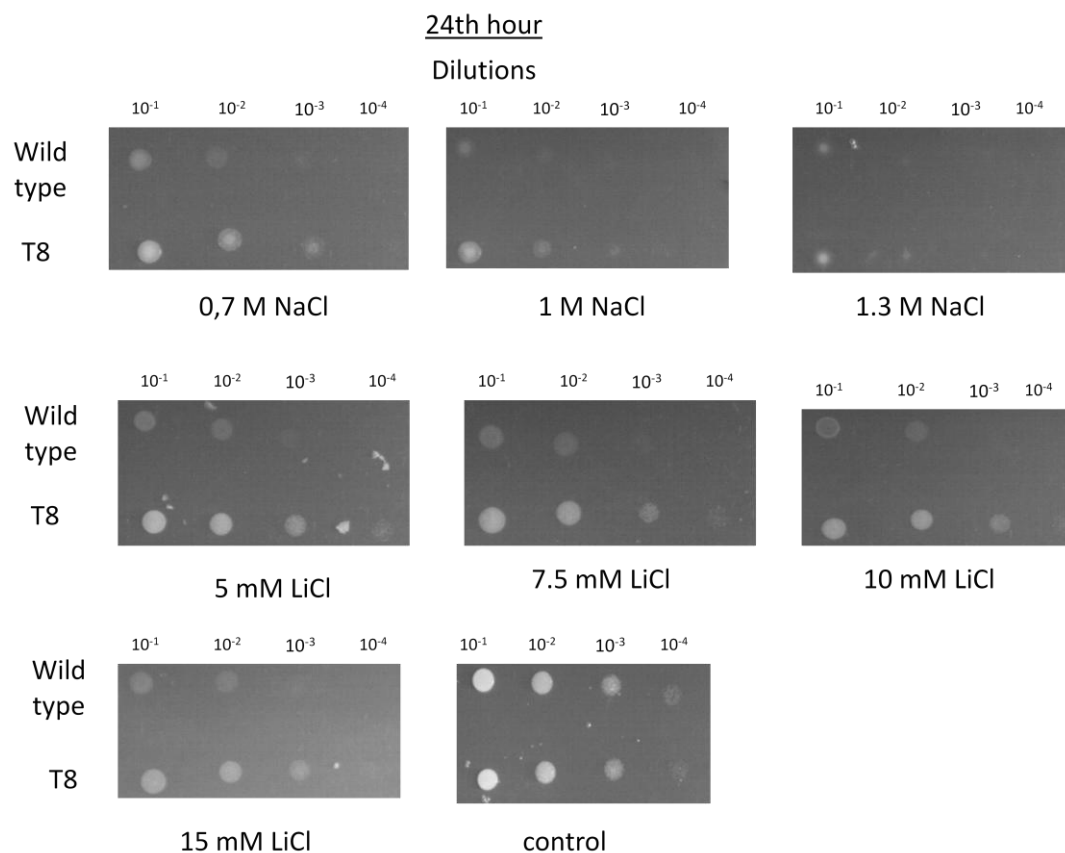


Figure 3.8 : Spot test results of wild type and T8 in YMM containing different amounts of NaCl and LiCl (24th hour).

Salt resistant mutant showed resistance to 0.7 M, 1 M and 1.3 M NaCl. T8 was resistant to LiCl. Wild type was even sensitive to 5 mM LiCl, however T8 was resistant to 15 mM LiCl (Figure 3.8 - Figure 3.11).

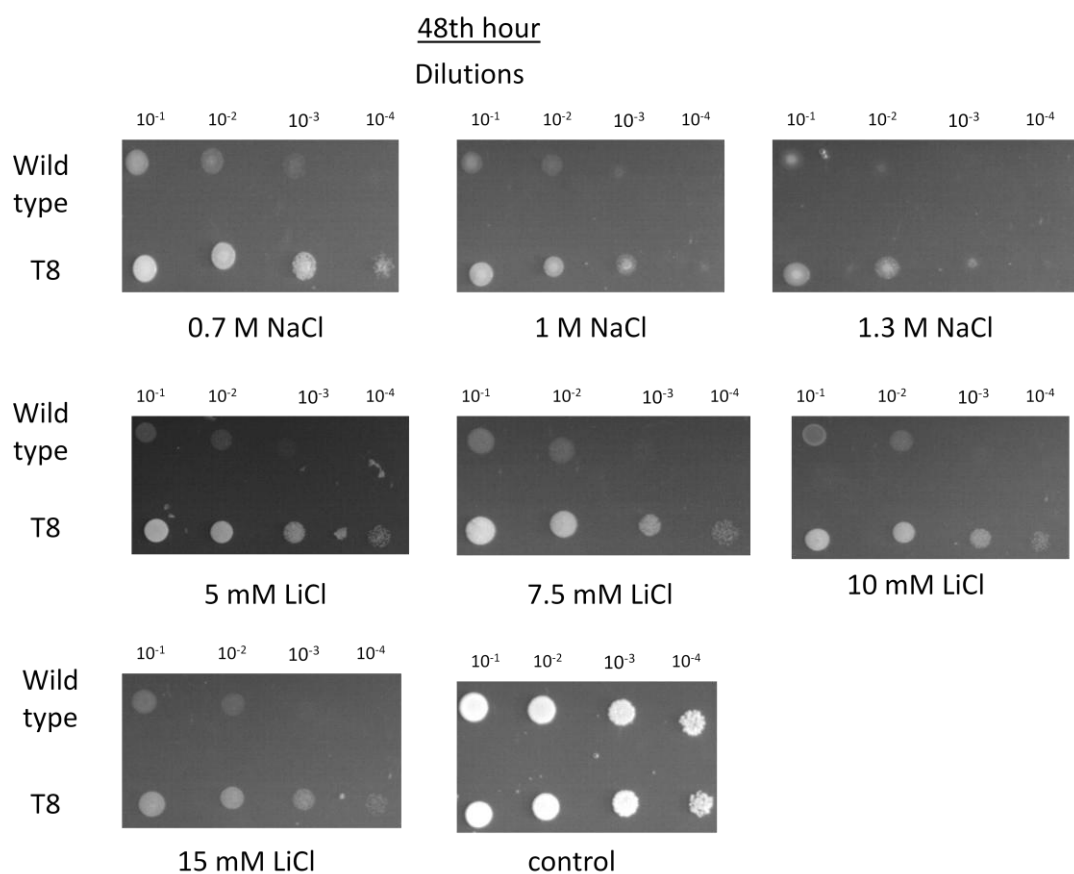


Figure 3.9 : Spot test results of wild type and T8 in YMM containing different amounts of NaCl and LiCl (48th hour).

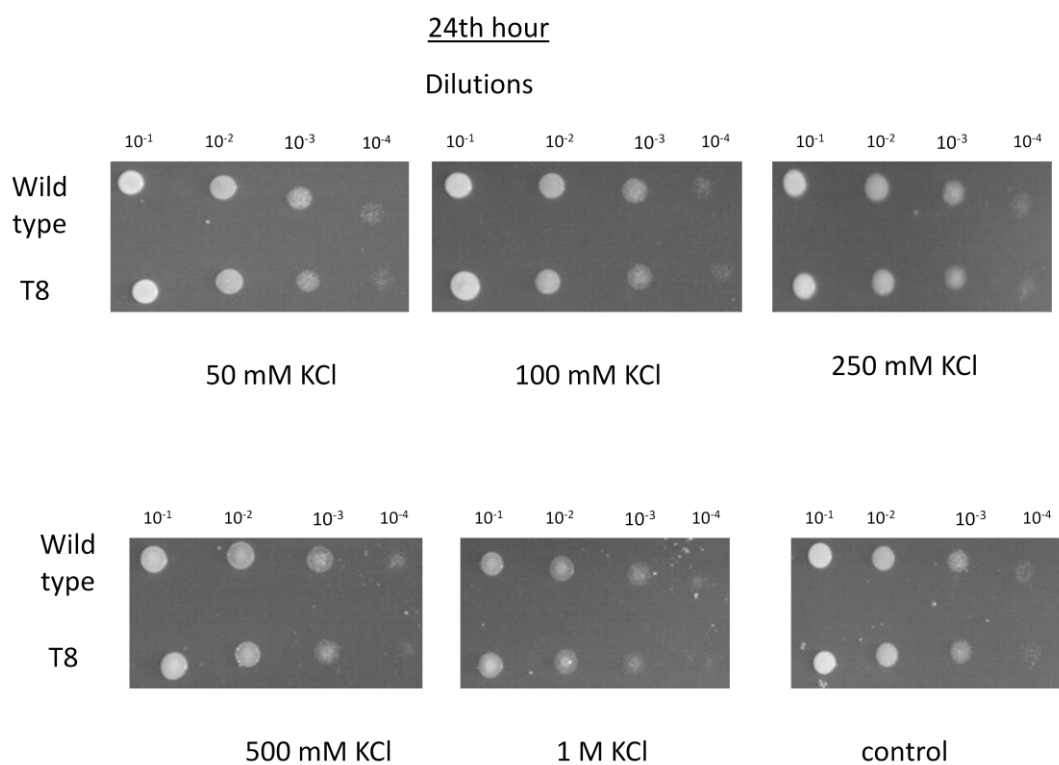


Figure 3.10 : Spot test results of wild type and T8 in YMM containing different amounts of KCl (24th hour).

KCl concentrations of 50 mM, 100 mM, 250 mM, 500 mM and 1 M were tested on wild type and T8. There was no significant difference for KCl stress between T8 and the wild type (Figure 3.10).

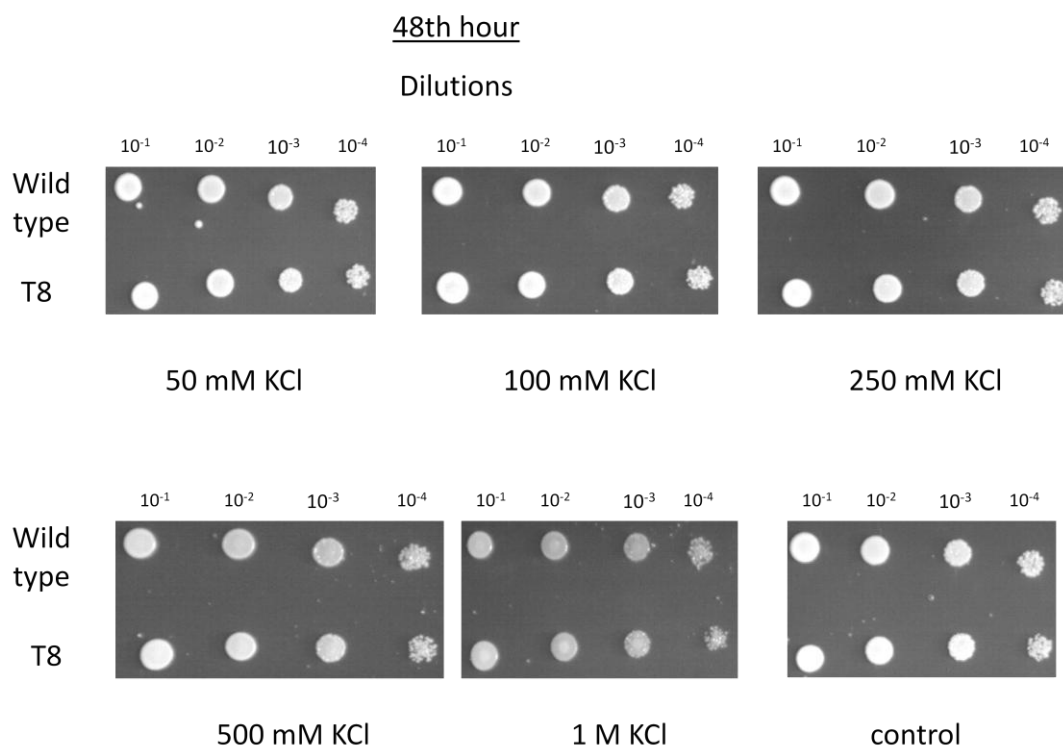


Figure 3.11 : Spot test results of wild type and T8 in YMM containing different amounts of KCl (48th hour).

There was no significant difference between T8 and the wild type for KCl stress. Concentration of 50 mM, 100 mM, 250 mM, 500 mM and 1 M KCl were tried on the wild type and T8.

3.2.3 Spot Test of CEN.PK 113.7D (wild type) and T8 with different concentrations of LiCl (YPD)

Wild type and T8 were grown until OD₆₀₀ 1.2 and equal numbers of yeast cells were harvested by centrifugation at 2500 rpm for 10 min at room temperature. Cells were diluted serially as 10⁰, 10⁻¹, 10⁻², 10⁻³, 10⁻⁴ in YPD plates without and with 5,10,15, 20 mM LiCl.

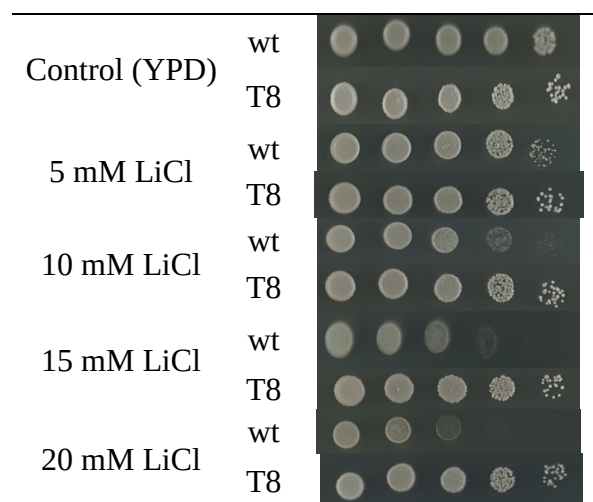


Figure 3.12 : Spot test results of wild type and T8 in YPD containing different amounts of LiCl.

In control plates (YPD), growth of wild type and T8 was almost the same. The increase in LiCl concentrations inhibited wild type's growth. However, the presence of LiCl did not reduce the growth of T8 significantly. These results clearly showed that T8 had cross-resistance to LiCl (Figure 3.12).

3.2.4 Spot Test of CEN.PK 113.7D (wild type) and T8 with KCl and Sorbitol (YMM)

Wild type and T8 were tested for their potential resistances to KCl and sorbitol. For that reason, T8 and wild type were grown in YMM until their exponential growth phase. Their diluted samples were then dropped to solid media supplemented with KCl (1.25, 1.5 and 1.75M) and sorbitol (%25, %30 and %35 w/v).

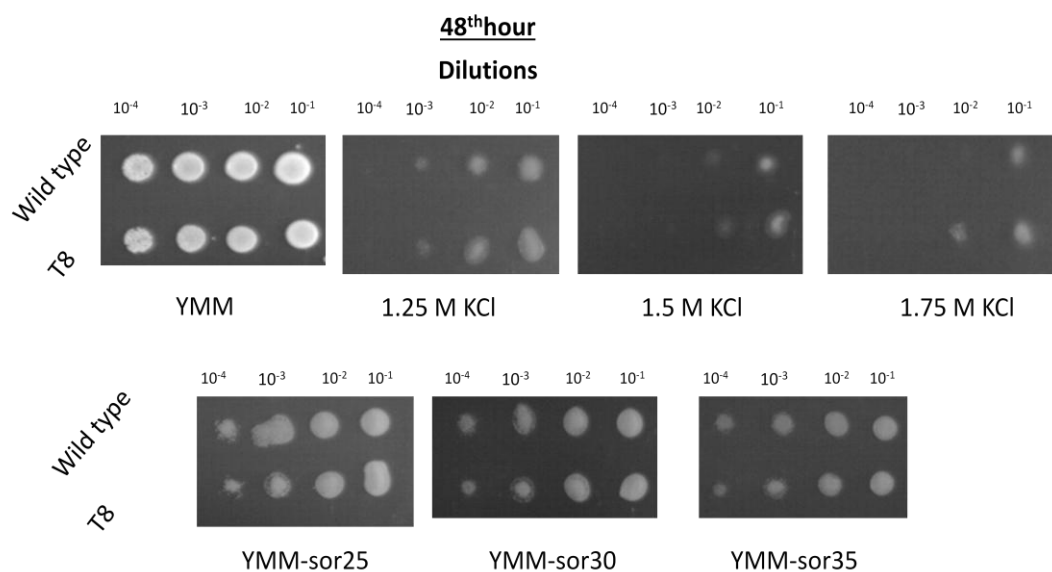


Figure 3.13 : Spot test results of wild type and T8 in YMM containing different amounts of KCl (48th hour).

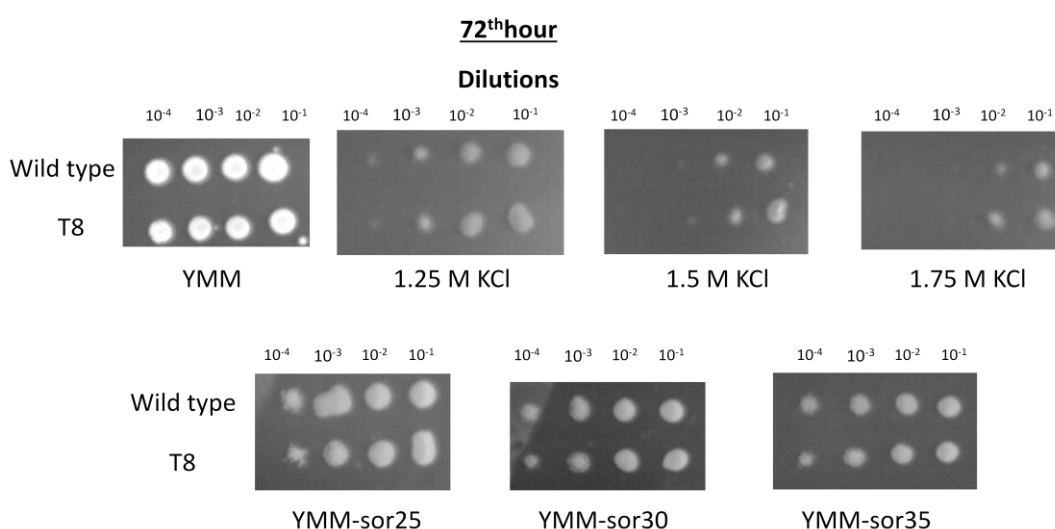


Figure 3.14 : Spot test results of wild type and T8 in YMM containing different amounts of KCl and sorbitol (72nd hour).

Salt resistant mutant grew better than the wild type at 1.75 M KCl (Figure 3.13). Although there was no significant difference for sorbitol resistance between T8 and the wild type, T8 colony size was smaller than that of the wild type (Figure 3.14). KCl and sorbitol only caused osmotic stress while NaCl also contributed to sodium toxicity. It could be shown that T8 was only resistant to sodium and lithium stress, but not to osmotic stress.

3.2.5 Spot Test of CEN.PK 113.7D (wild type) and T8 with combination of NaCl and KCl stress (YMM)

Wild type and T8 were tested for their possible resistances to KCl, NaCl and NaCl+KCl. T8 and wild type were grown in YMM until their exponential growth phase. Their diluted samples were then dropped to the media supplemented with NaCl (0.5 and 0.7 M), KCl (1 M) and both 0.5 M NaCl and 1 M KCl stress.

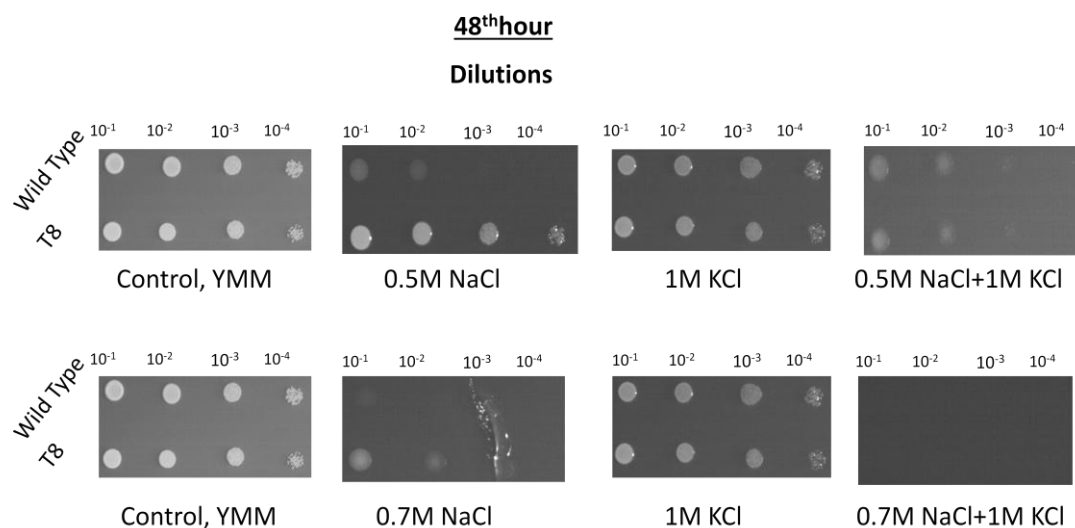


Figure 3.15 : Spot test results of wild type and T8 in YMM containing different amounts of KCl and NaCl (48th hour).

Salt resistant mutant was resistant to 0.5 and 0.7 M NaCl, but did not show any significant resistance to 1 M KCl. There was no difference between wild type and T8 resistance levels against 0.5M NaCl stress, when NaCl and KCl stress were applied at the same time to the cultures. However, both 0.7M NaCl and 1M KCl made T8 less resistant (Figure 3.15 and 3.16).

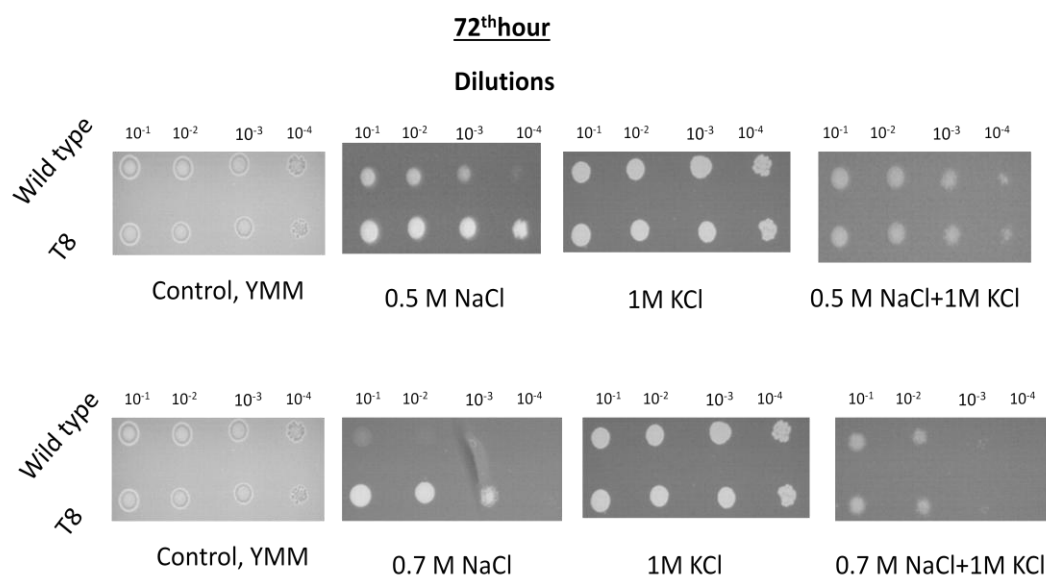


Figure 3.16 : Spot test results of wild type and T8 in YMM containing different amounts of KCl and NaCl (72nd hour).

3.2.6 Spot Test of CEN.PK 113.7D (wild type) and T8 with pH and 0.7 M NaCl stress effect (YMM)

The growth capacity of yeast strains in the presence of 0.7M NaCl was tested on solid YPD media inoculated with serial 10-fold dilutions of saturated cultures. Growth was recorded at 36th, 48th and 72nd h. Growth was tested at pH 3.6, 5.5, 6.6 and 7.9. To attain pH 3.6, HCl was added to the media, and for pH 5.5, 6.6 and 7.9. NaOH was used after autoclaving (Banuelos *et al.*, 1998).

T8 was much more resistant than the wild type in plates that contained 0.7 M NaCl at pH 3.6. T8 was more resistant than the wild type at pH 5.5 and 6.6 with 0.7 M NaCl stress. However, at pH 7.9 there was no resistance difference between T8 and the wild type. It was observed that T8 became more resistant to salt stress at acidic pH (Figure 3.17, 3.18 and 3.19).

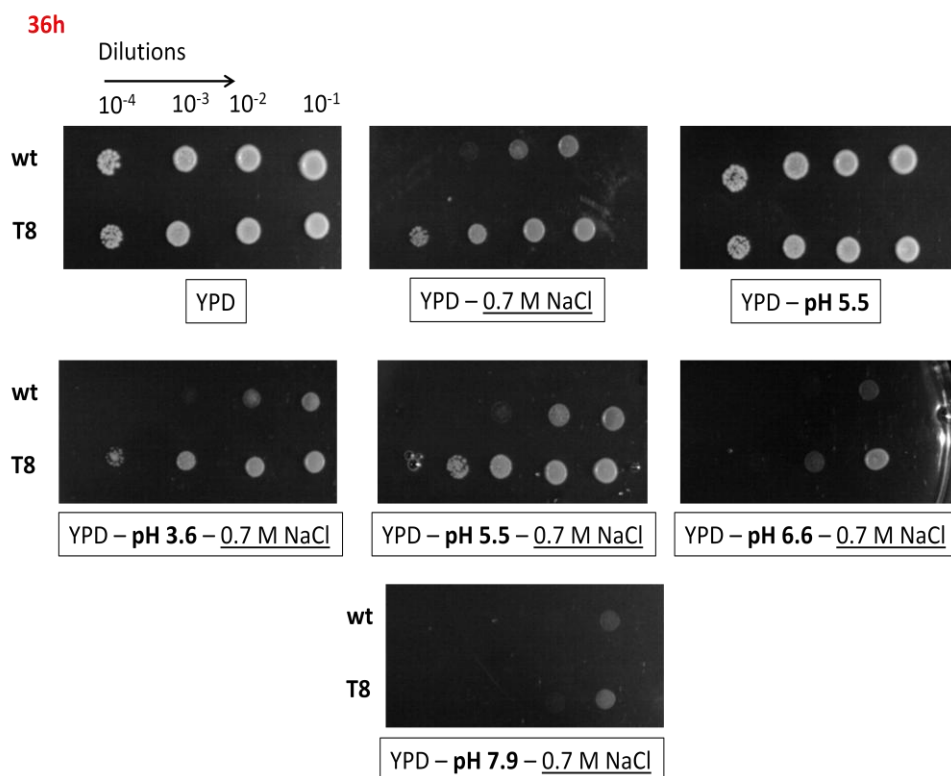


Figure 3.17 : Spot test results of wild type and T8 in YPD containing 0.7M NaCl and at different pH range (36th hour).

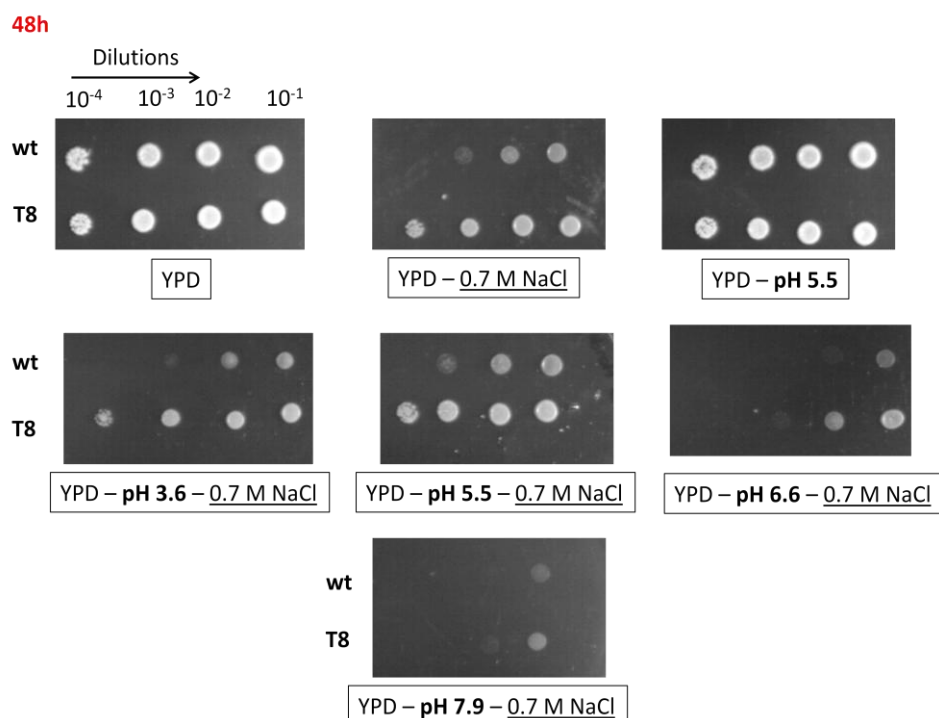


Figure 3.18 : Spot test results of wild type and T8 in YPD containing 0.7 M NaCl and at different pH range (48th hour).

72h

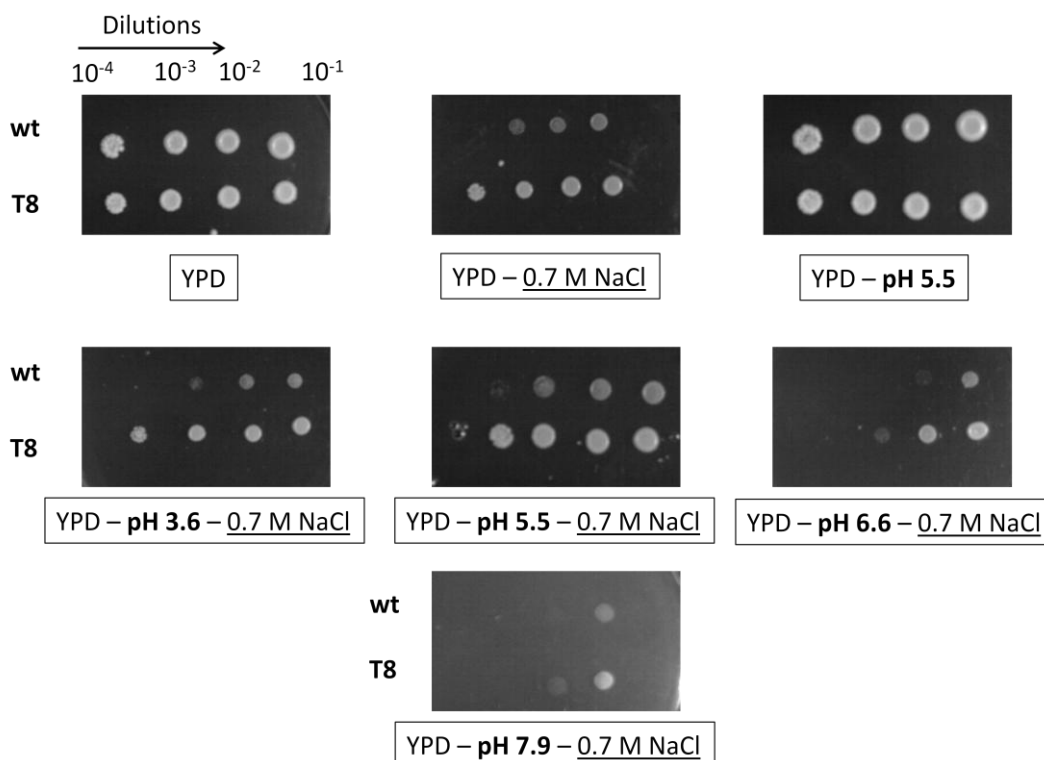


Figure 3.19 : Spot test results of wild type and T8 in YPD containing 0.7M NaCl and at different pH range (72nd hour).

3.2.7 Spot Test of CEN.PK 113.7D (wild type) and T8 with pH and 150 mM and 300 mM NaCl stress effect (YPD)

After wild type and T8 were grown until an OD₆₀₀ value of 1.2 in 10mL liquid YPD, equal numbers of yeast cells were harvested by centrifugation at 10000 *g* for 5 min and the supernatant was removed. The pellets were resuspended in 100 μ L YPD and diluted serially as 10⁻⁰, 10⁻¹, 10⁻², 10⁻³ and 10⁻⁴. Citric acid, anhydrous/ HEPES/ CHES system was used for pH adjustment.

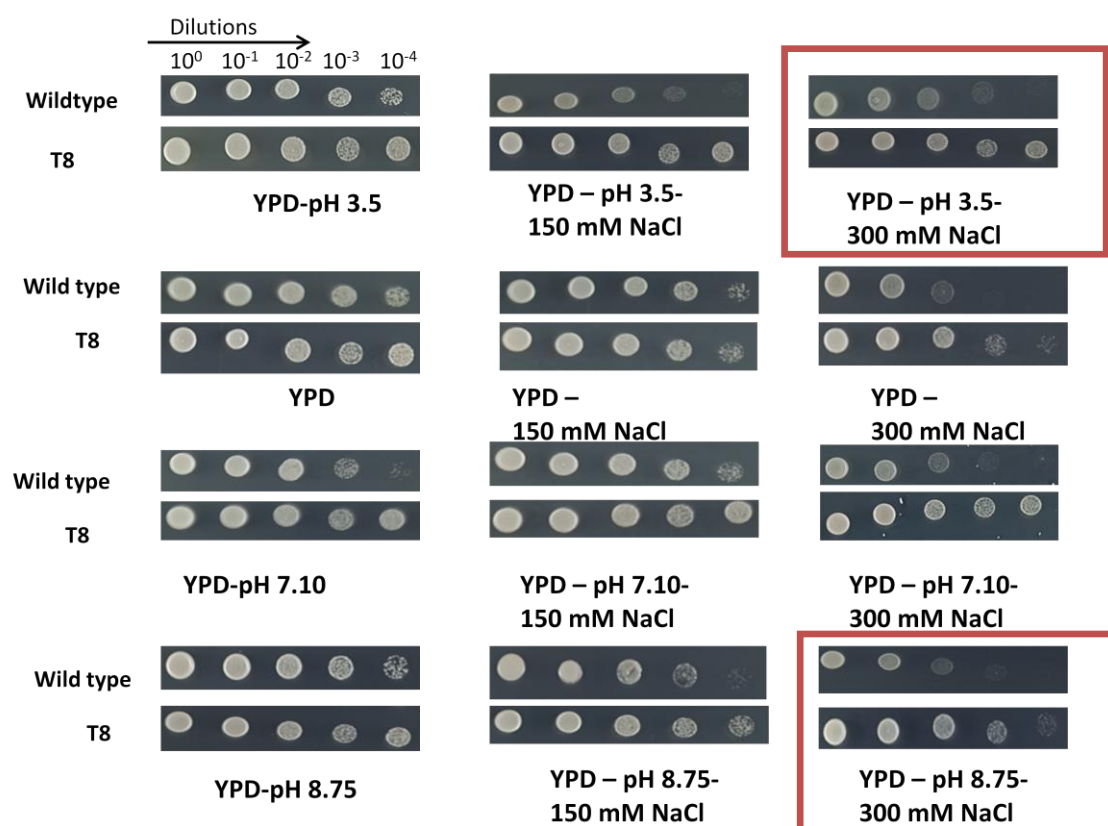


Figure 3.20 : Spot test of wild type and T8 with different concentrations of NaCl at different pH values.

Resistance of T8 to NaCl stress was remarkable at acidic (pH 3.5) and basic pH (pH 8.75) conditions. T8 has more resistance to NaCl at acidic pH (Figure 3.20).

3.2.8 Spot Test of CEN.PK 113.7D (wild type) and T8 with pH and LiCl stress effect (YPD)

After wild type and T8 were grown until $OD_{600}=1.2$ in 10-mL liquid YPD, equal numbers of yeast cells were harvested by centrifugation at 10000 *g* for 5 min and the supernatant was removed. The pellets were resuspended in 100 μ L YPD and diluted serially as $10^0, 10^{-1}, 10^{-2}, 10^{-3}$ and 10^{-4} . Citric acid, anhydrous/ HEPES/ CHES system was used for pH adjustment. Concentration for LiCl was chosen as 20 mM for pH test.

20mM LiCl	905					T8				
	10 ⁰	10 ⁻¹	10 ⁻²	10 ⁻³	10 ⁻⁴	10 ⁰	10 ⁻¹	10 ⁻²	10 ⁻³	10 ⁻⁴
pH 3.5										
pH 6.9										
pH 8.75										

Figure 3.21: Spot test of wild type and T8 with 20 mM LiCl at different pH values.

LiCl affected wild type's growth more in acidic conditions, and T8 grew better in acidic conditions than in basic conditions. So, T8 had resistance to LiCl in acidic conditions; however, it lost its resistance in basic conditions. At pH 8.75, the growth of strains seemed to be the same (Figure 3.21).

