ISTANBUL TECHNICAL UNIVERSITY ★ INSTITUTE OF SCIENCE AND TECHNOLOGY

EVOLUTIONARY ENGINEERING OF *SACCHAROMYCES CEREVISIAE* FOR IMPROVED INDUSTRIAL PROPERTIES

Ph. D. Thesis by

Department : Advanced Technologies

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Tuğba SEZGİN

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İSTANBUL TEKNİK ÜNİVERSİTESİ ★ FEN BİLİMLERİ ENSTİTÜSÜ

YÖNLENDİRİLMİŞ EVRİM YÖNTEMİYLE *SACCHAROMYCES CEREVISIAE*'NIN ENDÜSTRİYEL AÇIDAN ÖNEMLİ ÖZELLİKLERİNİN GELİŞTİRİLMESİ

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ARALIK 2010

FOREWORD

Like many other ones, this doctoral thesis is very much a team effort. Several people made such significant contributions that I feel compelled to single them out.

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ABBREVIATIONS

IME	**:** Inverse Metabolic Engineering
EMS	**:** Ethyl Methane Sulphonate
ESR	**:** Environmental Stress Response
STRE	**:** Stress Response Elements
HOG	**:** High Osmolarity Glycerol
HSP	**:** Heat Shock Protein
MAPK	**:** Mitogen Activated Protein Kinase
MAT	**:** Mating Type
PCR	**:** Polymerase Chain Reaction
RT-PCR	**:** Reverse Transcriptase Polymerase Chain Reaction
QRT-PCR	**:** Quantitative Real Time Polymerase Chain Reaction

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EVOLUTIONARY ENGINEERING OF *SACCHAROMYCES CEREVISIAE* FOR IMPROVED INDUSTRIAL PROPERTIES

SUMMARY

Generally, in many biotechnological processes such as baking, brewing and wine fermentation, yeast cells are exposed to a variety of stress conditions. Usage for thousands of years has domesticated *Saccharomyces cerevisiae* and resulted in the development of various strains for specific applications. However, obtaining multiple stress resistant yeast is still an important requirement of the industry. Since principles of stress adaptation are generally similar in eukaryotes, stress response mechanism of *Saccharomyces cerevisiae*, as a model organism, has attracted significant attention. On the other hand, multiple stress resistant phenotypes appear to have multi-factorial alterations which are not easy to obtain via classical metabolic engineering strategies. Thus, empirical procedures based on random mutation and selection are useful approaches to optimize robustness. In this study, 'evolutionary engineering' an inverse metabolic engineering strategy was applied to obtain osmotic, salt and acid stress resistant mutants. For this purpose, genetic diversity of *S. cerevisiae* initial population was increased by exposure to ethyl methane sulphonate mutagen. Batch selection strategies were applied to this mutant population by applying stress conditions continuously, at gradual increasing levels. Individuals were selected randomly from the final populations of osmotic, salt and acid stress selection, which have survived in the highest possible stress conditions. By most probable number 'MPN' and serial dilution method, salt and sorbitol resistant individuals were investigated for their resistance to the stress type at which they have been selected and to other industrially relevant stress types, to detect any potential cross-resistance. Those stresses included oxidative, ethanol, freezing- thawing, heat and some heavy metal stresses. Also catalase activities of the mutants were measured under control and oxidative stress conditions. Tetrad analysis of 'T8' mutant, which was one of the highest resistant individual to salt stress, indicated that a single recessive gene mutation was responsible for the resistance. Finally, expression levels of particular genes of mutant T8 in stress conditions were investigated quantitatively using real time PCR and compared to wild type expression levels. Interestingly, expression level of particular genes which were expected to be important for resistance had increased in the mutant compared to wild type, even in the absence of stress conditions. Future experimental studies including transcriptomic analysis could help to clarify the complex mechanism of stress resistance.

YÖNLENDİRİLMİŞ EVRİM YÖNTEMİYLE *SACCHAROMYCES CEREVISIAE*'NIN ENDÜSTRİYEL AÇIDAN ÖNEMLİ ÖZELLİKLERİNİN GELİŞTİRİLMESI

ÖZET

Genel olarak ekmek üretimi, bira üretimi ve fermentasyon gibi pek çok biyoteknolojik süreçte, maya hücreleri çok çeşitli stres koşullarına maruz kalmaktadır. Binlerce yıllık biyoteknolojik kullanım *Saccharomyces cerevisiae* mayasını evcilleştirerek çok çeşitli uygulamalar için farklı suşlarının gelişmesine yol açmıştır. Bununla birlikte çeşitli stres koşullarına dayanıklı maya suşlarının elde edilmesi halen endüstriyel uygulamaların en önemli gereksinimlerinden biridir. Stres adaptasyon mekanizmalarının ökaryotlar arasında genellikle benzer olması sebebiyle, bir model organizma olan *Saccharomyces cerevisiae*'nın stres tepki mekanizmaları önemli ölçüde ilgi çekmektedir. Diğer yandan, çeşitli streslere dayanıklı fenotipler klasik metabolik mühendislik stratejileri ile elde edilmesi kolay olmayan çok faktörlü genetik farklılaşmalara sahiptir. Bu nedenle, rastgele mutasyon ve seleksiyona dayalı deneysel prosedürler, stres koşullarına dayanıklılığın optimize edilmesi açısından yararlı yaklaşımlardır. Bu çalışmada ozmotik, tuz ve asit streslerine dayanıklı maya hücreleri elde etmek amacıyla, bir tersine metabolik mühendislik yaklaşımı olan evrimsel mühendislik stratejisi uygulanmıştır. Bu amaçla, *S. cerevisiae* başlangıç populasyonunun genetik çeşitliliği, etil metan sülfonata maruz bırakılarak arttırılmıştır. Bu mutant popülasyona, stres düzeyi sürekli ve kademeli olarak arttırılarak kesikli seleksiyon stratejisi olarak uygulanmıştır. Ozmotik, tuz ve asit stres seleksiyonlarının, uygulanan en yüksek stres koşulunda hayatta kalabilen son populasyonlarından rastgele bireyler seçilmiştir. En muhtemel sayı (MPN) ve seri seyreltme yöntemleriyle tuza ve sorbitole dirençli bireylerin, hem seçildikleri stres koşuluna hem de olası çapraz dirençleri belirlemek için endüstriyel açıdan önemli diğer stres türlerine dirençleri incelenmiştir. Bu streslere, oksidatif, etanol, donma-erime,ısı ve bazı ağır metal stresleri dahildir. Ayrıca oksidatif stres ve kontrol koşullarında mutantların katalaz aktiviteleri incelenmiştir. Tuz stresine en dirençli bireylerden biri olarak seçilen 'T8' mutantının tetrat analizi sonucu strese direncini sağlayan mutasyonun tek bir resesif gen mutasyonundan kaynaklandığı belirlenmiştir. Son olarak T8 mutantının, stres koşullarında belirli genlerinin ekspresyon düzeyleri kantitatif olarak gerçek zamanlı polimeraz zincir reaksiyonu ile incelenerek yaban türün ekspresyon düzeyleri ile karşılaştırılmıştır. İlginç olarak, stres direnci ile ilgili olduğu beklenen belirli genlerin ekspresyon düzeylerinin, mutantta stressiz koşullarda bile yaban türe kıyasla yüksek olduğu gözlenmiştir. Transkriptomik analizleri içerecek yeni deneysel çalışmalar, karmaşık direnç mekanizmasının aydınlatılmasına yardımcı olabilecektir.

1. INTRODUCTION

1.1 Engineering Strategies for Strain Improvement

1.1.1 Metabolic Engineering

Metabolic engineering is referred to as the directed improvement of cellular properties through the modification of specific enzymatic reactions, or the introduction of new ones, with the use of recombinant DNA technology (Bailey 1991; Stephanopoulos, 1999). The aim of metabolic engineering may be to increase a specific metabolic activity, to enhance product range including heterologous protein production, improvement of cellular properties and elimination of side products or extension of substrate range. In order to achieve this particular goal, traditionally engineering principles such as design and analysis are applied on metabolic pathways (Yang et al., 1998). Cellular properties are aimed to be improved via genetic engineering relying on biochemical information and theoretical analysis.

Metabolic engineering approaches consist of two parts 'Analytical part' and 'Synthetic part' (Ostergaard et al., 2000). Analytical part involves usage of chemical engineering concepts for metabolic flux analyses, molecular biology techniques for metabolic mapping and the synthesis part is based on transferring the heterologous genes to the organism of interest via genetic engineering methods (Figure 1.1).

Traditionally metabolic engineering is a deductive and rational methodology that enables the manipulation of the pathway of interest by a directed approach. One can modify the promoter of a gene, delete a gene or introduce new genes or create new pathways in cells. Several different algorithms and modeling frameworks has been developed resulting with successful applications.

Analysis *Synthesis*

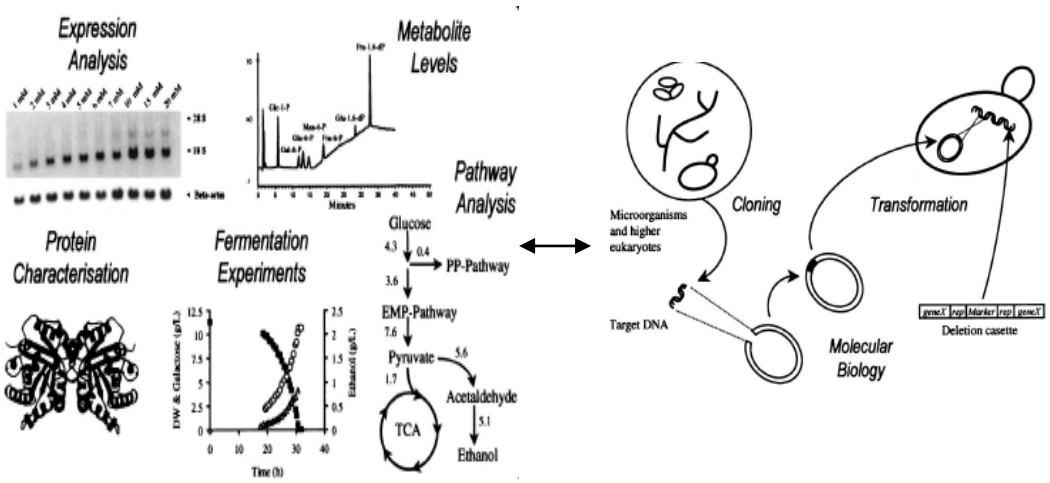


Figure 1.1 : Various aspects of Metabolic Engineering (Ostergaard et al., 2000).

Extension of substrate range may improve industrial production of desired metabolites such as ethanol from substrates in raw materials of waste stream from industrial processes. There has been many studies on utilization of starch, lactose, xylose and melibiose as substrate by *S. cerevisiae*. Hollenberg and Strasser obtained starch hydrolyzing *S. cerevisiae* strain by introduction of *Schwanniomyces occidentalis GAM1* and *AMY1* gene (Hollenberg and Strasser, 1990). Other studies include conversion of xylose to xylitol by transforming *XYL1* gene from *Pichia stipitis* to *S. cerevisiae* (Hallborn et. al 1991; Ostergaard et al., 2000) and ethanol production from xylose by transforming *XYL1* and *XYL2* gene from *Pichia stipitis* to *S. cerevisiae* and overexpression of *XKS1* gene (Ho, 1998; Ostergaard et al., 2000). Also there has been some attempts on introducing bacterial XI xylose isomerase (which converts xylose to xylulose) encoding gene from *Thermus thermolyticus* to *S. cerevisiae*. However, the ethanol productivity was not as much as expected and xylitol production could not be avoided. (Walfridsson et al., 1996)

Studies on yeast do not only aim the improvement of substrate range but also to increase product range. A metabolic engineering application of baker's yeast for extension of product range is on lactic acid production with acid tolerant yeast *S. cerevisiae* by lactate dehydrogenase gene transfer from bovine muscle (Porro et al.,

1995; Ostergaard et al., 2000). Also a number of heterologous proteins for medical purposes have been produced by *S. cerevisiae*. Human interferon (Glick and Pasternak, 1998) and hepatitis B surface antigen are the first genetically engineered vaccine products (Valenzuela et al., 1982; Ostergaard et al., 2000) .

Generally in metabolic engineering of yeast, the synthesis part is straightforward due to the availability of the genes to be expressed. However, the analysis part attempts usually fail, resulting with unpredicted phenotypes with unwanted side effects. Due to the complexity of the cellular metabolic networks, there is a need of extensive knowledge about the dynamics of metabolism and kinetics i.e. flux limiting steps of pathways. In many cases, multiple modifications are required and each modification brings unexpected changes in metabolism. There is a limiting information about concentrations of intermediate products of a pathway and their threshold levels for cellular toxicity. Also non-sequence and non-protein level related information is not well understood yet and there are still unknown pathways and secondary responses of cells upon modifications (Cakar et al., 2005). As a result, rigidity of the organism upon alterations of some metabolic functions limits rational metabolic engineering approaches.

Due to these reasons, to improve cellular activity a global view of the entire cell rather than isolated metabolic pathways is needed. This necessity has led to improvement of 'Systems Biology' which is based on first to collect experimental data of the whole cell and then construct a sophisticated mathematical model.

Many powerful techniques which serve metabolic engineering have been developed. These include DNA microarray technology, two dimensional gel electrophoresis, gas chromatography-mass spectrometry (GC-MS), liquid chromatography-mass spectrometry (LC-MS), ^{13}C-labeling and advanced online monitored fermentation systems (Ostergaard et al.,2000). However, it is also far from challenging goal since active collaboration of mathematical modelers, bioengineers and biologists is required. Thus an alternative methodology, a novel approach called 'Inverse metabolic engineering' was introduced in 1996's by James Bailey (Bailey et al.,1996).

1.1.2 Inverse Metabolic Engineering (IME)

The classical approach of metabolic engineering requires detailed knowledge of the enzyme kinetics, the systems network, and intermediate pools involved, and on such bases, a genetic manipulation is proposed for some presumed benefits. However limitations we have mentioned above result in fail of rational expectations. In order to overcome these problems, Inverse Metabolic Engineering (IME) was introduced as an alternative approach.

IME is a combinatorial strategy involving three steps. First step is to obtain desired phenotype, second step is to determine environmental or genetic conditions that confer this phenotype, and finally engineering, to transfer the phenotype to the industrial host organism via recombinant DNA technology. Fıgure 1.2 illustrates the flow diagram of information in inverse metabolic engineering (Bailey et al., 1996).

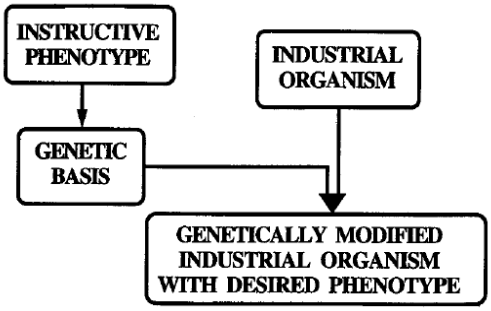


Figure 1.2 : Information flow diagram in inverse metabolic engineering (Bailey et al., 1996).

Usually the genetic variation is done by introduction of mutation via conjugative plasmids, transposons, transducing phage or by overexpression using plasmid library. The desired phenotypes are then screened and selected. For this purpose, cells can be exposed to a selection pressure that will favor the desired mutation. Finally, the selected mutant is characterized and the genotype underlying that specific phenotype is determined.

Not only in metabolic engineering, but also in protein engineering, rational deductive approach has brought similar problems within. Thus, evolutionary engineering strategies and methods were also improved for protein engineering studies. Traditionally rational design also known as computer modeling is carried out to modify or create proteins with the desired properties, via site directed mutagenesis.

However, predicting which amino acid sequence will produce the undesired protein is not so easy since there are a number of interdependent variables that have influence on the function of a protein. Starting from DNA there are many steps in peptide synthesis such as post-translational modifications, subcellular targeting, intermolecular binding and each step is regulated by multiple mechanisms. Also protein folding is related to many internal and external factors. The alternative approaches of irrational design aims to generate new proteins via iterative selection methods in the absence of detailed physicochemical knowledge. Directed Protein Evolution tools such as phage display and other in vitro screening methods such as DNA shuffling, family shuffling, are alternative methods to rational protein design.

In inverse metabolic engineering, the use of the advantage of cellular differences for screening and selection are needed. These phenotypes can result from the selection strategies based on (i) exposing to environmental conditions (ii) creating heterologous organisms via irrational protein engineering (iii) evolutionary engineering.

1.1.2.1 Evolutionary Engineering as an Inverse Metabolic Engineering (IME) strategy

Evolution of *S. cerevisiae* through thousands of years has domesticated this organism and resulted with the development of various strains for specific environmental conditions**.** However, obtaining multiple stress resistant yeast is still one of the most important requirements of industry. The difficulty is that a multiple stress resistant phenotype usually needs multi-factorial and highly complex alterations. These can be achieved via empirical procedures like adaptive preconditioning and evolutionary engineering.

Nature's design algorithm described as 'Evolution' is a repetitive process of 'genetic diversification and selection' arising from successive cycles of mutation and adaptation. However, in nature, adaptive mutations which increase an organism's ability to produce offspring are very rare and usually lost by random chance. In order to amplify these mutations and to obtain the desired cellular properties, evolutionary experiments can be applied.

Evolutionary engineering incorporates principles of this natural algorithm for biological design. It consists of creation of a genetic library, accumulation of beneficial genetic variants with iteration of directed evolution cycle and finally screening for selection of desired phenotype (Figure 1.3).

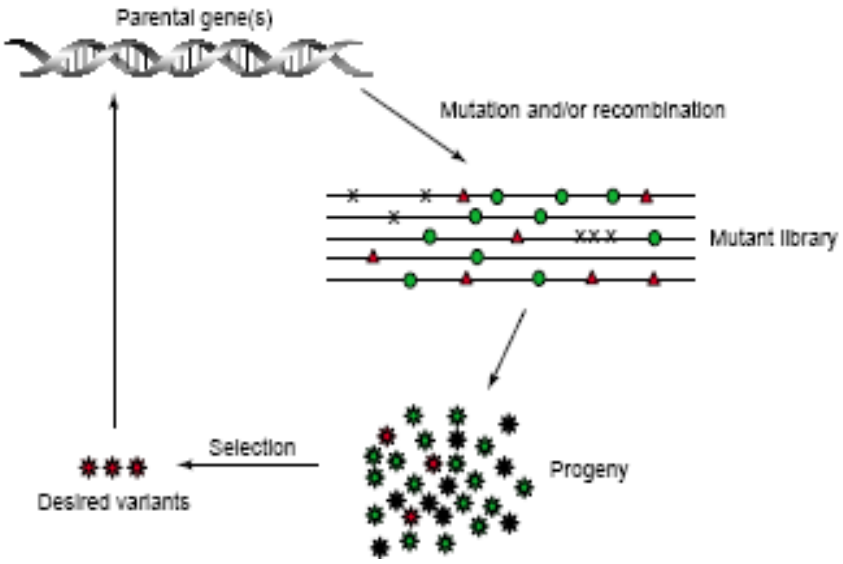


Figure 1. 3: Typical scheme for directed evolution (Chatterjee and Yuan, 2006).

Directed evolution differs from natural evolution in two key points: (i) in directed evolution, selection pressure for specific functions can be controlled whereas in natural evolution, there are multiple selection pressures (ii) in directed evolution non-natural functions can be obtained whereas in natural evolution, advantageous functions are favored (Chatterjee and Yuan, 2006).

Genetic library can be created by natural/induced mutagenesis, sequential random mutagenesis, sexual recombination or whole genome shuffling (WGS). Also, combinatorial libraries enhanced by recombination in yeast (CLERY) are particularly useful for directed pathway evolution studies.

There are many examples of evolutionary engineering applications that aim to improve stress resistant phenotypes. In one of these studies, Patnaik has used whole genome shuffling and obtained *Lactobacilli* strains with improved lactic acid production and low pH-tolerance. Parental population was exposed to five iterations of protoplast fusion. One of the final mutants produced lactic acid three-fold of the wild-type strains and some other mutants grew at pH 3.8, a pH at which wild-type strains could not survive (Patnaik, 2002).

Also Cakar et al. (2009) has started by ethyl methane sufonate (EMS) mutagenesis to a wild type *S. cerevisiae* strain, iteratively applied cobalt stress based on evolutionary engineering algorithm. Finally, they selected cobalt resistant mutant individuals having up to 3700-fold survival rate of the wild type in 8 mmol l^{-1} $CoCl_2$. These cobalt-resistant individuals had 2-4-times lower intracellular cobalt contents compared to wild-type, and also had cross-resistance to metals such as nickel, zinc, manganese (Cakar et al., 2009). Ethyl methane sufonate (EMS) is used as a chemical mutagenizing agent to create genetic libraries. It's role in mutations is alkylating and modifying mostly G nucleotides in DNA which will then result with G-T pairing instead of G-C pairing. Thus in the further replications, G is replaced with A and thus G-C pair is transferred to A-T pair. These mismatch mutations occur randomly in the whole genome increasing the mutation rate. EMS mutation principle is shown in Figure 1.4.

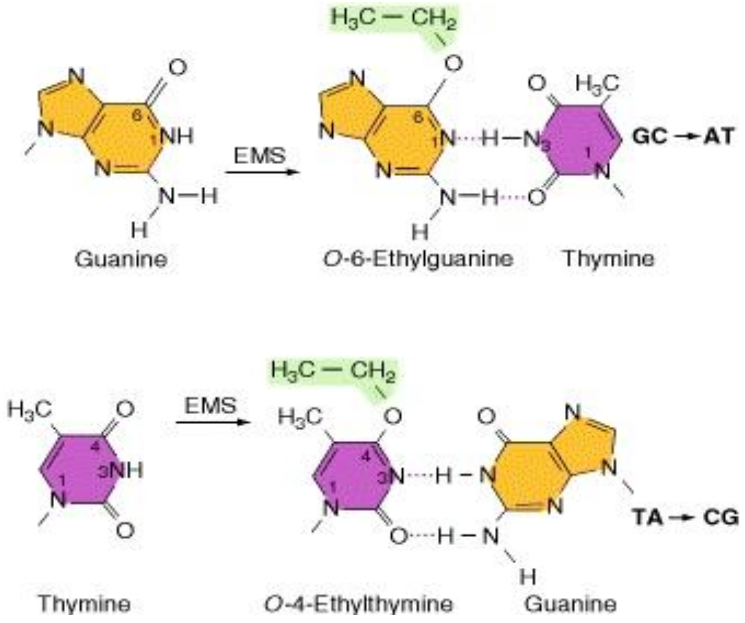


Figure 1.4 : EMS mutation on DNA (Griffiths A.J.F. et al, 2005)

Another advantage of inverse metabolic engineering approach is that even though the mechanism is not clarified yet, if the genetic locus of mutagenesis is identified, hypothesis-driven experiments can uncover the mechanism of the desired phenotype. And with more refined genetic pertubations, the desired phenotype can be improved. This approach is also advantageous since it enables screening to be done with

laboratory strains which are easy to work with and then to transfer the genetic changes to the organism of interest.

1.2 *Saccharomyces cerevisiae* and Its Life Cycle

Saccharomyces cerevisiae, a species of budding yeast is a unicellular fungi under the phylum ascomycetes. The name *"Saccharomyces"* derives from "sugar fungus." in Greek, the name "*cerevisiae*" derives from "of beer" in Latin. Other names include brewer's yeast , ale yeast, top-fermenting yeast baker's yeast and budding yeast. The scientific name and classification of it can be seen in Table 1.1.

Table 1.1 : Scientific classification of *S. cerevisiae* (Solomon et al., 1999).

Kingdom:	**Fungi**
Phylum:	Ascomycota
Class:	Hemiascomycetes
Order:	Saccharomycetales
Family:	Saccharomycetaceae
Genus:	*Saccharomyces*
Species:	*S. cerevisiae*

Major organelles of *S. cerevisiae* are nucleus, endoplasmic reticulum, vacuoles, mitochondria, Golgi apparatus, secretory vesicles, microbodies ribosomes and occasionally plasmids. The cellular contents are coated by plasma membrane, periplasm and cell wall. Since it involves mitochondria but not chloroplast, its evolution is more related to animals than plants (Alberts, et al., 2002) thus even though it has a cell wall which is as hard as other fungi, it has been considered as an eukaryote.