3.2.9 Spot Test of CEN.PK 113.7D (wild type) and T8 with pH and sorbitol stress effect (YPD)

50% (v/v) sorbitol	905					T8				
	10 ⁰	10 ⁻¹	10 ⁻²	10 ⁻³	10 ⁻⁴	10 ⁰	10 ⁻¹	10 ⁻²	10 ⁻³	10 ⁻⁴
pH 3.5										
pH 7.1										
pH 8.75										

Figure 3.22 : Spot test of wild type and T8 with 50% (v/v) sorbitol at different pH values.

Different pH values did not affect growth of 905 and T8. No strong T8 phenotype was observed on sorbitol plates (Figure 3.22).

3.2.10 Spot tests of FGY217, AB11c, KTA40-2 at pH3.5, pH 7.1, pH 8.75 with 150 mM, 300 mM, 400 mM NaCl

FGY217 is a *pep4Δ::hisG* strain (*MATα ura3-52 lys2Δ201 pep4Δ*). The AB11c strain is a pink haploid strain with deletion of genes for Na⁺transport (*ena1Δ::HIS3::ena4Δnha1Δ::LEU2 nhx1Δ::TRP1*). KTA40-2 strain resulted from a cross between AB11c and MA-KN (*ena1Δ::HIS3::ena4Δnha1Δ::LEU2 kha1Δ::KanMX*).

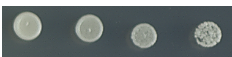
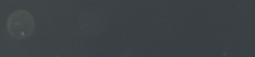
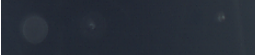
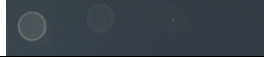
FGY217	0 mM NaCl 10 ⁰ 10 ⁻¹ 10 ⁻² 10 ⁻³	150 mM NaCl 10 ⁰ 10 ⁻¹ 10 ⁻² 10 ⁻³	300 mM NaCl 10 ⁰ 10 ⁻¹ 10 ⁻² 10 ⁻³
pH 3.5			
pH 7.1			
pH 8.75			
AB11c	0 mM NaCl 10 ⁰ 10 ⁻¹ 10 ⁻² 10 ⁻³	150 mM NaCl 10 ⁰ 10 ⁻¹ 10 ⁻² 10 ⁻³	300 mM NaCl 10 ⁰ 10 ⁻¹ 10 ⁻² 10 ⁻³
pH 3.5			
pH 7.1			
pH 8.75			
KTA40-2	0 mM NaCl 10 ⁰ 10 ⁻¹ 10 ⁻² 10 ⁻³	150 mM NaCl 10 ⁰ 10 ⁻¹ 10 ⁻² 10 ⁻³	300 mM NaCl 10 ⁰ 10 ⁻¹ 10 ⁻² 10 ⁻³
pH 3.5			
pH 7.1			
pH 8.75			

Figure 3.23 : Spot test of FGY217, AB11c, KTA40-2 at pH 3.5, pH 7.1, pH 8.75 with 150 mM, 300 mM, 400 mM NaCl.

While FGY217 could grow at different pH values, AB11c and KTA40-2 could not show the same growth attitude. The absence of *ENA1-4*, *NHA1*, *NHX1*, *KHA1* genes decreased Na⁺ tolerance. AB11c and KTA40-2 could grow at pH 3.5, 7.1 and 8.75 without NaCl stress. When 150 mM NaCl was added to solid medium (YPD), basic and acidic pH abolished growth of these strains. 150 mM NaCl did not affect the growth of AB11c and KTA40-2 at neutral pH. Although 300 mM NaCl did not affect the growth of FGY217, 300 mM NaCl abolished the growth of AB11c and KTA40-2 (Figure 3.23).

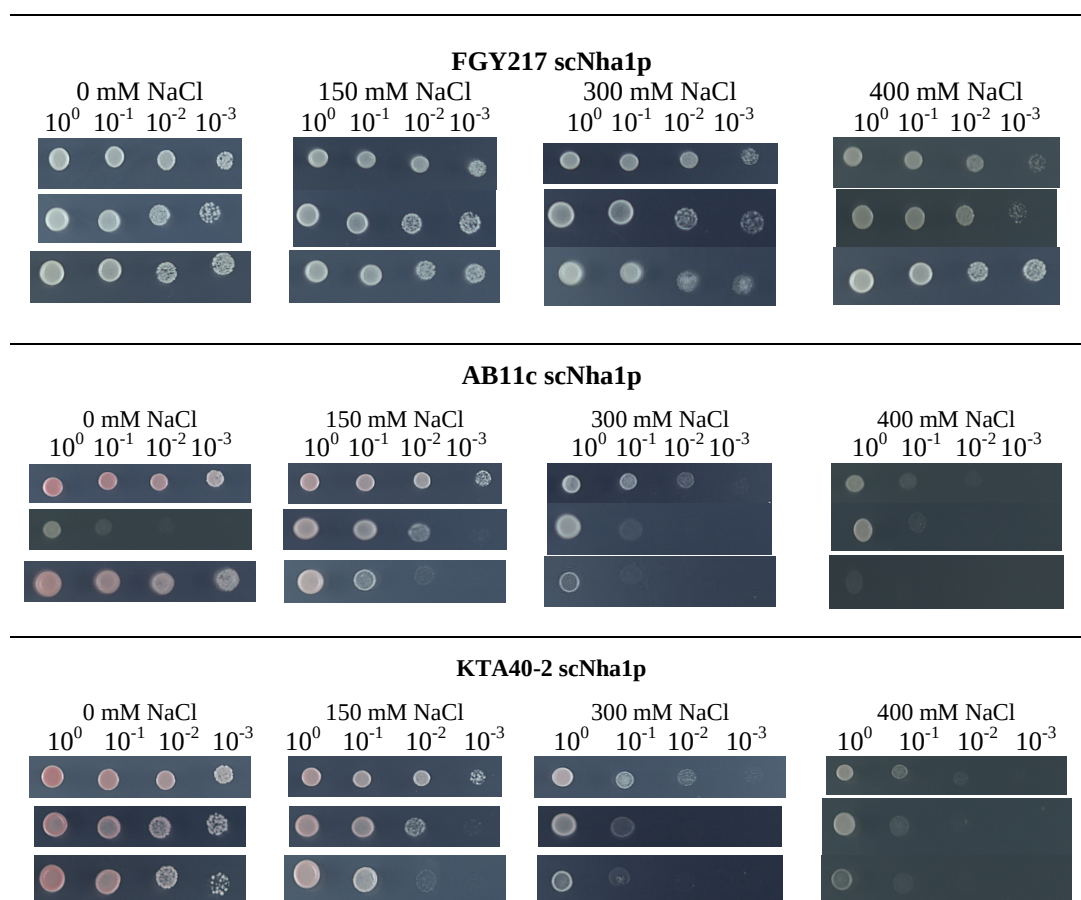


Figure 3.24 : Spot test of scNha1p in FGY217, AB11c, KTA40-2 at pH 3.5, pH 7.1, pH 8.75 from top to bottom, respectively, with 150 mM, 300 mM, 400 mM NaCl.

The complementation of Nha1p in Na^+ tolerance was evident at pH 3.5. Overexpression of Nha1p improved Na^+ tolerance in those strains. The effects of *NHA1* gene at pH values 3.5 were more pronounced than at pH 7.1 and 8.75. It showed that Nha1p had a better effect on Na^+ tolerance up to pH 7.1 (Figure 3.24). The results demonstrated the complementary function of the Nha1 antiporter for Na^+ tolerance in cells growing at different pH values.

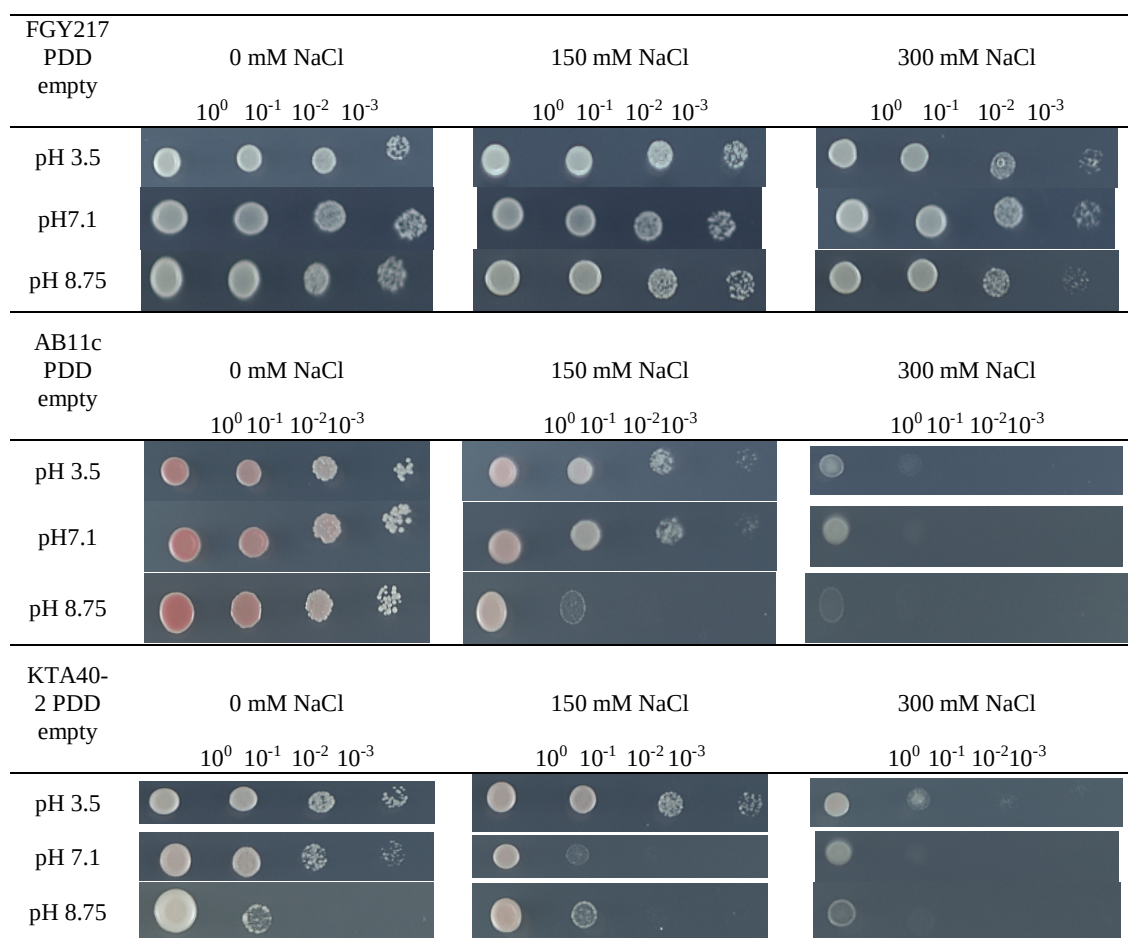


Figure 3.25 : Spot test of PDD empty in FGY217, AB11c, KTA40-2 at pH 3.5, pH 7.1, pH 8.75 with 150 mM, 300 mM.

NHA1 gene was overexpressed from PDD vector to verify the influence of Nha1p on NaCl tolerance. As a control, cells harboring empty plasmid were used. The tested protein increased NaCl tolerance of strains compared to cells harboring the empty plasmid (Figure 3.25).

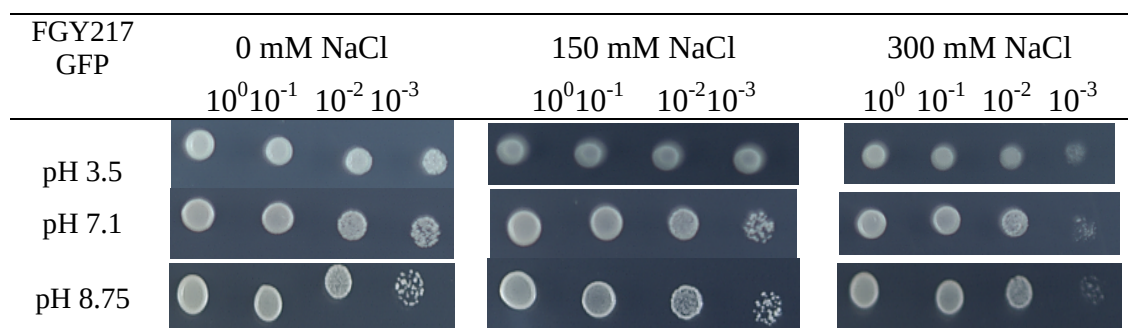


Figure 3.26 : Spot test of GFP in FGY217 at pH 3.5, pH 7.1, pH 8.75 with 150 mM, 300 mM NaCl.

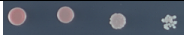
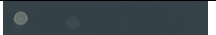
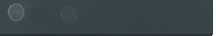
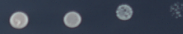
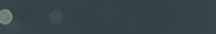
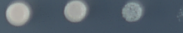
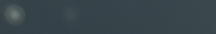
AB11c GFP	0 mM NaCl 10 ⁰ 10 ⁻¹ 10 ⁻² 10 ⁻³	150 mM NaCl 10 ⁰ 10 ⁻¹ 10 ⁻² 10 ⁻³	300 mM NaCl 10 ⁰ 10 ⁻¹ 10 ⁻² 10 ⁻³	400 mM NaCl 10 ⁰ 10 ⁻¹ 10 ⁻² 10 ⁻³
pH 3.5				
pH 7.1				
pH 8.75				
KTA40-2 GFP	0 mM NaCl 10 ⁰ 10 ⁻¹ 10 ⁻² 10 ⁻³	150 mM NaCl 10 ⁰ 10 ⁻¹ 10 ⁻² 10 ⁻³	300 mM NaCl 10 ⁰ 10 ⁻¹ 10 ⁻² 10 ⁻³	400 mM NaCl 10 ⁰ 10 ⁻¹ 10 ⁻² 10 ⁻³
pH 3.5				
pH 7.1				
pH 8.75				

Figure 3.26 (continued) : Spot test of GFP in AB11c, KTA40-2 at pH 3.5, pH 7.1, pH 8.75 with 150 mM, 300 mM, 400 mM NaCl.

Nha1p is a membrane protein in *S. cerevisiae*. Improving the success in obtaining pure membrane proteins for biochemical and structural analysis was to use GFP-based fusion technology. As the C-terminal GFP folded and became fluorescent only if the upstream membrane protein integrated into the membrane, the resultant fluorescence was a fast and accurate measure of membrane-integrated expression (Drew *et al.*, 2008). GFP fluorescence provided a unique and sensitive means for detecting tagged proteins within a cellular environment (Hammon *et al.*, 2009). PDD tagged with GFP gave the same result as PDD empty. There was no effect of GFP on the complementation of Nha1p (Figure 3.26).

3.2.11 Spot tests of FGY217, AB11c, KTA40-2 at pH 3.5, pH 7.1, pH 8.75 with 50% (v/v) sorbitol

FGY217 scNha	50% (v/v) sorbitol			
	10^0	10^{-1}	10^{-2}	10^{-3}
pH 3.5				
pH 7.1				
pH 8.75				
KTA40-2 scNha	50% (v/v) sorbitol			
	10^0	10^{-1}	10^{-2}	10^{-3}
pH 3.5				
pH 7.1				
pH 8.75				
AB11c scNha	50% (v/v) sorbitol			
	10^0	10^{-1}	10^{-2}	10^{-3}
pH 3.5				
pH 7.1				
pH 8.75				

Figure 3.27 : Spot test of scNha in AB11c, KTA40-2 at pH 3.5, pH 7.1, pH 8.75 with 50% (v/v) sorbitol.

Transformation with the *NHA1* gene on plasmid restored the ability of KTA40-2 to grow in the presence of 50% (v/v) sorbitol. The effect of *NHA1* mutation was not related to pH sensitivity in sorbitol stress (Figure 3.27).

KTA40-2 GFP	%50 sorbitol			
	10^0	10^{-1}	10^{-2}	10^{-3}
pH 3.5				
pH 7.1				
pH 8.75				
AB11c GFP	%50 sorbitol			
	10^0	10^{-1}	10^{-2}	10^{-3}
pH 3.5				
pH 7.1				
pH 8.75				

Figure 3.28 : Spot test of GFP in AB11c, KTA40-2 at pH 3.5, pH 7.1, pH 8.75 with 50% (v/v) sorbitol.

Spot test of GFP in KTA40-2 at pH 3.5, pH 7.1, pH 8.75 with 50% (v/v) sorbitol PDD tagged with GFP was used as a control for sorbitol stress effect on complementation of Nha1p (Figure 3.28).

3.3 pH Determination Experiment

Both wild type and T8 with and without 0.7 M NaCl were cultivated in 50 mL culture tubes containing 10mL liquid YMM and incubated at 30°C and 150 rpm. pH was measured at 6th, 24th, 48th and 72nd hour. OD₆₀₀ was measured at 6th, 24th, 48th and 72nd hour

Ena/Pmr2 ATPases are responsible for cell growth at high concentrations of NaCl and KCl at high external pH values, while the Nha1 antiporter is necessary at acidic pH levels. Nha1p is a typical secondary active transport system, which used the proton gradient created by Pma1 ATPase as the energy source for K⁺/(Na⁺) export (Maresova *et al.*, 2006). It was checked if NaCl made the medium more basic than medium without salt stress.

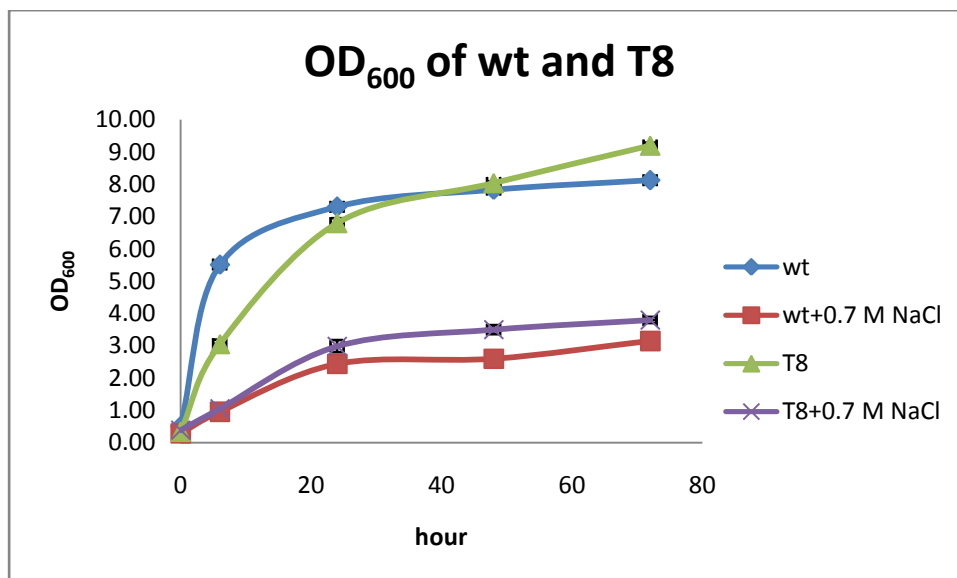


Figure 3.29 : OD₆₀₀ values of wild type, T8, wild type+0.7 M NaCl and T8+0.7 M NaCl.

OD₆₀₀ of T8 in the presence of 0.7 M NaCl was higher than that of the wild type. It can be concluded that T8 is resistant to 0.7 M NaCl. pH of wild type, T8, wild type with 0.7 M NaCl and T8 with 0.7 M NaCl became acidic during batch growth (Figure 3.29).

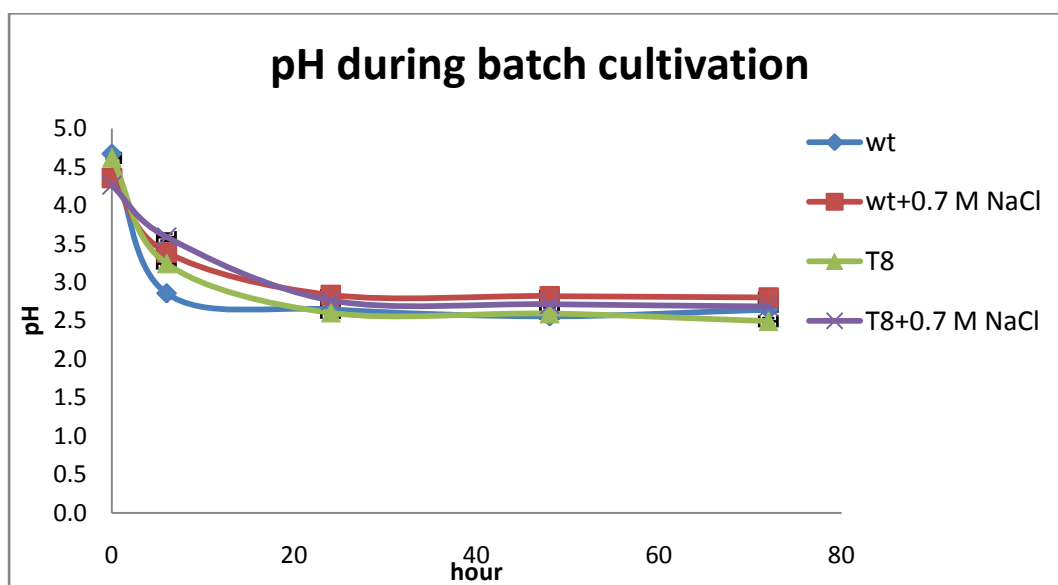


Figure 3.30 : pH values of wild type, T8, wild type+0.7 M NaCl and T8+0.7 M NaCl.

3.4 Proton Efflux

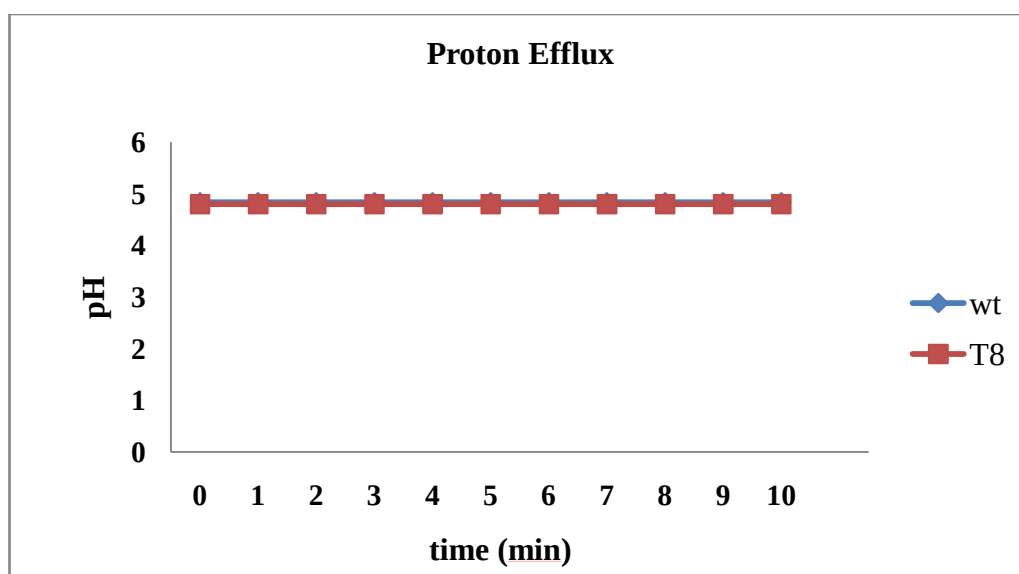


Figure 3.31: Proton efflux of wild type and T8.

There was no difference in proton extrusion capacity between salt-resistant mutant "T8" and the wild type (Figure 3.31). T8 was not more sensitive to cationic drugs such as spermine and tetramethylammonium (TMA) (Figure 3.6 and 3.7). Results of spot tests (Figure 3.6 and 3.7) confirmed the results of this proton efflux experiment.

3.5 Estimation Of Stress Resistance For CEN.PK 113.7D By Spot Test (Cross-resistance)

3.5.1 YMM

Wild type and T8 were tested for their potential cross-resistances to different types of stress on solid media. They were grown in YMM until their exponential growth phase. They were serially diluted and spotted onto the YMM agar plates supplemented with 5 mM LiCl, 8% ethanol, 0.15 mM CuCl₂, 3 mM CoCl₂, 5 mM NiCl₂, 1 mM H₂O₂, 2 mM CrCl₃, 7.5 mM AlCl₃, 10 mM ZnCl₂, 20 mM MnCl₂ stresses.

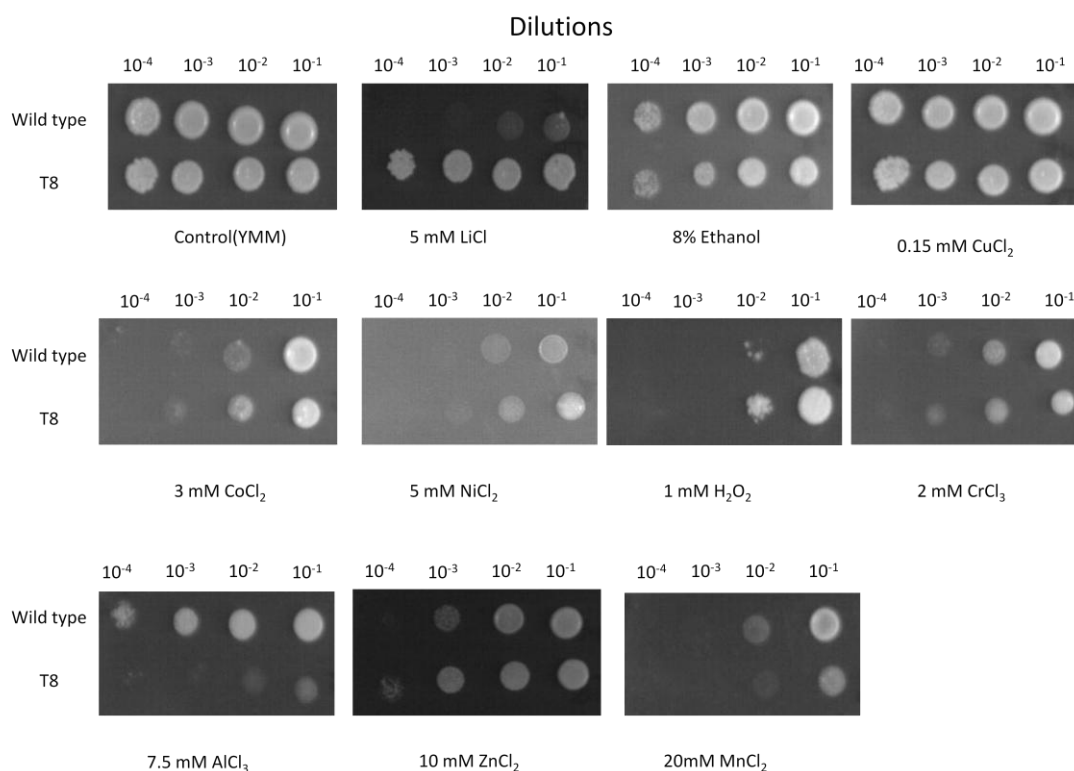


Figure 3.32 : Spot test results of wild type and T8 in YMM in the presence of different stress types.

T8 seemed to be slightly more resistant to NiCl₂, H₂O₂ and ZnCl₂ than the wild type according to the spot assay analyses. It was observed that the T8 mutant can survive in the presence of 5 mM LiCl stress, but the wild type could not survive (Figure 3.32). This data was consistent with the MPN method results for the 5 mM LiCl stress condition. Resistance of the NaCl-evolved strain contrasted with its hypersensitivity to aluminum and manganese.

3.5.2 YPD

Wild type and T8 were tested for their potential cross-resistances to different types of stress on solid media. They were grown in YPD until their exponential growth phase. They were serially diluted and spotted onto the YPD agar plates supplemented with 5 mM LiCl, 8% ethanol, 0.15 mM CuCl₂, 3 mM CoCl₂, 1 mM H₂O₂, 2 mM CrCl₃, 0.7 M NaCl, 7.5 mM AlCl₃, 10 mM ZnCl₂, 20 mM MnCl₂, 1 M KCl stresses.

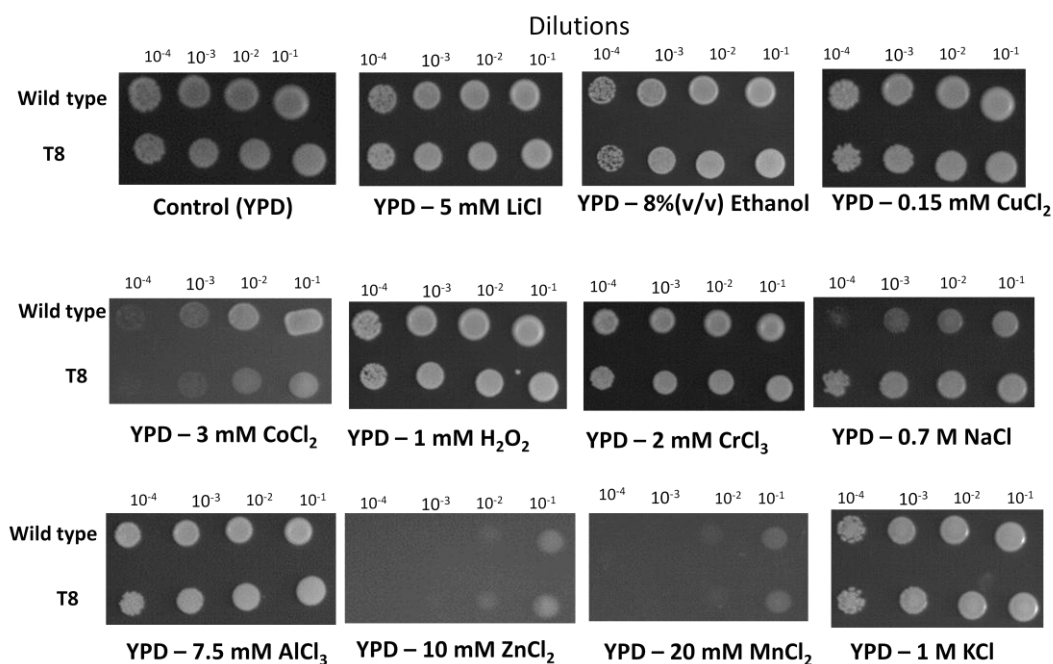


Figure 3.33 : Spot test results of wild type and T8 in YPD in the presence of different stress types.

When the same amount of stress were applied to wt and T8 on YPD plates as YMM, resistance of T8 was not obvious. For spot tests on YPD, higher stress levels seem to be necessary (Figure 3.33).

3.6 Most Probable Number (MPN) Assay

S. cerevisiae cells (CEN.PK 113.7D) and NaCl-resistant mutant individual T8 were screened at different stress conditions via 5-tube MPN methodology. The cross-resistance analyses were performed by using continuous stress strategy.

Wild-type and T8 were exposed to 5 mM LiCl, 0.7 M NaCl, 10 mM ZnCl₂, 0.8 mM H₂O₂, 4 mM CoCl₂, 1.5 M KCl, 6% (v/v) ethanol, 0.1 mM CuCl₂, 15 mM MnCl₂, 2 mM CrCl₃, 10 mM AlCl₃ stresses.

The MPN results and survival ratios for the applies stresses are shown in Tables 3.12, 3.13 and 3.14.

Table 3.10 : MPN cell number of wild type and T8 at NaCl, LiCl and KCl stresses.

Stress Conditions	Wild Type "Number of cells/mL "	T8 "Number of cells/mL "	Survival ratio of Wild Type	Survival Ratio of T8	Survival Ratio of T8 as fold of Wild Type
Control	920000	920000	-----	-----	-----
0.7 M NaCl	240	350000	0.000261	0.380435	1458
1 M KCl	210000	920000	0.23	1	4.38
5 mM LiCl	240	920000	0.00011	2.63	24115

Table 3.11: MPN cell number of wild type and T8 at different stress conditions.

Stress Conditions	Wild Type "Number of cells/mL "	T8 "Number of cells/mL "	Survival ratio of Wild Type	Survival Ratio of T8	Survival Ratio of T8 as fold of Wild Type
Control	540000	92000	-----	-----	-----
10 mM ZnCl₂	23	240	0.00004	0.003	61.2
0.8 mM H₂O₂	350	3500	0.0006	0.04	59
4 mM CoCl₂	23	23	0.00004	0.00025	5.87
6% ethanol	350000	92000	0.65	1	1.54
0.1 mM CuCl₂	2400000	350000	4.44	3.8	0.86
15 mM MnCl₂	2400	240	0.004	0.002	0.59
2 mM CrCl₃	240	23	0.0004	0.00025	0.56
10 mM AlCl₃	280000	23	0.52	0.0003	0.0005

Survival ratios of wild type and T8 were calculated according to a published MPN table (<http://www.jlindquist.net/generalmicro/102dil3a.html>)

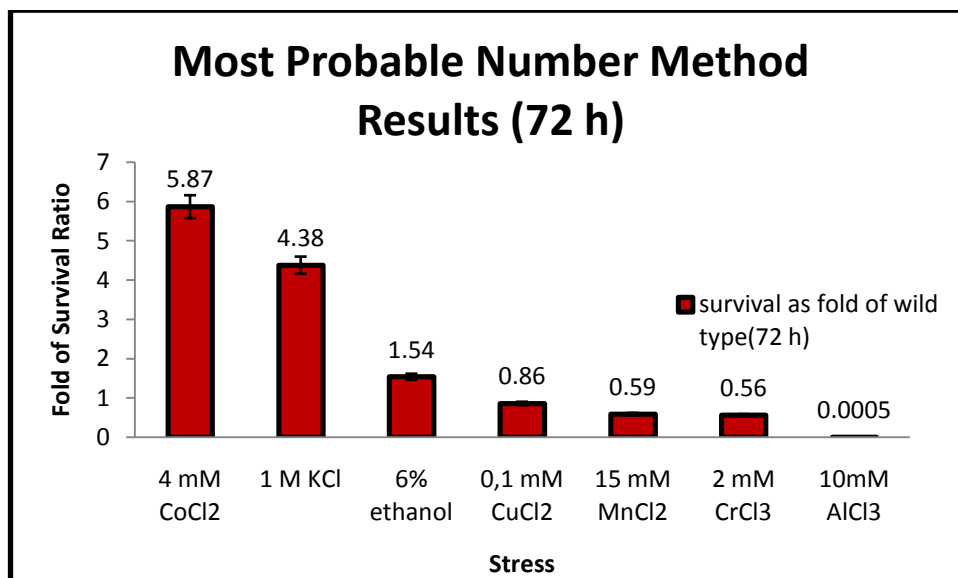


Figure 3.34: Determination of survival ratio of T8 normalized to wild type at 4 mM CoCl₂, 1.5 M KCl, 6%(v/v) ethanol, 15 mM MnCl₂, 2 mM CrCl₃, 10 mM AlCl₃.

In MPN results, T8 had slight sensitivity to CuCl₂, MnCl₂ and CrCl₃ and showed high sensitivity to AlCl₃.

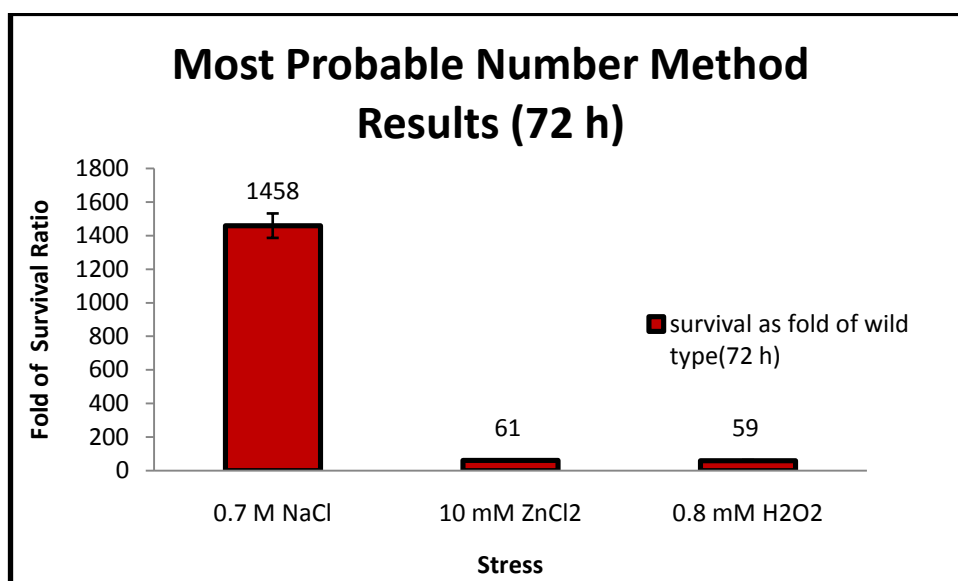


Figure 3.35 : Determination of survival ratio of T8 normalized to wild type at 0.7 M NaCl, 10 mM ZnCl₂, 0.8 mM H₂O₂.

T8 had resistance to KCl, CoCl₂, H₂O₂, ZnCl₂ and NaCl (Fig 3.34 and 3.35).

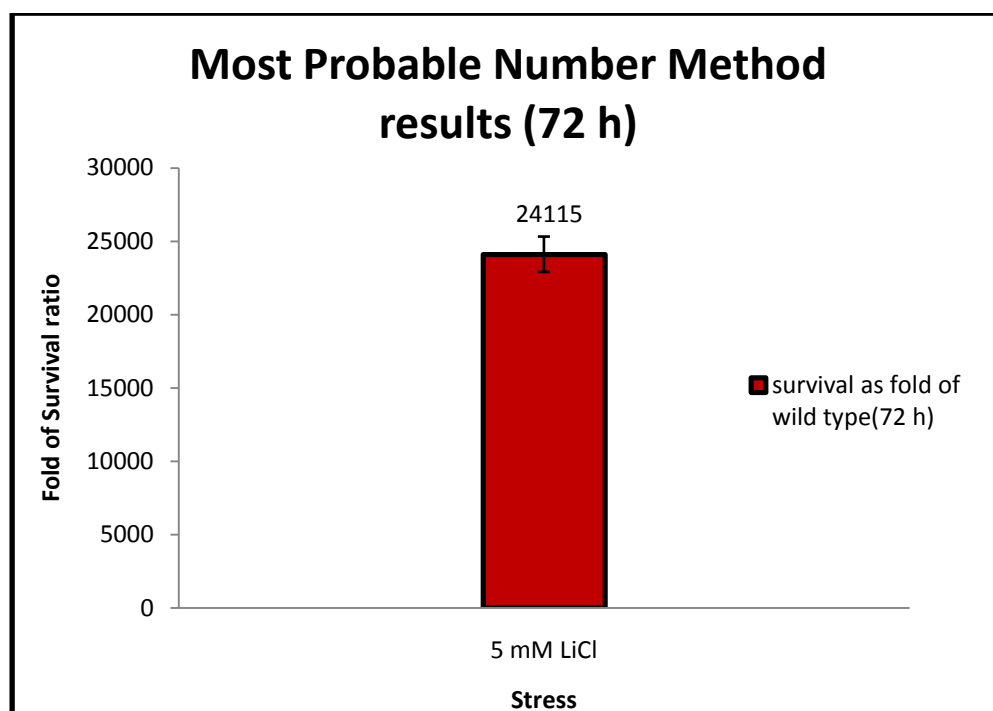


Figure 3.36 : Determination of survival ratio of T8 normalized to wild type at 5 mM LiCl.

T8 showed significantly high resistance to LiCl (Figure 3.36).

Table 3.12 : Survival ratios of T8 as fold of wild type in all stress conditions (MPN results).

Stress Conditions	Survival ratio of T8 as fold of wild type (72 h)
Control	1
5 mM LiCl	24115
0.7 M NaCl	1458
10 mM ZnCl ₂	61
0.8 mM H ₂ O ₂	59
4 mM CoCl ₂	5.87
1.5 M KCl	4.38
6% ethanol	1.54
0.1 mM CuCl ₂	0.86
15 mM MnCl ₂	0.59
2 mM CrCl ₃	0.56
10 mM AlCl ₃	0.0005

It could be clearly observed that NaCl-resistant mutant T8 had the highest fold of survival ratio in the presence of 0.7 M NaCl and 5 mM LiCl (Table 3.12). Results of MPN were in accordance with results of spot test.

3.7 Metal Content Determination by Flame Atomic Absorption Spectrometer (FAAS)

Cell dry weights (CDW) were measured for both T8 and wild type strains at different stress conditions (Table 3.13) in triplicates.

Table 3.13 : Cell dry weights (CDW) values.

Culture	AVG CDW (mg/ml)	STD AVG CDW (mg/ml)	OD ₆₀₀
wt control-1	2.46	0.31	6.01
wt 0.7 M NaCl-1	0.71	0.48	1.06
wt 5 mM LiCl-1	1.75	0.64	1.66
wt 1M KCl-1	1.76	0.71	2.06
wt 0.7 M NaCl+5 mM LiCl-1	1.1	0.73	0.80
wt 0.7 M NaCl+ 1 M KCl-1	0.97	0.39	0.68
wt 1 M KCl+ 5mM LiCl-1	1.3	0.58	1.60
T8 control-1	2.23	0.1	5.49
T8 0.7 M NaCl-1	0.96	0.26	1.88
T8 5 mM LiCl-1	2.22	1.02	3.81
T8 1M KCl-1	1.43	0.33	1.71
T8 0.7 M NaCl+5 mM LiCl-1	0.65	0.33	1.45
T8 0.7 M NaCl+ 1 M KCl-1	1.1	0.65	0.90
T8 1 M KCl+ 5 mM LiCl-1	1.41	0.3	2.14

According to results, mutant T8 was more tolerant than the wild type against sodium, lithium and potassium, as indicated by the CDW value (Table 3.13). Salt-resistant mutant ‘T8’ could survive under lithium and potassium stress. This showed that T8 had cross resistance to lithium and potassium.

Salt resistant mutant T8 mainly grew better than the wildtype under NaCl and LiCl stress conditions, but the wild type grew slightly better under KCl stress (Table 3.13). This may be a result of wild type yeast’s potassium tolerance, while toxic sodium and lithium levels were not tolerated. KCl caused osmotic stress while NaCl contributed to sodium toxicity (Rios *et al.*, 1996). Sodium toxicity affected wild type, however, osmotic stress was not that effective. Salt-resistant mutant yeast T8 were

more tolerant to NaCl and LiCl and they could survive under stress conditions (Figure 3.37).

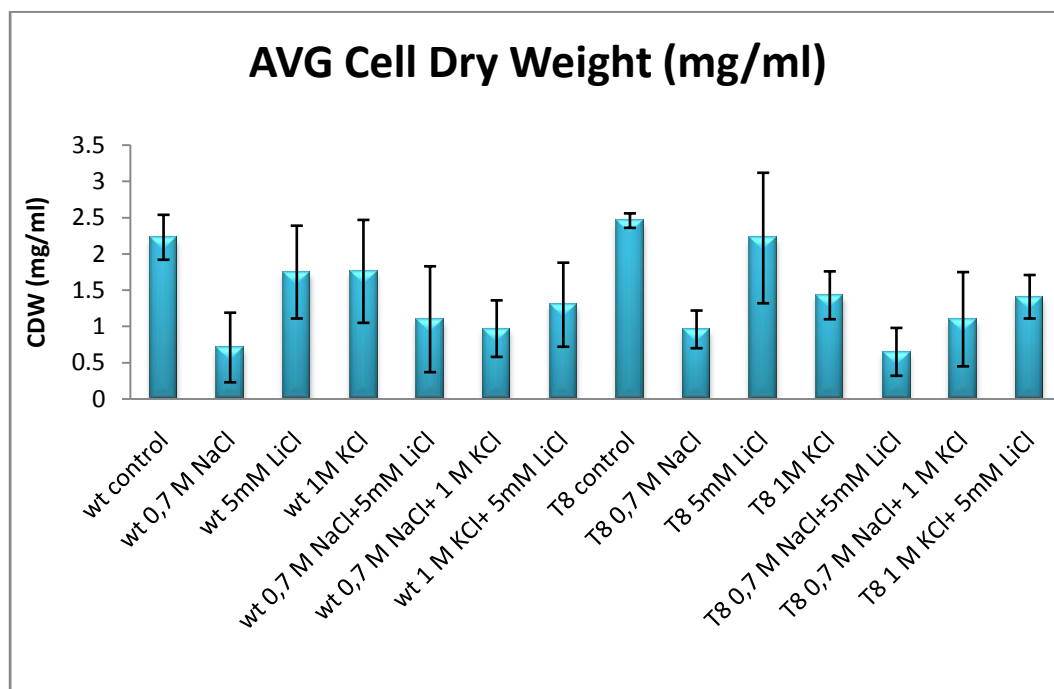


Figure 3.37: Cell dry weight measurement results.

3.7.1 Potassium and sodium content determination via Flame Atomic Absorption Spectrometer (FAAS)

Cells were grown in YMM at 30°C to an OD₆₀₀ of 3 to determine whether the cellular potassium and sodium contents were altered in response to NaCl (0.7 M), LiCl (5 mM) and KCl (1M), both NaCl and LiCl (0.7 M and 5 mM), both NaCl and KCl (0.7 M and 1M), both KCl and LiCl (1 M and 5 mM).

Potassium and sodium content of T8 and 905 are shown in Table 3.14.

Table 3.14 : Total cellular potassium, sodium and lithium amount ($\mu\text{g}/\text{mg}$) in wild type and T8 mutant, as determined by flame atomic absorption spectrometry.

	K^+ ($\mu\text{g}/\text{mg}$)	standard deviation	Na^+ ($\mu\text{g}/\text{mg}$)	standard deviation	Li^+ ($\mu\text{g}/\text{mg}$)	standard deviation
wt	7.45	0.68	2.43	0.25	0.12	0.09
Wt+0.7 M NaCl	1.53	0.61	7.45	0.56	1.29	0.41
Wt+5mM LiCl	4.14	0.94	1.24	0.52	0.07	0.04
Wt+1M KCl	12.82	0.97	2.40	0.65	0.23	0.2
Wt+5mM LiCl+0.7 M NaCl	0.89	0.62	14.03	0.52	1.04	0.57
Wt+1M KCl+0.7 M NaCl	6.70	0.70	5.14	0.68	0.91	0.69
Wt+1M KCl+5mM LiCl	7.92	0.05	0.86	0.44	0.87	0.21
T8	8.20	0.47	1.63	0.38	0.12	0.29
T8+0.7 M NaCl	2.48	0.43	9.81	0.66	0.17	0.35
T8+5mM LiCl	6.82	0.95	0.96	0.44	0.15	0.69
T8+1M KCl	157.44	0.95	25.16	0.67	0.24	0.51
T8+5mM LiCl+0.7 M NaCl	0.53	0.31	6.25	0.49	7.6	0.2
T8+1 M KCl+0.7 M NaCl	4.39	0.53	2.87	0.09	3.34	0.59
T8+1M KCl+5mM LiCl	8.66	0.95	0.78	0.22	1.81	0.45

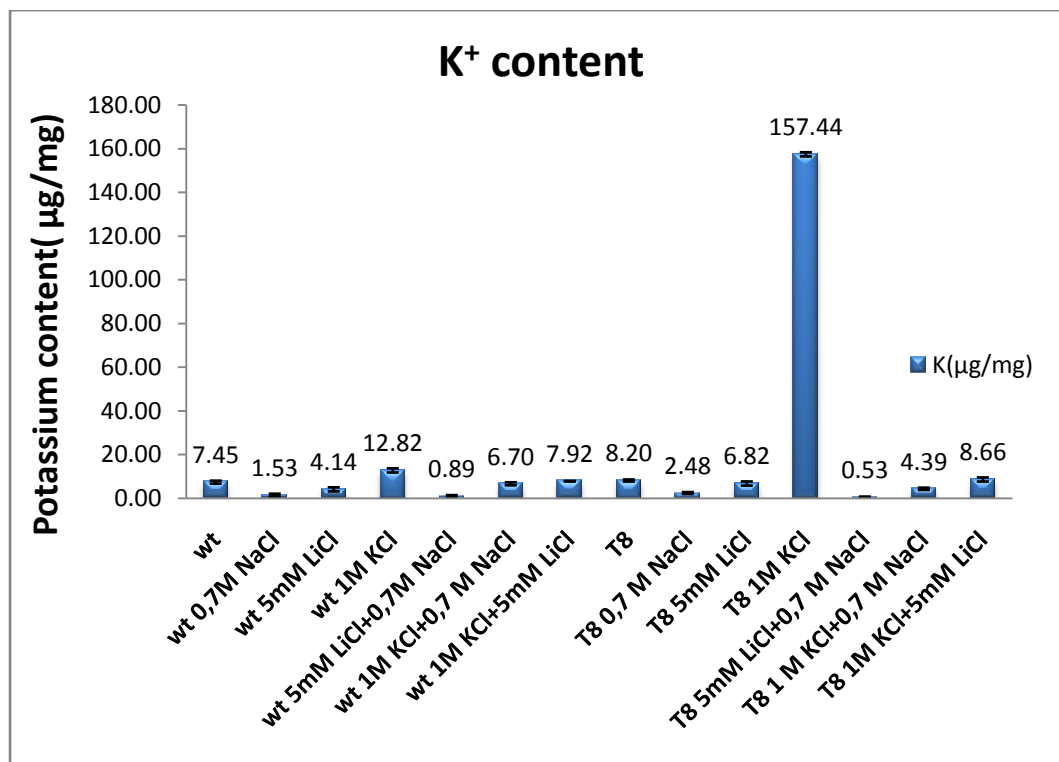


Figure 3.38: Total cellular potassium amount in wild type and T8 mutant.

The results showed that when there was sodium in the medium, the amount of sodium in T8 was always higher. In salt resistant mutant (T8) without any stress, K^+ amount was the same in T8 compared to wildtype. When 0.7 M NaCl was added to the medium, K^+ amount was the same in T8 compared to wild type. 5 mM LiCl increased potassium amount in T8. The co-presence of LiCl and NaCl and also the co-presence of KCl and LiCl did not affect the amount of potassium in T8 and in wild type (Figure 3.38).

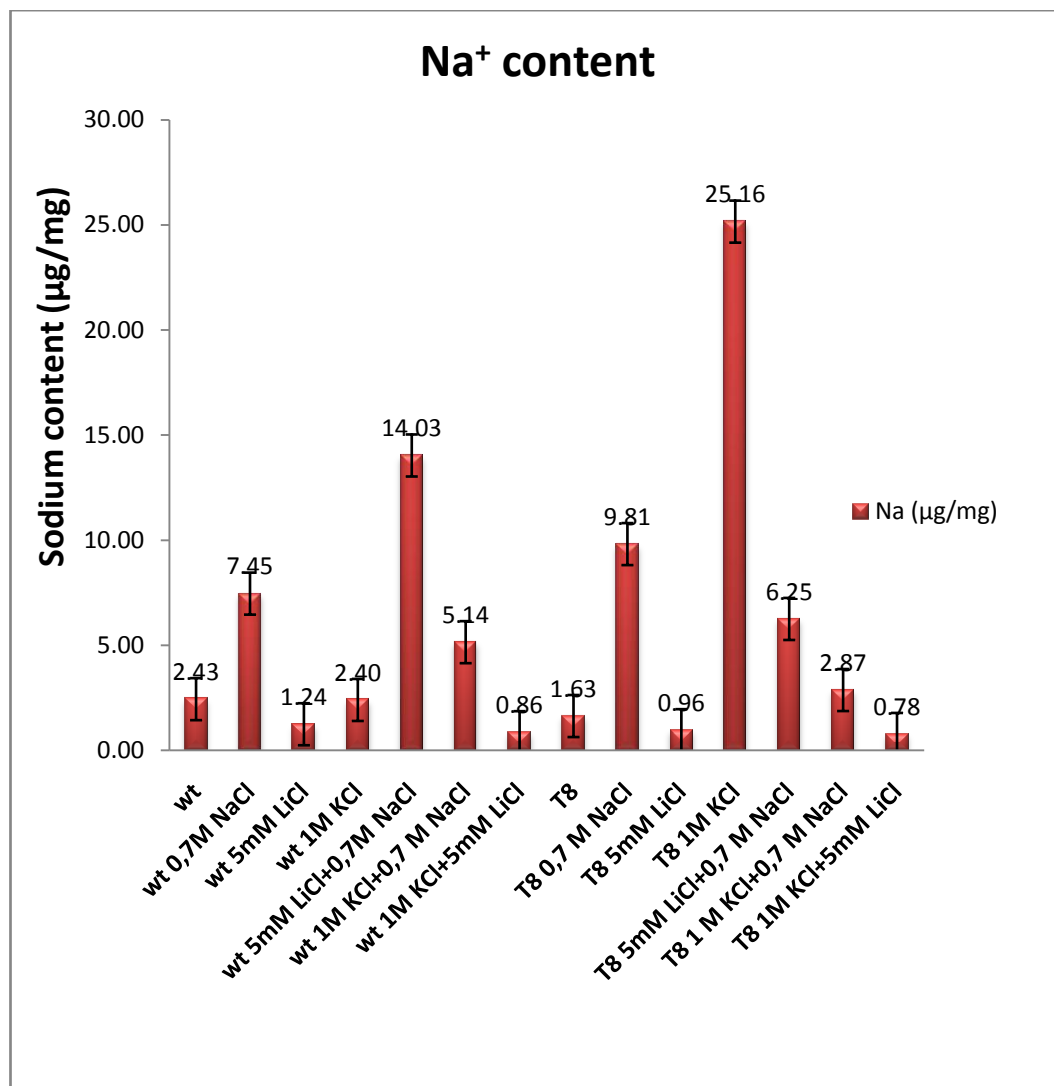


Figure 3.39 : Total cellular sodium amount in wild type and T8 mutant.

The results clearly showed that the sodium content of T8 cells was higher than that of the wild type in the presence of NaCl and KCl stress. According to these data, the NaCl-resistant mutant T8 could tolerate both 0.7 M NaCl and 1M KCl conditions. In the presence of 0.7 M NaCl, the amount of sodium was higher in T8 compared to the wild type. 5 mM LiCl stress affected the sodium amount in T8, and the amount of sodium is higher in T8 in the presence of 5 mM LiCl than in wild type. T8 held more sodium than the wild type. The amount of sodium was higher in wild type than in T8 in the co-presence of LiCl and KCl stress. When strains were incubated in the co-presence of KCl and LiCl, the amount of sodium was the same for both T8 and the wild type (Figure 3.39).

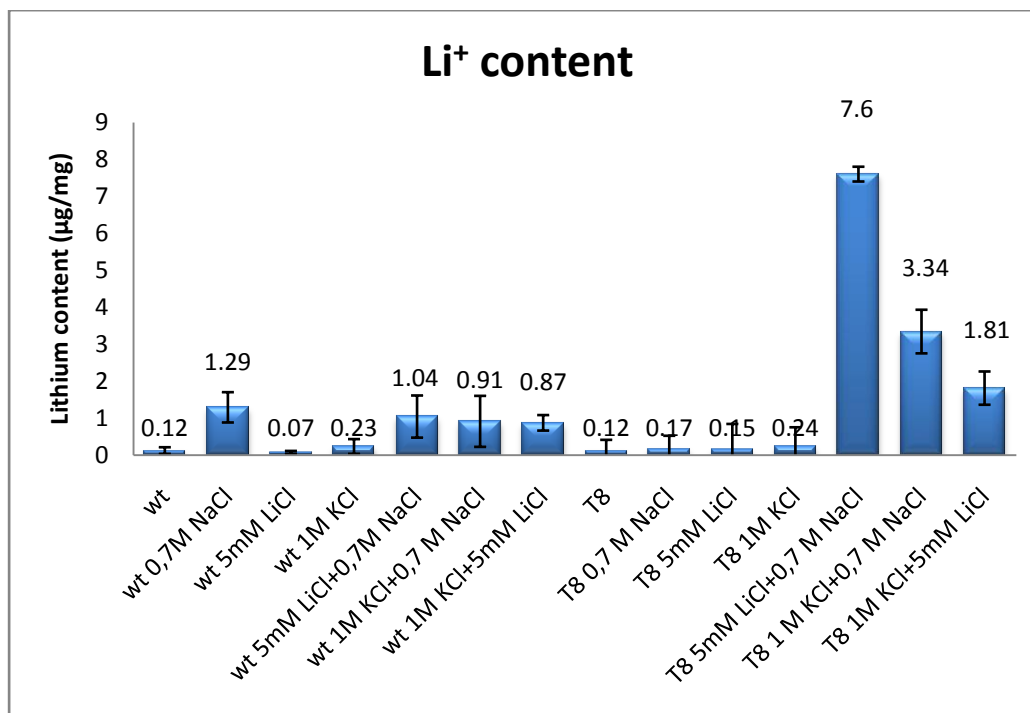


Figure 3.40 : Total cellular lithium amount in wild type and T8 mutant.

The lithium content of wild type and T8 were the same without stress. In the presence of 0.7 M NaCl, wild type had higher Na^+ content compared to T8. In the co-presence of LiCl+NaCl, NaCl+KCl, KCl+LiCl, T8 accumulated more Li^+ than the wild type (Fig 3.40).

Table 3.15: Total cellular potassium and sodium amount (mM) and Na^+/K^+ ratio

	K^+ (mM)	Na^+ (mM)	Li^+ (mM)	K^+/Na^+ ratio
wt	0.19	0.11	0.01	1.80
wt 0.7M NaCl	0.04	0.32	0.18	0.12
wt 5mM LiCl	0.11	0.05	0.01	1.97
wt 1M KCl	0.33	0.10	0.03	3.16
wt 5mM LiCl+0.7M NaCl	0.02	0.61	0.14	0.04
wt 1M KCl+0.7 M NaCl	0.17	0.22	0.13	0.77
wt 1M KCl+5mM LiCl	0.20	0.04	0.12	5.43
T8	0.21	0.07	0.01	2.97
T8 0.7 M NaCl	0.06	0.43	0.02	0.15
T8 5mM LiCl	0.17	0.04	0.02	4.21
T8 1M KCl	4.04	1.09	0.03	3.69
T8 5mM LiCl+0.7 M NaCl	0.01	0.27	1.08	0.05
T8 1 M KCl+0.7 M NaCl	0.11	0.12	0.47	0.90
T8 1M KCl+5mM LiCl	0.22	0.03	0.25	6.57

It was shown that the level of intra-cellular K^+/Na^+ ratio must be higher than 1:2 for normal growth of cells in the presence of Na^+ (Camacho *et al.*, 1981) and that the

Na^+/H^+ antiporter activity contributed in *S. cerevisiae* to maintaining the appropriate ratio (Banuelos *et al.*, 1998). The K^+/Na^+ ratio must be higher than 1:2 to guarantee normal growth of cells in the presence of Na^+ (Banuelos *et al.*, 1998). T8 had an elevated K^+/Na^+ ratio without stress conditions. The presence of 5 mM LiCl especially changed the K^+/Na^+ ratio of T8 significantly (Table 3.14). The high intra-cellular ratio of K^+/Na^+ was achieved by selectively accumulating K^+ , while actively extruding Na^+ .

3.8 Determination of Metabolite Contents by HPLC

3.8.1 HPLC (0.5 M NaCl)

Ethanol, acetate, glycerol, maltose and citric acid and the consumption of glucose were monitored along with the growth and quantified by HPLC.

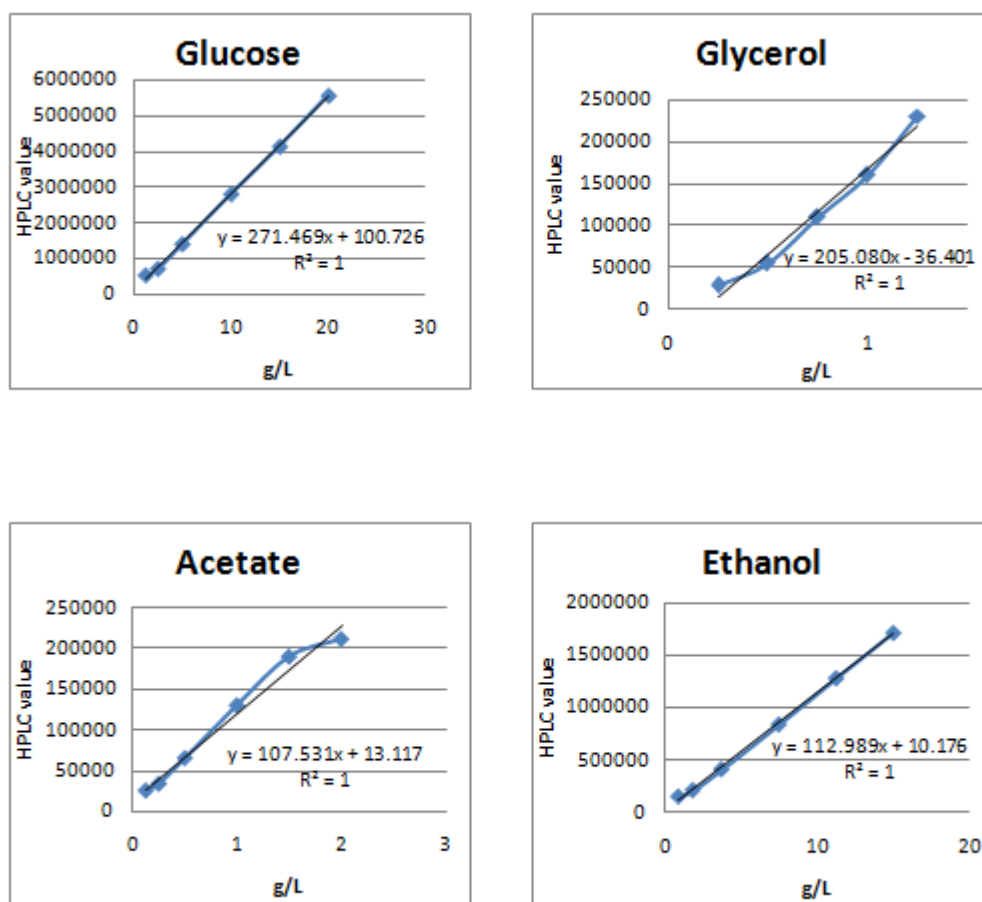


Figure 3.41: HPLC Standards for glucose, glycerol, acetate, ethanol.

Table 3.16 : Average amounts of metabolites (g/L) obtained from HPLC analysis for wild type.

Time(h)	Glucose(g/L)	Glycerol(g/L)	Acetate(g/L)	Ethanol(g/L)
0	20.30	0.24	0	0.29
1.5	20.31	0.27	0	0.37
3	19.53	0.30	0	0.57
4.5	18.42	0.32	0	0.88
6	15.60	0.39	0.03	1.57
7.5	12.10	0.54	0.24	2.49
9	8.79	0.68	0.30	3.03
10.5	4.77	0.85	0.43	3.66
24	0.00	0.97	0.39	4.07

Table 3.17 : Average amounts of metabolites (g/L) obtained from HPLC analysis for wild type+0.5 M NaCl.

Time (h)	Glucose(g/L)	Glycerol(g/L)	Acetate(g/L)	Ethanol(g/L)
0	19.94	0.42	0.01	0.27
1.5	19.76	0.47	0.04	0.32
3	19.38	0.54	0.10	0.42
4.5	18.97	0.60	0.13	0.49
6	18.53	0.70	0.14	0.54
7.5	17.89	0.74	0.13	0.70
9	17.42	0.83	0.15	0.73
10.5	16.93	0.92	0.17	0.75
24	11.18	2.11	1.14	1.84

Table 3.18 : Average amounts of metabolites (g/L) obtained from HPLC analysis for T8.

Hour	Glucose(g/L)	Glycerol(g/L)	Acetate(g/L)	Ethanol(g/L)
0	20.48	0.24	0	0.16
1.5	20.93	0.27	0	0.18
3	20.34	0.30	0	0.24
4.5	20.03	0.32	0	0.31
6	19.02	0.36	0	0.50
7.5	17.40	0.42	0.03	0.90
9	15.45	0.49	0.09	1.27
10.5	12.58	0.62	0.13	1.53
24	0.00	1.61	0	3.23

Table 3.19 : Average amounts of metabolites (g/L) obtained from HPLC analysis for T8+0.5 M NaCl.

Time (h)	Glucose(g/L)	Glycerol(g/L)	Acetate(g/L)	Ethanol(g/L)
0	20.16	0.44	0.02	0.13
1.5	20.21	0.47	0.02	0.15
3	20.04	0.51	0.07	0.17
4.5	19.81	0.57	0.11	0.22
6	19.18	0.64	0.15	0.34
7.5	18.21	0.74	0.17	0.51
9	17.28	0.88	0.18	0.55
10.5	15.81	1.06	0.25	0.78
24	7.74	2.53	1.49	2.39

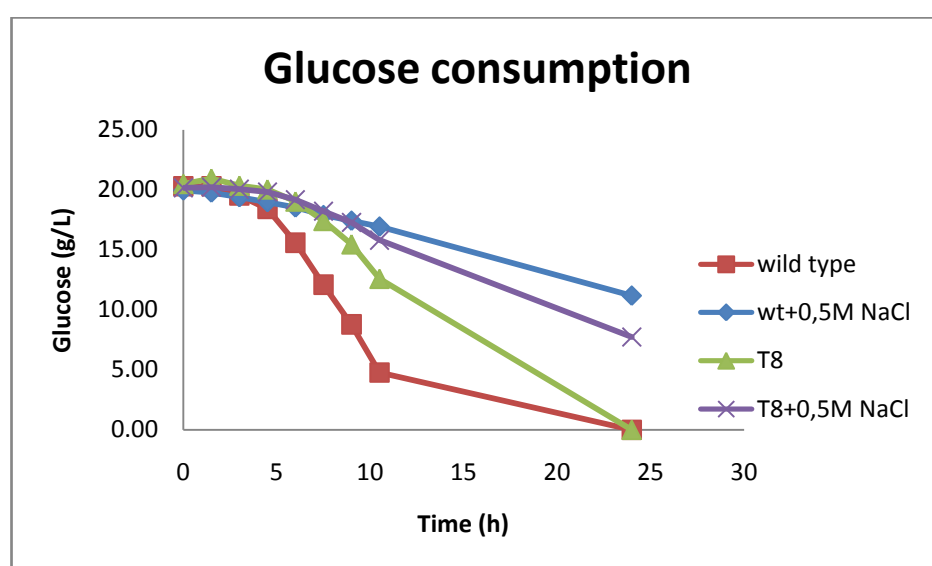


Figure 3.42 : Glucose utilization levels by wild type and T8 strain without and with 0.5 M NaCl stress conditions during 24 h incubation at 30°C, 150 rpm.

Figure 3.42 shows utilization of glucose (g/L) by time and rate of wild type's glucose consumption decreased under sodium stress. At control condition, all glucose was consumed by both wild type and mutant strains by the 24th hour of incubation. Wild type cultivated with 0.5 M NaCl did not utilize glucose.

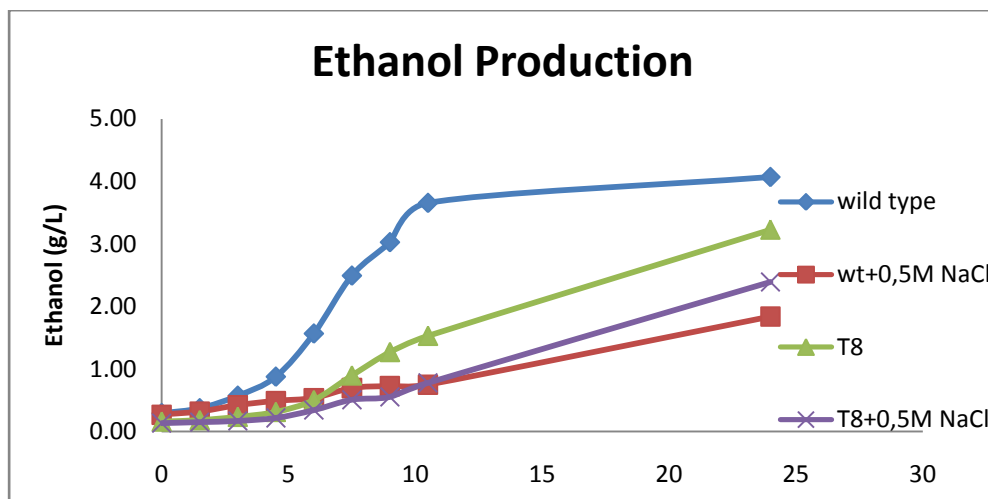


Figure 3.43 : Ethanol production levels by wild type and T8 strain at control and stress conditions during 24 h incubation at 30°C, 150 rpm.

At control condition, wild type reached approximately 4.00 (g/L) ethanol production at the end of 24 h of incubation. However, wildtype under stress condition could produce 1.84 (g/L) ethanol. Wild type and T8 strain produced less ethanol compared to ethanol production under 0.5 M NaCl stress condition (Figure 3.43).

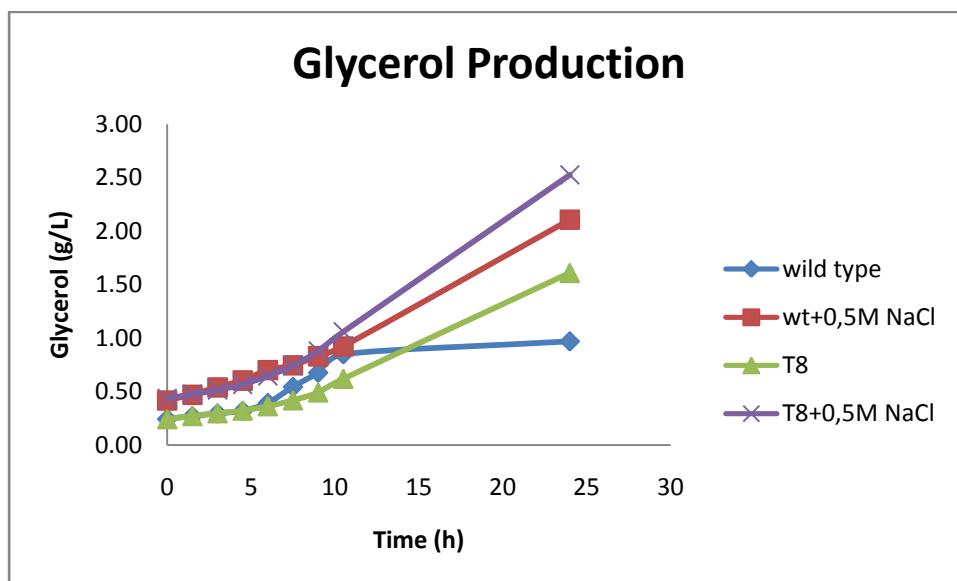


Figure 3.44 : Glycerol production by wild type and T8 strain at control and stress conditions during 24 h incubation at 30 °C, 150 rpm.

Glycerol amounts showed that T8 strain produced highest glycerol under NaCl stress (Figure 3.44).

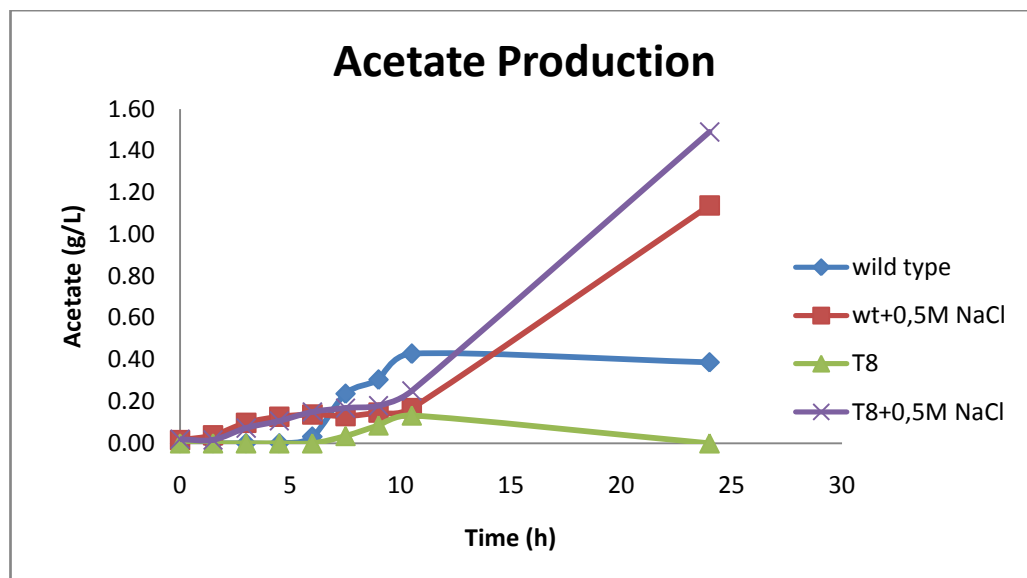


Figure 3.45 : Acetate production by wild type and T8 strain at control and stress conditions during 24 h incubation at 30°C, 150 rpm.

After 10.5th hour of incubation, while acetate production of wild type and T8 strain were increasing at stress conditions, acetate production of the wild type and T8 decreased at control conditions (Figure 3.45).

3.8.2 HPLC (5mM LiCl)

Ethanol, acetate, glycerol, maltose, citric acid and succinic acid, as well as glucose were monitored along with the growth and quantified by HPLC.