Microscopic view of *Saccharomyces cerevisiae* can be seen in Figure 1.5. It has elipsoidal structure generally with approximately 4.76 (long axis) to 4.19 μm (short axis) size for haploid cells and 6.01 to 5.06,μm for diploids (Herskovitz, 1988). On the other hand the mean cell size and morphology is variable relying on age and nutritional conditions.

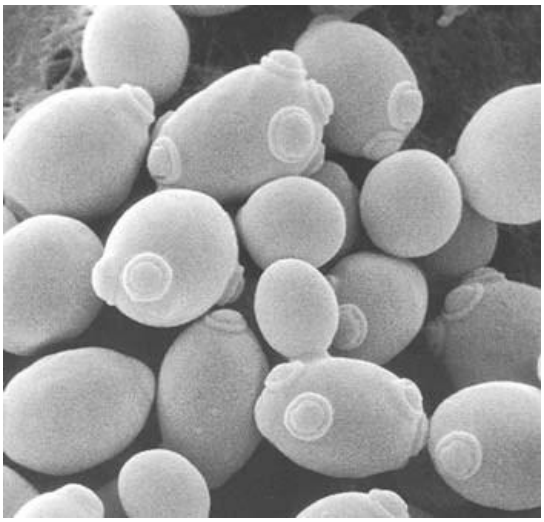


Figure 1.5 : Electron microscope view of Saccharomyces cerevisiae (Url-1)

It has 16 chromosomes with a 12068 kilobases (kb) genome size. 70% of the whole genome defines 5885 open reading frames (ORFs) potentially encodes 5885 protein-coding genes or protein products. Additionally ıt has 140 genes encoding ribosomal RNA (rRNA), 40 gene of them are for small nuclear RNA (snRNA), 275 genes are for transfer RNA (tRNA) **(**Goffeau et al., 1996). *S. cerevisiae* is called as 'homothallic', meaning that ıt can possibly regenerate diploid cell from haploid. However most of the *Saccharomyces* species exhibit heterothallic life cycle, unable to diploidize. At optimum growth temperature haploid strains duplicate in 90 minutes in rich media and at every 140 minutes in synthetic media.

S. cerevisiae can either reproduce vegetatively via budding or sexually. In vegetative reproduction, a small bud emerges from the surface of the parent cell, enlarges and separates. Budding cycle of cell starts in G1 phase by combination of cyclin dependent kinase Cdc28 with G1 cyclins (Cln1-2-3) enabling activation of transcription factor *SBF*. Then *SBF* activates genes predominantly involved in budding, in membrane and cell-wall biosynthesis (Iyer et al., 2001). Budding ceases at a certain stage of its growth, and vegetative cells become transformed into asci, each containing four haploid ascospores.

Haploid form has two sexes of mating types called as "a" and "α". Mating type a (MAT *a*) or mating type α (MAT α), can mate together to form a MAT *a/α* diploid strain which is stable under most conditions. Both sexes have typical pheromones

and surface pheromone receptors of opposite mating type. Sexual reproduction occurs between these two haploid cells fusing and forming a diploid cell.

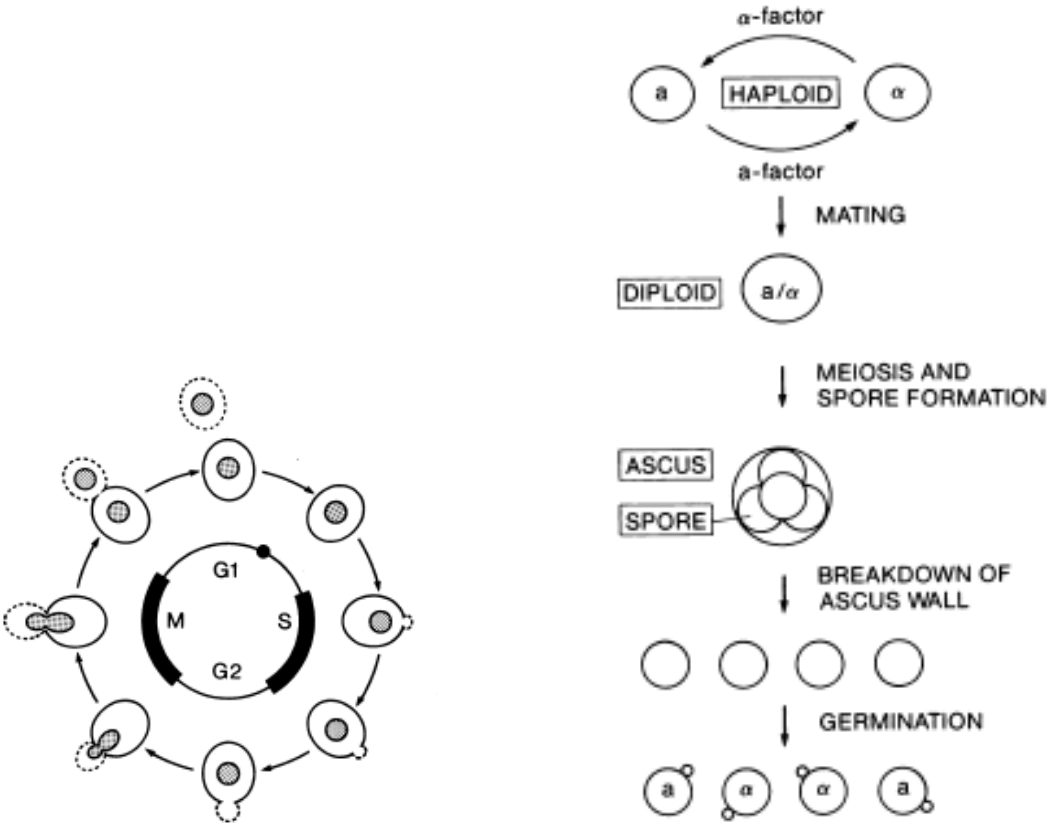


Figure 1.6 : Mitotic cell cycle of *S. cerevisiae* (left), transitions in ploidy (right) (Herskowitz, 1988)

Figure 1.6 illustrates Mitotic cell cycle of *S. cerevisiae* and transitions in ploidy that occur in the life cycle: diploid cells are formed from mating of haploids, and meiosis of diploid yields haploids. Ascus containing the four haploid (spores) is formed from a/a cells. For individual spore analysis, degradative enzymes can be used in order to breakdown the cell wall of ascus, leading to separation of individual spores by micromanipulation. The spores germinating on nutrient media can be tested for their mating type and for other markers (Herskovitz, 1988). Under sufficient environmental conditions, ascospores which are formed via sporulation can form haploid cells (Alberts et al., 2002; Lodish et al., 2000).

Possibility of sexual crossing allows more rapid changes in genetic inheritance than would be possible with mitosis, leading to valuable adaptations while eliminating harmful mutations. Also it is very advantageous in genetic manipulations and screening of yeast cells.

1.3 Biotechnological Importance of Yeast

Fermentation of sugars of barley, rice, wheat, and corn by '*Saccharomyces cerevisiae'* for alcoholic beverage production comprise the oldest application of microbial biotechnology. In archeological studies, some findings verified fermented beverage production in China at 7000bc and wine fermentation in Iran at 6000bc. These findings support that the expansion of wine fermentation has been through all Mediterranean Sea from Mesopotamia such as Greece at 2000 BC, Italy at 1000 BC, Northern Europe at 100 AD and America 1500 AD. (McGovern et al., 2004; Legras et al., 2007). Also various descriptions of beer recipes have been found in some Sumerian writings which are of the oldest known writing of any sort (Url-2). Nearly 5000 years ago it has been used in baking for flavouring and dough leavening. Also at 1st century AD, some Germanic and Celtic tribes have used *S. cerevisiae* as ale fermenting yeast for brewing.

In the 19th century usage of yeast from beer brewers for dough leavening has led to sweet-fermented breads such as the Imperial 'Kaiser-Semmel' roll which lacked the sour taste of *Lactobacillus*. However, switching from top-fermenting to bottom-fermenting yeast by beer producers caused a shortage of yeast for production of sweet-fermented breads, and this lead to development of 'Vienna Process' in 1846. After then, Pasteur found that aeration inhibits ethanol production and promotes yeast growth. In 1917, 'fed-batch technique' was described and industrial applications of *S. cerevisiae* were properly started (Ratledge et al., 2001).

In general, there are two types of yeast in brewing industry. One of them is bottom cropping (or fermenting) yeast called as 'Lager' and other is top fermenting 'Ale' yeast. The organism mostly used in ale brewing is *Saccharomyces cerevisiae* whereas in lager brewing its close relative *Saccharomyces pastorianus* is preferred. The difference of top fermenting yeasts is that, during fermentation they form foam at the top of the wort leading to a hydrophobic surface that causes the flocs to adhere to CO_2 and rise. Ale strains produce higher concentrations of ethanol at higher temperatures and produce fruitier, sweeter, full bodied ale-type beers. On the other hand, bottom-fermenting Lager strains need a long time for fermentation at lower temperatures than those typically used to brew Ales. In 1883, Dr. Dane Emil Christian Hansen used *S. carlsbergensis* for bottom fermentation and *S. cerevisiae*

for top fermentation in production of Lager beer which is named after as ‘Carlsberg Brewery’.

Today, yeast strains are not only used for traditional fermentation, they are also used in laboratory as model organism. It can be said that it is now a domesticated microorganism that has been derived from industrial strains during its usage for thousands of years. Several strains have been developed and optimized for specific applications. *S. cerevisiae* strains can also produce various by-products and secondary metabolites. It is not only appreciated as a model organism but also used in the investigation of a variety of biological problems involving the fields of genetics and molecular biology. In 1930’s following the studies on yeast physiology, and phylogeny; mating type system was demonstrated and genetic studies commenced. *S. cerevisiae* has some basic advantages for biological experiments such as its short generation time of about 1.5–2 hours at 30°C, easy cultivation of large populations in inexpensive media, and it is GRAS (‘Generally regarded as safe’) status.

Yeast cloning techniques have been expanded considerably after the first genetic transformation of yeast in 1978 (Hinnen et al., 1978). In 1985, first commercial pharmaceutical (hepatitis B vaccine) has been produced by recombinant yeast. (Hilleman, 1985). With the completion of yeast genome Project in 1996, *S. cerevisiae* has become an ideal eukaryotic model organism (Goffeau et al., 1996; Petranovich and Nielsen, 2009).

The yeast genome database has become the most important reference for developing basic knowledge about the function and organization of higher eukaryotic cell genetics that has been conserved through evolution. Many biotechnological applications have been improved including gene transfer, gene fusion and gene regulation. These techniques have been extensively used on yeast strains as an attractive host organism for production of heterologous proteins such as human serum albumin and human insulin.

Collection of single gene deletion mutants for diploid cells and nonessential genes of haploid cells are available. Also high-throughput data obtained from functional genomic tools (etc. metabolomics, flux analysis, proteomics) provides valuable comprehensive information. All these information enables us the understanding and

quantification dynamic networks which are conserved in higher eukaryotes etc., mechanisms underlying disease development and re-construction of similar pathways in them (Nielsen and Jewett, 2008).

The worldwide databases on baker's yeast that provides enormous information such as Saccharomyces Genome Database (Url-3), Comprehensive Yeast Genome Database (Url-4), European *S. cerevisiae* Archive for Functional Analysis (Url-5) and *Saccharomyces cerevisiae* Morphological Database (Url-6) provide enormous information.

1.4 Stress Responses of *S. cerevisae*

The word 'Stress' can be defined as extreme changes in the external surroundings that challenge all cell types both microorganisms and individual cells in multicellular organisms. These conditional fluctuations include temperature changes, starvation, pH, osmotic changes, desiccation, oxidative stress pressure, radiation, exposure to high salt or heavy metal ions (Figure 1.7). All these stress perturbations prevent enzyme functions, destabilize cellular structures and disrupt metabolic fluxes. As a result, they perturb the internal system of the cell leading to a reduced proliferation or its death.

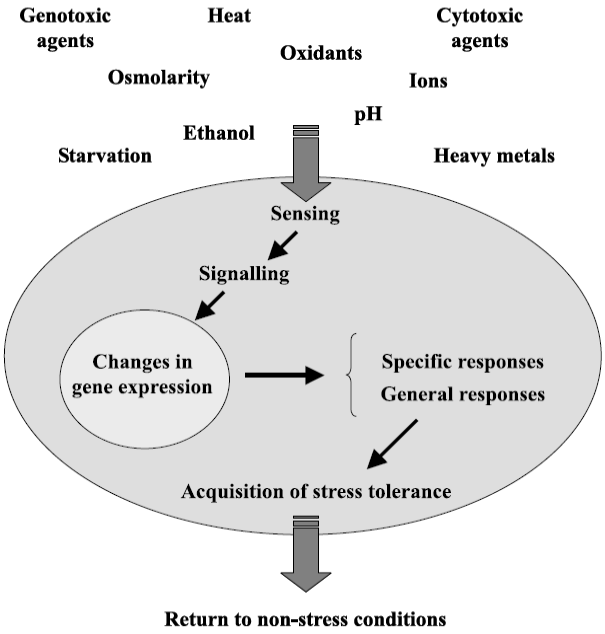


Figure 1.7 : Adaptive response system of yeast (Costa and Maradas-Ferreina, 2001)

In order to cope with these effects, yeast cells in wild life have evolved highly complex responses in time scale of minutes, hours or days. These responses and adaptation mechanisms of cells to different stress conditions are highly complex. They may be to prevent entrance of toxic compounds into the cell, to repair damages and maintain their internal homeostasis via production of protective biomolecules. Inducible responses of cells to these stresses are changing from one condition to another, however they are known to be overlapping. One of these stress responses is initiation of a common gene expression program referred as the ESR 'Environmental Stress Response' composed of approximately 900 genes whose expression is altered in response to environmental fluctuations. Change in expression of these genes is a common feature of the responses to various stress factors, however regulation of these expression changes is gene-specific and condition-specific. This indicates that initiation of the program is completely controlled in response to each specific condition. (Gasch et al., 2000)

Studies on yeast indicated that, cells exposed to one stress gains resistance to lethal doses of the same stress or other stresses (Costa and Maradas-Ferreina, 2001). This result has brought the idea that there is a general protection mechanism which is provoked in different stress conditions and provided discovery of 'Heat Shock Genes'. These genes were not only induced by temperature changes but also by other stress conditions (Kurtz et al., 1986; Gasch et al., 2000)

Also a sequence, common to the promoters of the stress-induced genes, referred to as the Stress Response Element (STRE) has been found, suggesting that the expression of these genes are controlled by a common factor. STRE is proved to be either of two related zinc-finger transcription factors, Msn2p and Msn4p (Martinez-Pastor et al., 1996; Schmitt and McEntee, 1996; Gasch et al.,2000) controlling the induction of a large number of genes in response to many different stresses. Deletion of these factors results with sensitivity to a variety of stresses. *MSN2* and *MSN4* are known to be general stress transcription factors which are activated to defend against environmental insult in various stress conditions (Kobayashi and McEntee, 1990, Gasch et al., 2000). However under certain conditions, targets of these transcription factors are controlled regardless of them, indicating that the stress response regulation is more complicated than thought (Schuller et al., 1994).

Environmental stress responses are commonly initiated after a shift to a new environment, and their expression levels return back to no stressed levels over time. These fluctuations of expression are not only related to the stress factor but also to the stress level. For example cells exposed to a high dose of stress will show more prolonged and bigger changes in ESR gene expression relative to cells which are exposed to a mild stress (Gasch et al., 2000). Approximately 600 genes which are usually related to protein synthesis in ESR are repressed in stress condition. 300 of them are characterized and more than 70% of them are declared by *Saccharomyces* Genome Database (SGD). Recently much research has been focused on yeast *Saccharomyces cerevisiae* in order to clarify these responses which have been evolved for millions of years. For this purpose, DNA microarrays give us the opportunity to quantify transcription levels in whole genome in certain stimuli, providing insights into overall response of the cell. Two dimensional gel electrophoresis or pulse labelled proteins complement these studies by measuring large scale fluctuations in protein synthesis.

1.5 General Responses of Organisms to Osmotic Stress

Osmotic stress is a result of a stimulus indicating an increase or decrease in the concentration of solutes outside, leading to various changes in metabolic state or activity of a cell. These changes may be in terms of enzyme production, movement, metabolite secretion, etc. (Url-3).

Osmotic stress can stem from hyper-osmotic or hypo-osmotic condition. Hyper osmotic stress is a result of accumulation of solute in the surrounding medium and hypo osmotic stress is formed in a reduction of solute in the medium. Both in hyper/hypo-osmotic environment, cells need to maintain cytoplasmic water activity lower than their surrounding medium. By this way water is driven inside the cell by a constant pressure along its concentration gradient until expansion of the plasma membrane and cell wall leading to turgor.

The first strategy of the cells to deal with osmo-stress is to accumulate ‘compatible solutes’ to decrease intracellular molecule concentrations below toxic level. These ‘compatible solutes’ range from sugars, sugar alcohols, ions to amino acid and their derivatives depending on the osmotic stress factor and organism. Also regulations in

membrane fluidity by change in sterol-phospholipid ratio and type is important in retention and flux of osmolytes. On the other hand, fatty acids have an important effect on membrane structure. Thus, changes in expression of fatty acid modifying enzymes are needed to modify membrane fluidity to deal with osmolarity. Also in *S. cerevisiae*, deletion of *FPS1* gene (which encodes main glycerol channel) leads to reduction of ergosterol content resulting with change of glycerol flux through membrane (Cimerman et al., 2009).

Cellular mechanisms to cope with osmotic stress include first sensation and quantification of the change in osmolarity by osmosensors, signal transduction cascade and activation of transcription factors. All eukaryotes respond to hyperosmotic stress by activating a conserved mitogen activated protein cascade, known in *S. cerevisiae* as high osmolarity glycerol (HOG) pathway.

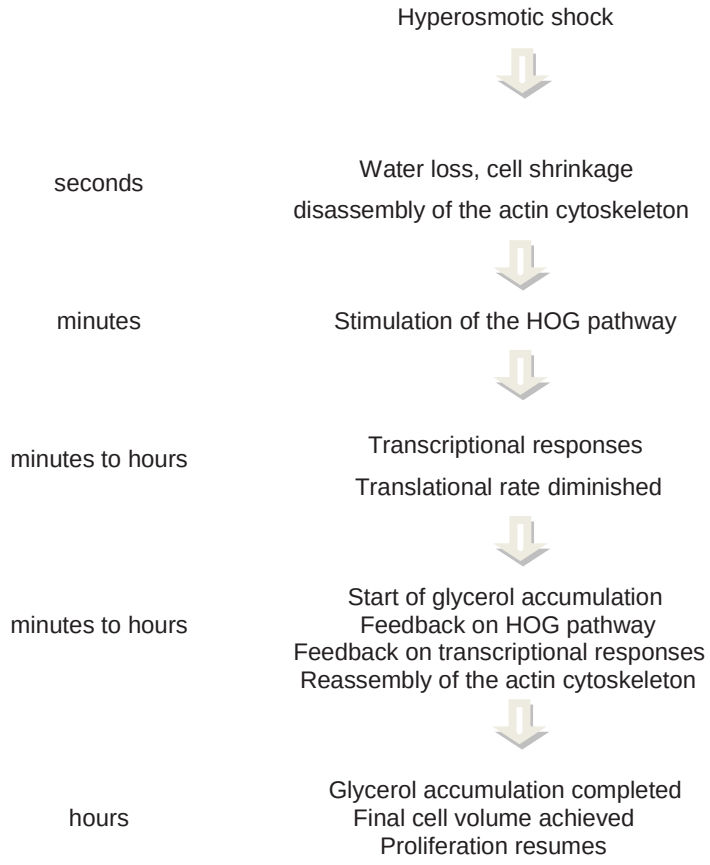


Figure 1.8 : Time course of different responses to osmotic stress (Hohmann and Mager, 2003).

Figure 1.8 illustrates the general time course of responses to osmotic stress. Intensive analyses on yeast have been bringing out prospects on osmosensing and downstream signalling components, transcriptional changes and their metabolic network. Also all these studies will help us to understand stress sensing and control of transcription by stress-activated mitogen-activated protein kinases in mammalian cells (Westfall et al., 2004)

1.6 Osmotic Stress Responses of Yeast *Saccharomyces cerevisiae*

S. cerevisiae is the most studied eukaryotic microorganism for the investigation of salt resistance, however, is not classified as a halotolerant organism (Serrano, 1996; Cimerman, 2009). In natural environments yeasts are exposed to a variety of sudden external adverse changes that evoke osmotic stress. For example, a yeast cell on a drying fruit will slowly lose water potential, with the rain it will be exposed to hypo-osmotic stress which will lead to swelling and finally turgor. Or opening of a fruit will lead to hyper-osmotic stress on yeast cell leading to rapid outflow of water and shrinkage. All these fluctuations lead cells to respond to maintain internal osmolarity higher than the external. (Url-8) Figure 1.9 shows the basic features of yeast to these fluctuations.

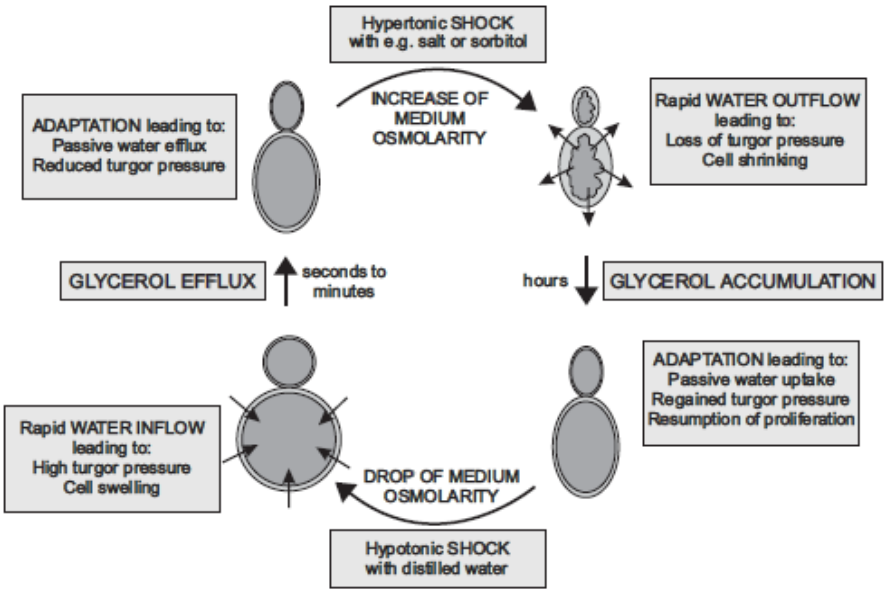


Figure 1.9 : Basic features of yeast osmotic stress response (Hohmann and Mager., 2003).

Not only in natural environment but also during high cell density fermentations, high salt concentration of the media formulation is a typical problem. For example, batch media usually induce osmotic stress on yeast cells with an osmolarity around 1000 mOsmol/l (comparable to a 0.5M NaCl concentration) that will decrease with the uptake of nutrients during growth (Mattanovich et al., 2004). Osmotic stress also perturbs the plasma membrane structure and permeability that are likely to affect various trans-membrane proteins such as transporters and osmo-sensors.