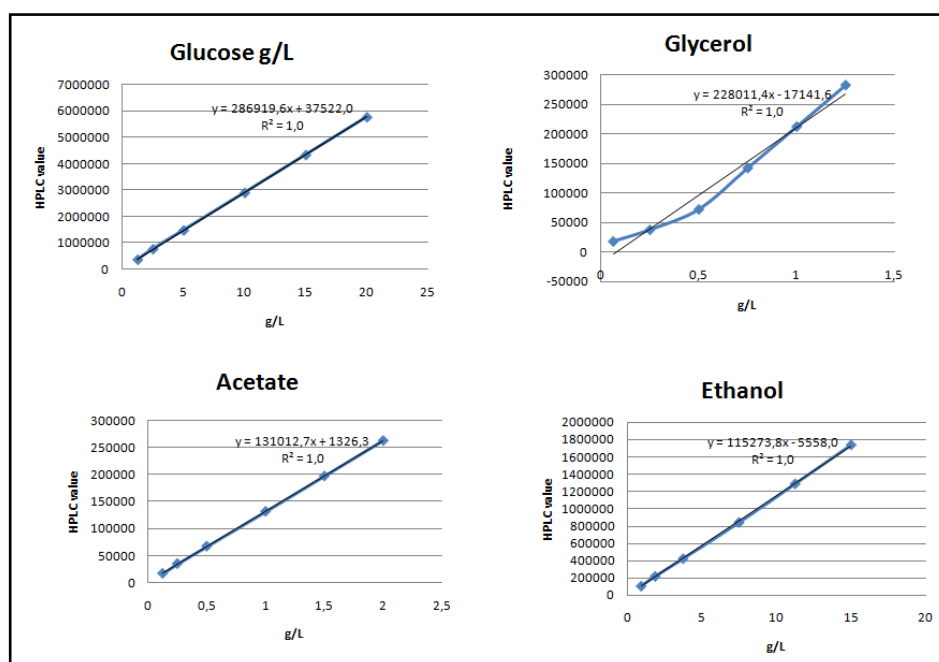


Figure 3.46: HPLC standards for glucose, glycerol, acetate, ethanol.

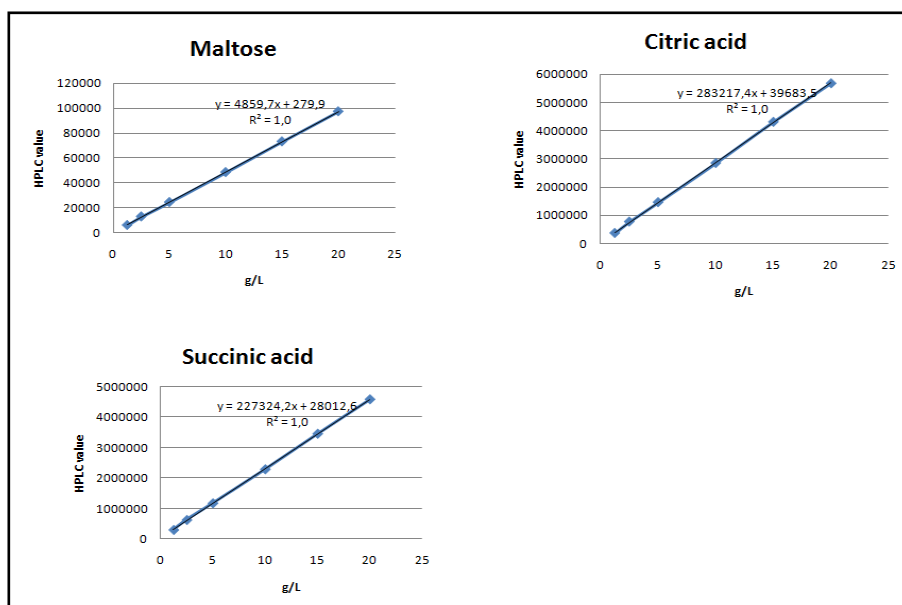


Figure 3.47: HPLC standards for maltose, citric acid, succinic acid.

Table 3.20 : Average amounts of metabolites (g/L) obtained from HPLC analysis for the wild type.

Time (h)	Glucose (g/L)	Glycerol (g/L)	Acetate (g/L)	Ethanol (g/L)	Maltose (g/L)	Citric acid (g/L)	Succinic acid (g/L)
0	20.74	0.26	0.01	0.43	0	0	0.75
1.5	20.62	0.28	0.03	0.49	0	0	0.75
3	20.03	0.29	0.05	0.64	0	0	0.74
4.5	18.72	0.32	0.09	0.96	0	0	0.75
6	16.15	0.37	0.14	1.54	0	0	0.73
7.5	12.13	0.45	0.20	2.56	0	0	0.74
9	8.39	0.56	0.27	3.16	0	0	0.76
10.5	4.99	0.68	0.43	3.85	0	0	0.79
24	0	0.78	0.59	4.50	0	0	0.81

Table 3.21: Average amounts of metabolites (g/L) obtained from HPLC analysis for the wild type in the presence of 5 mM LiCl.

Time (h)	Glucose (g/L)	Glycerol (g/L)	Acetate (g/L)	Ethanol (g/L)	Maltose (g/L)	Citric acid (g/L)	Succinic acid (g/L)
0	20.76	0.26	0.01	0.40	0	0	0.75
1.5	20.43	0.28	0.02	0.47	0	0	0.73
3	19.78	0.29	0.04	0.52	0	0	0.72
4.5	19.85	0.31	0.06	0.58	0	0	0.74
6	19.45	0.33	0.07	0.64	0	0	0.74
7.5	19.36	0.35	0.08	0.71	0	0	0.74
9	19.10	0.37	0.09	0.76	0	0	0.75
10.5	19.73	0.40	0.11	0.70	0	0	0.78
24	15.79	0.75	0.23	1.19	0	0	0.85

Table 3.22 : Average amounts of metabolites (g/L) obtained from HPLC analysis for T8.

Time (h)	Glucose (g/L)	Glycerol (g/L)	Acetate (g/L)	Ethanol (g/L)	Maltose (g/L)	Citric acid (g/L)	Succinic acid (g/L)
0	20.47	0.27	0	0.45	0	0	0.76
1.5	20.49	0.29	0.02	0.47	0	0	0.75
3	20.48	0.31	0.04	0.54	0	0	0.76
4.5	19.47	0.34	0.09	0.77	0	0	0.75
6	17.80	0.38	0.15	1.09	0	0	0.75
7.5	15.05	0.45	0.22	1.63	0	0	0.73
9	12.04	0.56	0.28	2.26	0	0	0.76
10.5	9.24	0.65	0.32	2.59	0	0	0.75
24	0	0.84	0.22	4.58	0	0	0.79

Table 3.23 : Average amounts of metabolites (g/L) obtained from HPLC analysis for T8 in the presence of 5 mM LiCl.

Time (h)	Glucose (g/L)	Glycerol (g/L)	Acetate (g/L)	Ethanol (g/L)	Maltose (g/L)	Citric acid (g/L)	Succinic acid (g/L)
0	20.12	0.27	0	0.47	0	0	0.73
1.5	20.29	0.29	0.02	0.50	0	0	0.73
3	19.92	0.31	0.05	0.55	0	0	0.73
4.5	19.53	0.35	0.09	0.72	0	0	0.76
6	17.28	0.39	0.15	1.01	0	0	0.73
7.5	14.97	0.46	0.22	1.58	0	0	0.73
9	11.97	0.55	0.25	2.20	0	0	0.72
10.5	9.57	0.65	0.28	2.53	0	0	0.74
24	0	0.87	0.11	4.47	0	0	0.79

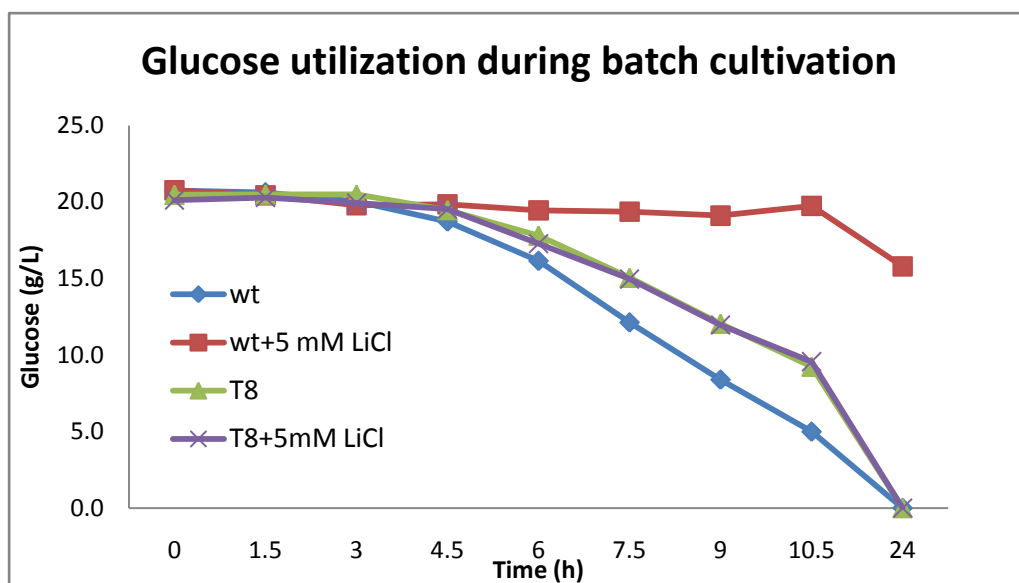


Figure 3.48 : Glucose utilization levels by wild type and T8 strain at control and stress conditions during 24 h incubation at 30°C, 150 rpm.

Figure 3.48 shows utilization of glucose (g/L) by time. Rate of wild type's glucose utilization decreased under lithium stress. At control condition, both wild type and mutant strains consumed all glucose by the 24th hour of incubation. Interestingly, T8 and T8 cultivated in the presence of 5 mM LiCl showed similar growth characteristics. Wild type cultivated with 5 mM LiCl could not utilize glucose (Figure 3.48).

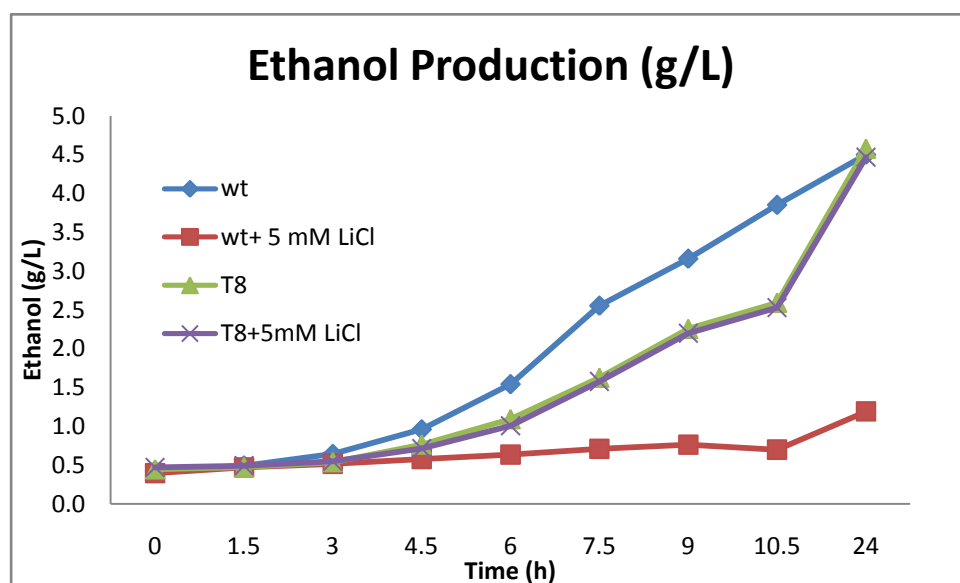


Figure 3.49 : Ethanol production levels by wild type and T8 strain at control and stress conditions during 24 h incubation at 30°C, 150 rpm.

At control conditions, wild type, T8 and T8 treated with 5mM LiCl reached approximately 4.50 (g/L) ethanol production at the end of 24 h of incubation. However, wild type under stress condition could produce 1.19 (g/L) ethanol (Figure 3.49).

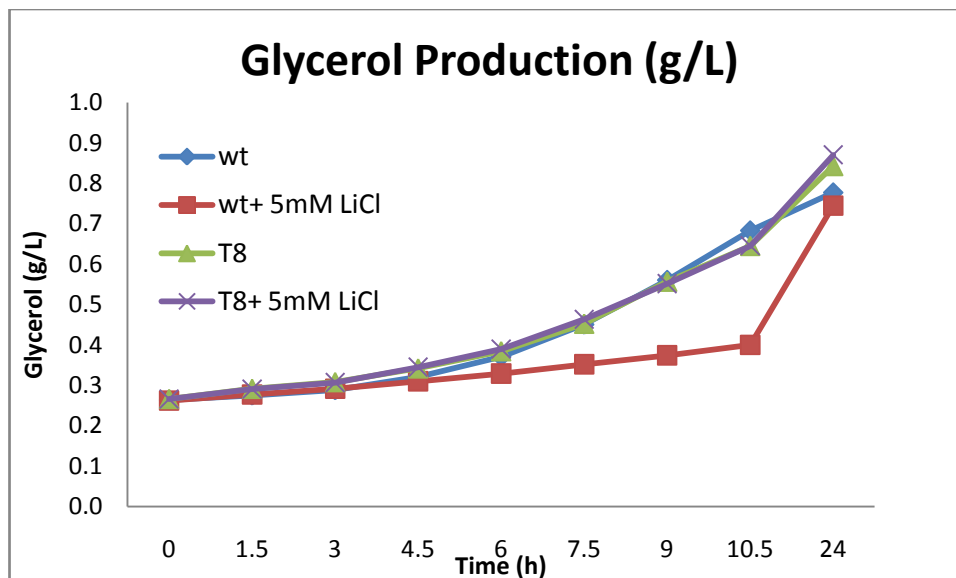


Figure 3.50 : Glycerol production by wild type and T8 strain at control and stress conditions during 24 h incubation at 30°C, 150 rpm.

Glycerol amounts showed that T8 strain produced the same amount of glycerol both at control and stress conditions. After 10.5 hours, T8 had more glycerol than the wild type (Figure 3.50).

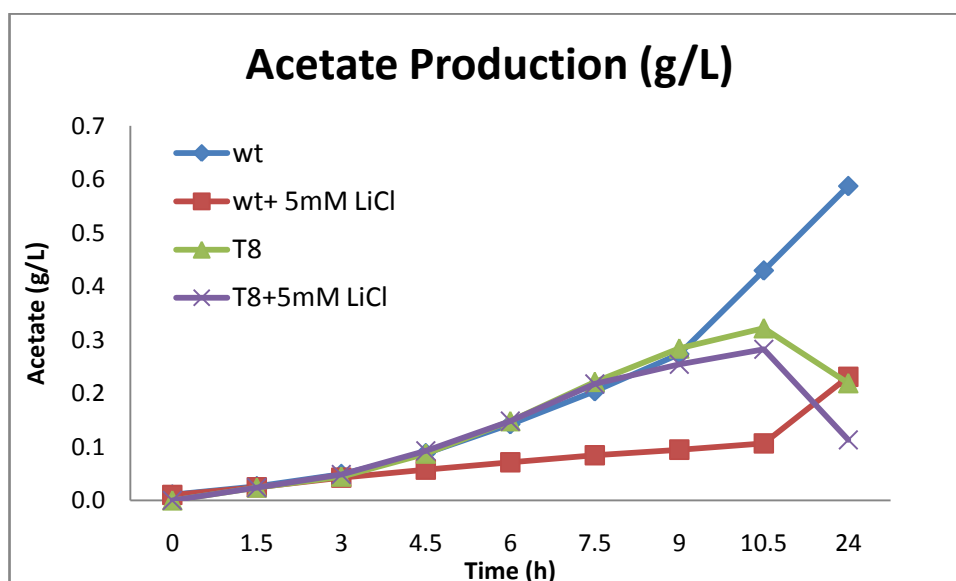


Figure 3.51 : Acetate production by wild type and T8 strain at control and stress conditions during 24 h incubation at 30°C, 150 rpm.

After 10.5th hour of incubation while acetate production of the wild type was increasing at stress conditions, acetate production of T8 decreased at control and stress conditions (Figure 3.51).

3.9 Quantitative assessment of glycogen and trehalose content

Trehalose and glycogen content of sodium-treated, lithium-treated and non-treated samples of the wild type and T8 were determined by procedure of Parrau and Francois (1997).

3.9.1 0.5 M NaCl

Trehalose and glycogen contents are shown at Table 3.24.

Table 3.24 : Relative trehalose and glycogen content of sodium-treated and non-treated samples of wild type and T8.

Trehalose content (mg glucose equivalents / mg CDW)				
Time(h)	wt+0.5M		T8+0.5M	
	wt	NaCl	T8	NaCl
24	0.01	0.06	0.22	0.15

Glycogen content (mg glucose equivalents / mg CDW)				
Time(h)	wt+0.5 M		T8+0.5 M	
	wt	NaCl	T8	NaCl
24	0.02	0.06	0.09	0.07

Sodium treatment caused an increase in the accumulation of both trehalose and glycogen for the wild type (Table 2.24).

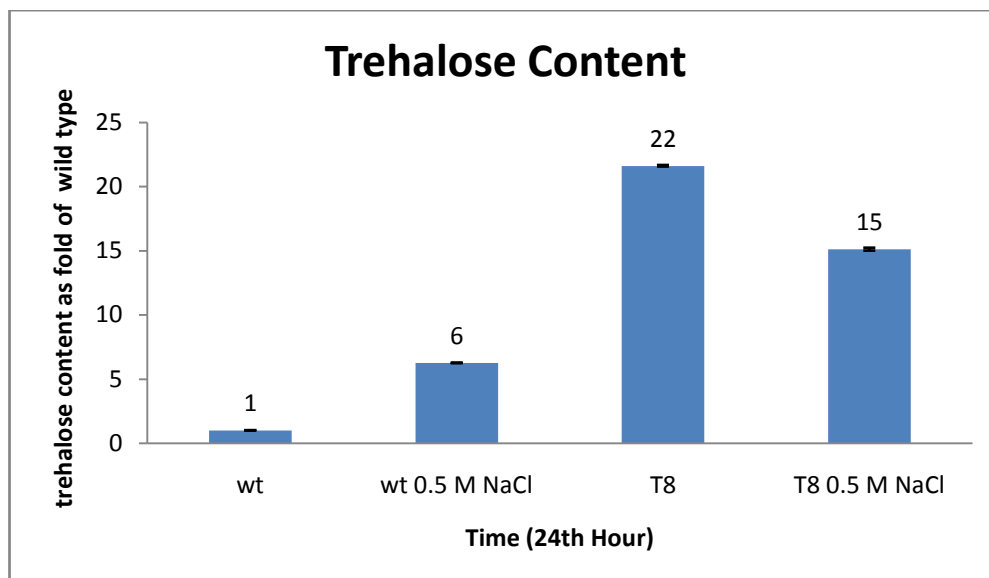


Figure 3.52 : Trehalose levels of 0.5 M sodium-treated wild type, T8 and non-treated T8 samples as fold of wild type.

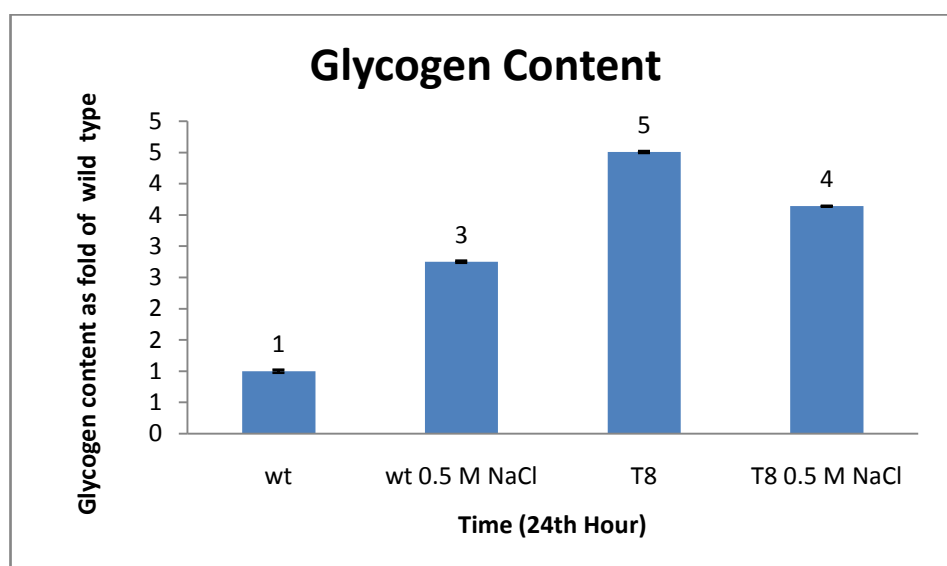


Figure 3.53 : Glycogen levels of 0.5 M sodium-treated wild type, T8 and non-treated T8 samples as fold of wild type.

When glycogen and trehalose levels of T8 with and without NaCl were compared, T8 had the highest trehalose and glycogen levels in the absence of NaCl. The presence of NaCl did not trigger trehalose and glycogen accumulation of T8 (Fig 3.52 and Fig. 3.53).

3.9.2 5 mM LiCl

Trehalose and glycogen contents of lithium treated and non-treated samples of wildtype and T8 are shown in Table 3.25.

Table 3.25 : Relative trehalose and glycogen contents of lithium-treated and non-treated samples of wild type and T8.

Time(h)	Trehalose content (mg glucose equivalents / mg CDW)			
	wt	wt+5mM LiCl	T8	T8+5mM LiCl
24	0.01	0.14	0.23	0.25
Time(h)	Glycogen content (mg glucose equivalents / mg CDW)			
	wt	wt+5mM LiCl	T8	T8+5mM LiCl
24	0.02	0.11	0.09	0.08

Applied to lithium treatment, wild type triggered an increase in the accumulation of both trehalose and glycogen for the wild type (Table 3.25).

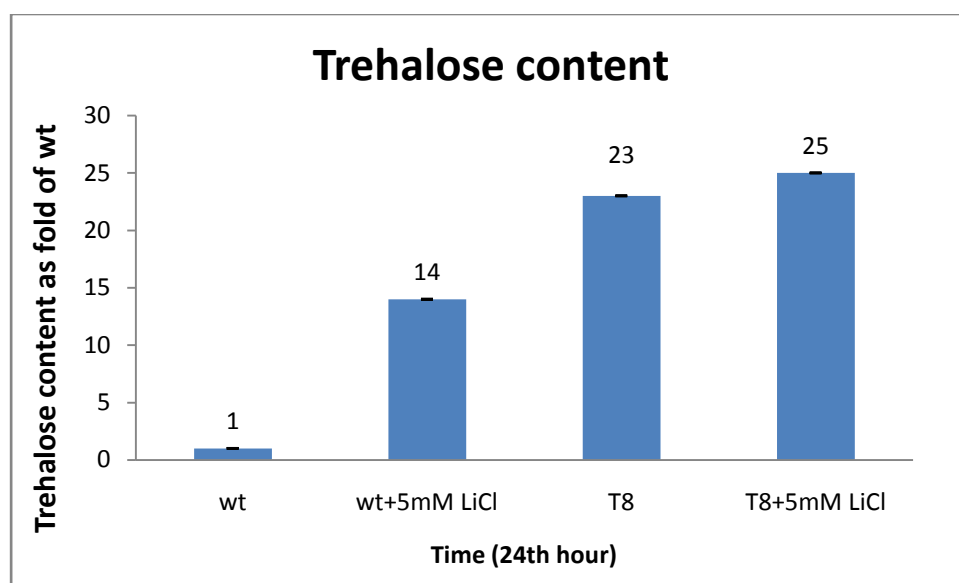


Figure 3.54 : Trehalose levels of 5 mM lithium-treated wild type, T8 and non-treated T8 samples as fold of wild type.

The intracellular trehalose levels of lithium-treated and non-treated samples of T8 were higher than those of the wild type. Lithium treatment did not increase trehalose concentrations of T8, unlike the wild type (Figure 3.54).

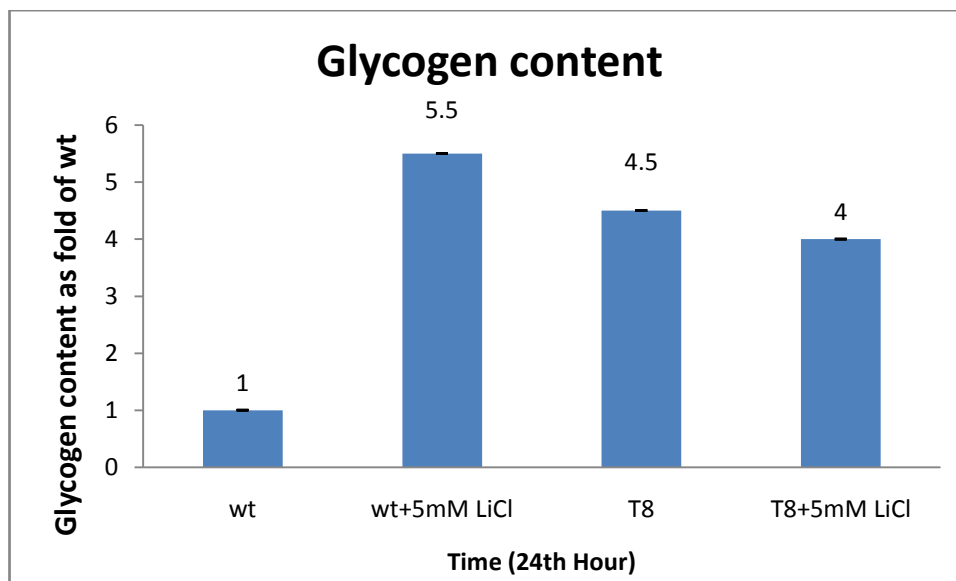


Figure 3.55 : Glycogen levels of 5 mM lithium-treated wild type, T8 and non-treated T8 samples as fold of wild type.

Lithium treatment caused an increase in the accumulation of both trehalose and glycogen for the wild type. Lithium-treated T8 samples had lower glycogen concentration compared to those of non-treated ones (Figure 3.55).

3.10 Whole genome transcriptomic analysis of salt resistant mutant

3.10.1 RNA Isolation and RIN Analysis

The cultures' initial OD_{600} and the OD_{600} value at which RNA isolation was started are shown in Table 3.26.

Table 3.26: Initial OD_{600} of the cultures and their OD_{600} values just before the RNA isolation.

Name	Initial OD_{600} of the cultures	OD_{600} of the cultures just before the RNA isolation				
		Culture 1	Culture 2	Culture 3	Culture 4	Culture 5
wt	0.12	1.16	1.08	1.09	1.12	1.13
T8	0.18	1.07	1.08	1.09	1.12	-

Agilent 2100 BioAnalyzer was used to identify RNA Integrity Number 'RIN'. RIN values of the cultures are shown in Table 3.26.

Table 3.27 : RNA Concentration (ng/μl) of the samples

Name	ng/μl of the cultures				
	Culture 1	Culture 2	Culture 3	Culture 4	Culture 5
wt	625	455	595	455	302
T8	304	335	325	297	---

Table 3.28 : RIN values for the parallel sets of cultures

Name	RIN of the cultures				
	Culture 1	Culture 2	Culture 3	Culture 4	Culture 5
wt	9.9	10.0	9.7	9.6	9.4
T8	9.7	9.7	9.8	9.6	

RNA concentration was determined by using NanoDrop instrument (Nano Drop 2000, Thermo Scientific) (Table 3.27). All RIN values were above 9. This showed that RNA of the cultures were stable and could be used for microarray analysis (Table 3.28).

3.10.2 Upregulated genes

Whole genome transcriptomic analyses were performed to compare the expression levels of genes in the NaCl-resistant mutant T8 and the reference strain. GeneSpring GX 12.5 software was used to analyze the microarray data. FunSpec and Funcat databases [(p-value) < 0.05] were used for the molecular and functional categorization of up- and down-regulated genes.

It was observed that 548 genes were upregulated and 22 genes were downregulated with two-fold expression change ($\log_2(\text{foldchange}) \geq 2$) t-test, $p < 0.05$).

The percentage of upregulated genes according to functional categories are shown in Figure 3.56. The analyses were performed using Funcat database.

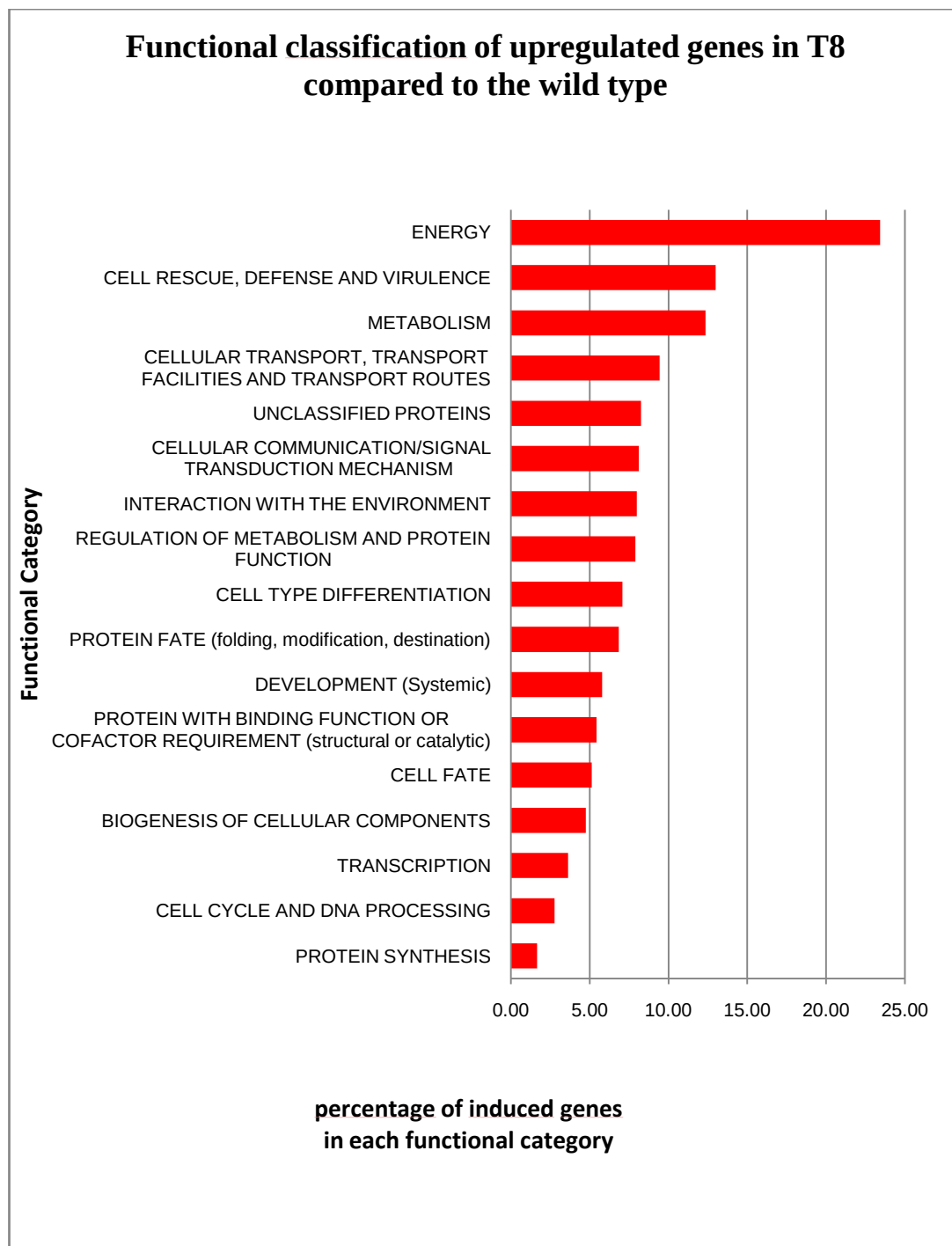


Figure 3.56:The percentage of upregulated genes to the total genes in each category (cut off value is ≥ 2 fold).

Classification of the genes by Funspec online software and FunCat database revealed that energy/cell rescue/defence and virulence metabolism and cellular transport-related genes were upregulated in T8, whereas protein synthesis and transcription genes were downregulated (Figure 3.56).

Upregulated genes were listed in Table 3.29. The cut-off value is set to ≥ 5 fold.

Table 3.29 : Upregulated genes (about five-fold and higher) in T8 based on microarray results.

Functional category	ORF Name	Gene Name	Fold Change
oxidation-reduction process	YAL061W	<i>BDH2</i>	35.66
	YBL064C	<i>PRX1</i>	5.03
	YEL070W	<i>DSF1</i>	10.14
	YGR088W	<i>CTT1</i>	13.31
	YHL021C	<i>AIM17</i>	8.27
	YIL155C	<i>GUT2</i>	5.62
	YML054C	<i>CYB2</i>	7.34
	YMR169C	<i>ALD3</i>	8.42
	YNR073C	<i>YNR073C</i>	9.88
	YOR374W	<i>ALD4</i>	9.75
	YPL171C	<i>OYE3</i>	5.66
Response to stress	YBR072W	<i>HSP26</i>	11.53
	YDL079C	<i>MRK1</i>	11.25
	YDL222C	<i>FMP45</i>	8.88
	YDR074W	<i>TPS2</i>	6.69
	YDR258C	<i>HSP78</i>	6.02
	YFL014W	<i>HSP12</i>	19.32
	YGR088W	<i>CTT1</i>	13.31
	YGR243W	<i>FMP43</i>	7.61
	YIL101C	<i>XBP1</i>	9.67
	YML100W	<i>TSL1</i>	30.42
	YOL052C-A	<i>DDR2</i>	7.93
	YOR049C	<i>RSB1</i>	8.90
metabolic process	YAL061W	<i>BDH2</i>	35.66
	YBR117C	<i>TKL2</i>	10.08
	YBR299W	<i>MAL32</i>	25.63
	YDL021W	<i>GPM2</i>	6.96
	YDR040C	<i>ENA1</i>	8.12
	YDR074W	<i>TPS2</i>	6.69
	YEL070W	<i>DSF1</i>	10.14
	YFR015C	<i>GSY1</i>	13.75
	YFR053C	<i>HXK1</i>	87.64
	YGR292W	<i>MAL12</i>	29.10
	YIL099W	<i>SGA1</i>	10.11
	YLR258W	<i>GSY2</i>	6.84
	YML054C	<i>CYB2</i>	7.34
	YMR169C	<i>ALD3</i>	8.42
	YNR073C	<i>YNR073C</i>	9.88
	YOR374W	<i>ALD4</i>	9.75
	YPL171C	<i>OYE3</i>	5.66
	YPR184W	<i>GDB1</i>	5.44

Table 3.29 (continued) : Upregulated genes (about five-fold and higher) in T8 based on microarray results.

Functional category	ORF Name	Gene Name	Fold Change
carbohydrate transport	YDL245C	<i>HXT15</i>	38.07
	YDR342C	<i>HXT7</i>	35.28
	YDR343C	<i>HXT6</i>	35.28
	YDR536W	<i>STL1</i>	9.8
	YGR289C	<i>MAL11</i>	11.71
	YHR096C	<i>HXT5</i>	5.02
	YJR158W	<i>HXT16</i>	32.13
	YMR011W	<i>HXT2</i>	14.34
	YNR072W	<i>HXT17</i>	14.65
glycogen biosynthetic process	YEL011W	<i>GLC3</i>	12.09
	YFR015C	<i>GSY1</i>	13.75
	YLR258W	<i>GSY2</i>	6.84
	YMR105C	<i>PGM2</i>	28.73
	YOR178C	<i>GAC1</i>	7.77
	YPR184W	<i>GDB1</i>	5.44
trehalose biosynthetic process	YDR074W	<i>TPS2</i>	6.69
	YML100W	<i>TSL1</i>	30.42
	YMR105C	<i>PGM2</i>	28.73
transmembrane transport	YDL245C	<i>HXT15</i>	38.07
	YDR039C	<i>ENA2</i>	9.66
	YDR040C	<i>ENA1</i>	8.12
	YDR342C	<i>HXT7</i>	35.28
	YDR343C	<i>HXT6</i>	35.28
	YDR536W	<i>STL1</i>	9.78
	YGR289C	<i>MAL11</i>	11.71
	YHR096C	<i>HXT5</i>	5.02
	YJL163C	<i>YJL163C</i>	8.31
	YJL212C	<i>OPT1</i>	5.20
	YJR158W	<i>HXT16</i>	32.13
	YLL055W	<i>YCT1</i>	6.51
	YMR011W	<i>HXT2</i>	14.34
	YNR072W	<i>HXT17</i>	14.65
	YOR348C	<i>PUT4</i>	7.97
	YPL265W	<i>DIP5</i>	10.83
	YPR194C	<i>OPT2</i>	10.43
glycolysis	YCL040W	<i>GLK1</i>	5.51
	YDL021W	<i>GPM2</i>	6.96
	YDR516C	<i>EMI2</i>	13.22
	YFR053C	<i>HXK1</i>	87.64
hexose transport	YDL245C	<i>HXT15</i>	38.07
	YDR342C	<i>HXT7</i>	35.28
	YDR343C	<i>HXT6</i>	35.28
	YHR096C	<i>HXT5</i>	5.02
	YJR158W	<i>HXT16</i>	32.13
	YNR072W	<i>HXT17</i>	14.65

Table 3.29 (continued) : Upregulated genes (about five-fold and higher) in T8 based on microarray results.

Functional category	ORF Name	Gene Name	Fold Change
cellular response to oxidative stress	YBL064C	<i>PRX1</i>	5.03
	YFL014W	<i>HSP12</i>	19.32
	YMR250W	<i>GAD1</i>	10.80
sucrose catabolic process	YBR299W	<i>MAL32</i>	25.63
glycogen catabolic process	YIL099W	<i>SGA1</i>	10.11
maltose metabolic process	YGR289C	<i>MAL11</i>	11.71
glucose transport	YHR096C	<i>HXT5</i>	5.02
	YMR011W	<i>HXT2</i>	14.34
transport	YDR039C	<i>ENA2</i>	9.66
	YDR040C	<i>ENA1</i>	8.12
	YDR536W	<i>STL1</i>	9.78
	YEL039C	<i>CYC7</i>	28.86
	YGR289C	<i>MAL11</i>	11.71
	YJL212C	<i>OPT1</i>	5.20
	YLL055W	<i>YCT1</i>	6.51
	YLR411W	<i>CTR3</i>	7.85
	YML054C	<i>CYB2</i>	7.34
	YMR011W	<i>HXT2</i>	14.34
	YNL093W	<i>YPT53</i>	5.80
	YNR072W	<i>HXT17</i>	14.65
	YOR049C	<i>RSB1</i>	8.90
	YOR100C	<i>CRC1</i>	5.99
	YOR348C	<i>PUT4</i>	7.97
	YPR194C	<i>OPT2</i>	10.43
phosphorylation	YCL040W	<i>GLK1</i>	5.51
	YDL079C	<i>MRK1</i>	11.25
	YDR516C	<i>EMI2</i>	13.22
	YGR194C	<i>XKS1</i>	5.14
	YJL164C	<i>TPK1</i>	6.75
ethanol metabolic process	YOR374W	<i>ALD4</i>	9.75
negative regulation of peptidase activity	YLR178C	<i>TFS1</i>	6.36
glucose 6-phosphate metabolic process	YMR105C	<i>PGM2</i>	28.73
pentose-phosphate shunt	YBR117C	<i>TKL2</i>	10.08
	YGR248W	<i>SOL4</i>	15.35

Table 3.29 (continued) : Upregulated genes (about five-fold and higher) in T8 based on microarray results.

Functional category	ORF Name	Gene Name	Fold Change
amino acid transport	YLL055W	<i>YCT1</i>	6.51
	YOR348C	<i>PUT4</i>	7.97
	YPL265W	<i>DIP5</i>	10.83
oligopeptide transport	YJL212C	<i>OPT1</i>	5.20
	YPR194C	<i>OPT2</i>	10.43
biofilm formation	YIR019C	<i>MUC1</i>	92.85
peptide transport	YJL212C	<i>OPT1</i>	5.20
ammonia assimilation cycle	YOR348C	<i>PUT4</i>	7.97
NADH oxidation	YIL155C	<i>GUT2</i>	5.62
maltose catabolic process	YBR299W	<i>MAL32</i>	25.63
regulation of proteolysis	YLR178C	<i>TFS1</i>	6.36
disaccharide catabolic process	YGR289C	<i>MAL11</i>	11.71
amino acid transmembrane transport	YOR348C	<i>PUT4</i>	7.97
	YPL265W	<i>DIP5</i>	10.83
ascospore formation	YDL222C	<i>FMP45</i>	8.88
	YIL099W	<i>SGA1</i>	10.11
	YNL194C	<i>YNL194C</i>	22.50
protein phosphorylation	YDL079C	<i>MRK1</i>	11.25
	YDL214C	<i>PRR2</i>	5.02
	YJL164C	<i>TPK1</i>	6.75

Many genes associated with stress response are strongly correlated with growth rate (Brauer *et al.*, 2008). Microarray data were analyzed by the Princeton growth rate/gene expression relationship database. The genes that were expressed under normal growth conditions in the cell cycle are indicated as a bar chart in Figure 3.57. the microarray data of upregulated genes are represented by the black line; whereas genes induced under stress are represented by the red line, and those repressed under stress by the green line (Fig 3.57).

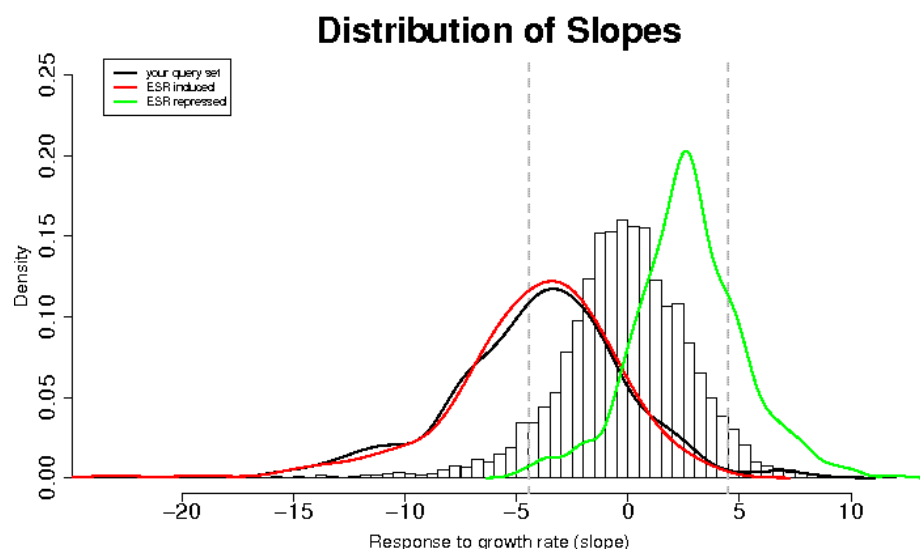


Figure 3.57 : The distribution of transcriptional response of stress-related and cell cycle-related genes. The red line shows induced genes, and the green line shows repressed genes under environmental stress conditions. Bar chart shows the genes that are expressed under normal growth conditions in cell cycle. The black line shows the upregulated genes of T8 under normal growth conditions in cell cycle.

Although no stress was applied, environmental stress response (ESR) gene expression was induced in T8 (Figure 3.57).

3.10.3 Downregulated genes

The percentage of downregulated genes according to functional categories are shown in Figure 3.58.

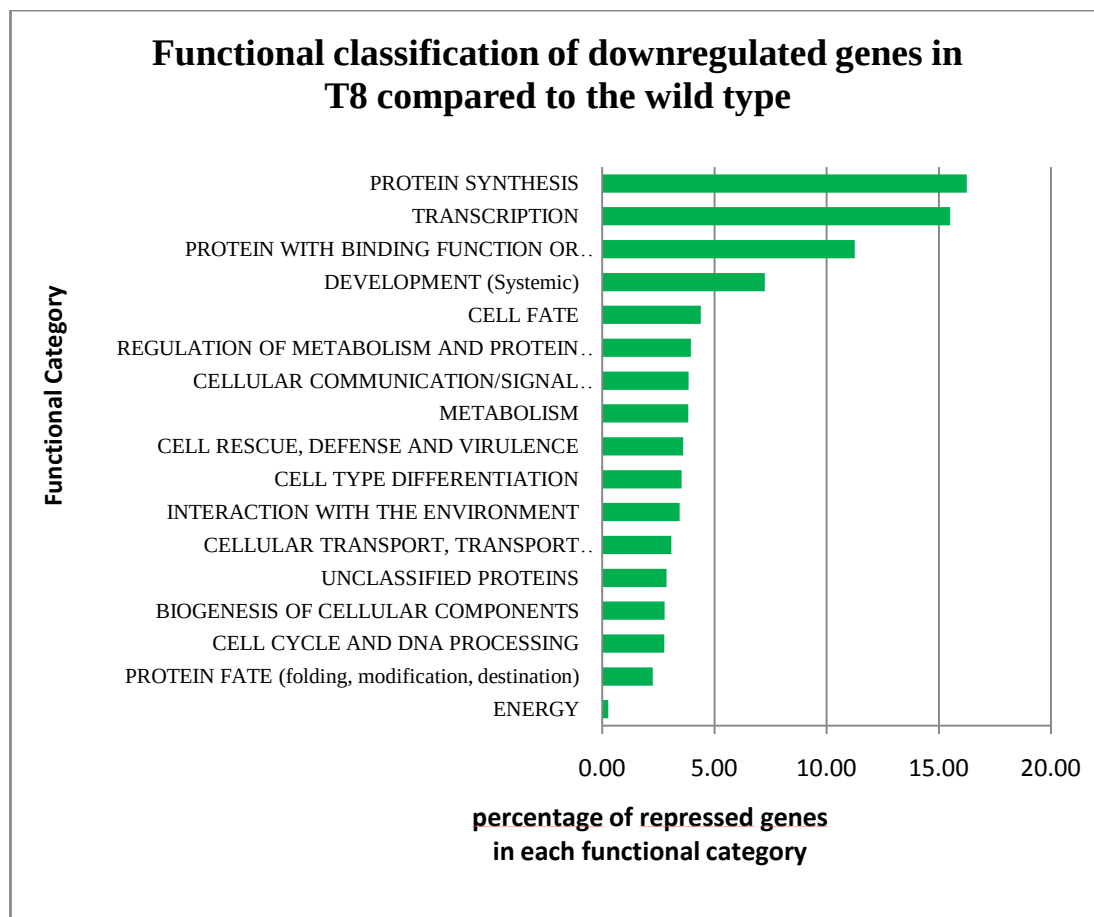


Figure 3.58 : The percentage of downregulated genes to total genes in each category (cut-off value is ≥ 2 fold).

Significantly, the genes under the functional categories of protein synthesis and transcription were downregulated.

Downregulated genes are listed in Table 3.30. The cut-off value was set to ≥ 2 fold.

Table 3.30 : Downregulated genes (about two-fold and higher) in T8 based on microarray results.

Functional category	ORF Name	Gene Name	Fold Change
transmembrane transport	YML123C	<i>PHO84</i>	-5.82
	ZRT1	<i>ZRT1</i>	-2.46
hexose transport	HXT3	<i>HXT3</i>	-3.27
carbohydrate metabolic process	MIG2	<i>MIG2</i>	-2.73
nucleotide metabolic process	AAH1	<i>AAH1</i>	-2.44
Biological process	SET6	<i>SET6</i>	-2.37
	YNL024C	<i>YNL024C</i>	-2.10
	GFD2	<i>GFD2</i>	-2.01
ribosome biogenesis	DBP2	<i>DBP2</i>	-2.29
	DBP7	<i>DBP7</i>	-2.22
	ALB1	<i>ALB1</i>	-2.20
	DHR2	<i>DHR2</i>	-2.09
	UTP23	<i>UTP23</i>	-2.09
	IPI3	<i>IPI3</i>	-2.06
	RLP24	<i>RLP24</i>	-2.02
methylation	HMT1	<i>HMT1</i>	-2.18
rRNA processing	REX4	<i>REX4</i>	-2.17
response to temperature stimulus	SPL2	<i>SPL2</i>	-2.04
budding cell bud growth	REI1	<i>REI1</i>	-2.00

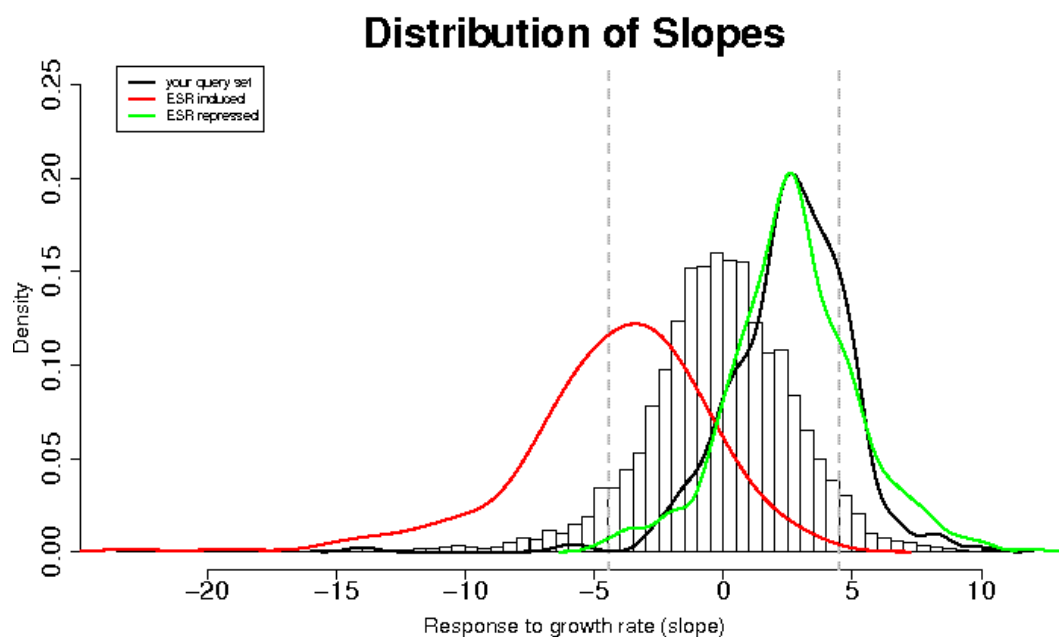


Figure 3.59 : The distribution of transcriptional response of stress-related and cell cycle-related genes. The red line shows induced genes, and the green line shows repressed genes under environmental stress conditions. Bar chart shows the genes that are expressed under normal growth conditions in cell cycle. The black line shows the downregulated genes of T8 under normal growth conditions in cell cycle.

When the growth rate decreased due to stress elements, some of the ESR genes were downregulated. Here, the ESR genes were repressed in T8 even when there was no stress (Figure 3.59).

PHO84 was the most downregulated gene in T8.

3.11 Expression Level Determination of *ENA6* and *NHA1* for both Wild Type and T8

ENA6 and *NHA1* gene expression levels were determined with and without stress. 5 mM LiCl and 0.7 M NaCl stresses were applied to both wild type and T8. When they reached $OD_{600}=1$, the culture was taken to prepare RNA samples. The housekeeping gene, beta actin (*ACT1*) was used as the endogenous control gene. Expression values of these genes were normalized to *ACT1*.

Table 3.31: Efficiency and error values for qPCR analysis.

	<i>ACT1</i>	<i>ENA6</i>	<i>NHA1</i>
Error	0.077	0.111	0.109
Efficiency	2.021	2.013	1.965

Relative expression profiles of wild-type and T8 for *ENA6* and *NHA1* genes with and without stress are shown in Figures 3.60 and 3.61.

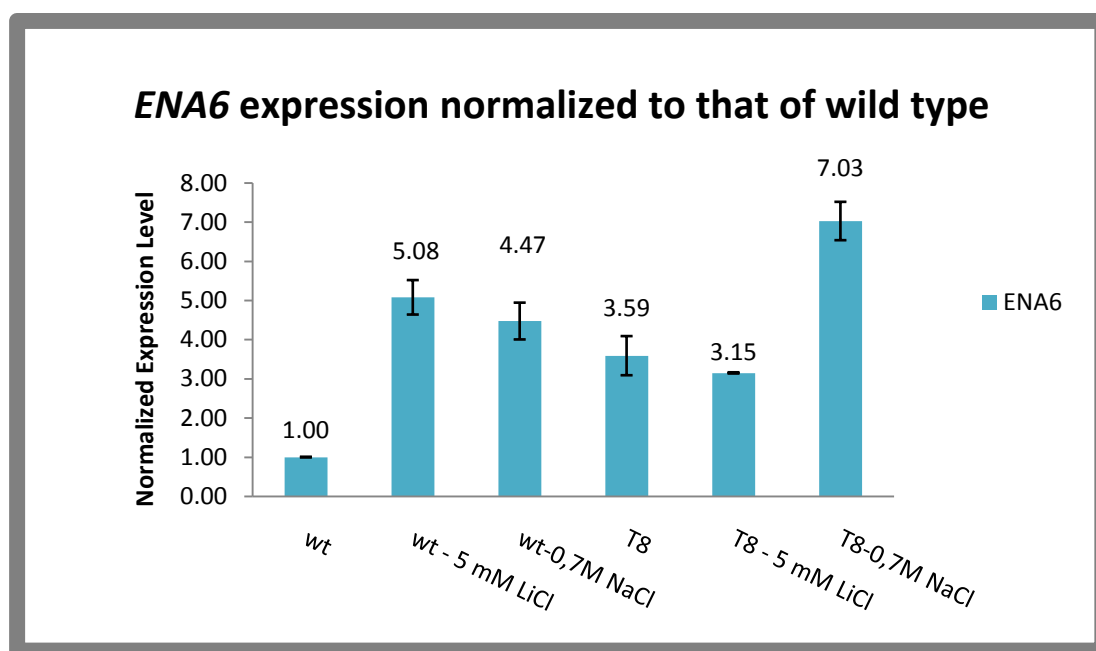


Figure 3.60: Expression level of *ENA6* gene in control, 0.7 M NaCl and 5 mM LiCl stress conditions.

Results showed that even without stress, *ENA6* gene of T8 was upregulated. NaCl stress induced *ENA6* more in T8. However, LiCl stress was more effective on wild type. 5 mM LiCl did not significantly change expression level of *ENA6* in T8.

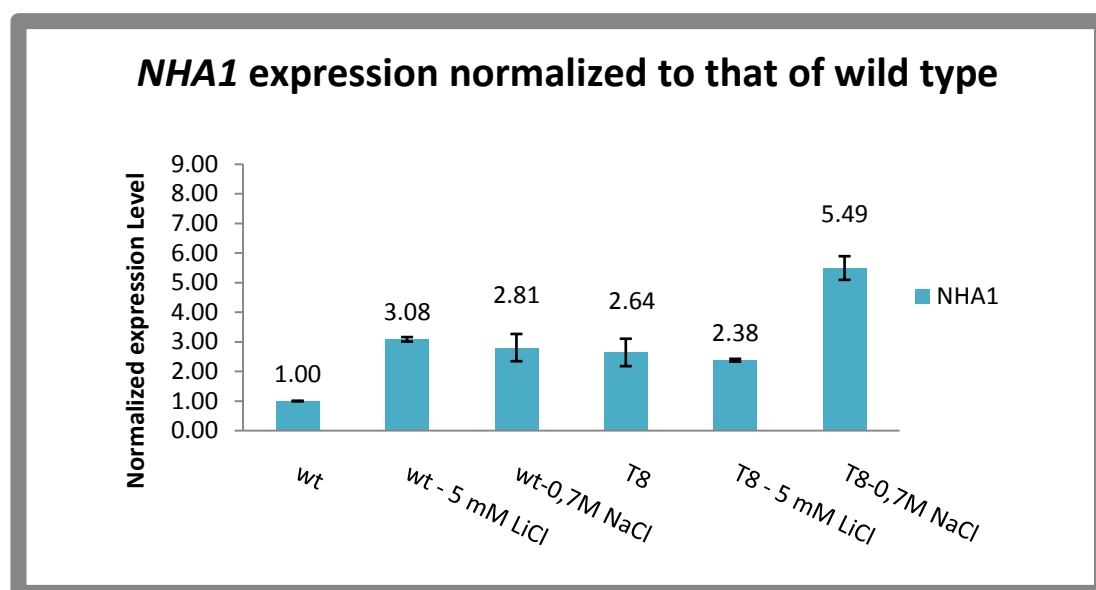


Figure 3.61 : Expression level of *NHA1* gene in control, 0.7 M NaCl and 5 mM LiCl stress conditions.

NHA1 was induced in T8 without stress. Expression level was highest in T8 when NaCl stress was applied. It was obvious that *ENA6* was induced more under each condition tested (Figure 3.61).

3.12 Proteomics Results of T8

The proteomics analysis was carried out among *S.cerevisiae* wild type and T8 mutant and the protein expression differences among these strains were investigated. The intracellular proteomics profile could reveal metabolic differences in *S. cerevisiae* strains with modified metabolic pathways. 22 proteins were identified with significantly different expression levels in T8, compared to the wild type. Ten of 22 protein levels were higher in T8 than the wild type. Twelve of 22 protein levels were lower in T8 than the wild type.

Table 3.32 : Relative changes in protein expression levels between *S. cerevisiae* wild type and T8.

Accession	Description	Ratio
P32288	GLN1 Glutamine synthetase	4
Q12497	FMP16 mitochondrial	4
P30614	PYK1 Pyruvate kinase	3.56
P20369	ADH1 Alcohol dehydrogenase 1	2.34
P04840	POR1 Mitochondrial outer membrane protein porin 1	1.8
Q6FQY4	ENO2 Enolase 2	1.8
O59841	G3P (TDH1) Glyceraldehyde-3-phosphate dehydrogenase	1.79
P00830	ATP2 ATP synthase subunit beta mitochondrial	1.72
P16474	KAR2 78 kDa glucose regulated protein homolog	1.67
P07251	ATP1 ATP synthase subunit alpha mitochondrial	1.58
P00360	G3P1 (TDH1) Glyceraldehyde 3 phosphate dehydrogenase 1	0.64
P07274	PFY1 Profilin	0.59
P53623	HSP72 Heat shock protein 70 2	0.55
P32471	EFB1 Elongation factor 1 beta	0.5
Q6FIS9	PK1 Pyruvate kinase 1	0.48
P00358	G3P2 (TDH2) Glyceraldehyde 3 phosphate dehydrogenase 2	0.44
Q8J2M3	HSP82 Heat shock protein HSP82	0.4
P12709	PGI1(CDC30) Glucose 6 phosphate isomerase	0.39
P14832	CPR1 Peptidyl prolyl cis trans isomerase	0.38
P14540	FBA1 Fructose biphosphate aldolase	0.36
Q03048	COFI Cofilin	0.33
P09435	SSA3 HSP73 Heat shock protein	0.28

Proteins of GLN1 and FMP16 exhibited 4-fold increase in T8. GLN1, FMP16, ADH1, GLN1 synthesize glutamine from glutamate and ammonia with Glt1p and form the secondary pathway for glutamate biosynthesis from ammonia. FMP16 was proposed to be involved in responding to conditions of stress. POR1 is the outer membrane protein required for maintenance of mitochondrial osmotic stability and mitochondrial membrane permeability. ATP2 is the Beta subunit of the F1 sector of mitochondrial F1F0 ATP synthase; which is a large, evolutionarily conserved enzyme complex required for ATP synthesis. KAR2 is the ATPase involved in protein import into the ER.

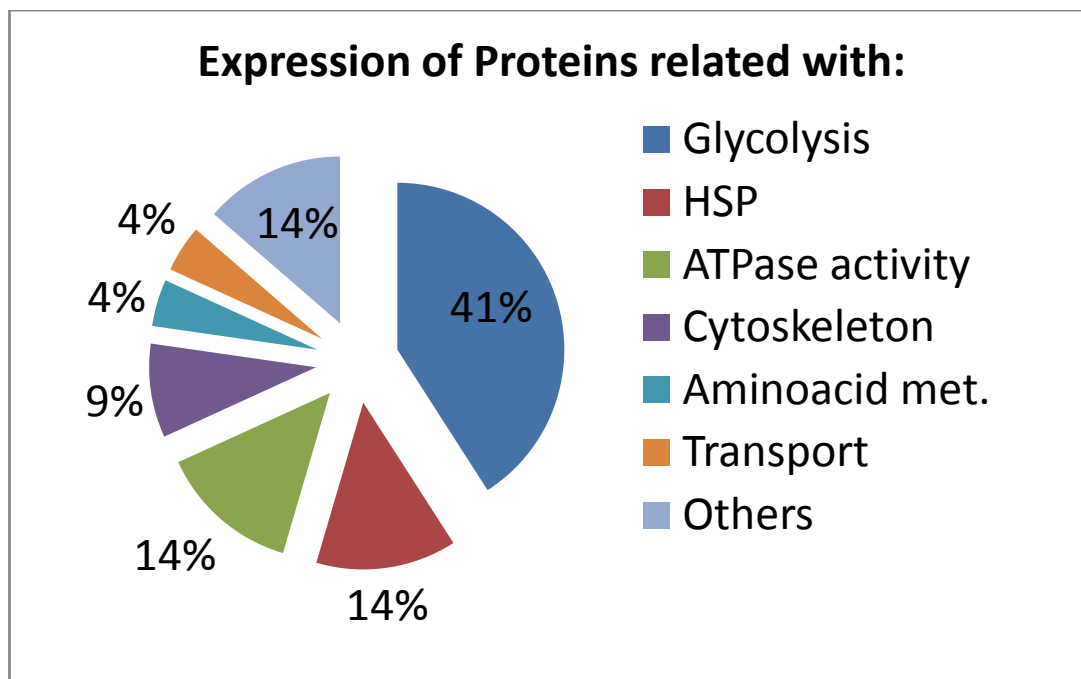


Figure 3.62: Expression of proteins related with metabolic pathways.

According to proteomic analysis, the protein expression levels were compared between NaCl- resistant mutant T8 and the reference strain. Processes from protein expression were implicated in glycolysis, HSP, ATPase activity, cytoskeleton, aminoacid metabolism and transport. The most prominent pathway identified was "glycolysis" (Figure 3.62).

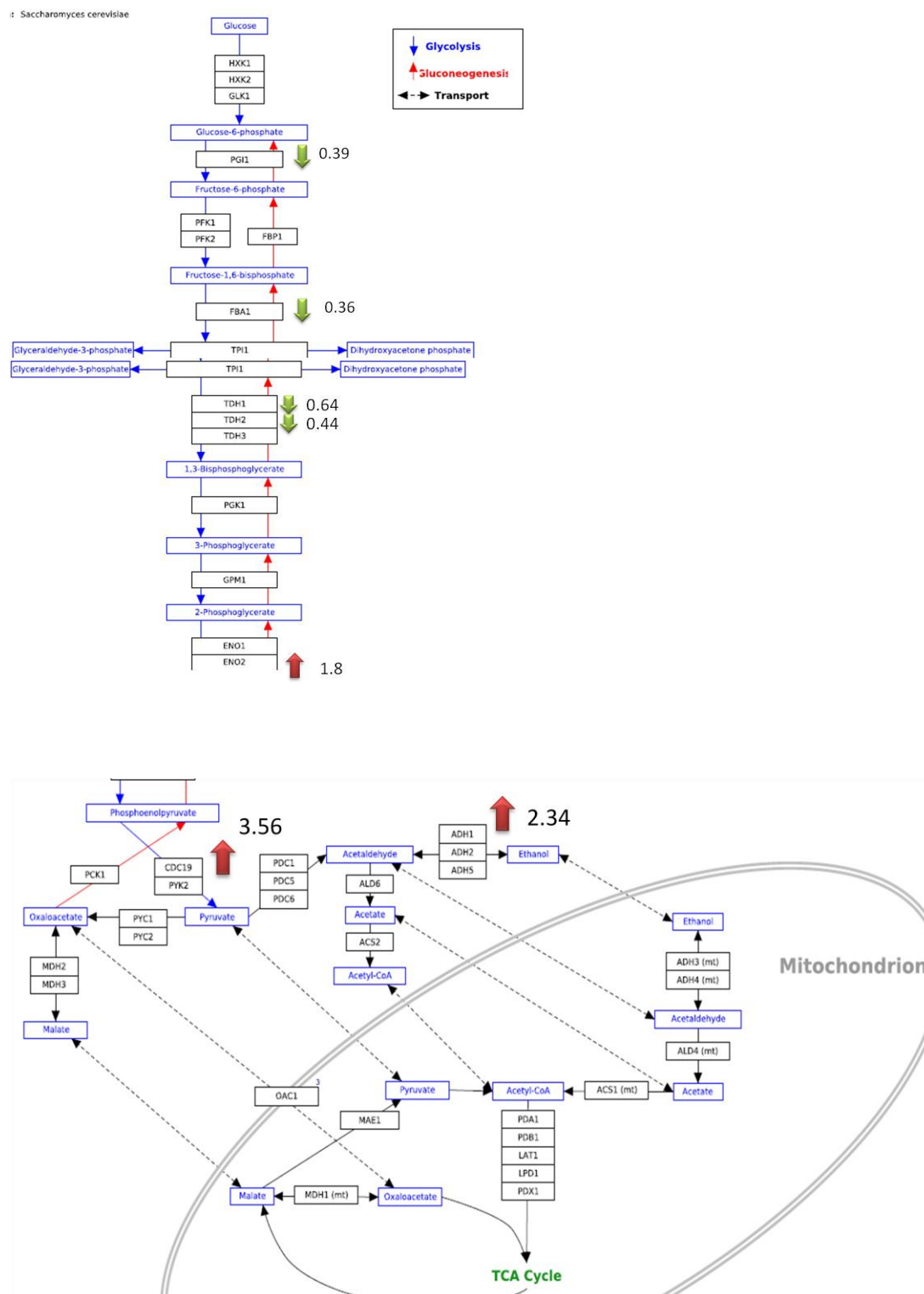


Figure 3.63 : Proteins whose expressions are changed in the glycolytic pathway of *S. cerevisiae*.

PYK1 (CDC19) showed the highest increased expression in the glycolytic pathway. It converts phosphoenolpyruvate to pyruvate. ENO2 and ADH1 protein expression also showed an increase. ENO2 converts 2-phosphoglycerate to phosphoenolpyruvate

(PEP) and it is a reversible dehydration reaction. ADH1 is required for the reduction of acetaldehyde to ethanol.

Expression of FBA1, TDH1, TDH2 and PGI1 decreased in T8. TDH1 showed highest decrease in the glycolysis pathway. PGI2 catalyzes the interconversion of glucose to glucose-6-phosphate and fructose 6-phosphate. FBA1 catalyzes conversion of fructose 1,6 bisphosphate to glyceraldehyde 3-P and dihydroxyacetone-P. TDH1 and TDH2 catalyze reaction of glyceraldehyde 3-phosphate to 1-3 bisphosphoglycerate.

Table 3.33: Important interactions according to the proteomic analysis results.

Symbol	Gene > name	sgdAlias	Organism	AnnotationType	InteractionType	InteractingGene	Experiment name	Name
HSP82	Heat Shock Protein	HSP90	S. cerevisiae	high-throughput	genetic interactions	ALR2	McClellan AJ, et al. (2007)-1	Aluminum Resistance
HSP82	Heat Shock Protein	HSP90	S. cerevisiae	high-throughput	physical interactions	HOG1	Millson SH, et al. (2005)-158	High Osmolarity Glycerol response
HSP82	Heat Shock Protein	HSP90	S. cerevisiae	manually curated	genetic interactions	HOG1	Yang XX, et al. (2006)-16487	High Osmolarity Glycerol response
HSP82	Heat Shock Protein	HSP90	S. cerevisiae	high-throughput	genetic interactions	HAL9	McClellan AJ, et al. (2007)-1	HALotolerance
SSA3	Stress-Seventy subfamily A	YG106	S. cerevisiae	high-throughput	physical interactions	SIS2	Krogan NJ, et al. (2006)-165	Slt4 Suppressor
ENO2	ENO2	enolase	S. cerevisiae	high-throughput	genetic interactions	PBS2	Costanzo M, et al. (2010)-20	Polymyxin B Sensitivity
TDH2	Triose-phosphate DeHydrogenase	GLD2 GAPDH	S. cerevisiae	high-throughput	physical interactions	PBS2	Krogan NJ, et al. (2006)-165	Polymyxin B Sensitivity
ATP1	ATP synthase		S. cerevisiae	high-throughput	genetic interactions	NHA1	Szappanos B, et al. (2011)-2	Na ⁺ /H ⁺ Antiporter

3.13 Sequence analysis of *NHA1*, *NHX1* and *KHA1*

NHA1, *NHX1* and *KHA1* are important genes for NaCl stress. Thus, the sequences of *NHA1*, *NHX1* and *KHA1* in T8 were compared with those of the wild type strain, to identify potential mutations. EMS and NaCl stress may cause a mutation or mutations in those gene sequences, which could cause their upregulation or downregulation. The corresponding sequences of T8 were aligned with that of the wild type. It was found that there were no differences in their nucleotide sequences.

3.13.1 Genomic DNA isolation

Genomic DNA isolation was carried out following the protocol of Small-scale preparation of yeast DNA in "Molecular Cloning A laboratory Manual (Green and Sambrook, 2001). The gel image of the isolated genomic DNA is shown in Figure 3.64.

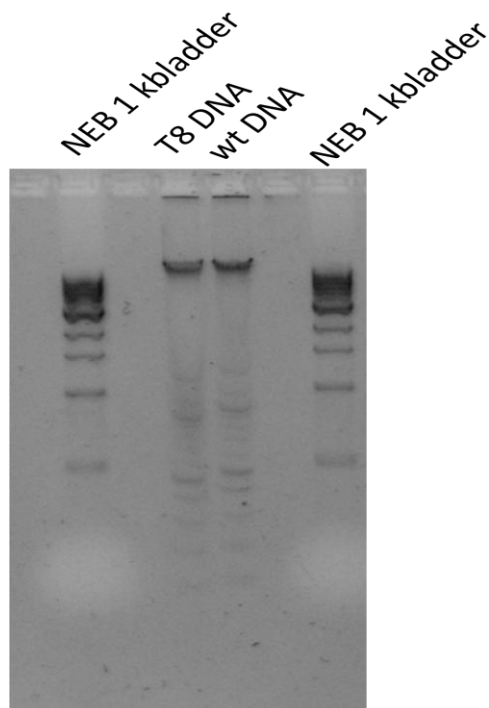


Figure 3.64: Gel image of DNA isolated from the wild type and T8.

3.13.2 Amplification of target sequences by PCR

DNA sequences of *NHA1*, *NHX1* and *KHA1* in wild type and T8 were amplified (Figure 3.65). *NHA1*, *NHX1* and *KHA1* genes were of 2958, 1902 and 2622 bp length.

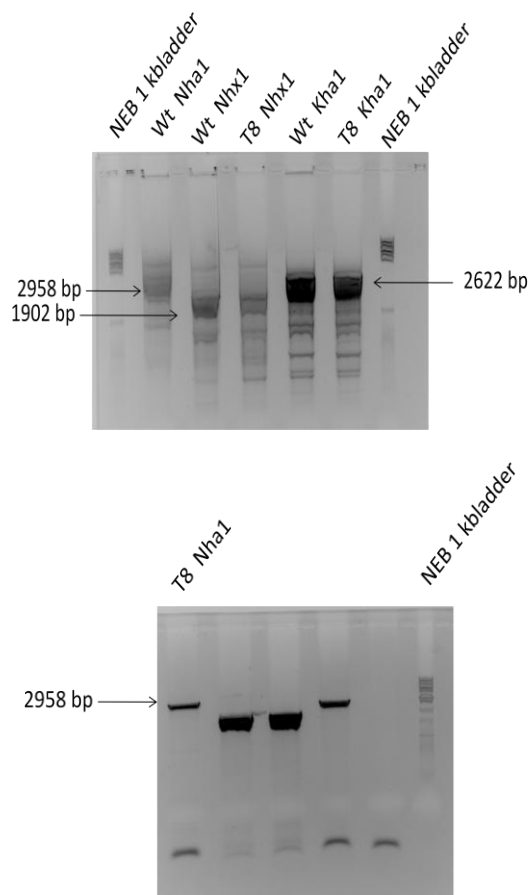


Figure 3.65: 1% (w/v) agarose gel image of amplification products of *NHA1*, *NHX1* and *KHA1*.

3.14 Site directed mutagenized sequences by PCR

Mutations [G372S (ggt → agc), D266H (gat → cat), D267A (gac → gcc), D266H/D267A (gat → cat, gac → gcc), D266N/D267N (gat → aat, gac → aac), N176 (aat → gat), D177N (gat → aat)] were chosen to investigate the pH sensor of *NHA1* (Figure 3.66).