Osmotic stress causes an impressive transcriptional response that affect expression of nearly 10% of the genes in yeast genome. Melamed et al.(2008) has investigated *S.cerevisiae* genes that are known to be down and up-regulated upon high salinity stress and grouped them based on their cellular functionalities via SGD GO Slim Mapper tool and literature data. Figure 1.10 illustrates the bar diagrams of these translationally regulated genes. (Melamed et al 2008)

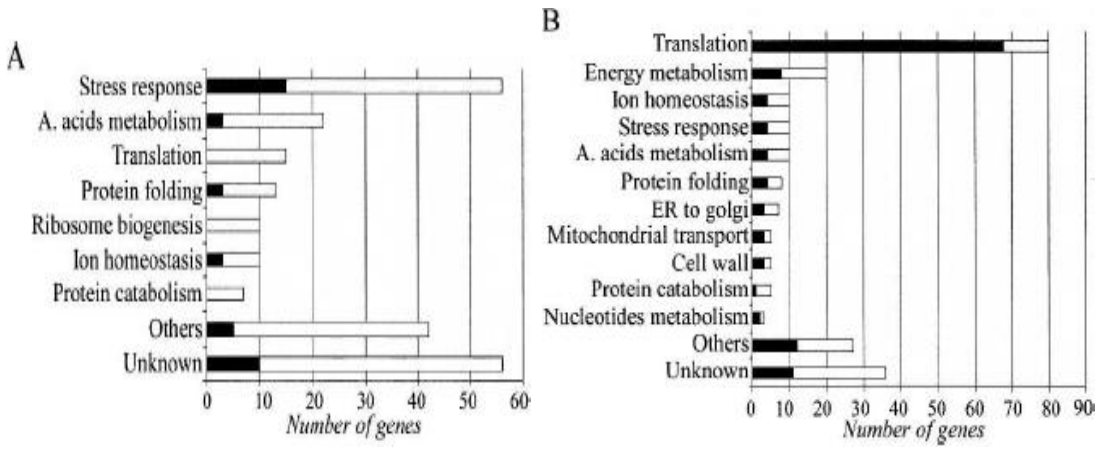


Figure 1.10 : Translationally regulated genes on osmotic stress. The bars indicate (A)total number of translationally induced genes (B) translationally repressed genes The black section in the bar indicates the proportion of genes having the highest change in transcript levels (Melamed et al., 2008).

S. cerevisiae has a variety of stress responses to compensate for differences between the intracellular and extracellular water potential such as transport of intracellular water from the vacuole to the cytoplasm, retention and over-synthesis of compatible solutes ‘osmolytes’, mainly glycerol and trehalose, and adjustment of cell wall organisation.

Generally the first response of yeast to high osmolarity is closure of glycerol export channel FPS1p localized on the plasma membrane. Then glycerol biosynthesis is stimulated via high osmolarity glycerol 'HOG' pathway which is a mitogen activated protein kinase 'MAPK' cascade and is known to be activated upon high osmolarity within one minute time scale. (Hohmann, 2002). This mechanism has been evolved by the extension of existing signaling modules in prokaryotes (two-component systems). In multicellular organisms, MAPK cascades have been adopted as signal-transduction mechanisms not only for intercellular communication and immunity, but also for carrying information about environmental stress (Kültz and Burg, 1998; Hohmann, 2002)

Studies of *S. cerevisiae* responses to osmotic stress have investigated many factors i.e. osmoticum (usually salt or sorbitol) types, time of stress exposure and strain types. These results have proved that the transcriptomic responses to this stress are highly dynamic to all these factors and osmo resistance characteristics in *S. cerevisiae* are a genetically complex phenotype. These experiments have focused on osmosensitivity trait, including vacuolar mutants (Coury et al., 1999; Hohmann, 2002), cytoskeletal mutants, phosphatase 2B (calcineurin) mutants (Lippuner et al.,1996), plasma membrane ATPase mutants (Sychrova et al., 1999, Escanciano, 2009)

1.6.1 Signalling pathways involved in osmotic and salt stress adaptation in *S. cerevisiae*

Fluctuations of gene expression are controlled by signaling pathways that sense osmotic changes and they transmit the signal leading to transcriptional responses. Osmolarity of the environment affect different signaling pathways in yeasts. HOG pathway is the best-characterized system, which is activated within less than 1 min by osmotic up shift (Hohmann, 2002).

1.6.1.1 High osmolarity glycerol (HOG) pathway

HOG 'High Osmolarity Glycerol' is the main pathway mediating hyperosmotic response in yeast and is the most studied and known osmo-response system in eukaryotes. Significant part of the transcriptional response of yeast cells to high

osmolarity and also some posttranscriptional effects are known to be mediated by this pathway (Hohmann, 2002).

HOG pathway is a member of MAP kinase cascade which control cell growth, morphogenesis, proliferation, and stress responses in eukaryotic systems. It's over activation causes lethality, while as lack of activation leads to osmosensitivity.

The HOG pathway is illustrated in Figure 1.11. It has two branches activated by different transmembrane osmosensor proteins: Sho1and Msb2 activated branch on one side, Sln1 on the other side. These osmosensor proteins act on different effector downstream proteins independent from each other but both activate the same MAPKK named as Pbs2. These two branches allow responding over a broad range of osmotic stress. Sln1 branch is known to be activated by a broad range of solute concentrations whereas Sho1 branch is dispensable (O'Rourke et al., 2002) . In a Sln1 branch deleted mutant Hog pathway can be activated over at least 300 mM NaCl stress. On the other hand in a mutant where Sho1 branch is deleted, Hog pathway activation starts at 100 mM NaCl concentration in as quick as one minute and reaches maximal level at 300 mM concentration (Hohmann, 2002). Thus Sln1 branch is apparently more sensitive to osmotic stress than Sho1 branch.

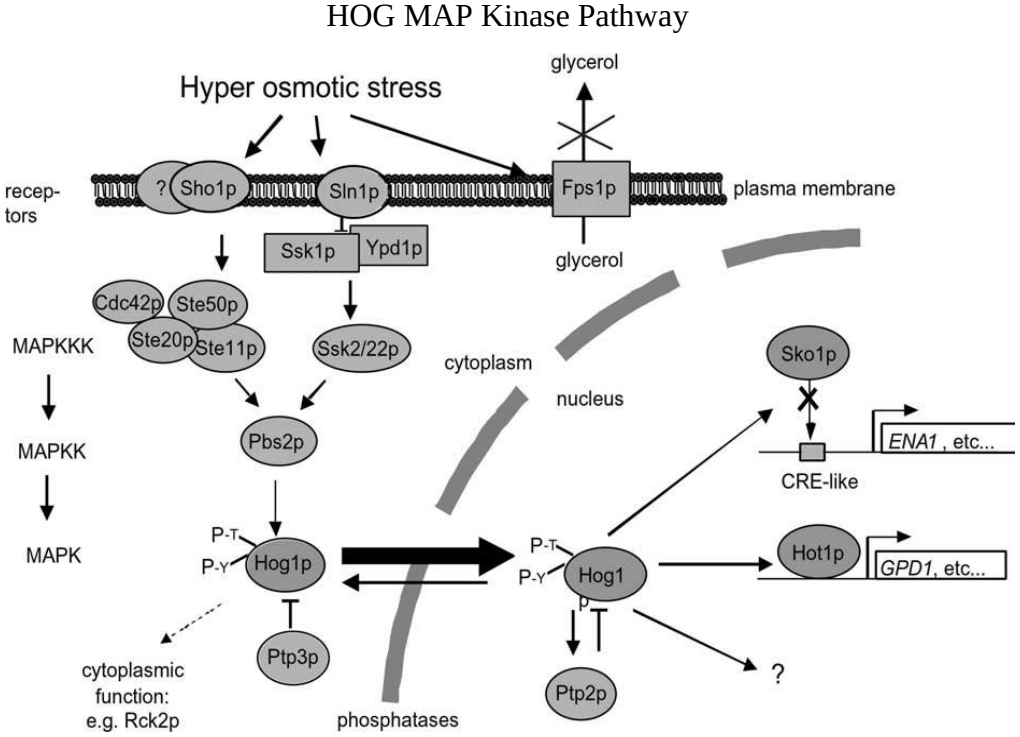


Figure 1.11 : *S. cerevisiae* HOG pathway (Mager and Siderius, 2002).

Sln1 branch regulates the activity of the two partially redundant MAPKKKs, Ssk2 and Ssk22. In constant osmotic conditions, Phosphotransfer between Sln1–Ypd1–Ssk1 occurs to keep Ssk1 inactive. The Sho1 branch uses a novel four membrane-spanning domain protein to recruit Pbs2 to the membrane through an SH3–polyproline interaction, after which the MAPKKK Ste11 is phosphorylated by Ste20. (O'Rourke et al., 2002)

(i) Sln1 branch of HOG pathway:

Sln1 branch is a two-component phosphorelay system which is not prevalent in eukaryotes but forms widespread bacterial response regulatory domains. It is composed of two transmembrane regions and a histidine kinase domain. General scheme for Sln1 branch of HOG pathway can be seen on Figure 1.12.

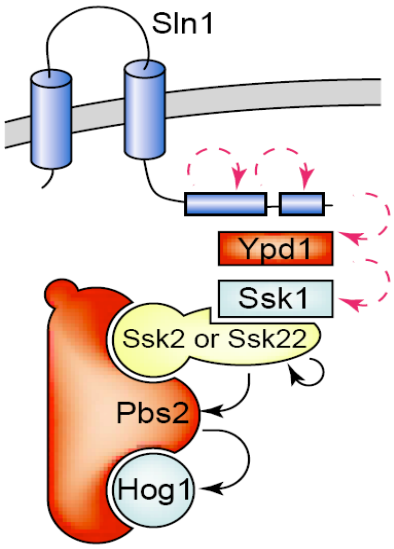


Figure 1.12 : Sln1 branch of *S. cerevisiae* HOG pathway (O'Rourke et al., 2002).

In normal osmolarity conditions, Sln1 domain is actively phosphorylating Ypd1 protein, which transfers phosphate group to Ssk1. Phosphorylation of Ssk1 is thought to be inhibiting the interaction of Ssk1 with Ssk2 and Ssk22. With the increase in external osmolarity, Sln1 activity is inhibited leading to dephosphorilation of Ypd1 and Ssk1, thus Ssk1 binds to Ssk2 which will then subsequently transfer phosphate to Pbs2 and activate Hog1. The mechanism how Sln1 senses osmolarity has not been clarified yet.Sln1 might not only be involved in sensing extracellular osmolytes but also in sensing intracellular glycerol amount (Tao et al., 1999; O'Rourke et al., 2002).

(ii) Sho1 branch of HOG pathway

Sho branch of HOG pathway is activated by high external osmolarities (0.5-1.0M NaCl). Sho1 protein is composed of four transmembrane regions and a transmembrane complex of Cdc42 and Ste20 with adaptor protein Ste50 (Tatebayashi et al., 2006; Wu et al 2006; Rensing and Ruoff, 2009). Schematic view of Sho1 branch can be seen on Figure 1.13.

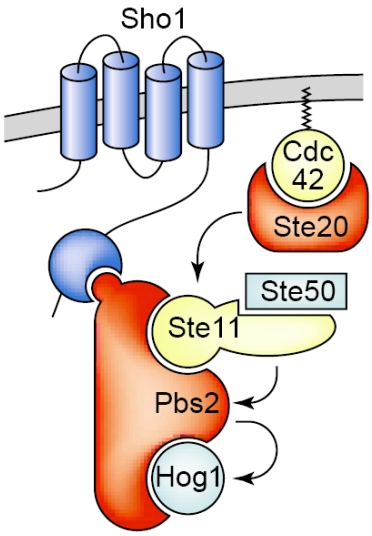


Figure 1.13 : Sho1 branch of *S. cerevisiae* HOG pathway (O'Rourke et al., 2002).

Ste50 is supposed to be a cofactor for Ste11 because their SAM domains are known to make a complex (Jansen et al., 2001; O'Rourke et al., 2002). In response to high salinity, Ste20 and in turn Ste11 phosphorylate Pbs2. Pbs2 then activates Hog1 (Hohmann, 2002). Pbs2 is supposed to act as a scaffold interacting with multiple proteins such as Sho1, Ste11, Hog1. Pbs2 is also known to have a role in inactivating other Ste11 dependent pathways by linking Sho1 to Ste11 (Posas et al., 1997; O'Rourke et al., 2002). Sho1 osmo-sensing mechanism has not been understood yet but it is thought that plasma membrane localized SH3 domain may have a role in connection with downstream proteins. Recently a third transmembrane protein Msb2 which is in connection with Sho1 branch, is thought to have a role in sensing osmolarity (Migdal et al., 2008).

1.6.1.2 Calcineurin pathway

The nature of the solute i.e. uncharged solute (sorbitol)/ charged solute (NaCl) has an important role as osmotic stress reagent. Ionizing compounds such as NaCl do not only create osmotic stress but also ionic toxicity. The data obtained from studies of *S.cerevisiae* indicate that upon exposure to sodium chloride stress, yeast signalling pathways activate salt responsive genes that are involved in modulating Na^+ influx/efflux, polarization of plasma membrane, and sequesteration of Na^+ in the vacuole.

There is a central role of calcium^{2+} dependent signaling 'Calcineurin' pathway as the main regulator of Na^+ influx and efflux. Thus it is a crucial intermediate that mediates K^+ and Na^+ homeostasis in yeast and mammals. Calcineurin has a heterotrimeric structure composed of a catalytic subunit (encoded by either *CNA1* or *CNA2*), a Ca^{2+}-binding regulatory subunit (encoded by *CNB1*) and Ca^{2+}/calmodulin. Binding of Ca^{2+}/calmodulin to the catalytic subunit gives an end to its autoinhibiton allowing calcineurin interaction with a variety of substrates that have a role in regulating salt response (Hamilton et al., 2002).

Figure 1.14 illustrates a hypothetical model of salt stress signal perception, transduction and regulation in yeast. Salinity stress is perceived by an unknown sensor which initiates the calcium dependent signal transduction. Calcium binds directly to CNB regulatory subunit which in turn binds and activates catalytic CNA subunit. CNA then activates transcription factor *TCN1* and some membrane transport proteins encoded by *ENA1, NHA1, SOD2, TRK1, TRK2* and *PMA1* (Bressan and Hassegawa, 1998).

If the conditions are alkaline or neutral, in the case of high salinity, Na^+ transport is mediated by plasma membrane Na^+-ATPase which is encoded by *PMR2/ENA1*. *ENA1* transcription is not only controlled by calcineurin but also by *HAL1, 8, 9, SNF1* and indirectly by *HAL3*. Also in *Schizosaccharomyces pombe AFT1* transcription factor is known to activate *ENA1* (Hamilton et al., 2002).

Coordinately with *ENA1* encoded Na^+-ATPase, Na+ influx is also reduced by K^+ uptake system which involves TRK1p and TRK2p (Mendoza, I. et al. 1994; Bressan and Hassegawa., 1998).

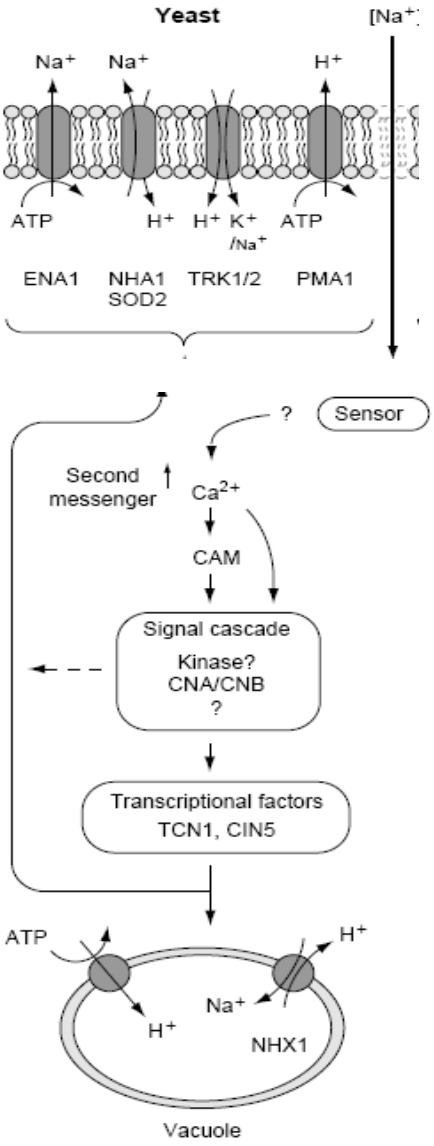


Figure 1.14 : A hypothetical model of yeast salt stress signal perception, transduction and regulation of effectors that mediate Na^+ homeostasis (Bressan and Hasegawa, 1998).

Not only the ion transport but also sequesteration of ions in the vacuole and depolarization of the cell membrane has an important role in salt stress resistance. Na^+ transportation to vacuole is controlled by an Na^+/H^+ antiporter NHX1 which is localized to a prevacuolar compartment (Hamilton et al., 2002). Depolarization of the plasma membrane is controlled by protein kinases *HAL4* and *HAL5* which are induced by high affinity K^+ channel, TRK1.

1.7 Hypo-osmotic Stress

Yeast responses to hypo-osmotic shock have been investigated less than hyper-osmotic shock. There are some studies on signal transduction MAP kinase cascade which is supposed to be proceeding via some PKC-like protein (Stadler and Schweyen, 2002). Some rapid posttranslational mechanisms are thought to be important, such as export of glycerol through Fps1 channel. Also it is known that upon osmotic downshift, cells increase uptake of calcium and accumulate inside.

Although the physiological significance has not been investigated yet, Sln1p, Skn1p and Ypd1p are known to be involved in a phosphorelay system that activates expression of several genes in hypoosmolarity (Hohmann, 2002).

1.8 Yeast Genes Involved in Hyperosmotic Response and Ion Toxicity

1.8.1 *HAL2 /YOL064C*

The yeast halotolerance gene *HAL2* (systematic name:*YOL064C)* encodes a nucleotidase that dephosphorylates 3'-phosphoadenosine 5'-phosphate (PAP) and 3'-phosphoadenosine 5'-phosphosulfate (PAPS), intermediates of the sulfate assimilation pathway. This nucleotidase enzyme is homologous to inositol phosphatases which are inhibited by Li^+. It is also known to be inhibited by Na^+ but not by K^+. *HAL2* expression is known to increase NaCl and LiCl stresses. *HAL2* gene is identical to *MET22* gene which is involved in methionine biosynthesis. Accordingly, methionine supplementation improves sodium and lithium tolerance of yeast (Glaser et al. 1993).

1.8.2 *HAL3/YKR072C*

As well as *HAL2, HAL3* (systematic name: *YKR072C)* gene also improves resistance of yeast cells to toxic concentrations of sodium and lithium. *HAL3* deletion is lethal in high sodium concentrations and even if there is a mutation in the calcineurin pathway it is known to support survival in saline stress. *HAL3* expression changes result with alterations in intracellular cation concentrations thus its activity is thought to directly increase cytoplasmic K^+ and decrease Na^+ and Li^+. Together with calcineurin, *HAL3* is necessary for activation of *ENA1/PMR24* gene which encodes plasma membrane Na^+-ATPase responsible for cation efflux. *HAL3* is also known to

have an effect on cell cycle and ion homeostasis as well as protein phosphatases, calcineurin and Sit4p (Ferrando et al 1995).

1.8.3 *HOG1/SSK3/YLR113W*

In the case of any increase in the osmolarity, *HOG1* (systematic ID: *YLR113W*, synonym: *SSK3*) encodes a mitogen-activated protein kinase involved in HOG pathway. It controls osmolarity of the cell via the stress response element (STRE) in promoters of target genes. In nonstressed conditions it is located in cytoplasm but if osmolarity increases, it rapidly concentrates within the nucleus. *HOG1* is known to have important roles in maintaining osmolarity, copper-resistance, hydrogen peroxide resistance, citric acid resistance, and repression of the mating pathway activity (Hohmann 2002).

In high osmolarity, *HOG1* associates with *SKO1-SSN6-TUP1* complex, phosphorylates *SKO1*, and converts it into an activator that subsequently recruits Swi/Snf and SAGA complexes. It is also known to activate the transcription factor SMP1 and the RCK2 kinase which are involved in the regulation of the expression of a subset of osmotic stress-related genes. At high osmolarity, phosphorylation of *HSL1* by *HOG1* leads to a G2 phase arrest which is essential for cell survival. *HOG1* also mediates cell-cycle arrest in G1 phase by the dual targeting of *SIC1*. *HOG1* targets *RDP3* histone deacetylase to osmotic stress related promoters. (Url-6). Another study indicates that *HOG1* is also activated by heat stress (Winkler et al., 2002).

1.8.4 *PBS2/SSK4/HOG4/YJL128C*

PBS2 (Systematic name: *YLJ128C*, synonym: *SSK4, HOG4*) encodes the MAP kinase kinase involved in HOG transduction pathway which is known to have a role in phosphorylating *HOG1* on a tyrosine residue. Pbs2p is phosphorylated by Ste11p and Ssk2/22 which are activated by two independent signals emanated from Sho1p and Sln1p osmosensors. Mutations in *PBS2* cause osmosensitivity and reduce glycerol accumulation in yeast (Hohmann 2002).

1.8.5 *ALR1/ YOL130W* and *ALR2/YFL050C*

ALR1 (Systematic name: *YOL130W*) and *ALR2* (Systematic name: *YOL130W*) overexpression protected *S. cerevisiae* cells against Al^{3+} toxicity (Macdiarmid and Gardner 1998). Their role is known to encode transmembrane protein involved in transport of mainly Mg^{2+} and other divalent cations (Graschopf et al., 2001). They are homologous to the Salmonella typhimurium CorA protein responsible for transport of Mg^{2+} and Co^{2+} through the membrane. Overexpression of Alr proteins causes resistance to Al^{3+} and Ga^{3+}, but sensitivity to several other metal cations such as Zn^{2+}, Mn^{2+}, Ni^{2+}, Cu^{2+}, Ca^{2+} and Li^{3+} ions. Also studies of Kern A.L. (2005) and his friends have indicated that Alr1p has a central role in the cell survival in cadmium stress (Kern, 2005).

Mg^{2+} has roles in cellular systems such as is binding negatively charged ligands, phosphorylating ATP, RNA, and DNA. MacDiarmid and Gardner (1998) have shown that Al^{3+} ion inhibits the Mg^{2+} and Co^{2+} uptake system thus induces Mg^{2+} deficiency in yeast. This indicated that the primary cause of aluminium toxicity is due to the inhibition of Mg^{2+} uptake (MacDiarmid and Gardner, 1998). Their study also revealed that increased activity of Mg^{2+} uptake system confers resistance to Al^{3+} stress and overcomes uptake of other cations. Studies with labeled cobalt has shown that cobalt is likely to be transported via Mg^{2+} transporters and Co^{2+} accumulation increases with overexpression of Alr proteins (MacDiarmid and Gardner, 1998; Grashopf et al., 2001).

ALR2 is known to encode an homolog of Alr1p protein. Yeast cell growth does not depend on Alr2p but when the overexpression of Alr2p can compensate for the absence of Alr1p. (Grashopf et al., 2001)

1.8.6 *NHA1 /YLR138W*

NHA1 (Systematic name: *YLR138W*) is the gene that mediates intracellular cation homeostasis by encoding a putative Na^+/H^+ antiporter and has important role in maintaining intracellular pH and Na^+ homeostasis. Nha1p, Na^+/H^+ antiporter also controls K^+ transport under specific conditions. *NHA1* overexpression is known to increase sodium and lithium tolerance mainly at acidic and neutral conditions (Hamilton et. al, 2002). It is known to encode a protein containing 12 putative

transmembrane regions and a long hydrophobic C-terminus that is thought to be cytosolic (Hohmann and Magers, 2003). With the increase in osmolarity, HOG1 phosphorylation stimulates at least two proteins in the plasma membrane, Nha1 Na^+/H^+ antiporter and Tok1 K^+ channel (Proft and Struhl, 2004).

Nha1p regulates internal cation concentrations via electrochemical proton gradient across plasma membrane. C-terminus of Nha1p seems to be involved also in the cell cycle regulation (Hohmann and Magers, 2003).

1.8.7 *CTT1 / YGR088W*

The relationship between osmotic stress and oxidative stress in *S. cerevisiae* depends on promoter region of the cytoplasmic catalase encoding gene *CTT1* and Hog1 protein HOG pathway (O'Rourke et al., 2002). Osmotic stress dependent transcriptional activation of *CTT1* can be seen on Figure 1.15.