CLUSTAL 2.1 multiple sequence alignment

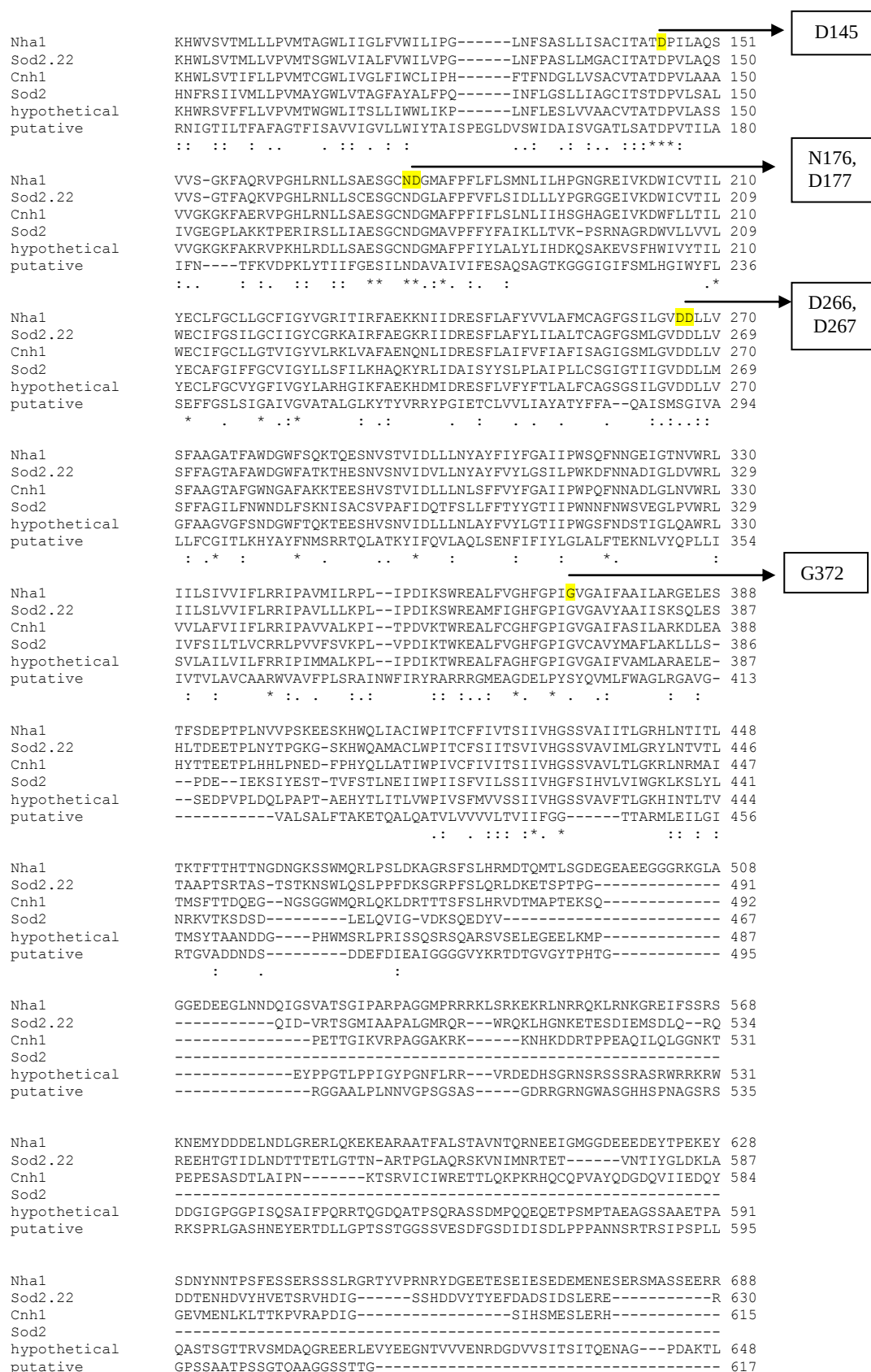
```

Nha1      -----MAIWEQLEVSKAHVAYACVGVFSSIFSLVSLYVKEKLY  38
Sod2.22   -----MVWRQLEVTKAHVAYSCLGIFSSIFSLVSLFVKERLY  37
Cnh1      -----MAWSQLELEPPHIAYACVGIFSTLFSLSLVFVKERLY  37
Sod2      -----GWRQLDIDKVHLALIVAGGFITFFCYFSEVFRKLL  36
hypothetical putative  -----MAWDKLSITGPHLVYIILGGFTSLFMLCSSI FKERMY  37
                               MLAQTLFSSLLELAKRDEEDPDASDPKQENNASWAIFILIVLLIVAFCTSYIMQQRKIT  60
                               : .                               : *               : : :

Nha1      IGESTVAGIFGLIVGPVCLNWFNPLKWGN-SDSITLEITRIVLCLQIFAVAVELPRKMYL  97
Sod2.22   IGESMVASVFGLIVGPHCLNWFNPLSWGN-TDSITLEISRILLCLQVFAVSVELPRKYM  96
Cnh1      IGEATVASIAGLILGPHCLNWFDPVSWGN-SDYITLIEICRIVLCIQIVAVAVELPKKMYM  96
Sod2      VGEAVLGSITGLIFGPHAACLVDPFWSGDHGDYLTVEICRIVLDVRVFASAIELPGAYFQ  96
hypothetical putative  IGEATVATICGVIFGPHAANLINPASWGG-VDIITIEFSRIVLVVQCFAVGVELPKYYME  96
                               AIHETVVSIFGGMIVGLGLRISGFDSIRDLVNFNYQYFFNLLLPPIILSSGYELHQGNFF 120
                               . : : * : . . . . : : : * . : . ** :

```

Figure 3.66: Multiple sequence alignment results.



Nha1	IRKMKEEEMKPGTAYLDGNRMIIENKQGEILNQVDIEDRNEARDDEVSDSTAHSSTTT	748
Sod2.22	IKLLREQEQQAYIAYTEDNQVI IENRQGEILEYARSHN-DGVRDAEAG-----	677
Cnh1	-----LTQYSTGS-----GEYTTDEDTHG-----	634
Sod2	-----	
hypothetical	EMKLKSEAGASGWTYEALKRRIMNWREAEMEKRKGKEKMF RKSEPARAFQFGNTIILENE	708
putative	-----	
Nha1	MTNLSSSSGGRLKRILTPTSLGKIHSVLVDKGKDKNKNSK-----YHAFKIDN	795
Sod2.22	-----SHNQGRHKRASSPP-LERLRQITN----EACKTK-----YYAYKVGN	714
Cnh1	-----LQEKLSRSLSRRSYKKD----DPNKRK-----VYAHRVDD	666
Sod2	-----	
hypothetical	DGDVIKKYDLP SDKSQSDLVAQGLKYMGMGIFVKVLDKAGGAAEKPQGEGALVAQRGKK	768
putative	-----QQPLTATAAIRQLLSAEN-----P	636
Nha1	LLIENEDGDVIKRYKINPHKSDDDKSKNRPND SVSRALTAVGLKSKANS GVPVPVDE	855
Sod2.22	DLIVEDESGEEFRYRISPHGGKRRIKKKNINN--PVSSVLSSMGITK-----PRRVPE	766
Cnh1	LIV IENEDGDIIRRYKVNKHAPAPRARSGSIMG----MVKSMVGIKP-----PPEISV	715
Sod2	-----	
hypothetical	KSLPNEQDEEDDKHIRFTIGGIGKRLTKEDFLN---EMKKLDKNQAKALAQQMTQSKANA	825
putative	TALLRQLDEEVIKPKLLLDAGSGRGGPGSGSH-----	669
Nha1	EKAIEGPSRKGPMLKKRTLT PAPPRGVQDSL DLEDEPSSEEDLGDSYNMDDSEDYDDNA	915
Sod2.22	-----RKNLSLHS----EDEMADDEAESEENYGDS-----DDLA	797
Cnh1	TDLEHGTQHKI ILPEEENTPMSS---KTEQKLED TIANILQENHTQPIS-----EESA	765
Sod2	-----	
hypothetical	SPSRSAAGKTGSSSAPAATTMPCIQVTPDVDTTKAEGETDAERKRRLAALKGMQNLSEEA	885
putative	-----	
Nha1	YESETEFERQRRNLALGEMTAPADQDDEELPPLPVEAQTGNDGPGTAEGKKKQKSAAVKS	975
Sod2.22	LFVKDHAD-----	805
Cnh1	DEEETEVEKKRRLQALGYLPSSRRDREDEEE-----	796
Sod2	-----	
hypothetical	HEIETEAEERRRREAAALGLRPHGAEDSEDDT PRLPPSRRSIRFAAAPNKERKDSA----	941
putative	-----	
Nha1	ALSKTLGLNK	985
Sod2.22	-----	
Cnh1	-----	
Sod2	-----	
hypothetical	-----	
putative	-----	

Figure 3.66 (continued) : Multiple sequence alignment results.

Asp266 and Asp267 are important aminoacids in proton translocation activity of the Na^+/H^+ exchanger (Dibrov *et al.*, 2000) and proper function of sod2 (Mager *et al.*, 2013). Asp266, Asp267, Gly372, Asn176, Asp177 were chosen for site-directed mutagenesis thus these sequences are highly conserved in *S. cerevisiae* (NHA1), *Zygosaccharomyces rouxii* (SOD2 and SOD22), *Candida albicans* (CNH1) and *Chaetomium thermophilum* (hypothetical and putative protein).

4. DISCUSSION

In this study, NaCl-resistant *Saccharomyces cerevisiae* was investigated at physiological and transcriptomic level. An individual mutant's salt resistance was investigated by comparing to the reference strain at physiological and transcriptomic level.

During evolutionary engineering studies, improved phenotype often means increased fitness. Maximum growth rate ($\mu_{\max}$) of microbial cells for toxic concentrations of certain chemical compounds are used as a fitness criteria for biotechnology (Dragosits and Mattanovich, 2013). Specific growth curves of wild type and "T8" in YMM were calculated by growing these strains in 5 mM LiCl, 0.5 M NaCl, 0.7 M NaCl and 0.9 M NaCl. In all of these conditions, T8 grew better than the wild type.

Organisms use several physiological mechanisms which can protect them against recurring stressors. One of these mechanisms is cross-protection which means one environmental stressor protects cells against a second stressor. However, not every stressor cross-protects against other stressors. The cross-resistance of T8 was assessed by growth on YPD and YMM plates contained LiCl, ZnCl₂, H₂O₂, CoCl₂, KCl, ethanol, CuCl₂, MnCl₂, CrCl₃, AlCl₃. T8 showed different resistance levels on YMM and YPD plates. Alkım *et al.* (2013) showed that higher resistance to these ions was achieved using a synthetic glucose mineral medium (SD) rather than a glucose-rich (YPD) medium. This sensitivity difference could be because of the former medium's poor content of growth factors and lack of amino acids.

Cakar *et al.* (2009) showed that cross-resistance is observed for *S.cerevisiae*. Cobalt resistance showed cross protection to thermal and oxidative stress. Cakar *et al.* (2005) also showed that freeze-thaw tolerance in *S.cerevisiae* is related to increased thermal, oxidative and ethanol stress resistance. T8 has significantly high cross-resistance to lithium. This cross-resistance might be due to the fact that sodium and lithium are mediated by the same transporters and ATPases such as ENA in *S. cerevisiae*.

Lithium is a widely used monovalent cation to treat manic bipolar disorder. Its antimanic and antidepressant effects require days to weeks to appear, and lithium affects gene expression in the central nervous system (Manji *et al.*, 1995; Masuda *et al.*, 2000). Homeostasis of Li^+ in yeast is maintained by multiple transport pathways that also transport Na^+ . Spot test results of T8 and the wild type (CENPK-7D) showed that resistance to sodium and lithium are obvious; however, potassium resistance is not obvious.

The lithium sensitivity of yeast cells reflects the toxicity of the cation, while high potassium sensitivity often mainly reflects the osmotic effect. NaCl sensitivity combines both, as the sodium cation is more toxic than potassium, but far less than lithium (Maresova and Sychrova, 2010). NaCl resistant mutant "T8" was resistant to sodium and lithium, while the wild type grew as well as T8 in the presence of potassium. This demonstrates that T8 had resistance to cation toxicity, but not to osmotic stress.

In a previous study, EMS-induced yeast mutants which were sensitive to aluminum were also found to be sensitive to NaCl, KCl and hydrogen peroxide (Schott and Gardner, 1997). 37 Al-tolerance genes were identified in *S. cerevisiae* (Kakimoto *et al.*, 2005). According to the microarray results of the present study, only one (*AGP2*) of the 37 genes was upregulated. These findings are not in accordance with the present results. Overexpressed *ALR1* and *ALR2* confer increased tolerance to Al^{+3} and Ga^{+3} ions, but increased sensitivity to Zn^{+2} , Mn^{+2} , Ni^{+2} , Cu^{+2} , Ca^{+2} , and La^{+3} ions (MacDiarmid and Gardner, 1998). Study of MacDiarmid and Gardner (1998) showed that the increased sensitivity of overexpressing strains to Co^{+2} , Mn^{+2} , Ni^{+2} , and Zn^{+2} was consistent with the Alr proteins directing the influx of these divalent cations system. Overexpression of the *ALR* genes increased Co^{+2} uptake, and loss of *ALR* gene activity reduced uptake. This study showed that the strains with aluminum resistance will show sensitivity to Co^{+2} , Mn^{+2} , Ni^{+2} , Zn^{+2} . T8 has slight resistance to Co^{+2} , Mn^{+2} , Ni^{+2} , Zn^{+2} and is sensitive to aluminum.

FAAS results showed that high extracellular Na^+ and Li^+ concentrations increase intracellular Na^+ and decrease intracellular K^+ levels of both wild type and T8. Goossens *et al* (2000) indicated that toxic cation tolerance may be due to reduced cellular uptake. However, according to AAS results, there is no reduced uptake of

K^+ , Na^+ or Li^+ in T8. Resistance of T8 cannot be explained by reduced uptake of cations.

NaCl resistant mutant "T8" did not exhibit significantly increased lithium accumulation compared to the wild type. The lithium resistance of T8 may not be caused by increased lithium efflux. "T8" accumulated slightly more Li^+ than the reference strain in double stress conditions especially in the presence of both NaCl and LiCl stresses.

S. cerevisiae has three mechanisms to prevent sodium accumulation and to maintain a stable high intracellular K^+/Na^+ ratio: restriction of influx, active efflux and compartmentation of Na^+ in the vacuole. FAAS results showed that T8 has higher K^+/Na^+ ratio at every stress condition tested, compared to wild type. The difference in K^+/Na^+ ratio between T8 and the wildtype is obvious at 5 mM LiCl. K^+/Na^+ ratio of T8 and the wildtype were 4.21 and 1.97, respectively, at 5 mM LiCl stress. This high K^+/Na^+ ratio showed that T8 actively extruded Na^+ .

Munson *et al.*, (2004) suggested that the increased sensitivity of the strains, which have a mutation for Na^+ pathway to cationic compounds such as hygromycin B or tetramethylammonium, might be due to the increased electrochemical potential at the plasma membrane. Wild type and "T8" were grown in plates containing different concentrations of spermine and TMA (tetramethylammonium). NaCl resistant mutant "T8" did not show any sensitivity to TMA and spermine, although these anionic drugs were shown to be the marker for membrane potential change (Kinclova-Zimmermannova *et al.*, 2006). NaCl resistant mutant T8 did not have altered membrane potential and there was no increased uptake of cationic drugs due to the membrane potential driving force. Kinclova-Zimmermannova *et al.* (2006) showed previously that neither the kha1 nor the nhx1 mutant, both sensitive to drugs such as hygromycin B, had an altered membrane potential. Maresova and Sychrova (2010) showed that arl1 mutants did not have increased membrane potential although they were sensitive to hygromycin B. Our results was consistent with Kinclova-Zimmermannova *et al.* (2006) and Maresova and Sychrova (2010).

Maresova and Sychrova (2010) indicated that Golgi proteins also take part in the complex process of intracellular detoxification, not only for inorganic cations but also for various organic drugs. There is no significant difference in the membrane

potential or the toxic cation content between "T8" and reference strain. The phenotype differences can be primarily caused by an altered uptake and/or an efflux of the chemicals.

Hernandez *et al.*, (2009) suggested that the late endosomal/prevacuolar compartment itself is a target of salt toxicity and Nhx1p has an important role in late endosome. Vesicular transport such as vacuolar sequestration of undesired compounds or the correct trafficking of the enzymes necessary for detoxification in T8 can be affected.

The family of Na^+/H^+ exchangers (NHEs) have a role in control of cell cycle and cell proliferation, transepithelial Na^+ movement, salt tolerance, vesicle trafficking, and biogenesis in mammals and also pathophysiological conditions such as hypertension, epilepsy, post-ischemic myocardial arrhythmia, gastric and kidney disease, diarrhea, and glaucoma were found to be associated with NHE dysfunction (Brett *et al.*, 2005).

Relationship of NHE with disease makes the mechanism and structure of NHE important for drug formulations. For example, overactivation of the NHE1 antiporter in heart muscle cells during open-heart surgery in humans has harmful consequences, which are reduced by the use of drugs inhibiting the antiporter (Hunte *et al.*, 2005). The fungal *NHX1* and *NHA1* are the oldest members of the NHE gene family and it makes them an appropriate model to study molecular structure of these antiporters to understand NHE gene family. Yeast is a suitable model to study cation binding and selectivity, drug binding, pH sensitivity, and ion translocation of *NHA1*. Yeast contains a single ortholog of each of the three CPA (alkali-cation/ H^+ exchanger) families represented in eukaryotes: *NHX1*, *NHA1*, and *KHA1* (Brett *et al.*, 2005). While in fungi a proton motive force generated by the plasma membrane H^+ -ATPase PMA1 is used to expel Na^+ (and K^+) from the cytosol, thereby acting to acidify the cytoplasm. In mammalian cells, plasma membrane NHE acts to alkalinize the cytoplasm by coupling to the Na^+ electrochemical gradient maintained by the Na^+/K^+ -ATPase. Sequences of *NHX1*, *NHA1*, and *KHA1* genes were checked to be compared with the mutant strain "T8". The *S. cerevisiae* genome contains three genes encoding alkali metal cation/ H^+ antiporters (Nha1p, Nhx1p, Kha1p) that differ in cell localization, substrate specificity and physiological function. *NHA1*, *NHX1* and *KHA1* of are 985, 873 and 633 aa. These three genes (*NHA1*, *NHX1*, *KHA1*) of T8 and the wild type were sequenced to check if T8 has mutations in these three genes

that may result from the EMS treatment and/or selection. Sequencing results showed that there was no mutation in these antiporters, and EMS mutagenesis and NaCl stress did not affect the sequences of *NHX1*, *NHA1*, and *KHA1*.

Membrane proteins are the gateways for signals that pass between a cell and its surroundings and are central to ionic signaling mechanisms and the material transport required for nutrient uptake and toxin disposal. One of the goal of modern structural biology is to understand membrane protein molecular structure. Obtaining sufficient quantities of pure material for biochemical and structural analysis is difficult. Most membrane proteins are not found in abundance in native sources, which makes overproducing membrane protein constructs necessary. The ability to overproduce proteins in heterologous systems is a revolution for structural biology studies of soluble proteins (Hammon *et al.*, 2009). Nha1 is heterologously overexpressed and it is checked by complementation assays.

AB11c, the triple mutant (*ena1-4Δ nha1Δ nhx1Δ*) and KTA40-2 (*ena1-4Δ nha1Δ nhx1Δ kha1Δ*) are more severely affected in their secretory, vesicle recycling pathways and protein sorting. Both strains contain the *ena1-4* and *nha1* deletions; that means they lack the main active sodium and potassium efflux systems. Overexpression of Nha1p showed that this protein complements better at pH 3.5.

Na^+/H^+ antiporters show a marked pH dependence, which is important for their physiological function in eukaryotic and prokaryotic cells. Hunte *et al.* (2005) described specific single site mutations which influence the pH dependence of NhaA. The *NHA1* of *Saccharomyces cerevisiae* has similarities to the Na^+/H^+ antiporters *sod2* of *Schizosaccharomyces pombe* (Jia *et al.*, 1992), Z-Sod2 and Z-Sod22 of *Zygosaccharomyces rouxii* (Banuelos *et al.*, 2002). Many single site mutations of the Na^+/H^+ exchanger have been described. Studies (Dibrov *et al.*, 1998; Wiebe *et al.*, 2003) of mutagenized *Schizosaccharomyces pombe* (SpoSOD2) revealed that five Asp are important for alkali metal cation and/or proton translocation. Five Asp corresponds to D145, D177, D241, D266 and D267 in Nha1. The neighbouring 6th TMD contains the conserved motif ESGCND (aa 172-177) in all Nha1 antiporters (Pribylova *et al.*, 2006). D177, D266, D267 and N176 were mutagenized to observe the effect of mutations. The fact that transport system were conserved through the evolution from bacteria to humans confirms the general need of a cell to efficiently

regulate the intracellular concentration of potassium and sodium (Pribylova *et al.*, 2006).

Nha1 gene is 2958 nucleotides long and is located on the right arm of chromosome XII between *TIS11* and *SLS1* genes. The Nha1p (985 amino acids long) is a typical hydrophobic membrane protein with a short hydrophilic N-terminus (12 amino acids), 12 transmembrane segments with short connecting loops, and an extremely long hydrophilic C-terminus (Sychrova, 20004). Systematic genomic sequencing of other species revealed highly conserved homologous ORFs in all of them (Pribylova *et al.*, 2006). *S. cerevisiae* (*NHA1*), *Zygosaccharomyces rouxii* (*SOD2* and *SOD22*), *Candida albicans* (*CNH1*) and *Chaetomium thermophilum* (hypothetical and putative protein) were compared to find conserved aminoacids. *Z.rouxii* has *SOD2* and *SOD22* that encodes Na⁺/H⁺ antiporter and *Candida albicans* encodes *CNH1* which functions as Na⁺/H⁺ antiporter. Specific single site mutations [G372S, D266H, D267A, D266H/D267A, D266N/D267N, N176, D177N] on Nha1 are applied to understand the influence of these mutations on pH.

Extremophiles use many of the same strategies observed in neutralophiles, further adapting them to respond to more extreme challenges. Typically, major pH homeostatic mechanisms of extremophiles are constitutively expressed, so that these bacteria are prepared for sudden shifts to the extreme end of the pH range. This preparedness often negatively impacts growth at near neutral pH because of the energetic cost of expressing proteins that are not useful at neutral pH and because some essential proteins are adapted to work at extreme pH values but function suboptimally at neutral pH (Krulwich *et al.*, 2011). NaCl-resistant mutant T8 showed same strategies as extremophiles. Microarray results indicated that T8 without NaCl stress has genes overexpressed compared to the wild type. These upregulated and downregulated genes adapted this mutant to high sodium and lithium stresses.

Glycerol is as a major player in the cell metabolism, a central element of the cell redox balance, essential precursor for phospholipids and triacylglycerolipids, and lastly an essential constituent of the cell stress resistance system. Tilloy *et al.* (2014) showed that it is possible to generate adaptive laboratory evolution strategies under 1.25 M KCl stress for generation of *S. cerevisiae* wine yeast strains with enhanced glycerol and reduced ethanol yields. In the present study it was shown that under 1.3

M NaCl, *S. cerevisiae* yeast strains can be selected by evolutionary engineering to have enhanced glycerol and reduced ethanol yields.

Tilloy *et al.* (2014) reported that the production of glycerol and ethanol by the NaCl-resistant evolved industrial strains was not affected. In the NaCl-resistant evolved *S. cerevisiae* strain "T8", production of glycerol is higher and production of ethanol is reduced, compared to the wildtype. Metabolism of evolved strain is redirected toward glycerol and away from ethanol production. There is great interest in using non-genetic modification to improve yeast strains because using DNA recombinant technology in food is difficult for commercialization. Cakar *et al.* (2005) showed that multiple-stress resistant *S. cerevisiae* can be obtained by evolutionary engineering. Yeast strains are improved by adaptive evolution, based on long-term adaptation of yeast under environmental or metabolic constraint (Dhar *et al.*, 2011; Gerstein *et al.*, 2011; McBryde *et al.*, 2006; Wisselink *et al.*, 2009; Stanley *et al.*, 2010; Kutyna *et al.*, 2012).

Dhar *et al.* (2011) and Gerstein *et al.* (2006) studied evolutionary processes by using experimental evolutions using sodium chloride to generate osmotic stress. The present study is the first one, in which EMS and increasing NaCl stress levels were used to obtain *S. cerevisiae* strains by evolutionary engineering. T8 has slower glucose uptake and no change in succinate production. Remize *et al.* (1999) showed that an engineered yeast strain for glycerol overproduction also accumulates acetate and succinate. However, T8 did not accumulate high levels of succinate and acetate. Tilloy *et al.* (2014) explained that it could be due to the smaller increase in glycerol production than that in the engineered strains or due to a different metabolic strategy.

Glycogen and trehalose are two important glucose stores of the yeast *S. cerevisiae*, and their production changes in response to a number of environmental stress conditions (François *et al.*, 2012). The disaccharide trehalose accumulates during salt adaptation. Increased production of trehalose is reflected in the reported salt-induced expression of the enzymes in the biosynthetic pathway from glucose-6-phosphate to trehalose. Expression analysis during salt stress revealed a dramatic increase in the expression of *TPS1*, which encodes a trehalose-6-phosphate synthase (Blomberg, 2000). Upregulation of *TPS1* was not observed in T8. However, upregulation of genes in trehalose pathway such as *TPS2*, *TSL1*, *PGM2*, *UGP1*, *GLK1*, *NTH1* and *ATH1* were observed. Assessment of glycogen and trehalose contents showed that

the amounts of trehalose and glycogen in T8 were prominently higher compared to the wild type levels without stress conditions. Lithium increased the amount of glycogen and trehalose more than sodium. It could be explained by higher toxicity of lithium. Trehalose accumulation was higher in T8 than glycogen, with and without sodium and lithium stress.

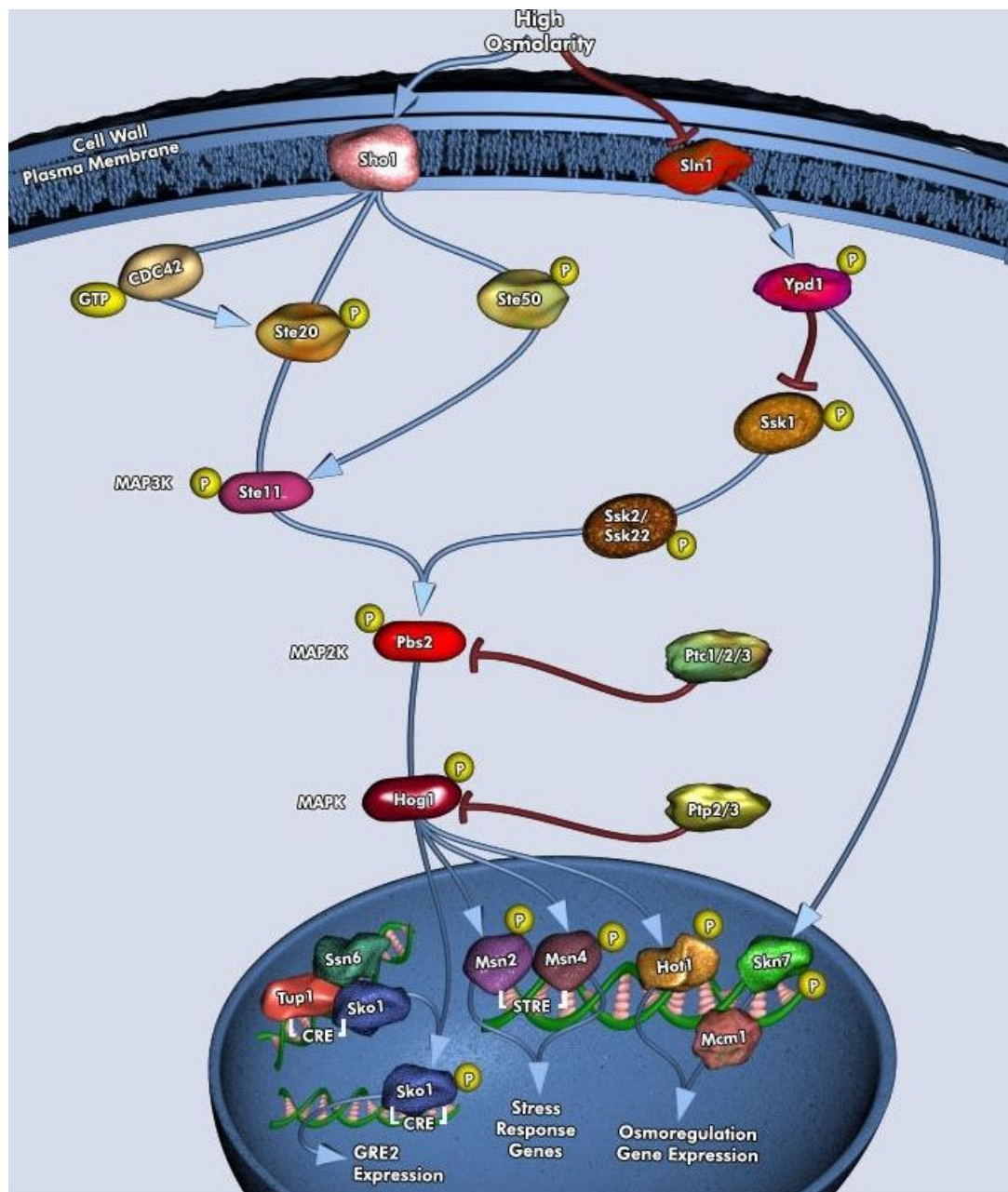


Figure 4.1: High Osmolarity pathway in *S.cerevisiae* (Url-1).

HOG1-mediated signaling pathway plays a key role in global stress conditions (Figure 4.1). However, in the present microarray results, the *HOG1* gene was not induced. Posas *et al.*, (2000) also observed the same result. *HOG1* pathway was

certainly involved in the transcriptional response of most salt responsive genes, but in many cases it was not the unique relevant signaling pathway (Posas *et al.*, 2000). The existence of different signaling pathways controlling the osmotic stress response had been documented in some detail in a few genes including the *ENA1* and *GPD1* genes (Posas *et al.*, 2000). Tilloy *et al.* (2014) suggested that the absence of a stress resistance phenotype and the improved fitness under carbon-restricted and stress conditions suggest that the primary target of evolution is not the *HOG* pathway. Our results of this thesis work are consistent with this suggestion. According to "T8" microarray results, *HOG* pathway was not induced.

Genes involved in the synthesis and degradation of glycogen such as *UGP1*, *GAC1*, *GPH1*, *GSY1*, *GLC3*, *GLG1* were induced under NaCl stress. Genes that were components of the vacuolar H-ATPase complex and required for sequestration of sodium into the vacuole such as *VMA6*, *VPH1*, *VMA7*, *VMA5*, *TFP1* were not induced in T8. These results were compatible with AAS results, sodium was not sequestered into the vacuole of the NaCl-resistant mutant T8. Synthesis of glycerol and trehalose was a major response of yeast cells to osmotic stress and expression of *GPD1* was induced in T8 and trehalose genes such as *TSL1*, *TPS1*, *TPS3* and *TPS2* were also upregulated. Higher glycerol levels in T8 were observed in HPLC results.

Microarray results of T8 was a genome-wide analysis of the transcriptional response of yeast cells to NaCl stress, followed by analysis of the response of *ENA6*, *PHO84*, *FLO11*, *TSL1*, *PGM2*, *GPH1* and *CTT1* genes, revealing that response involves different components.

Table 4.1 : Expression changes of glycerol transport related genes.

	Sytematic Name	Gene Name	Average Fold Change
Glycerol transport	YLL043W	<i>FPS1</i>	3.1
	YDL022W	<i>GPD1</i>	2.9
	YOL059W	<i>GPD2</i>	-
	YOR120W	<i>GCY1</i>	3.9

Glycerol-specific channel protein in the plasma membrane of *S. cerevisiae*, Fps1p, has been proven to be a regulated glycerol facilitator involved in the release of accumulated glycerol upon dilution stress (Blomberg, 2000). The key enzyme in

glycerol production in *S. cerevisiae* appeared to be the first enzyme redirecting the flux in glycolysis into glycerol, namely glycerol-3-phosphate dehydrogenase (GPD), since it was reported that this enzyme exhibits high controlling capacity in glycerol formation (Blomberg and Adler, 1989). There were two genes, *GPD1* and *GPD2*, encoding glycerol-3-phosphate dehydrogenase, and *GPD1* was only induced by osmotic stress and salt stress (Blomberg, 2000). Analysis at the transcript level clearly showed the putative dehydrogenase *GCY1* to be a salt upregulated gene (Norbeck and Blomberg, 1997). *FPS1*, *GPD1* and *GCY1* were induced in salt resistant mutant T8 (Table 4.1).

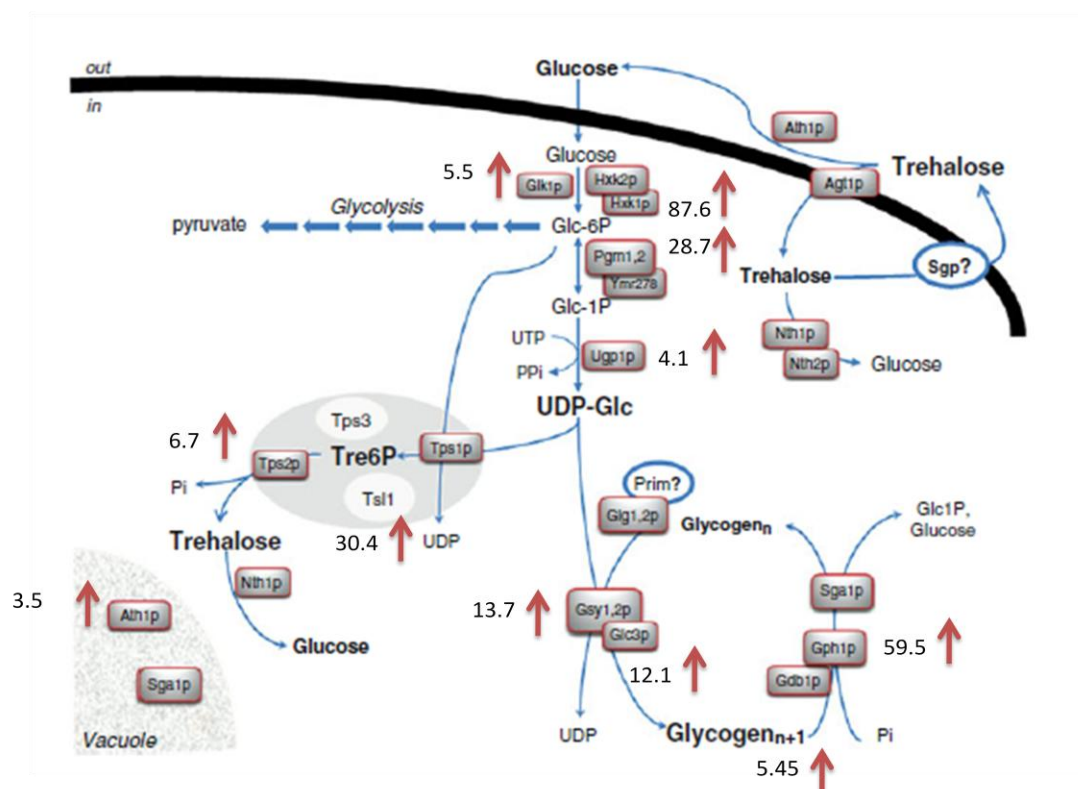


Figure 4.2 : Metabolic pathways for glycogen and trehalose (modified from François *et al.*, 2012).

Table 4.2 : Expression changes of trehalose biosynthetic process related genes

	Sytematic Name	Gene Name	Average Fold Change
trehalose biosynthetic process	YDR074W	<i>TPS2</i>	6.7
	YML100W	<i>TSL1</i>	30.4
	YMR105C	<i>PGM2</i>	28.7
	YKL035W	<i>UGP1</i>	4.1
	YCL040W	<i>GLK1</i>	5.5
	YDR001C	<i>NTH</i>	-
	YPR026W	<i>ATH1</i>	3.5
	YBR126C	<i>TPS1</i>	-

Intra-cellular levels of trehalose result from a well-controlled balance between enzymatic synthesis and degradation. In the yeast *S. cerevisiae*, the synthesis of trehalose is catalyzed by a UDP-glucose-dependent trehalose synthase (TPS) protein complex encoded by four genes. *TPS1* and *TPS2* encode trehalose-6-phosphate synthase and trehalose-6-phosphate phosphatase, respectively, while *TPS3* and *TSL1* code for two regulatory sub-units of the TPS complex (Francois and Parrou, 2001).

Ugp1p and Pgm2p are involved in the production of UDP-glucose, which was one of the substrates for trehalose-6-phosphate production via the Tps1 protein. The *UGP1*, *PGM2* and *TPS1* genes had recently also been shown to be induced during adaptation to salt. Trehalose-6-phosphatase (TPS2) had been reported to exhibit salt-stimulated expression (Winderickx *et al.* 1996). Tps1 protein itself plays a role in the inhibition of the glycolytic flux as part of a hypothetical general glucose-sensing complex formed by the association of Tps1, hexokinase and the glucose carrier (Parrou *et al.* 2005).

The upregulation of genes involved in trehalose synthesis of the cell was to produce and accumulate high amounts of trehalose and *TPS2*, *TSL1*, *PGM2* and *UGP1* were upregulated in T8 (Figure 4.2). Accumulation of the metabolite trehalose-6-phosphate (T6P) had inhibitory action on two of the three isoforms of the first enzymatic step in glycolysis, the hexokinases Hxk1p and Hxk2p. T6P had been proposed to exhibit the potential to act as a gate keeper in glycolysis via feedback inhibition (Blomberg, 2000). Fold change of *HXK1* was 87.6 in T8 (Table 3.28). The hypothesis on trehalose turnover suggests that not only the enzymes involved in the production of trehalose but also the *NTH1* encoded trehalase expression are salt-induced (Parrou *et al.*, 1997).

Hydrolysis of trehalose into glucose could be carried out by at least two enzymes: a cytosolic or neutral trehalase encoded by *NTH1* (Kopp *et al.*, 1993) and a vacuolar or acid trehalase encoded by *ATH1* (Destruelle *et al.*, 1995).