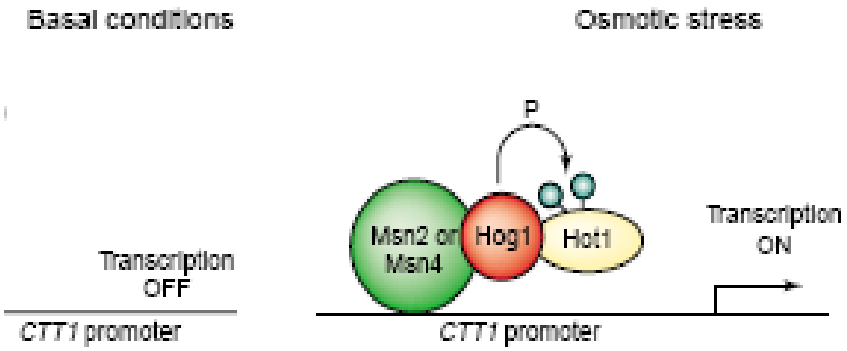


Figure 1.15 : Osmotic stress regulated transcriptional activation of *CTT1* gene (O'Rourke et al., 2002)

The *CTT1* promoter has binding sites for the Msn2 and Msn4 which are transcriptional activators. In response to osmotic stress, Msn2 and Msn4 enter the nucleus and bind the promoter region and through interaction with Msn2 and/or Msn4, Hog1 binds to DNA (O'Rourke et al., 2002).

1.8.8 *ENA1/ YRD040C/ HOR6/ PMR2*

The *ENA1* gene encodes plasma membrane ATPase which is the major Na^+, K^+ and Li^+ efflux system in *S. cerevisiae*. *S. cerevisiae* contains three genes in this cluster: *ENA1, ENA2, and ENA5*. The most well characterized member of this cluster is *ENA1* (Url-7). Expression of *ENA1* is strongly induced by Na^+ and Li^+ stress and by

alkaline conditions (Garciadeblas et al. 1993; Mendoza et al. 1994) and Ena1p is considered to be mainly responsible for the sodium export and Na^+ tolerance of *S. cerevisiae.* Its inactivation gives rise to NaCl sensitivity. *ENA1* transcription is also induced in glucose or nitrogen starvation or by high extracellular pH (Hohmann and Magers, 2003). Its transcription is controlled at transcriptional level by highly complex network of signaling pathways.

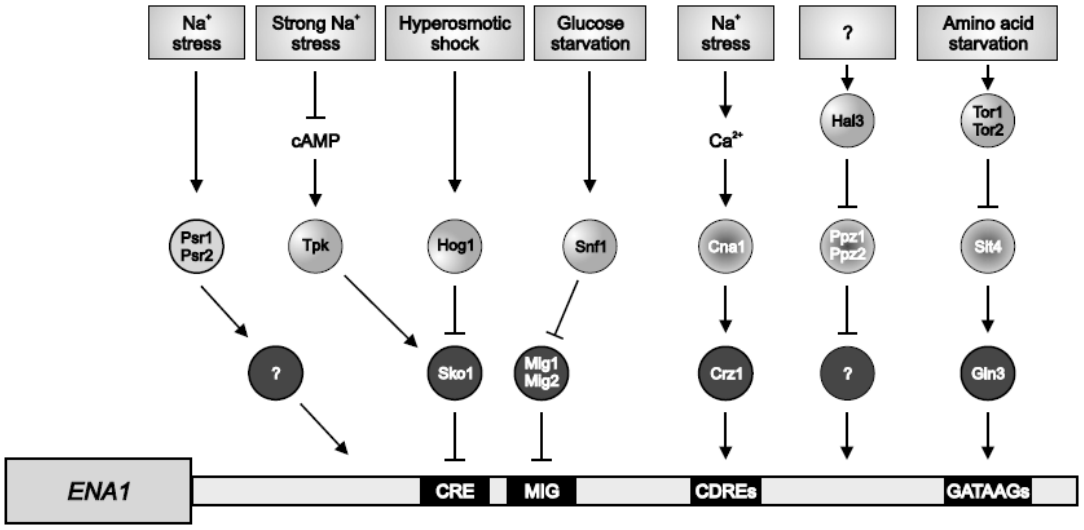


Figure 1.16 : Transcriptional regulation of *ENA1* gene (Hohmann and Magers, 2003).

In yeast, a transcription repressor, Sko1, mediates regulation of the sodium-pump *ENA1* gene by the Hog1 MAP kinase. And at high salinity, decreased *PKA* activity decreases Sko1p mediated repression by causing decreased nuclear localization of Sko1p. Also sodium stress stimulates *ENA1* expression via calcineurin provoked nuclear localization of the transcriptional activator Crz1p (Figure.1.16).

1.8.9 *AQY1/YPR192W* and *AQY2/YLL052C*

In *S. cerevisiae, AQY1* and *AQY2* encodes aquaporins, which are members of the major intrinsic protein (MIP) superfamily of integral membrane proteins (Tanghe et. al., 2005). Uptake or efflux of water, controlled by water channel proteins is believed to have major importance particularly under conditions where the passive diffusion of water through the lipid bilayer is insufficient.

Aqy1 is a spore-specific water channel whereas Aqy2 is only expressed in proliferating cells and is known to be controlled by osmotic signals. Aqy1 possibly

plays role in decreasing the spore water content, which is approximately half of that of vegetative cells (Pettersson et.al., 2005) . When cells are shifted to high osmolarity *AQY2* expression reduces. Down-regulation by osmoshock partly depends on the HOG pathway (Hohmann , 2002). *S. cerevisiae* aquaporin null strain displays a small but significant decrease in sensitivity to osmotic shock (Bonhiver et.al., 1998). Deletion of aquaporin also has minor effects on surface hydrophobicity, flocculation, cell aggregation and invasive growth (Carbrey et. al.,2001).

1.9 Acid Stress Responses of Yeast

In general, yeast cells usually maintain their pH around neutrality, however acid stress affect the cells' ability to maintain pH homeostasis leading to disruption of the cell membrane, cell wall, and the activity of cellular processes including the activity of enzymes and conductivity of ion channels. It may also affect ATP levels, and the cells' ability to maintain pH homeostasis, therefore leading to disruption of substrate transport and oxidative phosphorylation. Phenotypically resistant yeasts are known to have enhanced ability to catalyze energy-dependent extrusion of the acids.

Yeast cells have developed plasma membrane H^+ATPase dependent responses to acid stress (Serrano et al., 1986; Beales, 2003). H^+-ATPase is a member of a family of cation-translocating ATPase family found in a number of yeast and fungal species. They play a critical and energy-demanding role in adaptation to weak acid stress by extruding protons and retaining favorable internal conditions of the cell.

Under normal conditions, the cell uses 10% to 15% of the total ATP for the H+-ATPase activity. In high acidity, cell consume up to nearly 50% of cellular ATP for increased ATPase activity to keep pH homeostasis. Thus, under stress conditions the cell needs both to keep H^+-ATPase activity for maintaining homeostasis and appropriate ATP levels to enable cellular activities for growth. Under long term stress conditions, *HSP30* gene is thought to have a role in conserving the cellular ATP levels which is consumed by ATPase. *HSP30* has been shown to be induced by other stresses such as heat shock and ethanol (Panaretou and Piper, 1990). Henriques et al. (1997) have found another efflux system that actively extrude anions outside the cell called as ATP-binding cassette (ABC) transporter Pdr12 (Henriques et al.1997). Yeast cells not only have to extrude the anions but also to reduce the

diffusion coefficient of preservatives across the membrane avoiding their entry to the cell.

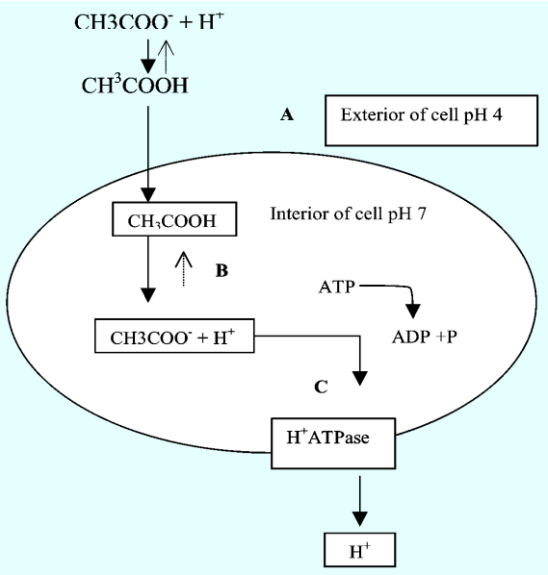


Figure 1.17 : Yeast Plasma-membrane H^+-ATPase (Beales, 2003).

Dissociation constant (pKa) determines the strength of an acid. pKa is the pH value when the dissociated and undissociated forms of the acid are in equal amounts in the medium. For example strong acids have a lower pKa value than weak acids which means that between pH: 3 – 6 they will be dissociated, whereas weak acids will be undissociated. This concept is important because undissociated acids can easily pass through the cell membrane and once inside the cell, they generally encounter a higher pH due to the cellular buffers which leads to dissociation inside the cell and thus acidification of the interior (Figure 1.17). Therefore, if the pH value is low, there will be greater proportion of acid in the undissociated form. Thus there will be a greater effect on the cell. Even though both the undissociated and dissociated forms result in a drop in internal pH (pHi), toxicity is predominantly due to the undissociated form of the weak acid (Beales, 2003).

Even at the same pH, different weak organic acids have different inhibitory effects on different organisms. Thus there is no single acid stress as for other stress kinds and their effects are dependent on the chemistry of each counter ion (Lawrence, 2004).

1.9.1 Citric acid stress response of *S. cerevisiae*

There are various kinds of studies on sorbic and benzoic acid responses of *S. cerevisiae.* In this study we aimed to obtain and investigate characteristics of citric acid stress resistant *S. cerevisiae* strains.

Citric acid ($C_6H_8O_7$) is a kind of weak organic acid that has wide application mainly as a natural preservative and flavor enhancer in food industry. It is also used in detergent and pharmaceutical industry as a metal chelating and pH buffering agent. Citric acid exists in a variety of fruits and vegetables, but in higher amounts it is manufactured by fermentation of molasses. It can exist both in monohydrate and anhydrous form. Biochemically citric acid is important as an intermediate in the metabolism of virtually all living things, namely in the citric acid cycle (also known as Krebs cycle,or Tri-Carboxylic Acid (TCA) cycle).

Lawrence et. al. (2004) have shown that HOG pathway regulates resistance to citric acid stress. Fig. 1.18 illustrates the relations between HOG pathway and citric acid adaptation. Proteins and genes which are sensitive to citric acid stress upon deletion or the ones that are up-regulated as response to citric acid are shown in the Fig. 1.14 (Lawrence et al., 2004). It was shown that citric acid phosphorylates both Thr174 and Tyr176 which then activates Hog1p. Also with the activation of HOG pathway, citric acid stress activates genes involved in glycerol biosynthesis and a set of transcription factors of stress response elements.

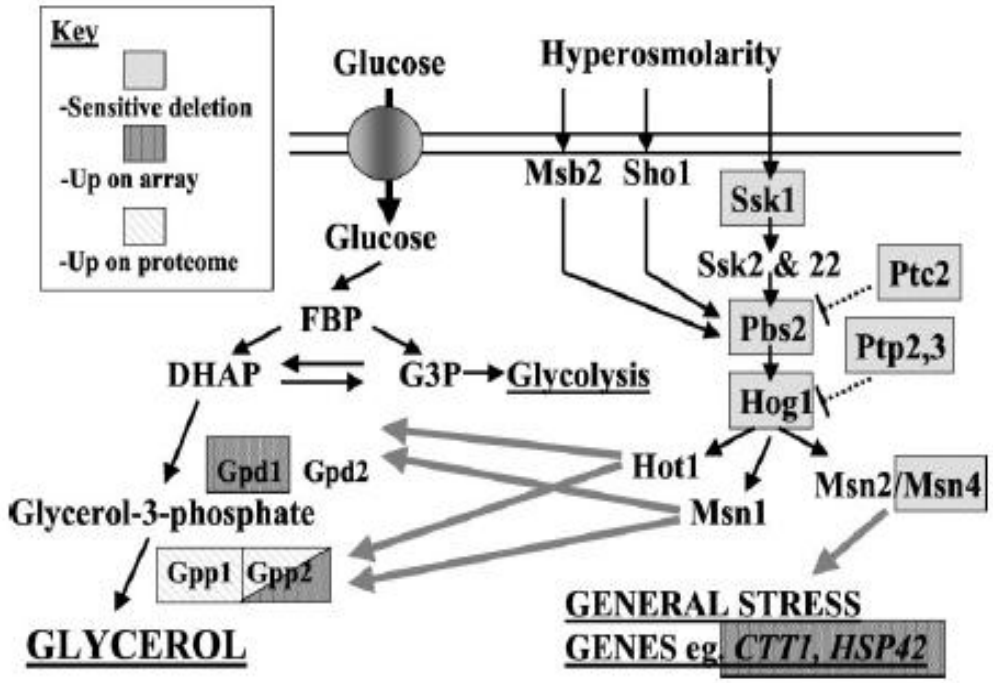


Figure 1.18 : The role of the HOG pathway in adaptation to citric acid stress in *S. cerevisiae.* (Lawrence, 2004).

Citric acid also induce expression of some genes that are known to be involved in other stress responses including Hsp40 (chaperone proteins) and Hsp70 family, Ssa1p, Ssb2p, Hsp26p, Sti1p, Ddr48, Sis1p, Hsp42, Ctt1 (cytoplasmic catalase) and trehalose biosynthesis related genes such as *UGP1, TPS1, TSL1* (Lawrence et al., 2004).

1.10 Heavy Metal Stress

Heavy metals have essential roles as trace elements in all living organisms. They play structural roles in biochemical reactions and as cofactors of protein molecules. They form complexes with ligands that contain nitrogen and sulphur centers. Trace amounts of them play role in biochemical reactions i.e. respiration, re-arrangement of C-C bonds, hydrogen assimilation, nitrogen fixation, cleavage of urea and transcription. However higher doses of them cause lethal effects on cell by blocking functions of some proteins such as membrane bound enzymes or by substitution of other essential metals.

As a stress factor they have both redox and non-redox effects. They can catalyze 'Fenton reactions' and the 'Haber-Weiss reactions' resulting in the production of

reactive oxygen species (ROS). Most of the enzymes that produce reactive oxygen species contain these heavy metals and their presence in biological systems is in an uncomplexed form (not in a protein or other protective metal complex) which causes oxidative stress.

These heavy metals also have high affinity for phosphates, purines, porphyrins, cysteinyl and histidyl side chains of proteins, thus may interact with active sites of protein structures and nucleic acids. For example, copper ions can react with nucleic acids in the active site of the enzymes and can induce oxidative stress Also cadmium ions can react with polythiol groups and have high affinity to zinc containing enzymes such as carboxypeptidases and metallothioneins (Howlett and Avery, 1997).

Cobalt is such a transition metal that causes degradation of hydrogen peroxide to hydroxyl radical (·OH). Therefore there seems to be a strong relationship between heavy metal stress and oxidative stress (Herrero et al., 2008). These reactive oxygen species produced are responsible of peroxidation of lipids causing disruption of organelles or cell membrane structure. (Gharieb et al.1998). On the other hand, cobalt can substitute magnesium and calcium which is involved in essential cellular reactions (Stadler and Schweyen, 2002).

Cells generally encounter with heavy metal stress either by prevention of enterence by reduction of uptake, activating efflux or via forming complexes outside the cell. They can compartmentate heavy metals in vacuole or sequesterate them in cytosol by intracellular chelation with organic or inorganic ligands which they synthesize. Also, some metal ions can be reduced to an oxidation state that has less toxic effect.

Yeast cells produce ‘Metallothionein’ proteins rich in cysteine. These low molecular weight proteins have high amount of sulfur (sulfhydryl) rich amino acids with a thiol group. These sulphydryl groups help to bind numerous metal ions in the form of metal–thiolate clusters (Avery et al., 2004; Presta and Stillman, 1997)

Glutathione is also another radical scavenging yeast protein, which is cysteine rich like metallothioneins. Sulphydryl groups react with an oxidant resulting in the formation of reduced gluthationein. (Avery et al., 2004)

Catalase and superoxide dismutase enzymes catalyze heavy metal defense system reactions. When metal concentration are at toxic level, free radicals are generated

resulting with protein disruption. Redox active metals like copper and iron cause Fenton reactions that generate reactive oxygen species (ROS) as result of a univalent reduction of H_2O_2 generating $OH^{\cdot}$ Radical

$Cu^{+2} + H_2O_2 \longrightarrow Cu^{+1} + OH^{\cdot} + OH^{-}$ (Fenton reaction).

Catalase has a role in breaking down of H_2O_2 to O_2 and H_2O, thus preventing Fenton reactions.

Another yeast enzyme called Superoxide dismutase is a homodimeric zinc and copper containing enzyme that catalyzes the decomposition of superoxide (O^{-2}) to hydrogen peroxide (H_2O_2) and oxygen. Superoxide dismutase binds to intracellular copper and functions as a buffering molecule when intracellular copper concentration is high (Avery et al., 2004).

Heavy metals are released to the environment from plastics, batteries, metal processing industries, mining and metallurgical applications. They enter all living organisms via food, drinking water and air and tend to bioaccumulate in bodies. Heavy metal resistance evolved by microorganisms may have a significant role in understanding tolerance mechanisms of higher organisms. Additionally, metal resistant microorganisms might find use in biotechnological applications i.e. in the fields of biomimetics, in bio-mining and bioremediation of metal contaminated environments (Nies, 1999).

1.11 Oxidative Stress

Oxidative stress is caused by reactive reactive oxygen species (ROS) including free radicals such as highly reactive superoxide anion radical, the hydroxyl radical and peroxides including hydrogen peroxide, and various organic peroxides (Steels et al. 1994). They are produced at a low level by normal aerobic metabolism as the result of changes in oxygen tension or by redox reactions and the damage they cause is constantly repaired by the cell.

Reactive oxygen species play important roles in cell signalling via a process termed redox signaling. Any disturbances in this normal redox homeostasis of cell between reactive oxygen production and consumption leads to toxic effects that damage a wide range of cellular molecules i.e. proteins, nucleic acids, and lipids.

These free radicals affect proteins via causing crosslinks between them which will cause enhanced proteolysis and damage tissue structure. Hydroxyl radical oxidizes aminoacyl residues such as tyrosine, cysteine, tryptophan and phenylalanine. And the protein-hydroperoxides which are reactive themselves decompose to free radicals that will lead to formation of modified, crosslinked and unfolded protein structures. Singlet oxygen and hydroxyl radical is also known to be directly affecting DNA, whereas superoxide radical and hydrogen peroxide cause damage via generation of these species. H_2O_2 is also known to cause interchromosomal recombination (Dickinson and Schweiser, 2004).

Cells respond to oxidative stress via both enzymatic and non-enzymatic ways including degredation of reactive oxygen species, maintaining metal ion homeostasis to prevent free metal ions that generate hydroxyl radicals and repair damaged structures within the cell.

Enzymatic defense systems are based on removal of the oxygen radicals and their products and/or repair their damages. In non-enzymatic way, small molecules which are either soluble in water or lipids have role as radical scavengers. They remove oxidants from the environment via oxidizing themselves by reactive oxygen species. Non enzymatic defense systems include production of some molecules such as phytochelatins, metallothioneins, polyamines, ascorbic acid, trehalose, and flavohaemoglobins.

Glutathione (GSH), glutaredoxin, metallothioneins, ascorbic acid and thioredoxins protect against hydrogen peroxide in both maintenance of redox homeostasis within the cell and in protein repair mechanism against oxidative stress. NADPH-dependent glutathione reductase prevents radical chain reactions by catalyzing reduction of oxidized glutathione (GSSG) to reduced glutathione (GSH), which then reacts with hydrogen peroxide and superoxide radicals. Other cellular enzymes with antioxidant role include superoxide dismutase (SOD), catalase, and glutathione peroxidase, peroxiredoxins and sulfiredoxin.

In medical point of view, oxidative stress is suspected to be important in chronic fatigue syndrome, ischemic cascade due to oxygen reperfusion injury following hypoxia and neurodegenerative diseases such as Lou Gehrig's disease, Parkinson's disease, Alzheimer's disease, and Huntington's disease. Thus, studies on oxidative

stress response of model organism such as yeast might be useful to understand more complex systems.

1.12 Freezing-Thawing stress

The tolerance of yeast cells to freeze-thaw stress is critical for frozen-dough technology in the baking industry. Freezing stress also itself is an extreme form of osmotic stress. It causes cells to lose water, lowering of internal pH, ionic toxicity, impairment of glycolysis, macromolecular damage, aggregation of cytoskeletal elements and disruption of organelles. Thus freezing-thawing responses of cells are related with most of the other types of stresses.

One of the physical factors that cells encounter during freezing is ice crystal formation and dehydration. In high freezing rates, intracellular ice crystal formation is the main reason that leads to cell damage. On the other hand, at low freezing rates extracellular ice formation leads to intracellular dehydration.

Viability of the cell depends on freezing parameters such as rate of freezing and physiological state of the cell. Each cell has its own specific freezing rate depending on shape, structure, surface area-to-volume ratio, and membrane permeability. For example, freezing rates below 7°C per min are known to have a slow-freezing effect on yeast. Another biochemical damage is due to the oxidative stress by reactive oxygen species formed during the thawing process.

Synthesis of stress proteins or metabolites such as trehalose and glycerol are some of the responses that cells encounter against freezing-thawing stress. The main role of trehalose is to stabilize intracellular water structure, protein structure and cell membranes under stress conditions.

There is a correlation between freezing resistance and aquaporin encoding genes such as *AQY1* and *AQY2*, thus it is supposed that plasma membrane water transport has a big role in freezing tolerance. (Ann et al., 2002; Dickinson and Schweiser, 2004).

Mutations in glycerol and trehalose pathways do not lead to a sensitivity to freezing. Thus, during slow freezing, osmostress defense system other than the HOG pathway

is supposed to be involved resulting with a significant change in intracellular salt concentration (Park et al., 1997; Dickinson and Schweiser, 2004).

Previous exposure to heat shock, H_2O_2 or NaCl is known lead cross protection against freezing-thawing stress (Lewis et al., 1995; Park et al., 1997; Dickinson and Schweiser, 2004).

1.13 Heat Stress

When yeast cells are exposed to mild heat treatment, they are known to gain resistance to certain degree of thermotolerance, which is thought to be related to heat shock factor and stress response pathways that regulate synthesis of heat shock proteins (Hsps). Hsps are the major thermotolerance factors in yeast cells (Guyot et. al., 2005). Addition to active heat stress response mechanisms such as such as Hsps or trehalose, yeast cells also have passive mechanisms that change plasma membrane properties. Yeast thermotolerance depends on the kinetics of temperature variation and membrane permeability (Martinez de Maranon et al., 1999; Guyot et. al., 2005). Rapid heat shock causes complete and simultaneous phospholipid disorganization, leading to increasing membrane permeability and cell death. Plasma membrane integrity which affect membrane protein activities are related to changes in membrane fluidity that is known to be depending on the composition of phospholipid bilayer and fatty acid saturation ratio (Beney and Gervais, 2001). Also measurements of the loss of intracellular solutes have shown that ion leakage (Ca^+, Na^+, and K^+) has important role after the heat shock stress (Guyot et. al., 2005).

1.14 Ethanol Stress

S. cerevisiae, one of the most ethanol-tolerant organisms for up to %15 (v/v) resistance is the predominant organism that is used in fermentation of alcoholic beverages (Cot et al., 2007). Eventhough ethanol is produced as the final product of anaerobic fermentation of sugars, %4-6 (v/v) concentration of it has a toxic effect that slow metabolism and reduce yeast growth rate to half percent of nonstressed cells (Piper, 1995; Kubota et al., 2004; Snowdon et al., 2009). Ethanol stress is thought to be one of the major causes of sub-optimal or stuck ferments. Since one of the major aims in fermentation industry is to keep viability of yeast cells high during

ethanol production, there have been many studies on yeast ethanol tolerance mechanisms.

It is reported that the primary target for ethanol toxicity is the plasma membrane which is crucial for solute transport and energy generating systems of yeast cells. Other disruptive effects of ethanol are on mitochondrial DNA, ATPases and some enzymes such as hexokinase and dehydrogenase. It is also known to affect many transport systems including aminoacid and glucose transport systems (You et al., 2003).

Although the complex signal network of ethanol stress responses in yeast is not clearly identified, several mechanisms have been reported related to this process. These studies have focused on gene expression changes, alterations of cellular lipid compositions, membrane structure and chaperone proteins.

Ethanol changes physical properties of membranes causing increased fluidity and permeability. Yeast cells compensate for the destabilising effects of ethanol by changing the lipid composition of their membranes such as increasing the mono-unsaturated fatty acid, oleic acid and ergosterol contents (Alexandre et al., 1994; Snowdon et al., 2009).

The amount of monounsaturated fatty acids in cellular lipids is known to be increased when cells are exposed to ethanol stress. Since cell membranes are the primary targets of ethanol stress, there seems to be a relationship between membrane fatty acid composition and ethanol tolerance. Also, when yeast cells are exposed to ethanol, an increase in the synthesis of a disaccharide, trehalose and various stress response proteins such as chaperone proteins has been observed (Piper, 1995).

Many research groups have attempted to identify genes that are up-regulated when cells are exposed to pulse ethanol stress. Early research in this area has shown that expression levels of yeast genes related to general stress response, lipid metabolism, energy consumption, cell surface interactions and transport mechanism increase. (Chandler et al., 2004). However, identification of ethanol tolerance related genes among thousands of genes that are up- or down-regulated in the microarray experiments requires considerable effort. With the help of classical genetics and molecular biology, evolutionary engineering may be used to obtain novel yeast

strains that might be of direct benefit to the wine industry and identify genes involved in ethanol tolerance.

1.15 RT PCR

RT-PCR (reverse transcription-polymerase chain reaction) is one of the techniques for detection and quantification of mRNA expression. In RT- PCR, mRNA is copied to cDNA by reverse transcriptase using an oligo dT primer. The polymerase chain reaction (PCR) allows the exponential copying of part of cDNA molecule. After certain rounds of cDNA synthesis, reaction products are usually visualized and quantified via agarose gel electrophoresis with the help of a nucleic acid dye especially ethidium bromide. Quantification of the product can be done using special commercial computer programmes such as Bio-Capt. However, this type of agarose gel-based analysis of cDNA products of reverse transcriptase-PCR does not allow accurate quantification of the differences in mRNA expression.

RT-PCR can be used to quantify mRNA levels from much smaller samples, compared to Northern blot analysis. However, in some procedures, only small amount of mRNA is available which will bring difficulty to get truly quantitative results using conventional PCR. Thus Real Time PCR which is relative ease and convenience of use gives more quantitative results.

1.16 Q-RT PCR

Real-time reverse-transcriptase qRT-PCR is a powerful tool that helps to quantify the initial amount of the template most specifically, sensitively. It allows to observe the ongoing PCR reaction and to detect the amount of final amplified product at the end-point. It is based on monitoring and quantifying the fluorescence emitted which is directly proportional to the amount of amplicon in each PCR cycle.

qRT-PCR probes can be (1) hydrolysis probes which are composed of a specific quencher and fluorescent reporter molecules such as TaqMan, molecular beacons, scorpions (2) hybridizing probes (3) fluorescent DNA binding dye such as SYBR green or ethidium bromide.

There are two types of quantification in qRT-PCR. One of them is relative quantification which is based on the relative expression of a target gene versus a reference gene. This method is useful if the chosen endogenous/internal control gene is more abundant and remains constant in proportion to total RNA. Mostly preferred control genes in this method are 18S RNA, *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase) and β-actin. The best normalizer is the one with constant expression under the experimental conditions designed for the target gene. Physiological changes in gene expression levels can be investigated via relative quantification. In relative quantification, relative fold change method is usually preferred for analyzing differences in gene expression. Absolute quantification is the other method that calculates amplicon size usually by comparing the PCR signal to a standard curve (Schmittgen and Livak, 2008). For this purpose, standard curve is constructed from an RNA of known concentration usually from cDNA plasmids.

The first significant increase of the amount of PCR product in exponential phase is correlated to the amount of target template at the beginning of the reaction. The higher the starting amount of nucleic acid target, the sooner will be the increase in fluorescence. Accumulated PCR product is quantified from the significant increase in fluorescence above the baseline value during the 3-15 cycles. The operator of the device sets a fixed fluorescence threshold above the baseline. The cycle number at which the fluorescence emission exceeds the fixed threshold gives us the C_T (threshold cycle) value which is quantitative endpoint for real-time PCR. The higher the initial amount of DNA, the sooner the product is detected by the system and the lower the C_T value. If the C_T value is equal to or over 40 it can be concluded that there is no amplification and this value cannot be used for the calculations. The amplification efficiency is calculated from the slope of the log-linear phase. In an acceptible PCR, efficiency of the reaction should be between 90% - 100% ($-3.6 > \text{slope} > -3.1$) and the error must be below 0.2. Efficiencies and errors of genes and house-keeping genes are calculated by standard curves in each reaction. Efficiency can be calculated from the formula ‘‘Efficiency = $10^{(-1/\text{slope})} - 1$ ’’ (Schmittgen and Livak, 2008). If there is no known amount of standard, Comparative Threshold (ΔC_T) method can be used by comparing the relative amount of the target sequence to any of the reference values chosen. The result is obtained as relative to the

reference value. The advantage of this method is that there is no need for a standard curve in each run.

1.16.1 Comparative threshold method (ΔC_T)

The mean C_T values of both the control and the samples are determined for each gene and C_T (reference-target) values are calculated. Assuming the efficiency is 2 for all genes, the fold increases at threshold cycles are determined by $2^{(\text{reference-target})}$. It is important to note that in this method, the efficiency of the target amplification must be approximately equal to the efficiency of the reference amplification.

1.16.2 Comparative CT method (ΔΔCT)

This method is used for relative quantification of gene expression without need for standard curves. For each gene investigated, expression levels of the samples are compared with those of the control. The comparison by $2^{-\Delta\Delta}C_T$ method is performed by normalizing the obtained values to the wild type C_T values. In this thesis, comparative C_T method ($\Delta\Delta C_T$) was used for relative quantification of gene expression. $\Delta\Delta C_T$ method is the most practical method, if the target and the control genes are in similar dynamic ranges.

1.17 Aim of This Study

In this study, 'evolutionary engineering' an inverse metabolic engineering strategy was applied to obtain *S. cerevisiae* mutants resistant to osmotic stress, salt stress and acid stress and to investigate the mutants at genetic level. For this purpose, firstly, the genetic diversity of *S. cerevisiae* population was increased via exposure to ethyl methane sufonate. Batch selection strategy was applied to the resulting initial mutant population by exposing to stress conditions continuously and at gradually increasing levels. Individuals were selected randomly from the final populations that have resisted the highest stress conditions. By most probable number (MPN) method and serial dilutions on solid plate, they were tested for their resistance to the stress condition at which they were selected and to the other stress types for a potential cross-resistance. Classical genetic tests such as tetrad analysis were performed to test if resistance to a particular stress is controlled by a single gene or more. Additionally

q-RT-PCR test was performed with the most resistant mutant and the wild type, to determine and compare the expression levels of different genes that might be involved in resistance to a particular stress type of interest. The results were compared with those found in the literature and discussed in detail.

2. MATERIALS AND METHODS

2.1 Materials

2.1.1 Yeast strain

Saccharomyces cerevisiae CEN.PK113-7D (named as 100 or 905) was kindly provided by Dr.Peter Kötter from Johann Wolfgang Goethe University, Frankfurt, Germany. The populations called 101 and 906 were obtained by applying the EMS mutagenesis method to the wild type populations 100 and 905 respectively.

2.1.2 Media compositions, chemicals, buffers and solutions

Compositions of the yeast minimal medium (YMM) used in MPN and stress application are given in Table 2.1.

Table 2.1 : Yeast minimal medium (YMM)

Yeast Nitrogen Base without aminoacids	6.7 g
Dextrose	20 g
Agar (for solid media)	20 g
in 1 liter of distilled water.	

Yeast complex medium (YPD) used for precultivations was prepared as in Table 2.2.

Table 2.2 : Yeast complex medium (YPD)

Bacto Yeast Extract	10 g
Dextrose	20 g
Bacto Peptone	20 g
Agar (for solid media)	20 g
Per 1 liter of distilled water	

Chemicals used in experiments are listed in Table 2.3.

Table 2.3 : Chemicals

Chemical	**Company**
NaCl	Carlo Erba (Italy)
Ethanol	J.T.Baker (The Netherlands)
D(+)-Trehalose dihydrate	Riedel-de Haën (Germany)
Hydrogen peroxide(% 35, v/v)	Merck (Germany)
Cobalt (II)- chloride–hexahydrate	Merck (Germany)
Nickel (II)- chloride - hexahydrate	Sigma Aldrich (USA)
Copper (III)- chloride	Merck (Germany)
Chromium (III)- chloride - hexahydrate	Acros Organics (USA)
Citric Acid (65%, v/v)	Merck (Germany)
Sorbitol	Merck (Germany)
Glycerol	Sigma Aldrich(USA)
Yeast Extract	Merck (Germany)
Tyrptone	Merck (Germany)
$ZnCl_2$	Riedel- de Haën (The Netherlands)
Bovine Serum Albumin (BSA)	Sigma Aldrich (USA)
DNA markers	Fermentas (Lithuania)
Agarose	Applichem (Germany)
RNA isolation kit	Roche (Switzerland)
SuperScript III One Step RT-PCR System	Invitrogen (USA)

2.1.2.1 Buffers and solutions

Phosphate buffer saline (PBS), which was used during RNA isolation procedure (Table 2.4) was prepared according to Good et al (1966).

DEPC (Diethylene Pyrocarbonate) at 1:1000 dilution with ddH_2O was used to denature RNases (Ambion, 2008).

The Phosphate buffer saline and buffers used for agarose gels are listed on Table 2.4 and 2.5.

Table 2.4 : Phosphate Buffer Saline (PBS)

NaCl (Sodium Chloride)	8 g
KCl (Potassium Chloride)	0.2 g
Na_2HPO_4 (Disodium Hydrogen Phosphate)	1.44 g
KH_2PO_4 (Potassium Dihydrogen Phosphate)	0.24 g
pH was buffered to 7.4 with HCl and total volume was completed to 1 liter with DEPC-H_2O	

Table 2.5 : Agarose gel content

Agarose	1 g	1 or 1.5% Agarose in TBE with 0.5-0.8 μg/ml Ethidium Bromide
Ethidium Bromide	~7 μl	10mg/ml
10X TBE	100 ml	1 M Tris-(hydroxymethyl) amino methane (pH 7.8) -1 M Borate (Boric Acid) 2 mM EDTA dissolved in DEPC-H_2O

Laboratory equipments and kits used are listed in Table 2.6 and Table 2.7.

Table 2.6 : Laboratory equipment

Thermomixer	Eppendorf, Thermomixer Comfort 1.5-2 ml, (Germany)
Light Microscope	Olympus CH30
Microfuge	Eppendorf Centrifuge 5424
Ultracentrifuge	Beckman Coulter, Avanti J-30I (USA)
Rotor	Beckman Coulter JA-30.50i (USA)
UV-Visible Spectrophotometer	Shimadzu UV-1700 (Japan)

Table 2.6 : Laboratory equipment (continued)

Ultrapure Water System	USF-Elga UHQ (USA)
Microplate Reader	Biorad Model 3550 UV (USA)
Micropipettes	Eppendorf (Germany)
pH meter	Mettler Toledo MP220 (Switzerland)
Water Bath	Memmert wb-22 (Switzerland) Nüve BS402 (Turkey)
Balances	Precisa BJ 610C (Switzerland) Precisa 620C SCS (Switzerland)
Laminar Flow	Özge (Turkey) Faster BH-EN 2003 (Italy)
Autoclaves	Tuttnauer Systec Autoclave 2540ml (Switzerland) Tuttnauer Systec Autoclave 2870ELVC (Switzerland) NüveOT 4060 Steam Sterilizer(Turkey)
Deep Freezes and Refrigerators	Sanyo (-80^{o}C)Ultra Low MDT- U40865 (Japan) -20˚C Arçelik(Turkey) +4˚C Arçelik (Turkey)
Incubators	Nüve EN400 (Turkey) Nüve EN500 (Turkey)
Orbital Shaker Incubators	Certomat S II Sartorius (Germany)
Elecrophoresis Tank	Midicell PrimoTM EC 330 (USA) Midicell PrimoTM EC 320 (USA)
Power supply for Electrophoresis	EC Apparatus Corporation EC 250-90
Fluorometer	Qubit Invitrogen (USA)
Real Time PCR	LightCycler 480 II Roche (Switzerland)
HPLC (High Performance Liquid Chromatography) System	
- System Controller	Shimadzu SCL10A) (Japan)
- Liquid Chromatography	Shimadzu LC-10AD (Japan)
- Column Oven	Shimadzu CTO-10AC (Japan)

Table 2.7 : List of kits

SuperScript III One Step RT-PCR System	Invitrogen (USA)
High Pure RNA Isolation Kit	Roche (Switzerland)
Transcriptor High Fidelity cDNA Synthesis Kit	Roche (Switzerland)
Light Cycler 480 SYBR Green I Master	Roche (Switzerland)

Websites and programmes used are listed in Table 2.8.

Table 2.8 : Websites and programmes

Software	Company/ Website
Primer3Plus	http://www.bioinformatics.nl/cgibin/primer3plus/primer3plus.cgi)
S. cerevisiae Database	http://www.yeastgenome.org
Bio – Capt	Vilber Loumat (France)
Oligo Analyser	Integrated DNA Technologies http://eu.idtdna.com/analyzer/Applications/OligoAnalyzer/

2.2 Methods

2.2.1 Cultivation of yeast cells

Wild type strain CEN.PK 113-7D (named as 100 and 905) and EMS mutagenized populations 101 and 906 and mutants obtained from selections were used throughout this study. The culture stocks kept at -20 ^{o}C or 80^{o}C were inoculated in 10ml YPD in 50ml culture tubes as precultures. They were incubated at 30 ^{o}C, 150rpm for 1 to 2 days. The cells were precultured with an OD_{600} of 0.3 unless stated otherwise. Culture stocks were prepared for each population by mixing 500 µl of cells with 60% (v/v) sterile glycerol and kept at -80^{o}C.

2.2.2 Ethyl methane sulfonate (EMS) mutagenesis

Saccharomyces cerevisiae CEN.PK 113-7D culture, approximately at a concentration of $1x10^{6}$ cells/ml; was inoculated into 10 ml YPD, and incubated overnight at 30ºC and 150 rpm to have a cell concentration of approximately $2x10^{8}$ cells/ml. 2.5 ml of this culture was washed twice with 50 mM potassium phosphate

buffer (pH 7) and resuspended in the same buffer to obtain a final concentration of $5x10^7$ cells/ml. Resuspended cells are placed on ice for 15 sec and sonicated for 5-10 sec. 300 µl of EMS was added into each 10 ml of cell suspension in a screw-cap glass tube. The tube was vortexed and incubated for 30 min at 30º C. To stop EMS mutagenesis, an equal volume of freshly made and filter-sterilized sodium thiosulfate solution (10%, w/v) was added into the tube. The solution was mixed well with vortex and the cells were centrifuged at 10,000 rpm for 10 min (Beckman Coulter, JA 30.50i rotor). The supernatant was discarded and the cells were washed twice with yeast minimal medium without dextrose. The mutated cells were then inoculated into YPD and this culture was named as *101*. The original wild-type cells were named as *100*.

2.2.3 Selection of mutant populations

Generations were obtained via applying the initial stress condition to the mutated culture *101* and transferring the survivors of this present stress condition to the next stress condition. Fresh mutated culture *101* was used for the first stress application and the rest of the steps continued over the newly obtained generations. In increasing stress selections, cells were exposed to an increasing level of stress at each passage. Stress conditions were applied continuously throughout the whole cultivation. In continuous stress selection, the initial stress level was applied to the cells repeatedly from the first generation to the last generation. Optical density values obtained upon 24 h of cultivation were used for survival fitness analysis.

2.2.4 Selection of individual mutants and stock culture preparation

Final populations of each stress selection were cultivated overnight and inoculated into YMM agar plates by spreading with an appropriate dilution of cells. Following 48 h of cultivation, randomly selected individuals were transferred into fresh 10 ml of YMM for stress resistance determination. After stress application, all individual mutants were washed twice by centrifuging at 10,000 rpm for 5 min and resuspended in fresh sterile YMM without dextrose. Following the washing step, frozen stocks of cultures were prepared by addition of an equal volume of 60 % (v/v) glycerol to the liquid culture to have a final ratio of 30 % (v/v) glycerol, and placed at -20^0C and -80^0C for long-term preservation.

2.2.5 Characterization of individual mutants

Resistance of individual mutants against various stress conditions were screened either continuously or as pulse exposure. These stress conditions were freezing-thawing, heat stress, ethanol stress, oxidative stress and heavy metal stresses. Resistance to the stress condition at which the selection was done and to other different stress conditions were tested via 5-tube MPN (most probable number) method in 96-well plates.

MPN method was used to determine the number of the microorganisms in successively parallel dilutions. Twenty µl of stress applied cells were inoculated into the first five columns of microplate containing 180µl YMM. With a multipipette, 20µl of the cultures (OD_{600} values between 1.2 and 1.6) were transferred from the first to the second row in order to obtain a ten-fold dilution. This was repeated up to 10^{-8} dilution. Stress application was performed either in 96-well plates continuously throughout the cultivation or just before inoculation to the plates. After 48 and 72 h of incubation, growth in last three rows was recorded and correlated with Five Tube MPN Table (Appendix A)

2.2.6 Application of stress conditions

500 µl from the frozen stock samples of the strains kept at -80°C were inoculated into 10 ml of YMM, and the culture was incubated overnight their OD_{600} values has reached to 1.4- 1.6. One ml aliquots were then transferred to sterile 1.5 ml microfuge tubes and harvested by centrifugation at 10000 rpm for 10 min. The supernatant was discarded and the remaining cell pellet was resuspended in 1 ml yeast minimal medium without dextrose. This step was repeated twice. After the washing step, the cells were exposed to stress condition either continuous or pulse stress conditions.

2.2.6.1 Salt stress application

Yeast cells were harvested and washed as described previously. NaCl was selected as the salt stress factor. Following washing step, 500 µl of the cell suspension was inoculated into YMM involving varying percentages of NaCl (0%, 5%, 10%, 15%) and incubated at 30°C 150 rpm. Stress conditions were applied for 72 h continuously and the OD_{600} values of cells were measured regularly.

2.2.6.2 Sorbitol stress appllication

Yeast cells were harvested and washed as described previously. Sorbitol was used as the osmotic stress factor. Following washing step, 500 µl of the cell suspension was inoculated to YMM with varying concentrations of sorbitol and incubated at 30°C, 150 rpm. Stress conditions were applied for 72 h continuously and the OD_{600} values of cells were measured regularly.

2.2.6.3 Freezing-thawing stress application

Yeast cells were harvested and washed as described previously. Freezing- thawing stress was applied as a pulse stress by immersing 1ml aliquots of cells in liquid nitrogen (-196^{o}C) for 25 min and thawing at 30^{o}C for 20 min. Harvested cells (14000rpm, 5min) were then washed twice and resuspended in 1ml dextrose free minimal medium. Following washing step, 500 µl of the cell suspension was inoculated into YMM and incubated at 30°C, 150rpm.

2.2.6.4 Heat stress application

Yeast cells were harvested and washed as described previously. Heat stress was applied as pulse exposure of 1ml cell aliquots to 60^{o}C for 10 min. The cells were collected immediately by centrifugation at 14000 rpm for 5 min, washed twice and resuspended in 1 ml dextrose free minimal medium. Following washing step 500 µl of the cell suspension was inoculated to 10ml YMM and incubated at 30°C, 150rpm. The growth of cells was tested following overnight incubation.

2.2.6.5 Ethanol stress application

Cells were exposed to ethanol stress (6% v/v) for 1 h at 30^{o}C in 1.5 ml microfuge tubes. The cells were collected by centrifugation at 14000 rpm for 5 min and washed twice with YMM without dextrose. A cell suspension of 500 µl was inoculated into 10 ml of YMM and incubated at 30^{o}C, 150 rpm. The growth of the culture was tested following overnight incubation.

2.2.6.6 Oxidative stress application

Varying amounts of hydrogen peroxide were added from a 5M H_2O_2 stock solution into the liquid yeast culture of 1.5 ml. The cell pellets in microfuge tubes were

resuspended in dextrose-free yeast minimal medium. The cells were incubated at 30^{o}C, 150 rpm. After this stress application period, they were centrifuged at 10,000 rpm for 10 min using a Beckman Coulter benchtop centrifuge. Following centrifugation, the supernatant was discarded and the cell pellet was resuspended in 1.5 ml dextrose-free yeast minimal medium. A cell suspension of 500 µl was inoculated into 10 ml of liquid yeast minimal medium. The growth of the culture was tested following overnight incubation.

2.2.7 Catalase activity assay

Catalase activities were determined spectrophotometrically. Firstly, cultures grown overnight in YMM were inoculated at an initial OD_{600} of 0.4 into YMM medium with 1mM hydrogen peroxide, and incubated at 30^{o}C, 150 rpm until their OD_{600} values reached between 5 and 6. One ml of precultures was transferred to microfuge tubes and centrifuged at 14000 rpm for 5 minutes. Pellets were washed twice with 1 ml distilled water. Washing steps was performed by centrifugation at 14000 rpm for 5 min followed by resuspension of cell pellets in 300µl 50mM phosphate buffer (pH 7.2). Sterile glass beads were added until 1 ml volume line indicated on microfuge tubes. Vortex and -80^{o}C freezing steps were then performed for 1 min. This treatment was repeated sequentially for 10 times. Cell extracts were transferred into new microfuge tubes and centrifugation was applied at 12000 rpm for 10min. In separate microfuge tubes, 0.68 ml phosphate buffer and 0.48 ml 40 mM H_2O_2 were mixed and kept at 30^{o}C for 2.5 min. Then 40µl cell extracts were added to the mixture and the difference in absorbance was determined immediately for 2 min at 240 nm, using quartz tubes.

In parallel with the above procedure, protein amount of the cell extracts was determined via Bradford assay (Bradford, 1976). For this purpose 5µl samples were loaded into a 96- well microplate and 195µl Bradford reagent was added. After approximately 5 min of incubation, spectrophotometric measurements at 595nm were performed using a microplate reader. In addition for each measurement standard curves were obtained by using bovine serum albumin (BSA) standard solutions. (Concentrations of BSA solutions were as follows: 0.125mg/ml, 0.25mg/ml, 0.5mg/ml, 0.75mg/ml, 1mg/ml, 1.5mg/ml and 2mg/ml). According to

this standard curve obtained, total protein concentrations were calculated. Catalase activity was calculated as the ratio of absorbance difference to total protein amount.

2.2.8 Genetic stability analyses

The aim of the genetic stability test was to investigate if the obtained stress resistance was the result of a permanent mutation or a transient adaptation. For this purpose, individual mutants were grown overnight for five successive cultivations in the absence of selective pressure. After each cultivation the stress resistance was determined via MPN. Plates were examined after 96 h of incubation in stress conditions and viable number of cells were determined via 5-tube MPN methodology. The procedure was applied for five times including five successive cultivations and five sequential MPN application.