Two distinct pathways were found for trehalose assimilation in the yeast *S. cerevisiae*. One of them, the Agt1p- Nth1p-dependent route, is dependent on the genetic background of the strains and could be almost completely inactivated. The acid trehalase-dependent pathway thus appeared to be the most important system for trehalose assimilation. As yeast cells had very efficient hexose transporters, the rate

of extracellular trehalose hydrolysis into extracellular glucose, which could be easily monitored by the amount of Ath1p at the level of gene expression, limited glucose flux into yeast cells (Jules *et al.* 2004). In the microarray results, both *NTH1* and *ATH1* are upregulated.

Hirayama *et al.* (1995) reported that Glk1p was strongly induced under salt stress. As for glycerol, there was evidence for salt-stimulated turnover of the stress responsive metabolite trehalose.

Once again, this turnover would be at the expense of ATP, since going from glucose, to glucose-6-phosphate, trehalose and back again to glucose would effectively consume three ATP molecules. The Ugp1 protein utilized UTP instead of ATP for the activation of the glucose moiety, but these two nucleotide pools communicated via the exchange factor Ura6p (Blomberg, 2000).

The trehalose biosynthetic pathway is an attractive potential specific target for the development of antifungal drugs due to its absence in vertebrates; therefore, the effects of the *tps1* mutation (and that of *tps2*) had been studied in some pathogenic yeasts (Gancedo and Flores, 2004).

It had been reported that a *tps1* mutant exhibited lower expression of several genes involved in the osmotic or heat shock response (Hazell *et al.*, 1995, 1997). This implied a regulatory role of Tps1p on stress response, ion homeostasis and tolerance to toxic cations (Mulet *et al.*, 2004).

Table 4.3: Expression changes of glycogen biosynthetic process related genes.

	Sytematic Name	Gene Name	Average Fold Change
glycogen biosynthetic process	YPR160W	<i>GPH1</i>	59.5
	YEL011W	<i>GLC3</i>	12.1
	YFR015C	<i>GSY1</i>	13.7
	YLR258W	<i>GSY2</i>	6.8
	YOR178C	<i>GAC1</i>	7.7
	YPR184W	<i>GDB1</i>	5.4

The expression of genes involved in synthesis and degradation of glycogen, and the regulation of these pathways, was induced in early stationary phase. Regulated genes under stress were *GSY1* and *GSY2*, encoding glycogen synthase; *GPH1*, encoding glycogen phosphorylase; *GLC3*, encoding the glycogen branching enzyme and

GAC1, encoding the glycogen-binding sub-unit of protein phosphatase I (Sunnarborg *et al.*, 2001). The gene *GAC1* was also induced during osmoadaptation (Posas *et al.*, 2000). These genes were upregulated in T8 (Table 4.3).

Table 4.4: Expression changes of response to stress-related genes.

Functional Category	Systematic Name	Gene Name	Fold Change
Response to Stress	YBR072W	<i>HSP26</i>	11.53
	YDL079C	<i>MRK1</i>	11.25
	YDL222C	<i>FMP45</i>	8.88
	YDR074W	<i>TPS2</i>	6.69
	YDR258C	<i>HSP78</i>	6.02
	YFL014W	<i>HSP12</i>	19.32
	YGR088W	<i>CTT1</i>	13.31
	YGR243W	<i>FMP43</i>	7.61
	YIL101C	<i>XBP1</i>	9.67
	YML100W	<i>TSL1</i>	30.42
	YOL052C-A	<i>DDR2</i>	7.93
	YOR049C	<i>RSB1</i>	8.90
	YLL039C	<i>UBI4</i>	3.89

One of the well established stress response genes, *CTT1*, which encodes a cytosolic catalase, showed an increase in NaCl-resistant mutant T8. *CTT1* was induced 13 times more than the wild type. This gene had been shown to be upregulated in five previous studies of physiological stress response and salt stress response (Gasch *et al.*, 2000; Rep *et al.*, 2000; Causton *et al.*, 2001; Yale & Bohnert, 2001; Dhar *et al.* 2011).

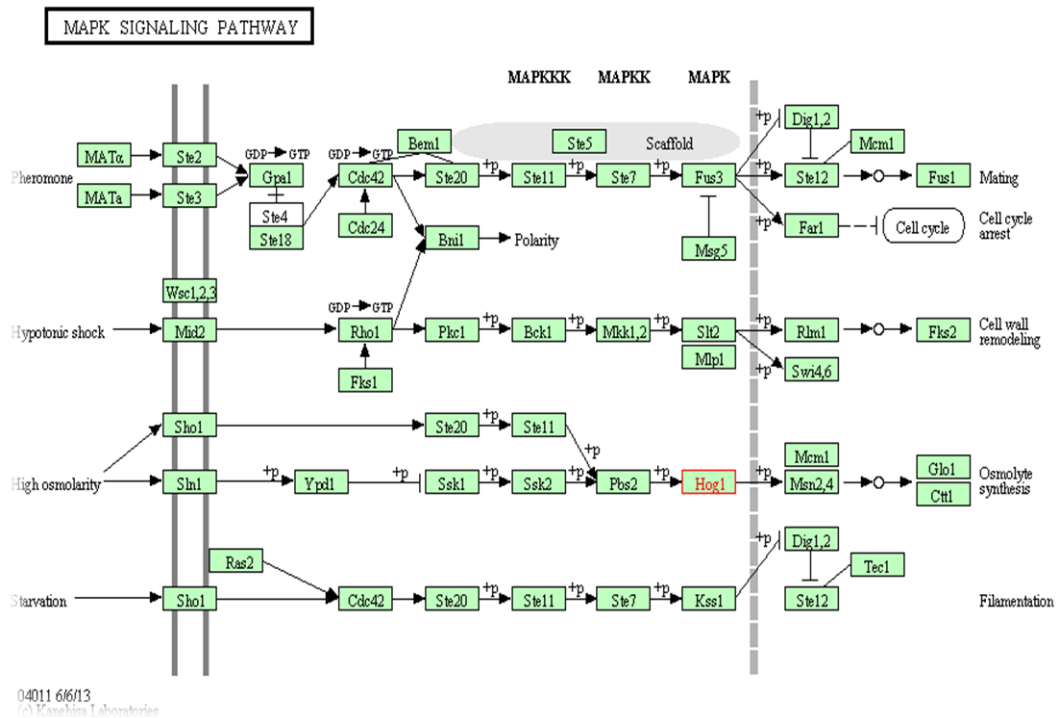


Figure 4.3: *HOG-1* Pathway related to *CTT1* (Wan *et al.*, 2008).

This gene contained stress-responsive elements (STRE) in their promoter. *CTT1* contained stress-responsive elements (STRE) in their promoter. Stress-responsive genes were frequently those whose products exerted a protective effect against damage to the cells by the stressor including *CTT1*, encoding catalase; *UBI4*, involved in ubiquitin-mediated protein degradation; *DDR2*, which was induced in response to DNA damage and *HSP12*, encoding a heat shock protein (Sunnarborg *et al.*, 2001). Posas *et al.*, (2000) indicated that, in many cases, *HOG1*-mediated signaling pathway was not the unique relevant signaling pathway and the induction of this set of genes appeared to be largely independent of the *HOG1*-mediated signaling pathway. The existence of different signaling pathways controlling the osmotic stress response had been found, including the *ENA1* and the *GPD1* genes. Also, the general response of genes encoding genes of trehalose, glycogen and glycerol pathways were important in T8. *CTT1*, *UBI4*, *DDR2*, *HSP12* were highly upregulated in T8 (Figure 4.3 and Table 4.4).

Table 4.5:Expression changes of *ENA6*.

	Sytematic Name	Gene Name	Average Fold Change
Ion transport	YDR536W	<i>ENA6</i>	8.13

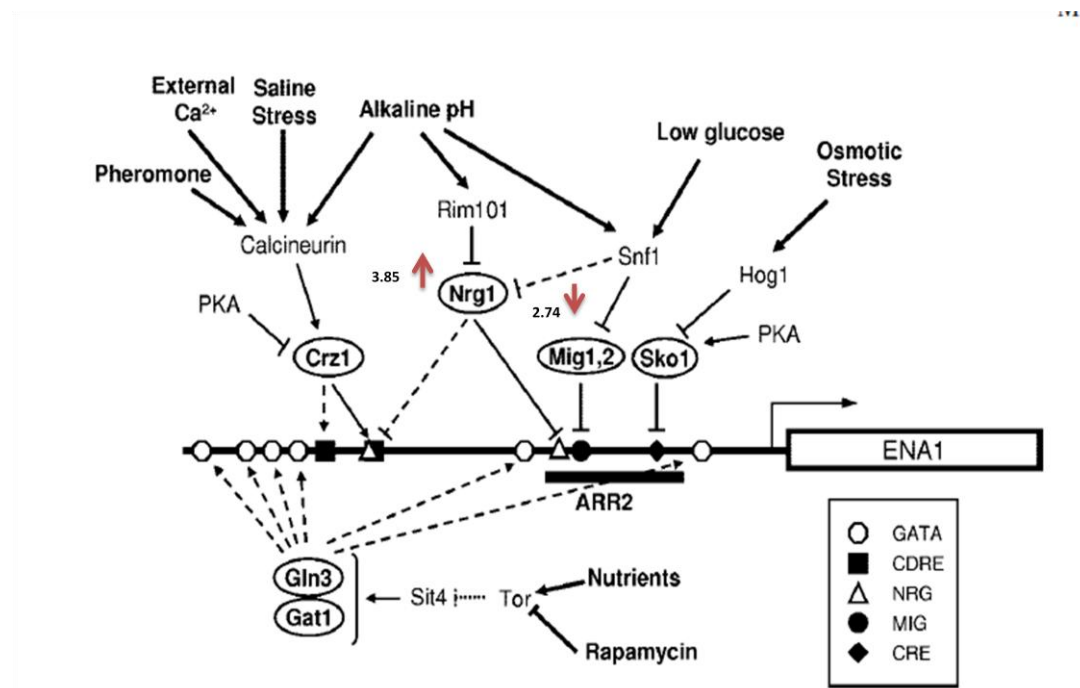


Figure 4.4: Regulation of *ENA1* in *S.cerevisiae* (modified from Ruiz and Arino, 2007).

Microarray results of T8 showed that *ENA6* could be regulated by Rim101 pathway (Figure 4.4).

Pho84p, the protein responsible for the high-affinity uptake and transport of inorganic phosphate across the plasma membrane, is also involved in the low-affinity uptake of heavy metals in the *S. cerevisiae* cells (Ofiteru *et al.* 2012). Pho84p is homologous to transporters from mammalian, plant, fungal, and bacterial sources and includes transporters for both phosphate and sugars. Although Pho84p was the first eukaryotic phosphate transporter described with a role in metal transport, it was likely that this type of system was present in higher organisms, including humans (Jensen *et al.*, 2003). The Pho84 protein catalyzed a proton-coupled phosphate transport at acidic pH, while the Pho89 protein catalyzed a sodium-dependent phosphate uptake at alkaline pH (Persson *et al.* 1998). Pho 84 was repressed in T8,

thus it was possible that it could not accumulate metals. Jensen *et al.* (2003) stated that Pho84 functions in manganese homeostasis, and when challenged with high concentrations of metals, *pho84*Δ yeast had reduced metal ion accumulation, suggesting that manganese resistance was due to reduced uptake of metal ions and disruption of Pho84. However, although Pho84 was repressed according to microarray results, T8 was sensitive to manganese.

S. cerevisiae has 20 genes that encode proteins of glucose (hexose) transporters (*HXT1* to *HXT17*, *GAL2*, *SNF3*, and *RGT2*) (Özcan and Johnston, 1999). The hexose transporters of *S. cerevisiae* are encoded by HXT genes. The two glucose sensors Snf3 and Rgt2 are also closely related to these transporters (Luyten *et al.*, 2002). *S. cerevisiae* has two uptake systems: low affinity (15 to 20 mM) and high affinity (1 to 2 mM) (Özcan and Johnston, 1999). The proteins encoded by *HXT1* to *HXT4* and *HXT6* to *HXT7* are considered to be the major hexose transporters in *S. cerevisiae* (Rintala *et al.*, 2008). These transporters are regulated by glucose levels. Hxt1p and Hxt3p are the low-affinity carriers. Hxt6p and Hxt7p have a high affinity for their substrate, whereas Hxt2p and Hxt4p display only moderate–low affinity (Luyten *et al.*, 2002). *HXT1* and *HXT3* transcription is induced only by high concentrations of glucose, *HXT2*, *HXT6*, and *HXT7* expression is induced only by low concentrations of glucose. Expression of *HXT5* is regulated not only by external glucose concentration, but also by growth rate (Rintala *et al.*, 2008).

HXT1 and *HXT5* are not only upregulated by glucose concentration, but also by NaCl and sorbitol (Gasch *et al.*, 2000; Posas *et al.*, 2000; Yale and Bohnert, 2001; Rep *et al.*, 2000). In microarray results of T8, *HXT2*, *HXT5*, *HXT6*, *HXT7*, *HXT15*, *HXT16*, *HXT17* were upregulated. *HXT1* and *HXT3* were downregulated. Downregulation of low affinity transporters (*HXT1* and *HXT3*) and upregulation of *HXT2*, *HXT6* and *HXT7* indicated that glucose concentration was low or glucose could not be taken into the cell in the presence of NaCl.

Yeast has two transport systems that pump out Na^+ that has entered the cell as follows: a cluster of up to five P-ATPases *PMR2A/ENA1-4* and a Na^+/H^+ antiporter, encoded by *NHA*. Ena1p is the most highly expressed. These two transport systems are differently regulated. *ENA1* is induced by osmotic stress, starvation, or high extracellular pH. *NHA1* increases sodium and lithium tolerance at an acidic or neutral

pH of the external medium (Masuda *et al.*, 2000). Expression of *ENA6* is inducible by Na^+ , Li^+ or high pH values, whereas expression of *NHA1* seem to be not inducible by salts, pH changes or osmotic shocks (Sychrova, 20004). Banuelos *et al.* (1998) suggested that the *NHA1* transcription is constitutive and very low. *ENA6* and *NHA1* genes' expression are lower at LiCl stress compared to NaCl stress. The reason why K^+/Na^+ ratio is higher at LiCl stress can be lower expression of these efflux genes. T8 may not have active efflux at LiCl stress as at NaCl stress. According to real-time PCR results, expression of *NHA1* and *ENA6* in T8 were lower under LiCl stress than NaCl stress.

In microarray results, T8 has induced *ENA6* expression, however, *NHA1* is not induced. Real-time PCR results, both *ENA6* and *NHA1* genes are induced. This may result from the differences in sensitivities of microarray and real-time PCR, the latter being more sensitive. In Real-Time PCR, the response of *NHA1* was weaker than those of *ENA6*. Expression of *NHA1* and *ENA6* were upregulated at Na^+ and Li^+ concentrations. LiCl affect T8 expression of *NHA1* and *ENA6* in T8 less than in wild type. The reason of this conclusion may be that resistance of T8 to LiCl.

The transcriptional response to high NaCl concentration stress in the yeast *S. cerevisiae* was analyzed by using DNA microarray to construct yeast strains showing tolerance to high salt concentration stress. In microarray results, several hundred genes were identified associated with NaCl tolerance involving a broad range of functional categories including energy; cell rescue, defense and virulence; metabolism; cellular transport, transport facilities and transport routes; cellular communication/signal transduction mechanism. Ma and Liu (2010) showed that many genes related to vacuolar protein sorting machinery, vacuolar membrane structure and function were essential for ethanol stress tolerance. 44 genes were found for ethanol resistance, and peroxisomal function also appeared to be associated with ethanol tolerance in yeast. *PEX1*, *PEX2*, *PEX3*, *PEX4*, *PEX5*, *PEX8*, *PEX10*, *PEX12*, *PEX14*, *PEX15*, *PEX17*, *PEX19*, and *PEX22*, encoding proteins of peroxisome transport machinery and peroxisomal membrane protein import machinery required for peroxisome organization and biogenesis, were found to be important to ethanol stress (Teixeira *et al.* 2009; Yoshikawa *et al.* 2009). Peroxisomal function is important for synthesis or degradation of membrane phospholipids in cell membrane remodeling (Ma and Liu, 2010). *VPS73* and *VPS62*

are upregulated in NaCl resistant mutant "T8". VPS73 is a mitochondrial protein and its mutation affects vacuolar protein sorting, and VPS62 is a vacuolar protein sorting (VPS) protein, required for the cytoplasm-to-vacuole targeting of proteins. Induction of *PEX17* and *PEX18* genes were observed in T8. *PEX17* is required for peroxisome biogenesis and *PEX18* is required of peroxisomal matrix proteins containing PTS2.

HSPs, mainly acting as chaperones, can ensure proper folding or refolding of nascent or denatured proteins and enzymes in order to maintain a functional conformation (Ma and Liu, 2010). Genes encoding HSPs are induced under NaCl stress. Significant up-regulated transcripts in T8 include 8 HSP genes: *HSP12*, *HSP26*, *HSP32*, *HSP33*, *HSP42*, *HSP82*, *HSP104*, and *HSP78*. Hsp82p has been reported to activate many other key proteins having cellular regulatory and signaling functions, including transcription factors and regulatory kinases (McClellan *et al.* 2007).

Chaperones are also involved in protein folding and refolding and besides HSPs, other genes encoding chaperones, such as *SSA1*, *SSA4*, *SSE1*, *SSE2*, *APJ1* were also up-regulated under NaCl stress conditions, according to microarray results of T8. The heat shock protein genes *HSP12*, *HSP42*, *SSA4*, and *HSP26*, as well as *GPD1*, are all well-known stress response genes (Hirasawa *et al.*, 2006). Gong *et al.* 2009 reported that interactions between chaperones exist widely and correct folding or stability of certain proteins may require more than two chaperones in participation for efficient function. Multiple chaperones may be induced to counteract NaCl stress. NaCl stress can affect protein conformation and cause accumulation of denatured proteins. Multiple chaperones repair protein functions and this mechanism appears to be important for yeast tolerance to NaCl. Up-regulated HSP genes influence cell functions at multiple locations including facilitating the functions of transcription factors in nucleus, improving ATP energy generation in metabolic processes, maintaining enzyme functions involving biosynthesis, and catabolism (Ma and Liu, 2010).

After trehalose prevented protein denaturation, subsequently, HSPs prevented protein aggregation and mediated refolding into functional conformations (Singer and Lindquist 1998). Gene expression levels of genes responsible for trehalose synthesis increased, triggering accumulation of trehalose, and accumulation of trehalose might create an optimal intracellular environment under NaCl stress

conditions. Thus, trehalose degradation genes are co-induced to mediate optimal concentrations of trehalose for its proper functions.

Simultaneously, expression of genes involved in glycogen biosynthesis and glycogen degradation were induced. Yeast cells seemed to adapt to NaCl stress by actively balancing trehalose or glycogen concentrations which restored proper cellular functions and enhanced glucose metabolism.

Redox balance cofactors NADH and NADPH and their regeneration has important roles in yeast metabolism (Bruinenberg *et al.* 1983) Genes involved in NADH/ NADPH regeneration reaction steps were up-regulated in T8 such as *GND2*, *ALD4*, and *GCY1*. Ma and Liu (2010) explained that up-regulated *GND2* and in oxidative phase of pentose phosphate pathway, *ALD4* in acetic acid production, and *GCY1* in glycerol metabolism were distinct characteristics of the tolerant yeast under ethanol stress condition. T8 showed same upregulation behaviour for NaCl resistance.

Upregulation of *SOL4* and *GND2* were observed in T8. *SOL4* encodes 6-phosphogluconolactonase, which is an enzyme in the pentose phosphate pathway, and *GND2* encodes 6-phosphogluconate dehydrogenase, which catalyzes an NADPH regenerating reaction in the pentose phosphate pathway. Products of enhanced expression of *SOL4* could provide sufficient substrate and accelerate downstream decarboxylation reactions to regenerate more NADPH by 6-phosphogluconate dehydrogenase encoded by *GND2*.

The reduction of ethanol and carbohydrates in alcoholic beverages is of considerable commercial interest because of the increasing demand for healthier food and beverages, as well as the preference for reduced ethanol content. However, removal of ethanol results in either a wort-like taste or a loss of aroma components. By-products such as glycerol would be acceptable, because they do not spoil the taste of the beverage (Saerens *et al.* 2010).

Nevoigt *et al.* (2002) showed that by overexpressing the *GPD1* gene in *S. cerevisiae ssp. carlsbergensis*, ethanol content can be decreased in beer. However, the concentration of several unwanted by-products, particularly acetaldehyde, considerably increased besides glycerol (Saerens *et al.* 2010). *GPD1* was upregulated in T8 and the production of ethanol was low, but acetaldehyde production was not observed.

Clearly, it was observed that in the present study NaCl tolerance in yeast is an event involving many genes in complex networks at the genome level. This is reflected by the high number and functional diversity of genes that were upregulated in T8: flocculation was a reversible, asexual and calcium-dependent process in which cells adhere to form flocs consisting of thousands of cells (Verstrepen *et al.* 2003). Flocculation is important for the beer production process which can be used for an efficient removal of yeast biomass (Saerens *et al.* 2010). Flocculation is a cost-effective and environmentally-friendly technique to separate cells from beer. Yeast flocculation depends on the interaction of with mannan carbohydrates adhesins or flocculins, both of which are found in the yeast cell wall. Adhesins or flocculins are cell wall glycoproteins that are encoded by the *FLO* (flocculation) genes (Verstrepen and Klis 2006). Dequin (2001) and Van Mulders *et al.* (2009) improved flocculation by overexpressing specific *FLO* genes. The *FLO11* gene encodes GPI-anchored cell surface glycoprotein (flocculin), which is required for flocculation and biofilm. *FLO11* gene is the gene with the highest upregulation level in T8, according to the present microarray study.

Glycogen and trehalose are the two major reserve carbohydrates in the yeast *S. cerevisiae* and could represent up to 25% of the dry cell mass, depending on the environmental conditions (Parrou *et al.*, 1997). Trehalose accumulates in the cells in response to a number of stress conditions and has been shown to protect cells against high temperatures by stabilizing proteins, and against cellular desiccation by helping to both stabilize proteins and to maintain membrane integrity. Increased production of trehalose was reflected in the reported salt-induced expression of the enzymes in the biosynthetic pathway from glucose-6-phosphate to trehalose (Blomberg, 2000).

Table 4.6: Expression changes of biofilm formation related genes.

	Systematic Name	Gene Name	Average Fold Change
biofilm formation	YIR019C	<i>MUC1</i>	92.85

FLO11 (*MUC1*) was the most upregulated gene in T8. It was upregulated 92 times higher than the wild type. *FLO11* belongs to a gene family that encodes glycosyl-phosphatidylinositol (GPI)-linked glycoproteins of domain structure similar to the adhesins of pathogenic fungi. Flo11p is localized to the cell surface and is required

for nutritionally induced cell-cell and cell-surface adhesion during invasive growth, biofilm formation, and pseudohyphal development (Braus *et al.*, 2003). It was previously shown that cations have a central role on *S. cerevisiae* flocculation. Rb^+ , Cs^+ , Fe^{2+} , Co^{2+} , Cu^{2+} , Ni^{2+} , Zn^{2+} , Cd^{2+} , Al^{3+} and mainly Mg^{2+} and Mn^{2+} had also been described as flocculent inducers (Soares, 2010). Additionally, Na^+ and K^+ have an effect on yeast flocculation. At low concentration, Na^+ and K^+ induce flocculation most likely because of the reduction of the electrostatic repulsive forces between the yeasts and/or stimulation of the leakage of intracellular Ca^{2+} (Stratford 1989). At high concentrations, it seemed that these ions provoked the distortion of the lectins and antagonized the calcium-induced flocculation (Stratford, 1992).

Besides the surface action, the presence of cations in the culture medium (Mg^{2+} or the $\text{Ca}^{2+}/\text{K}^+$ ratio) as micro-nutrients seemed to be essential for the expression of flocculation (Stratford, 1992). Thus, flocculation could act as a communitarian mechanism of survival: the external cells from the floc structure could protect the interior cells against a harmful environment by physical shielding (Soares, 2010).

FLO11-dependent flocculation is triggered by acidic pH (Bayly *et al.*, 2005). *FLO11* expression in yeast is regulated by external acidity changes (Barreles *et al.*, 2008) and Rim101p which is the main transcription factor involved in the pH response of *S. cerevisiae* has a role in *FLO11* activation (Lamb and Mitchell, 2003). Marques *et al.*, 2015 revealed the importance of a functional Rim101 complex for the correct accumulation of the Ena1 Na^+ -ATPase protein in response to NaCl stress in *S. cerevisiae*.

Gonzalez *et al.* 2013 suggested that a decrease in potassium influx can trigger flocculation and invasive growth by triggering *FLO11* expression. The association between potassium homeostasis and *FLO11* were explained (Ruiz *et al.*, 2004; Gonzalez *et al.* 2013). This study is the first one that shows a relationship between *FLO11* and NaCl homeostasis.

5. CONCLUSION

In this study, physiological and transcriptomic investigation of a NaCl-resistant strain obtained by evolutionary engineering was made. Dysfunction of Na^+/H^+ hydrogen exchangers (NHE) are associated with hypertension, epilepsy, post-ischemic myocardial arrhythmia, gastric and kidney disease, diarrhea, and glaucoma in mammals (Brett *et al.*, 2005; Hunte *et al.*, 2005). 1000 yeast genes which are members of orthologous gene families are found to be associated with human disease. Thus, the salt-resistant yeast mutant could be used as a model organism to understand sodium and lithium transport pathways and the functions of *NHA1* and *ENA6*, with the ultimate purpose of understanding disease mechanisms. According to microarray results of the NaCl-resistant mutant, *FLO11* was the most upregulated gene. This is the first study which found an association between *FLO11* and sodium resistance. As *FLO11* is the major gene involved in yeast flocculation and biofilm formation, the study of NaCl-resistant yeast mutants can lead to variety of industrial microbiology applications related to flocculation and/or biofilm formation. Further studies at system biology level are required to clarify the complex relationship between NaCl-resistance and these physiological processes.

REFERENCES

- Adamo, G.M., Brocca, S., Passolunghi, S., Salvato, B., Lotti, M.** (2012). Laboratory evolution of copper tolerant yeast strains. *Microb Cell Fact*, 11,1.
- Alizadeh, P. and Klionsky, D.J.** (1996). Purification and biochemical characterization of the *ATH1* gene product, vacuolar acid trehalase, from *Saccharomyces cerevisiae*. *FEBS Letters*, 391,273-278.
- Alkim, C., Benbadis, L., Yilmaz, U., Cakar, Z.P., François, J.M.** (2013). Mechanisms other than activation of the iron regulon account for the hyper-resistance to cobalt of a *Saccharomyces cerevisiae* strain obtained by evolutionary engineering. *Metallomics*, 5(8),1043-60.
- Aramburu, J., Rao, A., Klee, C.B.** (2000). Calcineurin: from structure to function. *Curr Top Cell Regul*, 36:237-95.
- Arino, J., Ramos, J., Sychrova, H.,** (2010). Alkali metal cation transport and homeostasis in yeasts, *Microbiol. Mol. Biol. Rev.*, 74, 95–120.
- Avrahami-Moyal, L, Engelberg, D, Wenger, JW, Sherlock, G, Braun, S** (2012). Turbidostat culture of *Saccharomyces cerevisiae* W303-1A under selective pressure elicited by ethanol selects for mutations in SSD1 and UTH1. *FEMS Yeast Res*, 12, 521–533.
- Bailey, J.E.** (1991). Toward a science of metabolic engineering. *Science*, 252,1668-1674.
- Banuelos, M.A., Sychrova, H., Bleykasten-Grosshans, C., Souciet, J.L., Potier S.** (1998). The Nha1 antiporter of *Saccharomyces cerevisiae* mediates sodium and potassium efflux. *Microbiology*, 144 (10), 2749-58.
- Barnett, J.A.** (2010). A history of research on yeasts 14: medical yeasts part 2, *Cryptococcus neoformans*. *Yeast*, 11, 875-904.
- Barreles, RR, Jimenez, J, Ibeas, JI.** (2008). Identification of Novel Activation Mechanisms for *FLO11* Regulation in *Saccharomyces cerevisiae*. *Genetics*, 178(1), 145–156.
- Bayly, JC, Douglas, LM, Pretorius, IS, Bauer, FF, Dranginis, AM** (2005). Characteristics of Flo11-dependent flocculation in *Saccharomyces cerevisiae*. *FEMS Yeast Res*, 5, 1151–1156.

- Bennett, AF, Dao, KM, Lenski, RE**(1990). Rapid evolution in response to high temperature selection. *Nature* 346, 79–81.
- Bennett, A.F., Hughes, B.S.** (2009). Microbial experimental evolution. *Am J Physiol Regul Integr Comp Physiol*, 297, 17–25.
- Blomberg, A., and Adler L.** (1992). Physiology of osmotolerance in fungi. *Adv. Microb. Physiol.*, 33,145–212.
- Blomberg, A.** (2000).Metabolic surprises in *Saccharomyces cerevisiae* during adaptation to salineconditions: questions, some answers and a model. *FEMS Microbiology Letters*, 18, 1-8.
- Blomberg, A. and Adler, L.** (1989). Roles of glycerol and glycerol-3- phosphate dehydrogenase (NAD) in acquired osmotolerance of *Saccharomyces cerevisiae*. *J. Bacteriol.*, 171, 1087-1092.
- Boekhout, T., Robert, R.** (2003). Yeasts and Food. Behr's Verlag Hamburg, Germany, pp. 1-488.
- Brett, C.L., Tukaye, D.N., Mukherjee, S., Rao, R.** (2005). The yeast endosomal $\text{Na}^+\text{K}^+/\text{H}^+$ exchanger Nhx1 regulates cellular pH to control vesicle trafficking. *Mol Biol Cell*, 16(3), 1396-405.
- Brewster, J.L., de Valoir, T., Dwyer, N.D., Winter, E., Gustin, M.C.** (1993). An osmosensing signal transduction pathway in yeast. *Science* , 259(5102), 1760-3.
- Braus, G.H., Grundmann, O., Brückner, S., and Mösch, H.U.** (2003). Amino Acid Starvation and Gcn4p Regulate Adhesive Growth and *FLO11* Gene Expression in *Saccharomyces cerevisiae*.*Mol Biol Cell*, 14(10), 4272–4284.
- Bruinenberg, P.M., Van Dijken, J.P., Scheffers, W.A.** (1983). A theoretical analysis of NADPH production and consumption in yeasts. *J Gen Microbiol*, 129, 953–964.
- Bowers, K., Levi, B. P., Patel, F. I., Stevens, T. H.** (2000). The sodium/proton exchanger Nhx1p is required for endosomal protein trafficking in the yeast *Saccharomyces cerevisiae*. *Mol. Biol. Cell*, 11, 4277-4294.
- Çakar, Z.P. , Seker, U.O., Tamerler, C., Sonderegger, M. and Sauer, U.** (2005). Evolutionary engineering of multiple-stress resistant *Saccharomyces cerevisiae*. *FEMS Yeast Res.*, 2, 569-78.
- Çakar, Z.P., Alkim, C., Turanli, B., Tokman, N., Akman, S., Sarikaya, M., Tamerler, C., Benbadis, L., François, J.M.** (2009). Isolation of cobalt hyper-resistant mutants of *Saccharomyces cerevisiae* by in vivo evolutionary engineering approach. *J Biotechnol*, 143,130–138.

- Çakar, Z.P., Turanlı-Yıldız, B., Alkım, C., Yılmaz, U.** (2012). Evolutionary engineering of *Saccharomyces cerevisiae* for improved industrially important properties. *FEMS Yeast Res*, 12(2), 171-82.
- Camacho, M., Ramos, J., Rodriguez-Navarro, A.** (1981). Potassium requirements of *Saccharomyces cerevisiae*. *Current Microbiology*, 6(5), 295-299.
- Causton, H.C., Ren, B., Koh, S.S., Harbison, C.T., Kanin, E., Jennings, E.G. , Lee T.I., True H.L., Lander E.S. and Young R.A.** (2001). Remodeling of yeast genome expression in response to environmental changes. *Mol. Biol. Cell*, 2, 323–337.
- Chen, M.** (2011). Glycogen Storage Diseases. *Molecular Pathology Library*, 5, 677-681.
- Conrad, T.M., Lewis, N.E., Palsson, B.** (2011). Microbial laboratory evolution in the era of genome-scale science. *Mol Syst Biol*, 7, 509.
- Cowen, L.E., Lindquist, S.** (2005). Hsp90 potentiates the rapid evolution of new traits: drug resistance in diverse fungi. *Science*, 309, 2185–2189.
- Cramp, D.G.** (1967). New automated method for measuring glucose by glucose oxidase. *J ClinPathol.*, 20(6), 910-2.
- Cyert, M.S., and Philpott, C.C.** (2013). Regulation of Cation Balance in *Saccharomyces cerevisiae*. *Genetics*, 193, 677–713.
- Daran-Lapujade, P., Daran, J.M., Luttik, M. A. H., Almering, M. J.H., Jack, T. Pronk, J. T. and Kötte, P.** (2009). Atypical PMR2 locus is responsible for hypersensitivity to sodium and lithium cations in the laboratory strain *Saccharomyces cerevisiae* CEN.PK113-7D. *FEMS Yeast Res*, 9, 789–792.
- Davenport, K.R., Sohaskey, M., Kamada, Y., Levin, D.E., Gustin, M.C.** (1995). A second osmosensing signal transduction pathway in yeast. *J Biol Chem*, 270, 30157-30161.
- Dhar, R., Sagesser, R., Weikert, C., and Wagner, A.** (2013). Yeast Adapts to a Changing Stressful Environment by Evolving Cross-Protection and Anticipatory Gene Regulation. *Mol Biol Evol.*, 30(3), 573-88.
- Dhar, R., Sägesser, R., Weikert, C., Yuan, J., Wagner, A.** (2011). Adaptation of *Saccharomyces cerevisiae* to saline stress through laboratory evolution. *J Evol Biol*, 24, 1135–1153.
- Dibrov, E., Fu, S., Lemire, B.D.** (1998). The *Saccharomyces cerevisiae* TCM62 gene encodes a chaperone necessary for the assembly of the mitochondrial succinate dehydrogenase (complex II). *J Biol Chem*, 273(48), 32042-8.

- Dibrov, P., Murtazina, R., Kinsella, J., and Fliegel, L.** (2000). Characterization of a histidine rich cluster of amino acids in the cytoplasmic domain of the Na⁺/H⁺ exchanger, *Bioscience reports*, 20, 185-197.
- Drew, D., Newstead, S., Sonoda, Y., Kim, H., Heijne G., Iwata S.** (2008). GFP-based optimization scheme for the overexpression and purification of eukaryotic membrane proteins in *Saccharomyces cerevisiae*. *Nat Protoc.*, 3(5), 784–798.
- Destruelle, M., H. Holzer, and D. J. Klionsky.** (1995). Isolation and characterization of a novel yeast gene, *ATH1*, that is required for vacuolar acid trehalase activity. *Yeast*, 11, 1015–1025.
- Dequin S.** (2001). The potential of genetic engineering for improving brewing, wine-making and baking yeasts. *Appl Microbiol Biotechnol*, 56, 577–588.
- Dragosits M. and Mattanovich D** (2013). Adaptive laboratory evolution - principles and applications for biotechnology. *Microbial Cell Factories*, 12, 64. doi:10.1186/1475-2859-12-64.
- Elion E.A.** (2000). Pheromone response, mating and cell biology. *Curr Opin Microbio*, 3, 573-81.
- Francois, J., and J. L. Parrou.** (2001). Reserve carbohydrates metabolism in the yeast *Saccharomyces cerevisiae*. *FEMS Microbiol. Rev.*, 25, 125–145.
- François, J.M., Walther T., Parrou J.L.** (2012). Genetics and Regulation of Glycogen and Trehalose Metabolism in *Saccharomyces cerevisiae*. In Liu L. (Eds.), *Microbial Stress Tolerance for Biofuels* (Vol.22, pp.29-55). Berlin Heidelberg: Springer-Verlag.
- Gasch, A.P., Spellman, P.T., Kao, C.M., Carmel-Harel, O., Eisen, M.B., Storz, Botstein D., Brown P.O.** (2000). Genomic expression programs in the response of yeast cells to environmental changes. *Mol. Biol. Cell*, 11, 4241–4257.
- Gancedo, C., Flores, C.L.** (2004). The importance of a functional trehalose biosynthetic pathway for the life of yeasts and fungi. *FEMS Yeast Res.*, 4(4-5), 351-9.
- Garty H. and Palmer L.G.** (1997). Epithelial sodium channels: function, structure and regulation. *Physiol Revs*, 77, 359–396.
- Gerstein, A.C., Chun, H.J.E., Grant, A. & Otto, S.P.** (2006). Genomic convergence toward diploidy in *Saccharomyces cerevisiae*. *PLoS Genet.*, 2(9), e145.
- Gerstein, A.C., Cleathero, L.A., Mandegar, M.A., Otto, S.P.** (2011). Haploids adapt faster than diploids across a range of environments. *J Evol Biol.*, 24(3), 531-40.