2.2.9 RNA isolation from yeast cells

Pre-cultures of wild type and mutants were inoculated with an OD_{600} of 0.4 in 10 ml YMM for pulse salt stress application. RNA isolation was done after 1h. One ml of the cell culture with an $OD_{600} \approx 1$ (approximately $2x10^7$ cells) was used for RNA extraction. Yeast RNA was isolated using High Pure RNA Isolation Kit (Roche).Briefly cells were centrifuged at 2000xg for 5 min, and the supernatant was discarded. The pellet was resuspended in 200 µl PBS, 4 µl zymolyase was added and incubated for 15 min at 30°C. After incubation, 400µl lysis/binding buffer was added and vortexed for 15 s. A high filter tube was put in a collection tube and all samples were filtered through. The collection tube with high filter tube was then centrifuged at 8000xg for 15 s. The filter was taken out of the collection tube. The liquid in the collection tube was discarded and the filter tube was reinserted in the collection tube. 90µl DNAse incubation buffer and 10 µl DNAse I mixture were pipetted through filter and incubated for 15 min at +15 to +25°C. 500 µl washing buffer I was added and centrifuged at 8000xg for 15 s. Flowthrough was discarded and 500µl washing buffer II was added and centrifuged for 15s at 8000xg. The flowthrough was then discarded and 200 µl washing buffer II was added. After centrifugation at 13000xg for 2 min the filter tube was transferred into a 1.5ml microfuge tube. 50-100µl Elution buffer was added and centrifuged at 8000xg for 1 min. Isolated RNA was

aliquoted and kept at -80°C. RNA concentrations were determined by using Qubit® Flourometer and Quant-iT™ RNA assay kit (Invitrogen).

2.2.10 Primer design

Coding gene sequences were retrieved from NCBI Map Viewer and approved by *Saccharomyces* Genome Database (SGD Gene/Sequence Resources). *ACT1 (YFL039C)* gene was selected as the housekeeping gene. Complementary DNA sequences of the genes were used for primer design via "Primer 3" The primer sets were analyzed for efficiency of binding sites in terms of dimer formation, non-specific binding and hairpin formation via Amplify3X (Amplify MacOS X) software programme. The results were confirmed with SciTools on the IDT DNA website and the best primer set was selected. Primer sets used in PCR applications are listed in table 2.9.

Table 2.9 : Oligonucleotide primers

Gene	Primer	Primer Sequence	T_m (˚C)	GC%	Amplicon Size (bp)
HOG1	F	GATGCCGTAGACCTTTTGGA	60.07	50	342
	R	CGCAGCCATGTTTAACTGA	58.43	47	
HAL2	F	AGGACGTCAGGCAAATCATC	60.1	50	176
	R	GTTGGGGCATCCAATACAAC	60.1	50	
PBS2	F	AGCGGTCAAATTACCACCAG	60.0	50	157
	R	AGAACCCGGATTCGATCTTT	59.9	45	
ACT1	F	CTTTCAACGTTCCAGCCTTC	59.9	50	94
	R	TCACCGGAATCCAAAACAAT	60.2	40	
ENA1	F	GTCTGGTGAAGGTCGGTGAT	56.8	55	155
	R	ACCCACGGAGGTTTCTTCTT	56,2	50	
NHA1	F	ACATTTGCATTGAGCACAGC	54.6	45	237
	R	TTCCATCTCGTCTTCGCTTT	54.1	45	
HAL3	F	CAACACCAGGACAATGCAAC	54.8	50	213
	R	TCAGAAGATTGTCGCACAGG	54.9	50	
CTT1	F	TGCAAGACTTCCATCTGCTG	55.3	50	193
	R	ACGGTGGAAAAACGAACAAG	53.8	45	

Table 2.10 : Oligonucleotide primers (continued)

Gene	Primer	Primer Sequence	T_m (˚C)	GC%	Amplicon Size (bp)
AQY1	F	TCGGCATCTCCCTGTTTATC	68	45	244
	R	TGAGCTTTTTCCTTGGTGCT	66	45	
AQY2	F	ACCATGATGTGGCTTTGAC	65	47	212
	R	ATCCCAGCAATGATCTGAGG	68	50	

2.2.11 RNA isolation and reverse-transcriptase polymerase chain reaction

For expression analysis of related genes, RT-PCR was applied. Expression levels of salt and osmotic stress related genes were investigated after exposure to 0.7M and 0.9M salt stress to a selected mutant and the wild type. They were incubated overnight and washed twice with dextrose free minimal medium. They were then inoculated into YMM with 0.7M and 0.9M NaCl and YMM only as a control, at an initial OD_{600} of 0.4. Following 1.5 h of incubation, total RNA was isolated via High Pure RNA Isolation Kit (Roche) and their purity were checked using Qubit® Flourometer and Quant-iT™ RNA assay kit (Invitrogen)

RT-PCR was performed using SuperScript^{TM} III One-Step RT-PCR System with Platinum® Taq DNA Polymerase kit (Invitrogen). In the experiments β-actin was used as the reference gene. Standard RT-PCR conditions are shown in Table 2.10. For each gene investigated, annealing temperature was optimized for primer sets.

Table 2.10 : RT-PCR concentrations and reaction conditions

Ingredients volume (μl)	Wild Type (control)	Wild type (stressed)	Mutant (control)	Mutant (stressed)
H_20	21-x	21-x	21-x	21-x
Reaction mixture	25	25	25	25
Primer (F+R)	1+1	1+1	1+1	1+1
RNA	x	x	x	x
Enzyme	2	2	2	2
Total	50	50	50	50

'x' is expressed as the RNA volume equivalent to 1μg RNA

Table 2.11 : RT-PCR concentrations and reaction conditions (continued)

Phase	Temperature(^{o}C)	Time	Cycle Number
Cdna synthesis	55	30 min	1
Initial Denaturation	94	2 min	1
Denaturation	94	15 s	36
Annealing	54 -56	90 s	
Extension	68	50 s	
Final Extension	68	5 min	1

2.2.12 Agarose gel electrophoresis of PCR products

To observe PCR products, 1,5% mini or midi agarose gels were prepared. Mini gels were prepared with 0.9 g agarose and 3 µl ethidium bromide added into 60 mL of 1x TBE buffer, which was diluted from 10x stock TBE. 1.5 % midi gels were prepared with 2.25 g agarose and 7.5 µL ethidium bromide was added into 150 mL of 1x TBE buffer. Ten µL of PCR product was mixed with 5 µL 1X loading dye and loaded into the wells. To calculate the length of the PCR products, 10µl of DNA ladder markers (fermentas # SM0338 and SM1293) were also loaded into the remaining wells. The gels were run at 110 V for 45 min, examined under UV light with a transilluminator, and image were obtained using a UV PhotoMW software.

2.2.13 Image Analysis

Image analysis of agarose gel photographs following electrophoresis was done by using Bio-Capt Software (Vilber-Lourmat, Marne LaValle, France). The volume of bands which correspond to mRNA levels were quantified using this program. The volume of a band was defined as the sum of all the intensities included in the defined area of the corresponding band.

2.2.14 cDNA synthesis and real time PCR (Q RT PCR) studies

For the related genes, qRT-PCR was applied to wild type and a selected mutant. β-actin was used as the reference gene and some stress related genes were analyzed. All q-RT PCR experiments were performed in duplicate using specific 96-well plates. 100ng RNA sample was reverse transcribed to corresponding cDNA via Transcriptor High Fidelity cDNA Synthesis Kit (Roche). For each 100 ng RNA

sample, 2µl of random hexamer primers were added and the mixture was completed to 11.4 µl with PCR-grade water. The mixture was denatured at 65^{o}C for 10 min in the thermal cycler with heated lid and cooled immediately on an ice block. Thereafter, 4µl of Transcriptor High Fidelity Reverse Transcriptase Reaction Buffer (5x), 0.5µl Protector RNase Inhibitor, 2µl Deoxynucleotide Mix (10mM), 1µl DTT and 1.1µl l Transcriptor High Fidelity Reverse Transcriptase were added sequentially. The content was mixed carefully and incubated at 55^{o}C for 30 min with a preheated lid. Finally, the reaction was stopped by heating to 85^{o}C for 5 min. Obtained cDNAs were kept at -20^{o}C for Real Time PCR application.

For QRT-PCR, cDNA standards of 1:10; 1:100 and 1:1000 dilution were prepared for each gene. Then 3µl of PCR grade water, 1µl of each forward and reverse primers, 10µl SYBR Green I Master and 5µl cDNA template and standards were mixed in 96-well plates. qRT-PCR reaction conditions are shown in Table 2.11. Relative quantification of expression levels was analyzed using C_T values via $2^{-\Delta\Delta}C_T$ method (Schmittgen *et al*. 2008).

Table 2.12: qRT-PCR conditions

Phase	**Temperature(**°C)	**Time**	**Cycle Number**
Initial Denaturation	95	5 min	1
Denaturation	95	10 s	
Annealing	54 -56	15 s	45
Extension	72	15 s	
Melting	95	5 s	1
Final Extension	68	5 min	1

2.2.15 Relative quantification of Q RT-PCR

Relative gene expression levels were compared by C_T values which is referred as the $2^{-\Delta\Delta}C_T$ method. For reliability of this method, efficiencies of the target genes and reference gene should be very close to each other. This is based on the assumption that each PCR cycle results in duplication of the amount, thus the increase in fold after a certain number of cycles can be calculated. First, the mean C_T values were calculated for both the control sample and the mutants, for each gene. Then ΔC_T values were calculated by subtracting the average C_T values of stressed gene from the previously obtained average control C_T values (C_TReference- C_TTarget).

Efficiency was assumed to be 2 for all genes and the fold increases at threshold cycles were determined by $2^{(\text{reference-target})}$. This calculation is ΔC_T method. For each gene, the expression levels of mutants were compared with those of the wild type. The obtained ΔC_T values were placed as the power of 2 ($2^{\Delta CT}$) and the expression levels were again calculated by dividing the $2^{\Delta CT}$ of "Target gene" with the $2^{\Delta CT}$of "Reference gene". (Schmittgen and Livak, 2008).

2.2.16 Yeast spore dissection and crossing experiments

'Mat a' and 'Mat α' yeast cells were incubated in YMM agar plates for 24 h and transferred to 3g/L potassium acetate-containing agar and mixed with a loop. After an incubation time of 24 h at 30°C, the mixed culture was observed by a light microscope for zigote formation. In the case of zigote formation, they were transferred to 90μl zymolase and 0.5 mg/ml 1M sorbitol mixture and incubated for 1 h at 30^{o}C. Tetrad formation was observed under light microscope. When tetrads are formed, the culture was transferred to a YPD plate and dissected with the help of a micromanipulator.

3. RESULTS

3.1 Obtaining Stress-resistant Generations via Evolutionary Engineering

In this study, for the improvement of osmotic and acid stress resistance in the yeast *Saccharomyces cerevisiae* , evolutionary engineering was applied. For this purpose, ethyl methane sulfonate (EMS) mutagenesis was used to increase the genetic diversity of the initial yeast population. This diverse population was exposed to increasing levels of stress conditions, such as salt, sorbitol and citric acid stresses. Finally populations were obtained that resisted much higher stress levels than the wild type did, and individual mutants were selected randomly from the final populations of each stress selection. To determine relative survival levels compared to wild type *S. cerevisiae*, a high throughput procedure in 96-well plates based on the most probable number (MPN) method was applied. Mutant individuals selected randomly from final mutant populations were tested for their resistance against the stress factor at which they had been selected and some other stress factors which are in particular relevance for yeast industry such as freezing-thawing, heat stress, salt stress, ethanol stress and oxidative stress. Resistances of these strains were compared with those of the wild type strain in terms of percent survival.

3.2 Continuously Increasing NaCl Stress Generations

3.2.1 Sodium chloride stress screening to determine the initial stress levels for selection

EMS mutagenized initial population of *S. cerevisiae* wild-type strain CEN.PK 113.7D was grown in a rotary shaker at 30^0 C and 200 rpm, in 50 ml culture tubes containing 10 ml yeast minimal medium (YMM) with 6.7 g /L yeast nitrogen base without amino acids (Difco) and 20 g /L glucose. To determine the initial stress levels for selection, both wild type and EMS mutagenized culture were screened in

various sodium chloride (NaCl) concentrations and their survival ratio was calculated based on OD_{600} values at 48h. (Fig 3.1)

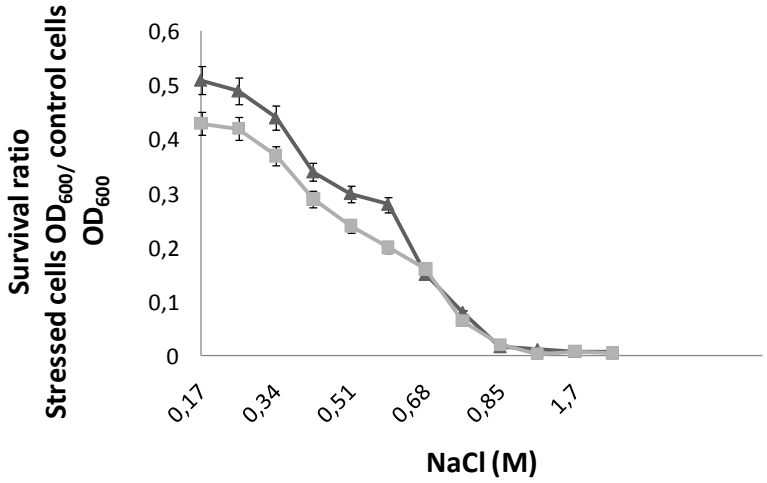


Figure 3.1 : Initial screening of wild type 100 (■) and EMS mutagenized culture 101 (▲) for resistance at varying levels of NaCl stress.

3.2.2 Sodium chloride stress application and obtaining generations

One % (w/v) NaCl initial stress condition was applied to the EMS mutagenized initial population. Each generation was transferred to higher stress levels with an initial OD_{600} of 0.2. In each step of stress application, cells incubated in control condition were used as control groups to calculate survival ratios. OD_{600} measurements were taken at the end of 24^{th} hour of cultivation. Three culture stocks were prepared for each generation and kept at $-80^{o}C$. After application of initial stress condition, the surviving population was exposed to a higher stress level. By this way 40 generations were obtained by increasing salt stress concentration up to a final level of 8.5 % NaCl (1.45M) at generation number 40. That final population which resisted 8.5 % NaCl was named as MO40, spread on YMM agar on Petri plates, and 23 colonies were selected randomly for further analyses. Optical densities and survival ratios of each generation of increasing salt stress selection are shown in Table 3.1 and Figure 3.2 respectively.

Table 3.1 : Optical densities (OD_{600})and survival ratios of continuously increasing salt (NaCl) stress selection after 24h cultivation in YMM with NaCl. NaCl stress levels are shown as (% w/v).

%NaCl (w/v)	**generation**	**OD_{600}control**	**OD_{600} stress**	**24h survival ratio**
1.00	*MO1*	5.32	2.66	0.50
2.00	*MO2*	4.61	3.69	0.80
2.50	*MO3*	4.90	4.31	0.88
3.00	*MO4*	4.02	2.41	0.60
3.50	*MO5*	3.90	2.65	0.68
4.00	*MO6*	3.00	1.62	0.54
4.40	*MO7*	3.70	1.45	0.39
4.80	*MO8*	4.60	1.60	0.35
5.00	*MO9*	5.50	1.25	0.23
5.20	*MO10*	4.40	1.30	0.30
5.40	*MO11*	4.00	1.06	0.27
5.60	*MO12*	3.63	1.02	0.28
5.80	*MO13*	4.30	2.30	0.54
6.00	*MO14*	5.16	2.62	0.54
6.40	*MO15*	3.80	2.10	0.55
6.80	*MO16*	2.75	1.72	0.63
7.20	*MO17*	2.54	1.50	0.60
7.60	*MO18*	5.22	2.68	0.51
5.00	*MO19*	5.01	1.16	0.23
5.25	*MO20*	6.30	2.16	0.34
5.50	*MO21*	5.40	1.40	0.26
5.75	*MO22*	4.60	1.32	0.29
6.00	*MO23*	5.17	1.73	0.33
6.25	*MO24*	5.87	1.42	0.24
6.50	*MO25*	5.35	1.96	0.37
6.75	*MO26*	5.00	1.20	0.24
7.00	*MO27*	5.70	1.44	0.25
7.25	*MO28*	5.28	0.72	0.14
7.50	*MO29*	6.04	1.08	0.18
7.60	*MO30*	6.10	1.20	0.20
7.70	*MO31*	5.63	1.08	0.19
7.80	*MO32*	4.30	1.21	0.28
7.90	*MO33*	6.35	1.40	0.22
8.00	*MO34*	6.80	0.86	0.13
8.10	*MO35*	5.50	0.89	0.16
8.20	*MO36*	4.89	0.80	0.16
8.30	*MO37*	5.02	0.75	0.15
8.40	*MO38*	5.22	0.73	0.14
8.45	*MO39*	4.90	0.76	0.15
8.50	*MO40*	5.10	0.65	0.13

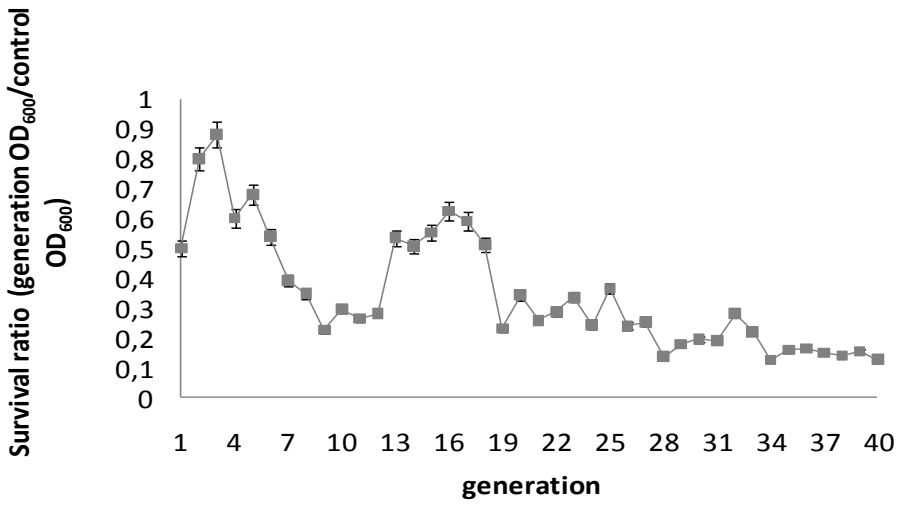


Figure 3.2 : Survival ratios of continuously increasing NaCl stress selection generations during selection procedure.

3. 3 Continuously Increasing Sorbitol Stress Generations

3.3.1 Sorbitol stress screening to determine the initial stress levels for selection

To determine the initial stress level to be used in selection, both wild type and EMS mutagenized culture were screened in various sorbitol concentrations and their survival ratios were calculated based on their OD_{600} values at 48h **(**Fig. 3.3**).**

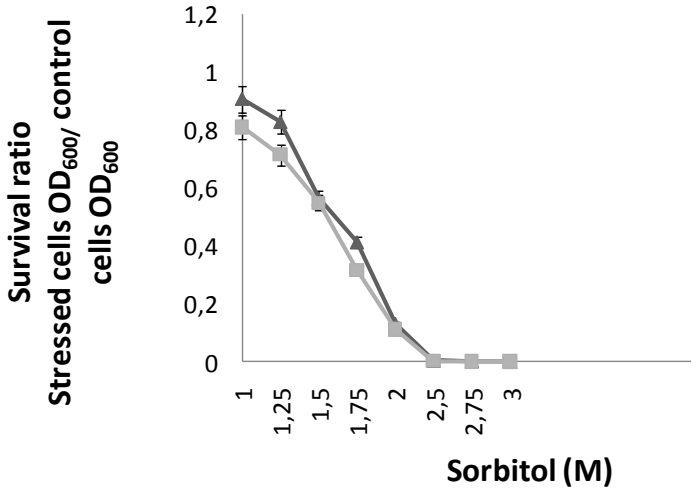


Figure 3.3 : Initial screening of wild type 905 (■) and EMS mutagenized culture 906 (▲) for sorbitol stress.

3.3.2 Sorbitol stress application and obtaining generations

EMS mutagenized culture of CEN.PK113.7D (906) was continuously exposed to increasing levels of sorbitol stress for successive generations. Selection strategy was applied as explained previously. For each generation, OD_{600} results were recorded at 24^{th} hour of cultivation. Three frozen stock samples from each generation were prepared and kept at $-80^{o}C$. Initial stress level of sorbitol was started with 1.2 M and was successively increased to a final concentration of 3M at the 17^{th} generation. Optical densities (OD_{600}) and survival ratios of these generations are shown in Table 3.2 and in Figure 3.4 respectively. The final population coded as SO17 was spread on YMM agar on Petri plates and 23 colonies were selected randomly for further investigations.

Table 3.2 : Optical densities (OD_{600}) and survival ratios of continuously increasing sorbitol stress selection generations after 24 hour of cultivation in YMM with sorbitol.

Sorbitol (M)	**generation**	**OD_{600}control**	**OD_{600} stress**	**24h survival ratio**
1.20	*SO1*	6.80	5.56	0.82
1.40	*SO2*	7.77	6.18	0.80
1.60	*SO3*	8.20	4.45	0.54
1.80	*SO4*	8.01	4.40	0.55
2.00	*SO5*	8.78	3.40	0.39
2.20	*SO6*	6.41	3.50	0.55
2.30	*SO7*	6.70	2.90	0.43
2.40	*SO8*	6.08	3.08	0.51
2.50	*SO9*	6.00	2.68	0.45
2.60	*SO10*	3.96	1.66	0.42
2.70	*SO11*	4.71	1.60	0.34
2.75	*SO12*	4.90	1.30	0.27
2.80	*SO13*	5.21	0.58	0.11
2.85	*SO14*	5.44	0.79	0.15
2.90	*SO15*	4.84	0.44	0.09
2.95	*SO16*	4.92	0.42	0.09
3.00	*SO17*	5.20	0.30	0.06

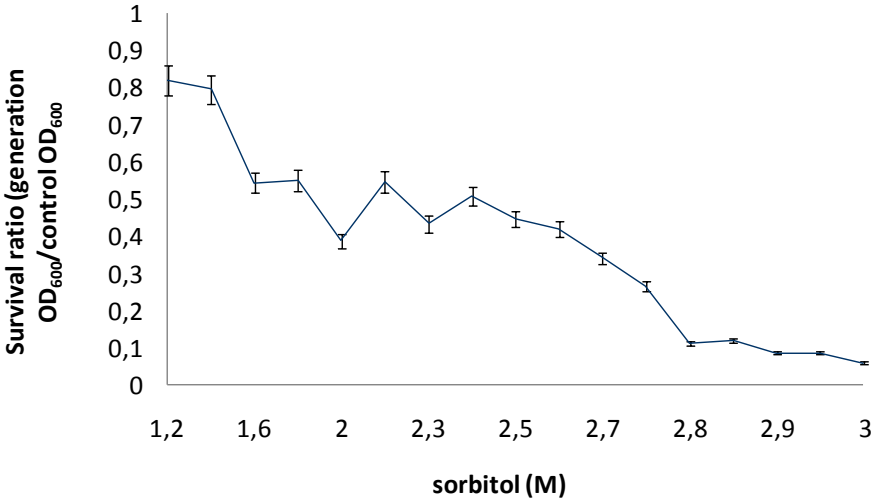


Figure 3.4 : Survival ratios of continuously increasing osmotic (sorbitol) stress selection generations during selection procedure.

3.4 Continuously Increasing Citric Acid Stress Generations

3.4.1 Citric acid screening to determine the initial stress levels for selection

In order to determine initial stress level of citric acid, both wild type and EMS mutagenized culture was screened in various concentrations and their survival ratio was calculated based on optical densities at 48h. (Fig. 3.5)

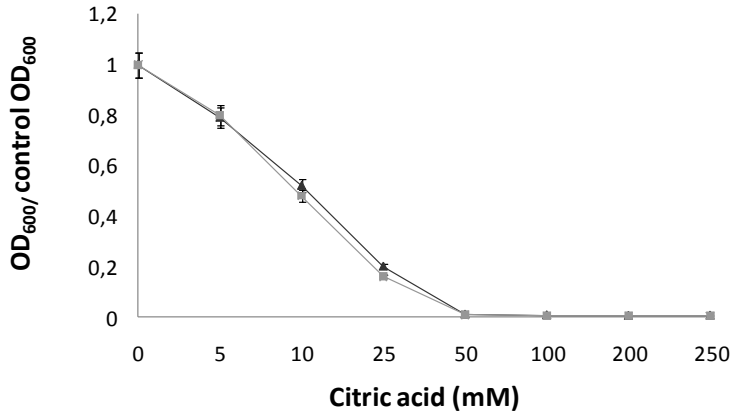


Figure 3.5 : Initial screening of wild type 905 (■) and EMS mutagenized culture 906 (▲) for citric acid stress.

3.4.2 Citric acid stress application and obtaining generations

EMS mutagenized culture of CEN.PK113.7D (906) was continuously exposed to increasing concentrations of citric acid stress for successive generations. Selection strategy was applied as explained previously. OD_{600} results were recorded at the 24^{th} hour of cultivation. Three frozen stock samples from each generation were prepared and kept at $-80^{o}C$. Citric acid concentration started with 10mM and was successively increased to 25mM during 23^{rd} generation. Final population coded as *CA23* was spread on YMM agar on Petri plates and 20 colonies were selected randomly for further analyses. Optical densities and survival ratios of generations are shown in Table 3.3 and Figure 3.6 respectively

Table 3.3 : Optical densities (OD_{600}) and survival ratios of continuously increasing citric acid stress selection generations after 24 hour of cultivation in YMM with citric acid.

Citric acid (mM)	generation	OD_{600}control	OD_{600} stress	survival ratio
10.0	*CA1*	4.10	2.08	0.507
11.0	*CA2*	4.80	2.55	0.531
12.0	*CA3*	4.70	2.68	0.570
13.0	*CA4*	5.21	2.90	0.557
14 .0	*CA5*	4.56	3.24	0.711
15.0	*CA6*	4.56	2.10	0.461
16.0	*CA7*	5.60	2.20	0.393
17.0	*CA8*	5.12	2.06	0.402
18.0	*CA9*	5.76	2.60	0.451
18.5	*CA10*	5.02	1.88	0.375
19.0	*CA11*	5.60	1.71	0.305
19.5	*CA12*	5.30	1.82	0.343
20.0	*CA13*	6.10	1.90	0.311
20.5	*CA14*	4.44	1.20	0.270
21.0	*CA15*	4.50	1.00	0.222
21.5	*CA16*	4.80	1.40	0.292
22.0	*CA17*	5.02	1.02	0.203
22.5	*CA18*	4.20	1.26	0.300
23.0	*CA19*	3.90	1.00	0.256
23.5	*CA20*	4.47	0.56	0.125
24.0	*CA21*	4.50	0.80	0.178
24.5	*CA22*	5.00	0.35	0.070
25.0	*CA23*	5.76	0.22	0.038

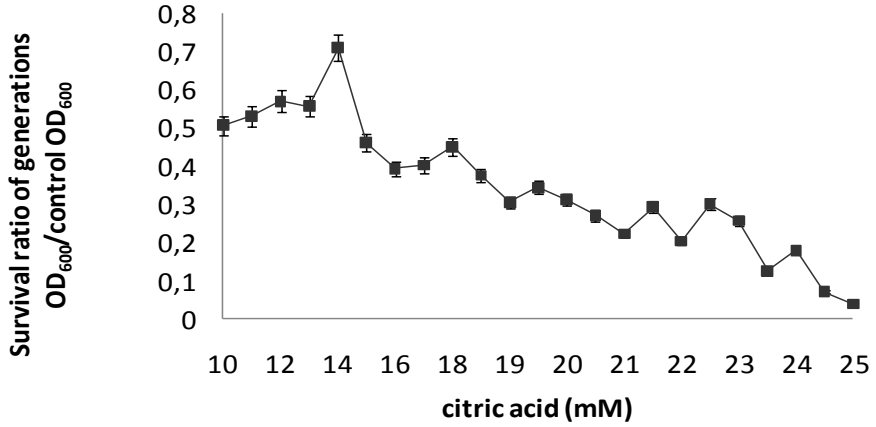


Figure 3.6 : Survival ratios of continuously increasing acid stress selection generations during selection procedure.

3.5 Characterization of Populations and Individual Mutants

3.5.1 Survival of sorbitol resistant mutants upon salt and sorbitol stress

Twenty-three individuals selected from sorbitol stress were investigated on solid YMM for their resistances against sorbitol stress conditions at which they had been selected (results not shown). Among these 23 individuals, 10 mutants were selected as the most resistant ones and tested via 5-tube MPN method for salt and sorbitol resistance. Stress conditions were 2 and 2.5 M for sorbitol, 5 and 8% (w/v) for NaCl. Stress conditions were applied on 96-well microplates continuously and survival ratios were determined at the 72nd h of cultivation upon comparison to the nonstressed control group.

Among continuous sorbitol stress selection mutants; *S17, S18, S15* were observed to have high resistance upon 2M sorbitol stress up to 200, 225 and 146 fold of the wild type resistance, respectively (Table 3.4).

Table 3.4 : % Survival values of sorbitol resistant mutants upon 2M sorbitol cultivation at 72nd hour, determined by 5-tube MPN method.

Sample	cell / ml (2M sorbitol)	cell / ml (0 M sorbitol	% survival (2M sorbitol)	Fold of WT
S6	$1.1x10^6$	$5.4 x10^6$	20.40	20.4
S22	$3.5 x10^6$	$3.5 x10^6$	100.0	100.0
S9	$7.9 x10^5$	$1.7 x10^6$	46.50	46.5
S17 *	$0.7 x10^7$	$3.5 x10^6$	200.0	200.0
S18 *	$5.4 x10^6$	$2.4 x10^6$	225.0	225.0
S15 *	$3.5 x10^6$	$2.4 x10^6$	145.8	145.8
S8	$2.2 x10^6$	$3.5 x10^6$	62.90	62.9
S3	$1.7 x10^6$	$3.5 x10^6$	48.60	48.6
S1	$7.9 x10^5$	$5.4 x10^6$	14.60	14.6
S19	$7.9 x10^4$	$5.4 x10^6$	1.50	1.5
SO17	$1.1 x10^6$	$3.5 x10^6$	31.40	31.4
905	$5.4 x10^4$	$5.4 x10^6$	1.00	-

*Mutants selected for cross-resistance tests

Upon 2.5M sorbitol stress S19 was the most resistant individual with 150 fold survival of the wild type and the survival of SO17 population was nearly 46 fold of the wild type (Table 3.5).

Table 3.5 : % Survival values of sorbitol resistant mutants upon 2.5M sorbitol cultivation at 72nd hour, determined by 5-tube MPN method.

Sample	cell / ml (2.5M sorbitol)	cell / ml (0 M sorbitol	% survival (2.5M sorbitol)	Fold of WT
S6	$1.3 x10^4$	$1.3 x10^6$	1.000	27.8
S22	$2.2 x10^4$	$3.5 x10^6$	0.629	17.5
S9	$2.8 x10^3$	$3.5 x10^6$	0.080	2.2
S17	$9.2 x10^3$	$1.3 x10^6$	0.708	19.7
S18	$2.8 x10^4$	$3.5 x10^6$	0.800	22.2
S15	$1.4 x10^4$	$1.7 x10^6$	0.824	22.9
S8	$3.4 x10^3$	$2.4 x10^6$	0.142	3.9
S3	$2.8 x10^3$	$3.5 x10^6$	0.080	2.2
S1	$1.4 x10^2$	$3.5 x10^6$	0.004	0.1
S19 *	$9.2 x10^4$	$1.7 x10^6$	5.412	150.3
SO17	$2.8 x10^4$	$1.7 x10^6$	1.647	45.8
905	$3.3 x10^3$	$9.2 x10^6$	0.036	-

*Mutant selected for cross-resistance tests

Sorbitol stress resistant mutants were investigated continuously in 5% w/v and 8% w/v NaCl concentrations. MPN results are shown in Table 3.6.

Table 3.6 : % Survival values of sorbitol resistant mutants upon 5% v/w NaCl ve 8% v/w NaCl stress cultivation at 72nd hour, determined by 5-tube MPN analyses.

Sample	**Cell / ml (5% NaCl)**	**Cell / ml (8% NaCl)**	**Cell / ml (0% NaCl)**	**% survival (5% NaCl)**	**% survival (8% NaCl)**	**Fold of WT (5% NaCl)**	**Fold of WT (8% NaCl)**
*S6 **	2.4×10^6	9.2×10^4	1.3×10^6	184.6	7.08	10.6	707.7
S22	1.7×10^6	2.4×10^3	3.5×10^6	48.6	0.07	2.8	6.9
*S9**	1.1×10^6	5.4×10^4	3.5×10^6	31.4	1.54	1.8	154.3
S17	3.5×10^5	1.3×10^2	1.3×10^6	26.9	0.01	1.5	1.0
S18	2.2×10^4	2.4×10^2	3.5×10^6	0.6	0.01	0.0	0.7
S15	2.3×10^1	3.5×10^2	1.7×10^6	0.0	0.02	0.0	2.1
S8	2.3×10^1	3.5×10^2	2.4×10^6	0.0	0.01	0.0	1.5
*S3 **	3.5×10^6	2.4×10^5	3.5×10^6	100.0	6.86	5.7	685.7
S1	9.2×10^5	2.4×10^2	3.5×10^6	26.3	0.01	1.5	0.7
S19	9.2×10^5	3.5×10^2	1.7×10^6	54.1	0.02	3.1	2.1
SO17	3.5×10^6	1.7×10^4	1.7×10^6	205.9	1.00	11.8	100.0
905	1.6×10^6	9.2×10^2	9.2×10^6	17.4	0.01	-	-

*Mutants selected for cross-resistance tests

Selected sorbitol resistant individuals were investigated via spotting assay on 2M sorbitol (YPD) plates. There was no significant resistance difference observed for for sorbitol stress. Results are shown in Fig. 3.7.

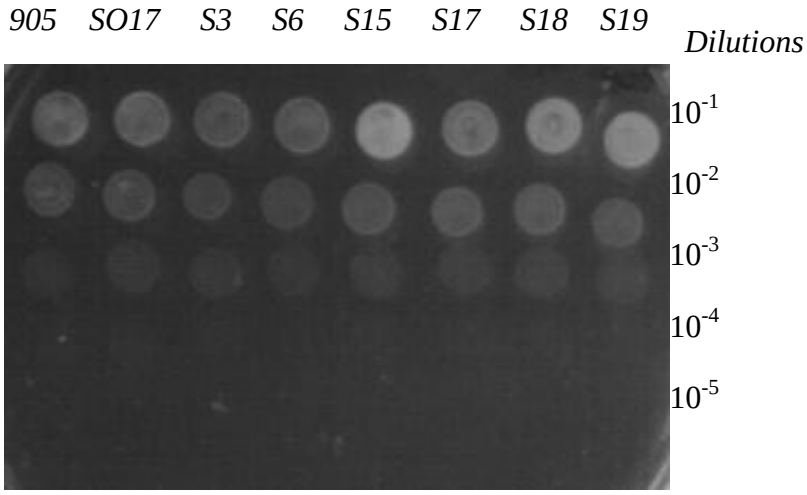


Figure 3.7 : Spotting assay resultsof selected sorbitol resistant individuals on 2M sorbitol (YPD) plates

S15, S18 and *S19* were also resistant upon spotting assay on 2M sorbitol agar. *S3, S6, S9* had high resistance upon both 5 and 8 % NaCl stress. Among sorbitol resistant mutants, *S6, S17, S18, S15, S19* were selected to be further investigated for cross-resistance tests

3.5.2 Survival of sodium chloride resistant mutants upon salt and sorbitol stress

Salt stress selection individuals were tested on solid YMM for their resistances against NaCl stress conditions at which they had been selected (data not shown). Ten of the most resistant individuals were then selected to be investigated via 5-tube MPN method for salt and sorbitol resistance. Stress concentrations were 5%w/v and 8% w/v for NaCl. All stress conditions were applied on 96-well microplates continuously and survival ratios were determined upon comparison to the nonstressed control group at 72^{nd} hour of cultivation. Results are shown on Table 3.7 and Table 3.8.

Table 3.7 : % Survival values of salt resistant mutants upon 5% NaCl (w/v) stress cultivation at 72^{nd} hour of cultivation, determined by 5-tube MPN method.

Sample	**Cell / ml (5% NaCl)**	**Cell / ml (0% NaCl)**	**% Survival (5% NaCl)**	**Fold of WT**
T12	3.5×10^5	7.0×10^5	50.0	22.9
T23	2.4×10^5	7.0×10^6	3.4	1.6
T4	1.7×10^6	3.5×10^6	48.6	22.3
*T13 **	3.5×10^6	3.5×10^6	100.0	45.9
*T19 **	5.4×10^6	1.7×10^6	317.6	145.7
T20	2.4×10^4	9.2×10^6	0.3	0.1
T8	1.7×10^6	3.5×10^6	48.6	22.3
T15	1.7×10^6	3.5×10^6	48.6	22.3
*T10 **	1.7×10^6	1.7×10^6	100.0	45.9
T11	2.4×10^5	1.3×10^6	18.5	8.5
MO40	1.7×10^6	3.5×10^6	48.6	22.3
905	2.4×10^4	1.1×10^6	2.2	-

*Mutants selected for cross-resistance tests

Table 3.8 : Survival values of salt resistant mutants upon 8% (w/v) NaCl stress at 72nd hour of cultivation, determined by 5-tube MPN method.

Sample	Cell / ml (8% NaCl)	Cell / ml (0% NaCl)	% Survival (8% NaCl)	Fold of WT
T12	2.4×10^4	3.5×10^6	0.69	68.6
T23	2.4×10^2	7.9×10^5	0.03	3.0
T4	2.4×10^4	2.4×10^6	1.00	100.0
T13 *	9.2×10^4	5.4×10^6	1.70	170.4
T19	1.6×10^4	5.4×10^6	0.30	29.6
T20	2.4×10^2	2.2×10^6	0.01	1.1
T8 *	2.4×10^5	3.5×10^6	6.86	685.7
T15 *	2.4×10^4	1.7×10^6	1.41	141.2
T10 *	2.4×10^5	5.4×10^6	4.44	444.4
T11	5.4×10^3	1.4×10^6	0.39	38.6
MO40	2.4×10^6	7.9×10^5	303.80	30379
905	2.4×10^2	1.7×10^6	0.01	-

* Mutants selected for cross-resistance tests

The most resistant individuals were selected and investigated via spotting assay on 0.7M NaCl (YPD) plates. Results are shown in Fig. 3.8.

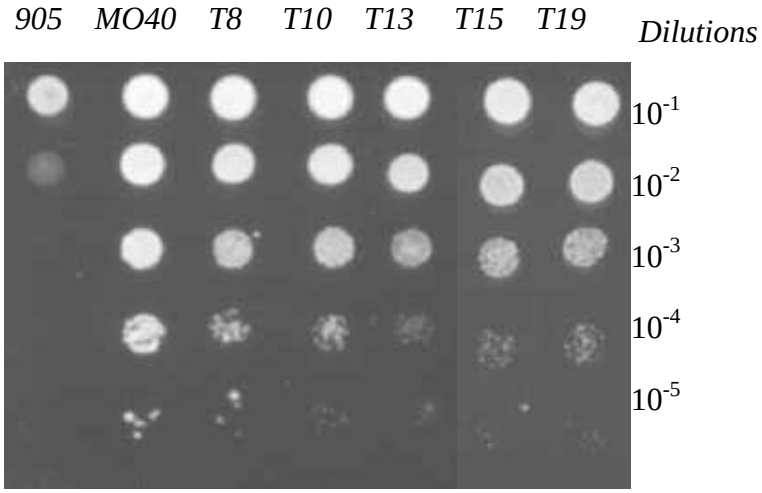


Figure 3.8 : Spotting assay results of selected salt resistant individuals on 0.7M NaCl (YPD) plates.

T13 was observed to have a high survival ratio in 5% (w/v) NaCl stress, whereas *T8* and *T10* have high survival ratios at 8% NaCl stress. Salt stress selection individuals were also tested in 2M and 2.5M sorbitol stress. Results are shown in Table 3.9 and Table 3.10.

Table 3.9 : Survival values of salt resistant mutants upon 2M sorbitol cultivation at 72nd hour, determined by 5-tube MPN method.

Sample	Cell / ml (2M Sorbitol)	Cell / ml (0M Sorbitol)	% Survival (2M Sorbitol)	Fold of WT
T12	1.1×10^6	7.0×10^5	157	1.3
T23	1.3×10^7	7.0×10^6	186	1.6
T4	2.2×10^5	3.5×10^6	6	0.1
T13	1.4×10^5	3.5×10^6	4	0.0
T19	1.3×10^6	1.7×10^6	76	0.6
T20	1.3×10^6	9.2×10^6	14	0.1
T8	1.7×10^6	3.5×10^6	49	0.4
T15	1.3×10^6	3.5×10^6	37	0.3
T10	7.9×10^5	1.7×10^6	46	0.4
T11	1.7×10^6	1.3×10^6	131	1.1
MO40	2.8×10^6	3.5×10^6	80	0.7
905	1.3×10^6	1.1×10^6	118	-

Table 3.10 : Survival values of salt resistant mutants upon 2.5M sorbitol cultivation at 72nd hour, as determined by 5-tube MPN method.

Sample	Cell / ml (2.5M Sorbitol)	Cell / ml (0M Sorbitol)	% Survival (2.5M Sorbitol)	Fold of WT
T12	280	3.5×10^6	0.008	0.11
T23	79	7.9×10^5	0.010	0.13
T4	79	2.4×10^6	0.003	0.04
T13	280	5.4×10^6	0.005	0.07
T19	79	5.4×10^6	0.001	0.02
T20	23	2.2×10^6	0.001	0.01
T8	23	3.5×10^6	0.001	0.01
T15	240	1.7×10^6	0.014	0.19
T10	23	5.4×10^6	0.000	0.01
T11	49	1.4×10^6	0.004	0.05
MO40	79	7.9×10^5	0.010	0.13
905	1300	1.7×10^6	0.076	-

3.5.3 Cross-resistance studies of sodium chloride and sorbitol stress resistant individuals

Five resistant individuals of salt stress (*T8, T10, T13, T15, T19*) were tested for cross-resistance. Among sorbitol stress individuals, six of the most resistant ones (*S3, S6, S15, S17, S18, S19*) were selected for cross-resistance tests. Ethanol and H_2O_2 stresses were applied continuously on 96-well plates, based on 5-tube MPN method. Freeze-thaw and heat stresses were applied as pulse stresses prior to MPN tests.

3.5.3.1 Ethanol stress resistance of sodium chloride stress resistant mutants

6% (v/v) ethanol resistance of NaCl stress resistant individuals were investigated. Cell growth at 48^{th} hour and 72^{nd} hour of incubation is shown in Table 3.12 and Table 3.13. At 6% (v/v) ethanol stress, *T8* and *T13* have shown the highest resistance levels. On the other hand, at 72 hour, 6% ethanol cross-resistance results showed that the differences in resistances became less significant between the mutants and the wild type. Especially, while *T8* and *T13* have higher resistance compared to wild type (905) after 48 h, survival of *905* has almost reached to the same levels as *T8* and *T13* after 72 h. Results are shown in Table 3.11 and Table 3.12.

Table 3.11 : MPN results of salt stress resistant individuals upon 6% EtOH stress (48^{th} hour)

Sample	**Cell / ml (6% EtOH)**	**Cell / ml (0% EtOH)**	**%Survival (6% EtOH)**	**Fold of WT**
T8	7.9×10^6	4.9×10^6	161	6.7
T10	1.7×10^6	3.3×10^6	52	2.1
T13	3.3×10^6	1.7×10^6	194	8.1
T15	2.4×10^5	4.9×10^6	5	0.2
T19	7.9×10^5	1.7×10^6	46	1.9
MO40	2.4×10^5	1.7×10^6	14	0.6
905	7.9×10^5	3.3×10^6	24	-

Table 3.12 : MPN results of salt stress resistant individuals upon 6% EtOH (v/v) stress (72nd hour).

Sample	Cell / ml (6% EtOH)	Cell / ml (0% EtOH)	%Survival (6% EtOH)	Fold of WT
T8	7.9 x10^6	4.9 x10^6	161	1,1
T10	1.7 x10^6	3.3 x10^6	52	0,3
T13	3.3 x10^6	1.7 x10^6	194	1,3
T15	3.3 x10^6	4.9 x10^6	67	0,5
T19	1.3 x10^6	1.7 x10^6	76	0,5
MO40	3.3 x10^5	1.7 x10^6	19	0,1
905	7.9 x10^5	3.3 x10^6	148	-

3.5.3.2 Ethanol stress resistance of sorbitol stress resistant mutants

Six of the most resistant sorbitol mutants *S3, S6, S15, S17, S18, S19* were investigated for ethanol stress at 6% (v/v) ethanol concentration.72 hour MPN results are shown in Table 3.13.

Table 3.13 : MPN results of sorbitol stress resistant individuals upon 6% EtOH stress (72nd hour).

Sample	Cell / ml (6% EtOH)	Cell / ml (0% EtOH)	%Survival (6% EtOH)	Fold of WT
S3	2.4 x10^5	1.3 x10^6	18.46	2.74
S6	1.3 x10^5	3.3 x10^6	3.94	0.59
S15	4.9 x10^5	9.2 x10^5	53.26	7.91
S17	7.9 x10^5	1.3 x10^6	60.77	9.03
S18	2.4 x10^6	1.7 x10^6	141.18	20.98
S19	3.3 x10^5	4.9 x10^6	6.73	1.00
SO17	3.3 x10^6	7.9 x10^6	41.77	6.21
905	3.3 x10^5	4.9x10^6	6.73	-

Generally, sorbitol resistant strains were more resistant than the wild type under ethanol stress conditions, except *S6* and *S19*.

3.5.3.3 Oxidative stress resistance of sodium chloride stress resistant mutants

Survival of NaCl stress resistant individuals was determined upon 0.8 mM hydrogen peroxide stress using five tube MPN methodology. Cell growth at the 72nd hour of incubation is shown in Table 3.14. In 0.8 mM hydrogen peroxide stress, salt stress

resistant individuals did not have a significantly high survival ratio. On the other hand, last population of salt stress had approximately 19.4 -fold survival compared to the wild type.

Table 3.14: MPN results of salt stress resistant individuals upon 0.8 mM H_2O_2 stress (72^{nd} hour).

Sample	**Cell / ml 0.8mM H_2O_2**	**Cell / ml 0mM H_2O_2**	**%Survival 0.8mM H_2O_2**	**Fold of WT**
T8	7.9×10^3	4.9×10^6	0.16	1.6
T10	3.3×10^3	3.3×10^6	0.10	1.0
T13	7.9×10^3	1.7×10^6	0.46	4.6
T15	3.3×10^2	4.9×10^6	0.01	0.1
T19	3.3×10^2	1.7×10^6	0.02	0.2
MO40	3.3×10^4	1.7×10^6	1.94	19.4
905	3.3×10^3	3.3×10^6	0.10	-

3.5.3.4 Oxidative stress resistance of sorbitol stress resistant mutants

Survival of sorbitol stress resistant individuals was investigated via 5-tube MPN methodology in 0.8 mM H_2O_2 stress conditions. Cell growth and the survival at the 72^{nd} hour of incubation is shown in Table 3.15.

Table 3.15 : MPN results of sorbitol stress resistant individuals upon 0.8 mM H_2O_2 stress (72^{nd} hour).

Sample	**Cell / ml 0.8 mM H_2O_2**	**Cell / ml 0 mM H_2O_2**	**%Survival 0.8 mM H_2O_2**	**Fold of WT**
S3	4.9×10^3	1.3×10^6	0.3769	838
S6	1.3×10^3	3.3×10^6	0.0394	88
S15	7.9×10^1	9.2×10^5	0.0086	19
S17	7.9×10^2	1.3×10^6	0.0608	135
S18	5.4×10^2	1.7×10^6	0.0318	71
S19	7.9×10^4	4.9×10^6	1.6122	3583
SO17	1.1×10^5	7.9×10^6	1.3924	3094
905	2.2×10^3	4.9×10^6	0.00045	-

Generally, sorbitol stress resistant individuals had dramatically higher survival ratios under H_2O_2 stress, compared to wild type. Especially, *S19* has 3583-fold survival of the wild type strain (Table 3.15)

3.5.3.5 -196^{0}C freezing-thawing stress resistance of salt stress resistant mutants

Survival of salt stress-resistant individuals was investigated in pulse application of -196^{0}C freezing-thawing stress. 5-tube MPN results after 72 h of incubation are shown in Table 3.16.