- Gervais, P., Molin, P., Marechal, P. A., Herail-Foussereau, C.** (1996). Thermodynamics of yeast cell osmoregulation: Passive mechanisms *Journal of Biological Physics*, 22(2), 73-86.
- Gong Y., Kakiyama Y., Krogan, N., Greenblatt, J., Emili, A., Zhang, Z., Houry, W.A.** (2009). An atlas of chaperone-protein interactions in *Saccharomyces cerevisiae*: implications to protein folding pathways in the cell. *Mol Syst Biol*, 5, 275.
- Gonzalez, Casado C, Petrezselyova, S., Ruiz A, Arino J** (2013).Molecular analysis of a conditional hal3 vhs3 yeast mutant links potassium homeostasis with flocculation and invasiveness. *Fungal Genet Biol*, 53, 1-9.
- Goossens, A., de La Fuente, N., Forment J., Serrano, R., Portillo, F.,** (2000). Regulation of yeast H(+)-ATPase by protein kinases belonging to a family dedicated to activation of plasma membrane transporters, *Mol. Cell. Biol.*, 20,7654–7661.
- Gray, J.C., Goddard, M.R.** (2012). Sex enhances adaptation by unlinking beneficial from detrimental mutations in experimental yeast populations. *BMC EvolBiol*,12, 43.
- Gustin, M.C., Albertyn, J., Alexander, M., Davenport, K.** (1998). MAP kinase pathways in the yeast *Saccharomyces cerevisiae*. *Microbiol Mol Biol Rev.*, 62(4),1264-300.
- Haro, R. and Rodriguez-Navarro, A.** (2002). Molecular analysis of the mechanism of potassium uptake through the TRK1 transporter of *Saccharomyces cerevisiae*. *Biochim Biophys Acta*, 1564(1), 114-22.
- Hammon, J., Palanivelu, D. V., Chen, J., Patel, C., and Minor, Jr. D.L.** (2009). A green fluorescent protein screen for identification of well-expressed membrane proteins from a cohort of extremophilic organisms. *Protein Science*,18, 121-133.
- Hase, C.C., Fedorova, N.D., Galperin, M.Y., and Dibrov, P.A.** (2001).Sodium ion cycle in bacterial pathogens: evidence from cross-genome comparisons. *Microbiol Mol Biol Rev*, 65, 353–370.
- Hazell, B.W., Nevalainen, H., Attfield, P.V.**(1995). Evidence that the *Saccharomyces cerevisiae* CIF1 (GGS1/TPS1) gene modulates heat shock response positively. *FEBS Lett*, 377(3), 457-60.
- Hazell, B.W, Kletsas, S., Nevalainen, H., Attfield, P.V.** (1997). Involvement of CIF1 (GGS1/TPS1) in osmotic stress response in *Saccharomyces cerevisiae*. *FEBS Lett*, 414(2), 353-8.

- Hernandez, A., Jiang, X., Cubero, B., Nieto, P.M., Bressan, RA, Hasegawa, P.M., Pardo, J.M.**(2009). Mutants of the *Arabidopsis thaliana* Cation/H⁺ Antiporter AtNHX1 Conferring Increased Salt Tolerance in Yeast: THE ENDOSOME/PREVACUOLAR COMPARTMENT IS A TARGET FOR SALT TOXICITY. *J Biol Chem*, 284(21),14276-85.
- Herskowitz, I.** (1995). MAP kinase pathways in yeast: for mating and more. *Cell*, 80(2);,187-97.
- Hirayama, T., Maeda, T., Saito, H. and Shinozaki, K.** (1995). Cloning and characterization of seven cDNAs for hyperosmolarity-responsive (HOR) genes of *Saccharomyces cerevisiae*. *Mol. Gen. Genet.*, 249, 127-138.
- Hirasawa, T., Nakakura, Y., Yoshikawa, K., Ashitani, K., Nagahisa, K., Furusawa, C., Katakura, Y., Shimizu, H., Shioya, S.** (2006). Comparative analysis of transcriptional responses to saline stress in the laboratory and brewing strains of *Saccharomyces cerevisiae* with DNA microarray. *Appl Microbiol Biotechnol.*, 70(3):346-57.
- Hohmann, S., and Mager, W.H.** (1997). Yeast stress responses. New York, NY:Chapman & Hall,
- Hohmann, S.** (1997). Shaping up: The response of yeast to osmotic stress. In: Yeast Stress Responses (Hohmann, S. and Mager, W.H., Eds.), pp. 101–145. Springer-Verlag, Heidelberg.
- Hohmann, S.** (2002). Osmotic stress signaling and osmoadaptation in yeasts. *Microbiol Mol Biol Rev.* 66(2):300-72.
- Hounsa, C.G., Brandt, E.V., Thevelein, J., Hohmann, S., Prior, B.A.** (1998). Role of trehalose in survival of *Saccharomyces cerevisiae* under osmotic stress. *Microbiology*, 144 (3),671-80.
- Jarosz, D.F., Lindquist, S.** (2010). Hsp90 and environmental stress transform the adaptive value of natural genetic variation. *Science*, 330,1820–1824.
- Jennings, D. H. , Burke, R. M.** (1990). Compatible solutes- the mycological dimension and their role as physiological buffering agents. *New Phytol.*, 116, 277-283.
- Jensen, L.T., Ajua-Alemanji, M., Culotta, V.C.** (2003). The *Saccharomyces cerevisiae* high affinity phosphate transporter encoded by PHO84 also functions in manganese homeostasis. *J Biol Chem.*, 278(43), 42036-40.

- Jia, Z-P., McCullough, N., Martel, R., Hemmingsen, S., Young, P.G.**(1992). Gene amplification at a locus encoding a putative Na⁺/H⁺ antiporter confers sodium and lithium tolerance in fission yeast. *EMBO J.*, 11, 1631–1640.
- Johnston, M.** (2000). The yeast genome: on the road to the Golden Age *Current Opinion in Genetics & Development*,10, 617–623.
- Jules, M., Guillou, V., Francois, J., and Parrou, J.L.** (2004). Two Distinct Pathways for Trehalose Assimilation in the Yeast *Saccharomyces cerevisiae*. *Appl Environ Microbiol.*70(5): 2771–2778.
- Kakimoto, M., Kobayashi, A., Fukuda, R., Ono, Y., Ohta, A., Yoshimura, E.** (2005). Genome-wide screening of aluminum tolerance in *Saccharomyces cerevisiae*. *Biometals*, 18(5):467-74.
- Kallay, L.M., Brett, C.L., Tukaye, D.N., Wemmer, M.A., Chyou, A., Odorizzi, G., Rao, R.** (2011). Endosomal Na⁺(K⁺)/H⁺ exchanger Nhx1/Vps44 functions independently and downstream of multivesicular body formation. *J Biol Chem*, 286(51),44067-77.
- Kinclova, O, Ramos, J, Potier, S, Sychrova, H** (2001). Functional study of the *Saccharomyces cerevisiae* Nha1p C-terminus. *Mol Microbiol*, 40(3),656-68.
- Kinclova, O., Potier, S., Sychrova, H.** (2002). Difference in substrate specificity divides the yeast alkali-metal-cation/H(+) antiporters into two subfamilies. *Microbiology*, 148(4),1225-32.
- Kinclova-Zimmermannova, O., Gaskova, D. Sychrova, H.** (2006). The Na⁺, K⁺/H⁺-antiporter Nha1 influences the plasma membrane potential of *Saccharomyces cerevisiae*. *FEMS Yeast Res*,6,792-800.
- Kojima, A., Toshima, J.Y., Kanno, C., Kawata, C., Toshima, J.** (2012). Localization and functional requirement of yeast Na⁺/H⁺ exchanger, Nhx1p, in the endocytic and protein recycling pathway. *Biochim Biophys Acta*, 1823(2):534-43.
- Kopp, M., H. Muller, and H. Holzer.** (1993). Molecular analysis of the neutral trehalase gene from *Saccharomyces cerevisiae*. *J. Biol. Chem.*,268, 4766–4774.
- Koppram, R., Albers, E., Olsson, L.** (2012). Evolutionary engineering strategies to enhance tolerance of xylose utilizing recombinant yeast to inhibitors derived from spruce biomass. *Biotechnol Biofuels.*, 5:32 doi:10.1186/1754-6834-5-32.
- Kota, J., Melin-Larsson, M., Ljungdahl, P.O., Forsberg, H.** (2007). Ssh4, Rcr2 and Rcr1 Affect Plasma Membrane Transporter Activity in *Saccharomyces cerevisiae*. *Genetics*, 175(4),1681-94.

- Krulwich, T.A., Sachs, G., Padan, E.**(2011). Molecular aspects of bacterial pH sensing and homeostasis. *Nat Rev Microbiol.*, 9,330–343.
- Kutyna, D.R., Varela, C., Stanley, G.A., Borneman, A.R., Henschke, P.A., Chambers, P.J.**(2012). Adaptive evolution of *Saccharomyces cerevisiae* to generate strains with enhanced glycerol production. *Appl Microbiol Biotechnol*, 93(3),1175-84.
- Lee B.N., Elion E.A.** (1999). The MAPKKK Ste11 regulates vegetative growth through a kinase cascade of shared signaling components. *Proc Natl Acad Sci*, 96(22),12679-84.
- Lifton, R.P., Wilson, F.H., Choate, K.A., and Geller, D.S.** (2002).Salt and blood pressure: new insight from human genetic studies. *Cold Spring Harbor Symp Quant Biol*, 67, 445–450.
- Lindquist, M.A.** (2008).The Statistical Analysis of fMRI Data. *Statistical Science*,23(4), 439–464.
- Livak, K.J. & Schmittgen, T.D.** (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods.*, 25(4),402-8.
- Luyten K., Riou C., Blondin B.** (2002) The hexose transporters of *Saccharomyces cerevisiae* play different roles during enological fermentation. *Yeast*, 19, 713–726.
- Ma M. and Liu Z.L.** (2010). Mechanisms of ethanol tolerance in *Saccharomyces cerevisiae*. *Appl Microbiol Biotechnol*, 87,829–845.
- MacDiarmid, C.W.&Gardner, R.C.** (1998). Overexpression of the *Saccharomyces cerevisiae* magnesium transport system confers resistance to aluminum ion. *J Biol Chem*, 273(3),1727-32.
- Madrid, J. , Hernández, F. , Pulgar, M. A. , Cid, J. M.,**(1998). Effects of citrus by-product supplementation on the intake and digestibility of urea sodium hydroxide-treated barley straw in goats. *Small Rumin. Res.*, 28, 241-248.
- Maeda T., Wurgler-Murphy S.M., Saito H.** (1994). A two-component system that regulates an osmosensing MAP kinase cascade in yeast. *Nature*, 369(6477),242-5.
- Mager, W., Siderius, M.** (2002). Novel insights into the osmotic stress response of yeast. *FEMS Yeast Res.*, 2, 251–257.

- Mager, T., Braner, M., Kubsch, B., Hatahet, L., Alkoby, D., Rimon, A., Padan, E., and Fendler, K.** (2013). Differential effects of mutations on the transport properties of the Na^+/H^+ antiporter NhaA from *Escherichia coli*, *The Journal of biological chemistry*, 288, 24666-24675.
- Manji, H.K. & Lenox, R.H.** (1998). Lithium: A Molecular Transducer of Mood-Stabilization in the Treatment of Bipolar Disorder. *Neuropsychopharmacology*, 19, 161–166.
- Maresova, L. & Sychrova, H.** (2005). Physiological characterization of *Saccharomyces cerevisiae* kha1 deletion mutants. *Mol Microbiol*, 55(2), 588-600.
- Maresova L. and Sychrova H.** (2010). Genetic interactions between the Arl1 GTPase and intracellular Na^+/H^+ antiporters in pH homeostasis and cation detoxification. *FEMS Yeast Res*, 10, 802-811.
- Martinez, P., and B. L. Persson,** (1998). Identification, cloning and characterization of a derepressible Na^+ -coupled phosphate transporter in *Saccharomyces cerevisiae*. *Mol. Gen. Genet.*, 258, 628–638.
- Marquez, J. A., and Serrano, R.** (1996). Multiple transduction pathways regulate the sodium-extrusion gene PMR2/ENA1 during salt stress in yeast. *FEBS Lett.*, 382, 89–92.
- Marquina, M., González, A., Barreto, L., Gelis, S., Muñoz, I., Ruiz, A., Álvarez, M.C., Ramos, J., Ariño, J.** (2012). Modulation of Yeast Alkaline Cation Tolerance by Ypi1 Requires Calcineurin, *Genetics*, 190, 1355–1364.
- Masuda C.A., Ramirez J., Pena A., Montero-Lomelí M.** (2000). Regulation of monovalent ion homeostasis and pH by the Ser- Thr protein phosphatase SIT4 in *Saccharomyces cerevisiae*. *J Biol Chem.*, 275, 30957–30961.
- McBryde, C., Gardner, J.M., Lopes, M.B, Jiranek, V.** (2006). Generation of Novel Wine Yeast Strains by Adaptive Evolution. *Am. J. Enol. Vitic.*, 57, 423-430.
- McClellan, A.J., Xia, Y., Deutschbauer, A.M., Davis, R.W., Gerstein, M., Frydman, J.** (2007). Diverse cellular functions of the hsp90 molecular chaperone uncovered using systems approaches. *Cell*, 131(1), 121-35.
- Millar, J. B.** (1999). Stress-activated MAP kinase (mitogen-activated protein kinase) pathways of budding and fission yeasts. *Biochem. Soc. Symp.*, 64, 49–62.

- Mitsui, K., Yasui, H., Nakamura, N., Kanazawa, H.** (2005). Oligomerization of the *Saccharomyces cerevisiae* Na⁺/H⁺ antiporter Nha1p: implications for its antiporter activity. *Biochim Biophys Acta*, 1720(1-2),125-36.
- Mulet, J.M., Alejandro, S., Romero, C. and Serrano, R.** (2004). The trehalose pathway and intracellular glucose phosphates as modulators of potassium transport and general cation homeostasis in yeast. *Yeast*, 21, 569–582.
- Munson, A.M., Love, S.L., Shu, J., Palanivel, V.R., Rosenwald, A.G.** (2004). ARL1 participates with ATC1/LIC4 to regulate responses of yeast cells to ions. *Biochem Biophys Res Commun*, 315(3),617-23.
- Müller B., Grossniklaus U.** (2010). Model organisms — A historical perspective *Journal of Proteomics*,73,2054-2063.
- Navarrete, C, Petrezsélyová, S, Barreto, L, Martínez, JL, Zahrádka, J, Ariño, J, Sychrová, H, Ramos, J.** (2010). Lack of main K⁺uptake systems in *Saccharomyces cerevisiae* cells affects yeast performance in both potassium-sufficient and potassium-limiting conditions. *FEMS Yeast Res.*,10(5),508-17.
- Nakamura, T., Liu Y., Hirata D., Namba H., Harada S., Hirokawa T., Miyakawa T.** (1993). Protein phosphatase type 2B (calcineurin)-mediated, FK506-sensitive regulation of intracellular ions in yeast is an important determinant for adaptation to high salt stress conditions. *EMBO J*, 12(11):4063-71.
- Nass, R., Cunningham, K.W., Rao, R.** (1997). Intracellular sequestration of sodium by a novel Na⁺/H⁺ exchanger in yeast is enhanced by mutations in the plasma membrane H⁺-ATPase. Insights into mechanisms of sodium tolerance. *J Biol Chem*, 272(42):26145-52.
- Nass, R.& Rao, R.** (1998). Novel localization of a Na⁺/H⁺ exchanger in a late endosomal compartment of yeast. Implications for vacuole biogenesis. *J Biol Chem*, 273(33),21054-60.
- Nevoigt, E., Pilger, R., Mast-Gerlach, E., Schmidt, U., Freihammer, S.** (2002). Genetic engineering of brewing yeast to reduce the content of ethanol in beer. *FEMS Yeast Res*, 2,225–232.
- Nijkam, J.F., Broek, M., Datema, E., de Kok, S. , Bosman, L. , Luttik, M.A. , Daran-Lapujade, P., Vongsangnak, W., Nielsen, J., Heijne, W.H.M., Klaassen, P., Paddon, C.J., Platt, D., Kötter, P., van Ham, R.C., Reinders, M.J.T. , Pronk, J.T., de Ridder, D. and Daran, J.M.** (2012). De novo sequencing, assembly and analysis of the genome of the laboratory strain *Saccharomyces cerevisiae* CEN.PK113-7D, a model for modern industrial biotechnology. *Microbial Cell Factories*, 11,36.

- Nielsen, J** (1998). Metabolic engineering: techniques for analysis of targets for genetic manipulations *Biotechnol Bioeng*, 58,125–132.
- Norbeck, J. and Blomberg, A.** (1997). Metabolic and regulatory changes associated with growth of *Saccharomyces cerevisiae* in 1.4 M sodium chloride: Evidence for osmotic induction of glycerol dissimilation via the dihydroxyacetone pathway. *J. Biol. Chem.*,272(9),5544-54.
- Ofiteru, A.M., Ruta, L.L., Rotaru, C., Dumitru, I., Ene, C.D., Neagoe, A., Farcasanu, I.C.** (2012). Overexpression of the PHO84 gene causes heavy metal accumulation and induces Ire1p-dependent unfolded protein response in *Saccharomyces cerevisiae* cells. *Appl Microbiol Biotechnol.*, 94(2),425-35.
- Ohgaki, R., Nakamura, N., Mitsui, K., Kanazawa, H.** (2005). Characterization of the ion transport activity of the budding yeast Na⁺/H⁺ antiporter, Nha1p, using isolated secretory vesicles. *Biochim Biophys Acta*, 1712(2),185-96.
- O'Rourke, S. M., Herskowitz, I., O'Shea, E. K.** (2002). Yeast go the whole HOG for the hyperosmotic response. *Trends in Genetics*, 18(8), 405-412.
- Oren, A.** (1999). Bioenergetic aspects of halophilism. *Microbiology and Molecular Biology Reviews*, 63, 334–348.
- Otero, J.M., Vongsangnak, W., Asadollahi, M. A. , Olivares-Hernandes, R., Maury, J. , Farinelli, L., Barlocher, L. , Østerås, M. , Schalk, M., Clark A., Nielsen, J.** (2010). Whole genome sequencing of *Saccharomyces cerevisiae*: from genotype to phenotype for improved metabolic engineering applications. *BMC Genomics*, 11,723.
- Ozcan, S. and Johnston, M.** (1999). Function and Regulation of Yeast Hexose Transporters *Microbiol. Mol. Biol. Rev.* 63(3),554.
- Page, M.J. and Di Cera, E.** (2006). Role of Na⁺ and K⁺ in enzyme function. *Physiol Rev*,86, 1049-1092.
- Patnaik, R.**(2008).Engineering Complex Phenotypes in Industrial Strains. *Biotechnol. Prog.*, 24, 38-47.
- Park, J., Lappe, M., Teichmann, A.** (2001). Mapping protein family interactions: Intramolecular and intermolecular protein family interaction repertoires in the PDB and yeast. *J. Mol. Biol.*, 307, 929–938.
- Parrou, J. L., Jules, M., Beltran, G., Francois, J.** (2005). Acid trehalase in yeasts and filamentous fungi: Localization, regulation and physiological function. *FEMS Yeast Research*, 5,503–511.

- Parrou, J.L., François, J.** (1997). A Simplified Procedure for a Rapid and Reliable Assay of both Glycogen and Trehalose in Whole Yeast Cells. *Anal Biochem.*, 15, 186-188.
- Parrou, J.L., Teste, M.-A. and Francois, J.** (1997). Effects of various types of stress on the metabolism of reserve carbohydrates in *Saccharomyces cerevisiae*: genetic evidence for a stress-induced recycling of glycogen and trehalose. *Microbiology*, 143, 1891-1900.
- Paquin, C, and Adams, J** (1983). Frequency of fixation of adaptive mutations is higher in evolving diploid than haploid yeast populations. *Nature*, 302,495–500.
- Persson, B.L., Berhe, A., Fristedt, U., Martinez, P., Pattison, J., Petersson J., Weinander, R.** (1998). Phosphate permeases of *Saccharomyces cerevisiae*. *Biochim Biophys Acta.*, 1365(1-2),23-30.
- Page M.J. and Di Cera E.** (2006). Role of Na⁺ and K⁺ in Enzyme Function. *Physiol Rev*, 86, 1049–1092.
- Patnaik, R.** (2008). Engineering Complex Phenotypes in Industrial Strains. *Biotechnol. Prog.*, 24, 38-47.
- Pattison-Granberg, J., and Persson B.L.,** (2000). Regulation of cation- coupled high-affinity phosphate uptake in the yeast *Saccharomyces cerevisiae*. *J. Bacteriol.*,182, 5017–5019.
- Pribylová, L., Papoušková, K., Zavrel, M., Souciet, J.L., Sychrová, H.** (2006). Exploration of yeast alkali metal cation/H⁺ antiporters: sequence and structure comparison. *Folia Microbiol (Praha)*, 51(5), 413-24.
- Proft, M. & Struhl, K.** (2002). Hog1 kinase converts the Sko1-Cyc8-Tup1 repressor complex into an activator that recruits SAGA and SWI/SNF in response to osmotic stress. *Mol Cell*, 9(6),1307-17.
- Posas, F., Witten, E.A., Saito, H.** (1998). Requirement of STE50 for osmostress-induced activation of the STE11 mitogen-activated protein kinase kinase kinase in the high-osmolarity glycerol response pathway. *Mol Cell Biol*, 18(10),5788-96.
- Posas, F., Chambers, J.R., Heyman, J.A., Hoeffler, J.P., de Nadal, E., Arino, J.** (2000). The transcriptional response of yeast to saline stress. *J Biol Chem*, 275, 17249–17255.
- Qiu, Q.S.& Fratti, R.A.** (2010). The Na⁺/H⁺ exchanger Nhx1p regulates the initiation of *Saccharomyces cerevisiae* vacuole fusion. *J Cell Sci*, 123(19),3266-75.

- Quintero, F.J., Blatt, M.R., Pardo, J.M.** (2000). Functional conservation between yeast and plant endosomal Na⁽⁺⁾/H⁽⁺⁾ antiporters. *FEBS Lett*, 471, 224-228.
- Rep, M., Albertyn, J., Thevelein, J.M., Prior, B.A., Hohmann, S.** (1999). Different signalling pathways contribute to the control of GPD1 gene expression by osmotic stress in *Saccharomyces cerevisiae*. *Microbiology*, 145,715-27.
- Rep, M., Krantz, M., Thevelein, J.M. and Hohmann, S.** (2000). The transcriptional response of *Saccharomyces cerevisiae* to osmotic shock. *J. Biol. Chem.*, 275, 8290–8300.
- Remize, F, Roustan, J.L., Sablayrolles, J-M, Barre, P., Dequin D.** (1999). Glycerol overproduction by engineered *Saccharomyces cerevisiae* wine yeast strains leads to substantial changes in by-product formation and to a stimulation of fermentation rate in stationary phase. *Appl. Environ. Microbiol.*, 65,143–149.
- Rintala, E., Wiebe, M.G., Tamminen, A., Ruohonen, L., Penttilä, M.** (2008). Transcription of hexose transporters of *Saccharomyces cerevisiae* is affected by change in oxygen provision. *BMC Microbiology*, 8, 53.
- Rios, G., Ferrando, A., Serrano, R.,** (1997). Mechanisms of Salt Tolerance Conferred by Overexpression of the *HAL1* Gene in *Saccharomyces cerevisiae*. *Yeast*, 13(6) , 515–528.
- Ruis, H., Schüller, C.** (1995). Stress signalling in yeast. *Bioessays*,17, 959-965.
- Ruiz, A.& Arino, J.** (2007). Function and Regulation of the *Saccharomyces cerevisiae* ENA Sodium ATPase System. *Eukaryot Cell*, 6(12),2175-83.
- Ruiz, A., Munoz, I., Serrano, R., Gonzalez, A., Simon, E., Arino, J.**(2004).Functional characterization of the *Saccharomyces cerevisiae* VHS3 gene: a regulatory subunit of the Ppz1 protein phosphatase with novel, phosphatase-unrelated functions. *J Biol Chem*, 279(33),34421-30.
- Saerens, S.M., Duong, C.T., Nevoigt, E.** (2010). Genetic improvement of brewer's yeast: current state, perspectives and limits. *Appl Microbiol Biotechnol.*, 86(5),1195-212.
- Sambrook, J.F.and Russell, D.W.** (2001). Molecular Cloning: A Laboratory Manual (3rd ed.), Cold Spring Harbor Laboratory Press, New York.
- Sauer, U.** (2001). Evolutionary engineering of industrially important microbial phenotypes. *Adv Biochem Eng Biotechnol*, 7,129-169.

- Schott, E.J. & Gardner, R.C.** (1997). Aluminum-sensitive mutants of *Saccharomyces cerevisiae*. *Mol Gen Genet*, 254(1),63-72.
- Serrano R.** (1996). Salt tolerance in plants and microorganisms: toxicity targets and defense responses. *Int Rev Cytol.*, 165,1-52.
- Singer, M.A., Lindquist, S.** (1998). Multiple effects of trehalose on protein folding in vitro and in vivo. *Mol Cell*,1,639–648.
- Simon, E., A. Barcelo, A., Arino J.,** (2003). Mutagenesis analysis of the yeast Nha1 Na⁺/H⁺ antiporter carboxy-terminal tail reveals residues required for function in cell cycle. *FEBS Lett.*,545, 239–245.
- Sunnarborg, S.W., Miller, S.P., Unnikrishnan, I., LaPorte, D.C.** (2001). Expression of the yeast glycogen phosphorylase gene is regulated by stress-response elements and by the HOG MAP kinase pathway. *Yeast*,18 (16),1505-14.
- Soares, E.V.** (2011). Flocculation in *Saccharomyces cerevisiae*: a review. *J Appl Microbiol.*, 110, 1–18.
- Somero, G. N., and P. H. Yancey.** (1997). Osmolytes and cell volume regulation:physiological and evolutionary principles, p. 441–484. *In* J. F. Hoffmann and J. D. Jamieson (ed.), Handbook of physiology, section 14: cell physiology. Oxford University Press, New York, N.Y.
- Stephanopoulos, G., Vallino, J.J.** (1991). Network rigidity and metabolic engineering in metabolite overproduction. *Science*, 252,1675-1681.
- Stratford, M.** (1989). Yeast flocculation: calcium specificity. *Yeast*,5, 487–496.
- Stratford, M.** (1992). Yeast flocculation – a new perspective. *Adv Microb Physiol*,33, 1–71.
- Sunnarborg, S.W., Miller, S.P., Unnikrishnan, I., LaPorte, D.C.** (2001). Expression of the yeast glycogen phosphorylase gene is regulated by stress-response elements and by the HOG MAP kinase pathway *Yeast*,18,1505–1514.
- Schüller, C., Brewster, J.L., Alexander, M.R., Gustin, M.C., Ruis, H.** (1994). The HOG pathway controls osmotic regulation oftranscription via the stress response element (STRE) of the*Saccharomyces cerevisiae* CTT1 gene. *EMBO J*,13, 4382–4389.
- Serrano, R.** (1996). Salt tolerance in plants and microorganisms: toxicity targets and defense responses. *Int Rev Cytol*,165, 1–52.
- Selmecki, A.M., Dulmage, K., Cowen, L.E., Anderson, J.B., Berman, J.** (2009). Acquisition of aneuploidy provides increased fitness during the evolution of antifungal drug resistance. *PLoS Genet*, 5,e1000705.

- Stanley, D., Bandara, A., Fraser, S., Chambers, P.J., Stanley, G.A.** (2010). The ethanol stress response and ethanol tolerance of *Saccharomyces cerevisiae*. *J Appl Microbiol*, 109(1),13-24.
- Sychrova, H.** (2004). Yeast as a model organism to study transport and homeostasis of alkali metal cations. *Physiol Res*, 53(1),S91-8.
- Tamás, M.J, Rep, M., Thevelein, J.M., Hohmann, S.** (2000). Stimulation of the yeast high osmolarity glycerol (HOG) pathway: evidence for a signal generated by a change in turgor rather than by water stress. *FEBS Lett.*, 472(1),159-65.
- Teixeira, M.C., Raposo, L.R., Mira, N.P., Lourenço, A.B., Sá-Correia, I.** (2009). Genome-wide identification of *Saccharomyces cerevisiae* genes required for maximal tolerance to ethanol. *Appl Environ Microbiol*, 75,5761–5772.
- Tilloy, V., Ortiz-Julien, A. , Dequin, S.** (2014) . Reduction of Ethanol Yield and Improvement of Glycerol Formation by Adaptive Evolution of the Wine Yeast *Saccharomyces cerevisiae* under Hyperosmotic Conditions. *Appl. Environ. Microbiol.*,80 (8),2623.
- Url-1** <<https://www.qiagen.com/tr/products/genes%20and%20pathways/pathway%20details/?pwid=224>>, retrieved 16.05.2015.
- Van Mulders, S.E., Christianen, E., Sacerens, S.M., Daenen, L., Verbelen, P.J.** (2009). Phenotypic diversity of Flo protein family-mediated adhesion in *Saccharomyces cerevisiae*. *FEMS Yeast Res*,9,178– 190.
- Ventosa, A., Nieto, J.J., Oren, A.** (1998). Biology of moderately halophilic aerobic bacteria. *Microbiol Mol Biol Rev*,62,504–544.
- Verstrepen, K.J., Klis F.M.** (2006). Flocculation, adhesion and biofilm formation in yeasts. *Mol Microbiol*,60,5–15.
- Verstrepen, K.J., Derdelinckx, G., Verachtert, H., Delvaux, F.R.** (2003). Yeast flocculation: what brewers should know. *Appl Microbiol Biotechnol*,61,197–205.
- Vreeland, R. H.** (1987).Mechanisms of halotolerance in microorganisms. *Crit Rev Microbiol*, 14,311–356.
- Wallis, J.W., Chrebet, G., Brodsky, G., Rolfe, M., Rothstein, R.** (1989). A hyper-recombination mutation in *S. cerevisiae* identifies anovel eukaryotic topoisomerase. *Cell*, 58, 409–419.

- Wan, Y-L., Zhong, H., Wu, C.J., Yang, Y.W., Wang, J.W., Hong, T.** (1998). Regulation of cell signaling cascades by influenza A virus. *The Second International Symposium on Optimization and Systems Biology*, Lijiang, China, October 31– November 3.
- Wiebe, C.A., Rieder, C., Young, P.G., Dibrov, P., Fliegel, L.**(2003). Functional analysis of amino acids of the Na⁺/H⁺ exchanger that are important for proton translocation. *Mol Cell Biochem*, 254, 117-124.
- Winderickx, J., de Winde, J.H., Crauwels, M., Hino, A., Hohmann, S., Van Dijck, P., Thevelein, J.M.** (1996). Regulation of genes encoding subunits of the trehalose synthase complex in *Saccharomyces cerevisiae*: novel variations of STRE-mediated transcription control? *Mol. Gen. Genet.*,252, 470-482.
- Wisselink, H.W., Toirkens, M.J., Wu, Q., Pronk, J.T., van Maris, A.J.** (2009). Novel evolutionary engineering approach for accelerated utilization of glucose, xylose, and arabinose mixtures by engineered *Saccharomyces cerevisiae* strains. *Appl Environ Microbiol*,75(4),907-14.
- Woods, R. A.** (1969). Response of ad-2 mutants of *Saccharomyces cerevisiae* to carbon dioxide. *Mol. Gen. Genet.*, 105,314-316.
- Yale, J. and Bohnert, H.J.** (2001). Transcript expression in *Saccharomyces cerevisiae* at high salinity. *J. Biol. Chem.*,276, 15996–16007.
- Yanagida, M.** (2003). The model unicellular eukaryote, *Schizosaccharomyces pombe*.*Genome Biol.*, 3(3) doi:10.1186/gb-2002-3-3-comment2003.
- Yancey, P.H.** (2005).Organic osmolytes as compatible, metabolic and counteracting cytoprotectants in high osmolarity and other stresses. *J Exp Biol*, 208, 2819–2830.
- Yoshikawa, K., Tanaka, T., Furusawa, C., Nagahisa, K., Hirasawa, T., Shimizu, H.** (2009). Comprehensive phenotypic analysis for identification of genes affecting growth under ethanol stress in *Saccharomyces cerevisiae*. *FEMS Yeast Res*, 9,32–44.
- Zahradka, J.&Sychrova, H.** (2012). Plasma-membrane hyperpolarization diminishes the cation efflux via Nha1 antiporter and Ena ATPase under potassium-limiting conditions. *FEMS Yeast Res*, 12(4),439-46.
- Zaragoza, O., Blazquez, M.A., Gancedo, C.** (1998). Disruption of the *Candida albicans* *TPS1* gene encoding trehalose-6-phosphate synthase impairs formation of hyphae and decreases infectivity. *J. Bacteriol.*,180, 3809-3815.

CURRICULUM VITAE



Name Surname: Şeyma Hande TEKARSLAN

Place and Date of Birth: Istanbul - 07.06.1983

E-Mail: handetekarslan@gmail.com

EDUCATION:

B.Sc.: Istanbul University – Faculty of Science - Biology Department (2004-2007)

Istanbul University – Faculty of Business Administration (2002-2006)

M.Sc.: Istanbul Technical University – Graduate School of Science Engineering and Technology - Molecular Biology – Genetics and Biotechnology Program (2007-2009)

PROFESSIONAL EXPERIENCE AND REWARDS:

Research Assistant at Istanbul University - Faculty of Pharmacy - Pharmaceutical Technology Department since 2012

TUBITAK (Scientific and Technological Research Council of Turkey) Scholarship for successful Ph.D. students in Turkey (2009 - 2015)

Guest Scholar at University of Freiburg Institute for Biochemistry and Molecular Biology for a total duration of six months between 2011 - 2014

PUBLICATIONS, PRESENTATIONS AND PATENTS ON THE THESIS:

- **Tekarslan S.H.**, Alkim C., Hunte C., Cakar Z.P., 2015. Physiological and genetic analysis of cellular sodium and lithium response/resistance behavior using the yeast *Saccharomyces cerevisiae* as a model organism. *Journal of Faculty of Pharmacy of Istanbul University*, Accepted in press.
- **Tekarslan S.H.**, Sezgin T., Yilmaz U., Alkim C., Ordek N., Tekin M.A., Gunduz S., Akman S., Cakar Z.P., 2013. Evolutionary engineering and molecular characterization of salt-resistant *Saccharomyces cerevisiae*, 5th Conference on Physiology of Yeast and Filamentous Fungi, PYFF 2013, Abstract Book, June 4-7, Montpellier, France.

▪ **Tekarslan S.H.**, Sezgin T.,Yılmaz U., Alkim C.,Gunduz S., Akman S.,Hunte C.,Cakar Z.P, 2013. Evolutionary engineering and molecular characterization of salt-resistant *Saccharomyces cerevisiae* COST ACTION:CM0902 Training School,Abstract Book, May 15-18, Lisbon, Portugal.

OTHER PUBLICATIONS, PRESENTATIONS AND PATENTS :

▪ Balaban B.G., **Tekarslan S.H.**, Aloglu-Sezgin T., Cakar Z.P. 2010. Characteristics of Iron resistant *Saccharomyces cerevisiae* obtained by Evolutionary Engineering. 4th Conference on Physiology of Yeast and Filamentous Fungi, Abstract Book June 1- 4, 2010, Rotterdam, the Netherlands.

▪ **Tekarslan S.H.**, Yılmaz U., Aksit A., Alkim C., Di Paolo F., Trotta M., Cakar Z.P, 2011. Evolutionary engineering of cobalt-resistant *Rhodobacter sphaeroides*, International Conference on Enzyme Science and Technology, Abstract Book, October 31- November 4, Kuşadası, Türkiye.

▪ **Tekarslan S.H.**, Akbal O., Cevher E., Yıldız-Pekoz A., 2014. Preparation of Doripenem loaded Microparticles for Pulmonary Delivery, 2nd International BAU Drug Design Congress 2014, Abstract Book, April 17-19, İstanbul, Türkiye.

▪ Yildiz-Peköz A., Akbal O., **Tekarslan S.H.**, Cevher E., 2014. Doripenem loaded microparticles delivery via pulmonary route for pneumonia treatment, DDL 25, December, Edinburgh , Scotland.

▪ **Tekarslan S.H.**, Akbal O., Cevher E., 2015. Development and Future of Therapeutic Proteins, World Conference on Technology, Innovation and Entrepreneurship, May 28-30, İstanbul, Türkiye.

▪ Akbal O., **Tekarslan S.H.**, Cevher E., 2015. Emerging trends in Pharmaceutical Industry: Biosimilars Position of Turkey, World Conference on Technology, Innovation and Entrepreneurship, May 28-30, İstanbul, Türkiye.