Table 3.16 : MPN results of salt stress resistant individuals upon -196^{0}C freezing-thawing stress (72nd hour).

Sample	Cell / ml Freeze-thaw (-196^{0}C)	Cell / ml control	%Survival Freeze-thaw (-196^{0}C)	Fold of WT
T8	3.3 x10^3	4.9 x10^6	0.07	0.01
T10	1.7 x10^3	3.3 x10^6	0.05	0.01
T13	7.9 x10^3	1.7 x10^6	0.46	0.06
T15	4.9 x10^3	4.9 x10^6	0.10	0.01
T19	7.9 x10^4	1.7 x10^6	4.65	0.64
MO40	4.9 x10^5	1.7 x10^6	28.82	3.96
905	2.4 x10^5	3.3 x10^6	7.27	-

At the end of 72 h of cultivation, none of the strains were found to be resistant. However the final population *MO40* from which the mutants had been selected, was approximately 4-fold more resistant than the wild type (Table 3.16).

3.5.3.6 -196^{0}C and -20^{0}C freezing-thawing stress resistance of sorbitol stress resistant mutants

Sorbitol stress resistant individuals were tested by pulse application of -196^{0}C and -20^{0}C freezing-thawing stress, followed by MPN analysis. 5-tube MPN results after 72 h of incubation are shown in Table 3.17.

Table 3.17 : MPN results of sorbitol stress resistant individuals upon -196^{0}C freezing-thawing stress (72nd hour).

Sample	Cell / ml Freeze-thaw (-196^{0}C)	Cell / ml control	%Survival Freeze-thaw (-196^{0}C)	Fold of WT
S3	4.9 x10^4	1.3 x10^6	3.77	26.9
S6	3.3 x10^1	3.3 x10^6	0.00	0.0
S15	9.2 x10^4	9.2 x10^5	10.00	71.4
S17	3.3 x10^4	1.3 x10^6	2.54	18.1
S18	1.3 x10^4	1.7 x10^6	0.76	5.5
S19	7.9 x10^4	4.9 x10^6	1.61	11.5
SO17	4.9 x10^4	7.9 x10^6	0.62	4.4
905	7.0 x10^3	4.9 x10^6	0.14	-

Generally, under -196^{0}C freeze-thaw stress, sorbitol resistant individuals were more resistant compared to the wild type (Table 3.17). Under -196^{0}C freeze-thaw stress, *S3*, *S19*, *S17* and especially *S15* individuals have shown resistance. Under -20^{0}C freeze thaw stress, however, only *S18* had almost the same resistance level as the wild type (905). All other mutants were found to be sensitive to -20^{0}C freeze-thaw stress (Table 3.18).

Table 3.18 : MPN results of sorbitol stress resistant individuals upon -20^{0}C freezing-thawing stress (72nd hour).

Sample	Cell / ml Freeze-thaw (-20^{0}C)	Cell / ml control	%Survival Freeze-thaw (-20^{0}C)	Fold of WT
S3	3.3 x10^5	3.3 x10^6	10.0	0.14
S6	4.9 x10^5	3.3 x10^6	14.8	0.20
S15	3.3 x10^5	1.3 x10^6	25.4	0.35
S17	1.3 x10^6	4.9 x10^6	26.5	0.36
S18	3.3 x10^6	3.3 x10^6	100.0	1.38
S19	3.3 x10^6	4.9 x10^6	67.3	0.93
SO17	1.3 x10^5	4.9 x10^6	2.7	0.04
905	2.4 x10^6	3.3 x10^6	72.7	-

3.5.3.7 60^0C Heat stress resistance of salt stress resistant mutants

Survival of salt stress resistant individuals was determined upon pulse application of 60^0C heat stress. 5-tube MPN results after 72 h of incubation are shown in Table 3.19.

Table 3.19 : MPN results of salt stress resistant individuals upon 60^0C heat stress (72^{nd} hour).

Sample	Cell / ml Heat (60^0C)	Cell / ml control	%Survival Heat (60^0C)	Fold of WT
T8	7.0×10^3	4.9×10^6	0.14	0.06
T10	2.1×10^3	3.3×10^6	0.06	0.03
T13	4.9×10^2	1.7×10^6	0.03	0.01
T15	7.0×10^3	4.9×10^6	0.14	0.06
T19	4.9×10^3	1.7×10^6	0.29	0.12
MO40	2.4×10^4	1.7×10^6	1.41	0.59
905	7.9×10^4	3.3×10^6	2.39	

In heat stress all of the salt stress resistant mutants and final population had lower survival compared to wild type

3.5.3.8 60^0C Heat stress resistance of sorbitol stress resistant mutants

Survival of sorbitol stress resistant individuals were investigated in pulse application of 60^0C heat stress. 5-tube MPN results after 72^{nd} hour of incubation is shown in Table 3.20.

Table 3.20 : MPN results of sorbitol stress resistant individuals upon 60^0C heat stress (72^{nd} hour).

Sample	Cell / ml Heat (60^0C)	Cell / ml control	%Survival Heat (60^0C)	Fold of WT
S3	4.9×10^3	1.3×10^6	0.38	0.038
S6	4.9×10^2	3.3×10^6	0.01	0.001
S15	2.4×10^5	9.2×10^5	26.09	2.609
S17	3.3×10^3	1.3×10^6	0.25	0.025
S18	1.7×10^2	1.7×10^6	0.01	0.001
S19	2.4×10^3	4.9×10^6	0.05	0.005
SO17	3.3×10^3	7.9×10^6	0.04	0.004
905	4.9×10^5	4.9×10^6	10.00	-

According to the results, five individuals and the final population of sorbitol stress had significantly lower survival ratios than the wild type. Only *S15* mutant had a higher survival of about 2.6-fold of the wild type.

3.5.3.9 Heavy metal resistance of the salt and sorbitol stress individuals

Heavy metal resistance of sorbitol and salt-stress resistant individuals were investigated by spotting assay. For this purpose, overnight cultures were adjusted to an OD_{600} unit of 8 by centrifugation and resuspended in minimal medium without dextrose. They were then diluted to 10^{-5} in 96-well plates. Five µl from each dilution were transferred and dropped on Petri plates containing specific heavy metal stress factors. Dilution factors of the cells increases from top to bottom on the Petri plates. Results were obtained after 48 h of incubation. Salt-stress resistant individuals were arranged from left to right as *T19, T15, T13, T10, T8, MO40* (final population), *905* (wild type). Sorbitol stress resistant individuals were arranged from left to right as *S19, S18, S17, S15, S6, S3, SO17* (final population), *905* (wild type) (Figure 3.9)

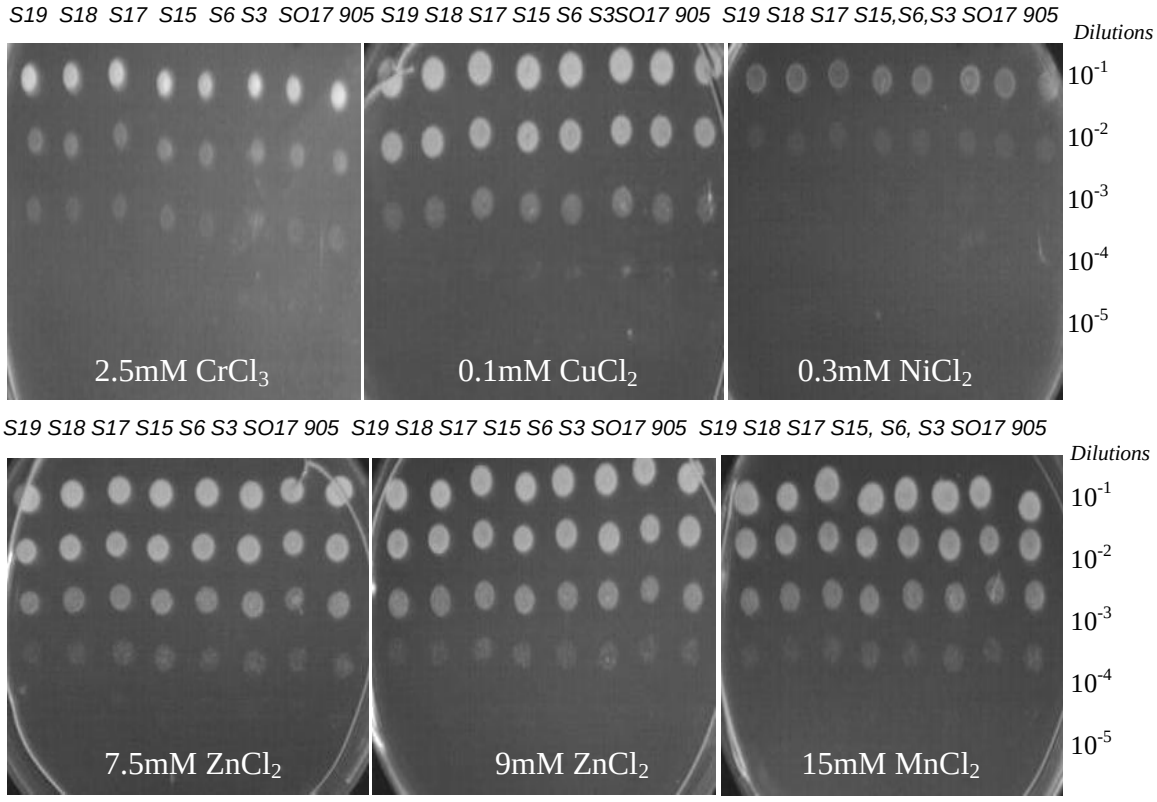


Figure 3.9 : Heavy metal resistance of sorbitol stress resistant individuals (48 hour).

Sorbitol stress-resistant individuals were investigated for heavy metal stress responses. Concentrations and types of these metals were 2.5mM $CrCl_3$, 0.1mM $CuCl_2$, 0.3mM $NiCl_2$, 7.5mM $ZnCl_2$, 9mM $ZnCl_2$ and 15mM $MnCl_2$. Compared to wild type strain, sorbitol stress-resistant individuals did not have a significantly different resistance to these heavy metals. Results are shown in Fig. 3.9.

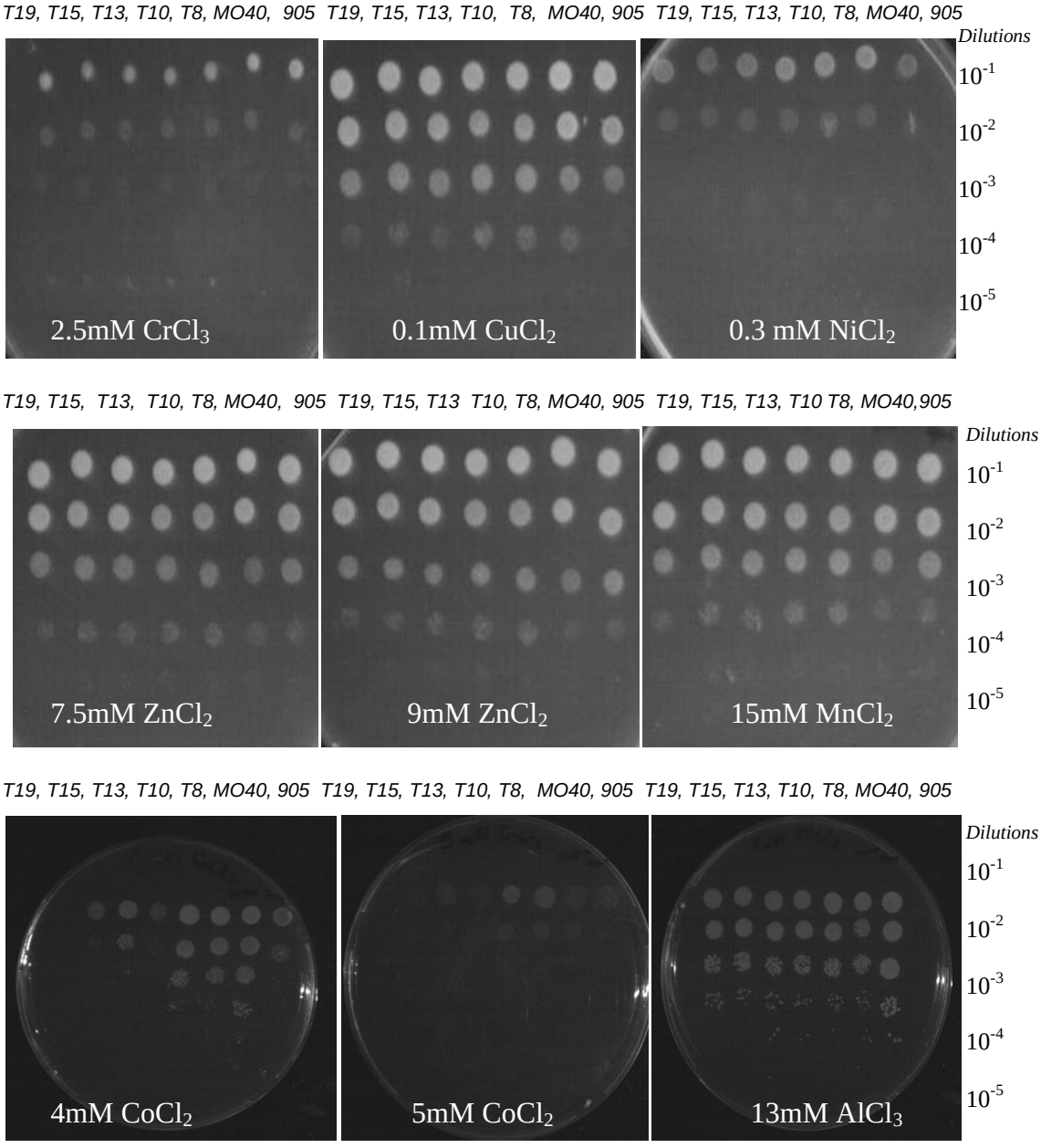


Figure 3.10 : Heavy metal resistance of salt stress resistant individuals (48 hour).

Salt stress-resistant individuals were also tested for heavy metal stress resistance. Concentrations and types of these metals were 2.5mM $CrCl_3$, 0.1mM $CuCl_2$, 0.3mM $NiCl_2$, 7.5mM $ZnCl_2$, 9mM $ZnCl_2$, 15mM $MnCl_2$, 4mM $CoCl_2$, 5mM $CoCl_2$ and 13mM $AlCl_3$. When compared to the wild type strain (905), salt resistant individuals did not have higher resistance to $CrCl_3$, $NiCl_2$, $ZnCl_2$ and $MnCl_2$ than the wild type. On the other hand, they had ten-fold higher resistance to $CuCl_2$, and up to 10^4-fold higher resistance to $CoCl_2$. However, under $AlCl_3$ stress conditions, salt-resistant individuals had lower resistance than the wild type, indicating sensitivity (Fig. 3.10).

3.5.4 Catalase Activity Analysis of Sorbitol-and Salt Stress-Resistant Individuals

In this part of the study, catalase activity tests were performed in order to obtain additonal information about oxidative stress resistance. Catalase activities of sorbitol and salt resistant individuals were determined both in 1mM H_2O_2 containing medium and yeast minimal medium without any stress. Catalase activity was calculated via the ratio of absorbance difference to total protein amount which was obtained by Bradford method using bovine serum albumin (BSA) standards (Li and Schellhorn, 2007).

Table 3.21 : Catalase activity of sorbitol-resistant individuals in YMM (control group).

	ΔOD_{240} /2min	**OD_{595}**	**Protein (mg/ml)**	**Activity ΔA_{240}/min/mg protein**	**Fold of WT**
905(wild type)	0.0075	0.214	0.561	0.0134	1.00
SO17	0.0030	0.121	0.139	0.0216	1.62
S3	0.0100	0.185	0.483	0.0207	1.55
S6	0.0050	0.143	0.369	0.0136	1.01
S9	0.0110	0.163	0.300	0.0367	2.74
S15	0.0050	0.119	0.131	0.0382	2.86
S17	0.0030	0.123	0.146	0.0205	1.54
S18	0.0125	0.115	0.293	0.0427	3.19
S19	0.0180	0.107	0.085	0.2118	15.85

Table 3.22 : Catalase activity of sorbitol resistant individuals under 1 mM H_2O_2 continuous stress conditions.

	ΔOD_{240} /2min	**OD_{595}**	**Protein (mg/ml)**	**Activity ΔA_{240}/min/mg protein**	**Fold of WT**
905(wild type)	0.007	0.190	0.657	0.0107	1.00
SO17	0.065	0.136	0.196	0.3316	30.99
S3	0.0032	0.150	0.510	0.0063	0.59
S6	0.0036	0.238	0.833	0.0043	0.40
S9	0.006	0.241	0.844	0.0071	0.66
S15	0.0024	0.086	0.274	0.0088	0.82
S17	0.0056	0.287	1.014	0.0055	0.51
S18	0.0184	0.174	0.598	0.0308	2.88
S19	0.012	0.202	0.701	0.0171	1.60

Catalase activities of sorbitol stress-resistant mutants in control medium were approximately two-or three-fold of the wild type. Only *S19* had a significantly higher activity about 19 fold of the wild type (Table 3.21). On the other hand upon exposure to 1mM H_2O_2 stress, *S18* had 3 fold activity than of wild type. However, other mutants did not have a significant catalase activity, when compared to the wild type (Table 3.22).

Catalase activities of salt sress-resistant individuals were also investigated in control conditions and upon exposure to 1 mM H_2O_2 continuous stress. Compared to wild type, all salt stress resistant individuals had higher catalase activity both in control and stress conditions. *T8* seems to be one of the highest catalase-producing individual under both control and oxidative stress conditions. As expected, catalase activity increased for each culture upon addition of H_2O_2 to the medium (Table 3.23, Table 3.24).

Table 3.23 : Catalase activity of salt resistant individuals in YMM (control group).

	ΔOD_{240}/ 2min	OD_{595}	Protein (mg/ml)	Activity ΔA_{240}/min/ mg protein	Fold of WT
905(wild type)	0.0015	0.234	0.674	0.0022	1
MO40(final pop.)	0.0045	0.210	0.597	0.0075	3.41
T8	0.0175	0.243	0.702	0.0249	11.32
T10	0.007	0.213	0.607	0.0115	5.23
T13	0.012	0.255	0.740	0.0162	7.36
T15	0.0225	0.231	0.664	0.0339	15.41
T19	0.0095	0.209	0.594	0.0160	7.27

Table 3.24 : Catalase activity of salt resistant individuals in 1 mM H_2O_2 continuous stress.

	ΔOD_{240}/ 2min	OD_{595}	Protein (mg/ml)	Activity ΔA_{240}/min/ mg protein	Fold of WT
905(wild type)	0.004	0.211	0.608	0.0066	1
MO40(final pop.)	0.013	0.197	0.565	0.0230	3.48
T8	0.028	0.184	0.525	0.0533	8.08
T10	0.021	0.205	0.589	0.0356	5.39
T13	0.027	0.17	0.482	0.0560	8.48
T15	0.009	0.161	0.455	0.0198	3.00
T19	0.014	0.162	0.458	0.0306	4.64

3.5.5 Genetic stability of resistant individuals

Among sorbitol, salt and citric acid- resistant individuals, genetic stability of *S3, S18, S19, T8, T10, T19, C1,C4* and *C6* were investigated. To test their genetic stability, they were cultivated in YMM for successive times overnight, without any stress condition. After each sequential cultivation of resistant strains, MPN was applied continuously at 8% (w/v) NaCl stress for salt-resistant individuals, 2.5 M sorbitol stress for sorbitol-resistant individuals and 25mM citric acid for acid resistant mutants. At the end of 96 h of cultivations, plates were observed. Survived cell number and genetic stability of cells were determined according to 5-tube MPN table. Genetic stability results for sorbitol stress-resistant individuals are shown in Fig 3.11, 3.12 and 3.13.

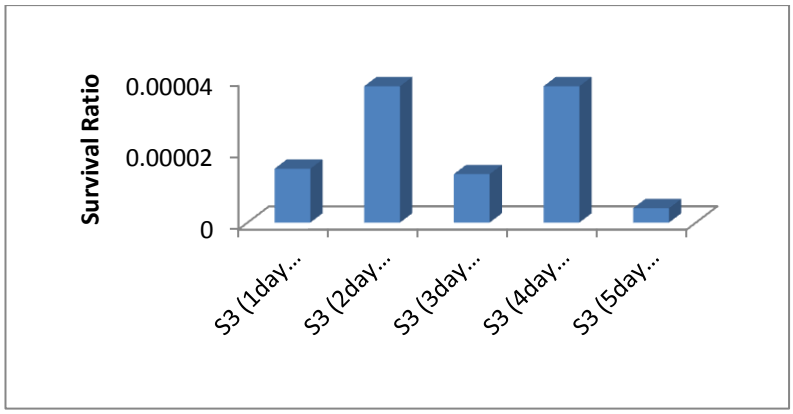


Figure 3. 11 : Survival ratios of *S3* individual through 5 successive cultivations in nonselective medium, when exposed to 2.5 M sorbitol stress for MPN assay.

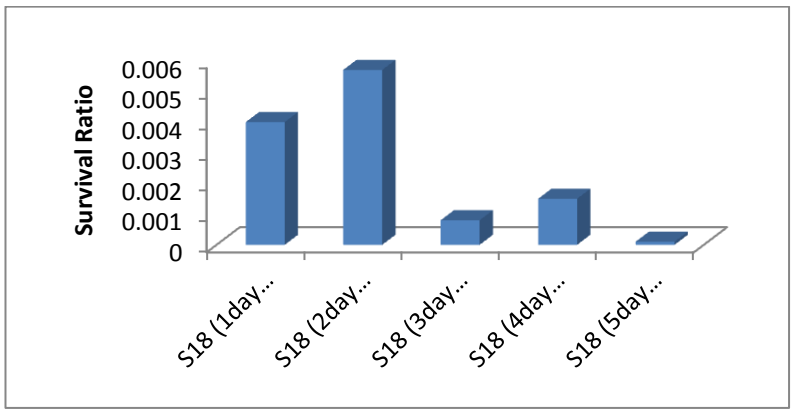


Figure 3.12 : Survival ratios of *S18* individual at each of the 5 successive cultivations in nonselective medium, when exposed to 2.5 M sorbitol stress for MPN assay.

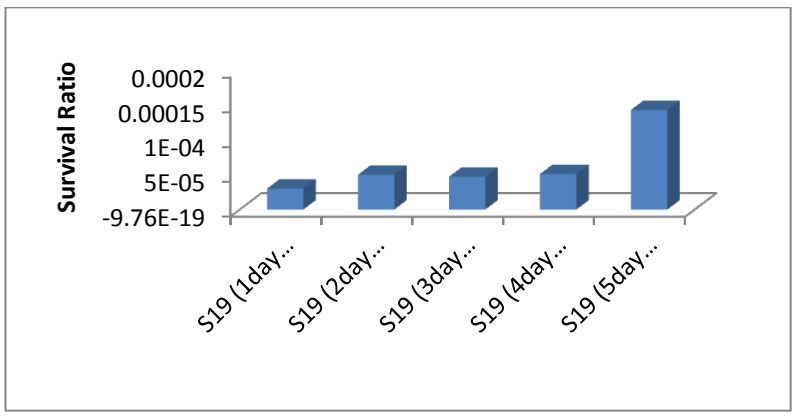


Figure 3.13 : Survival ratios of *S19* individual at each of the 5 successive cultivations in nonselective medium, when exposed to 2.5 M sorbitol stress for MPN assay.

Among sorbitol resistant individuals, *S19* seems to keep its high resistance level better than *S3* and *S18.* Stability results for salt stress-resistant individuals are shown Figures 3.14, 3.15 and 3.16.

Among salt resistant individuals, *T8* and *T19* seem to have a genetically stable resistance to NaCl stress. (Figures 3.14, 3.15 and 3.16)

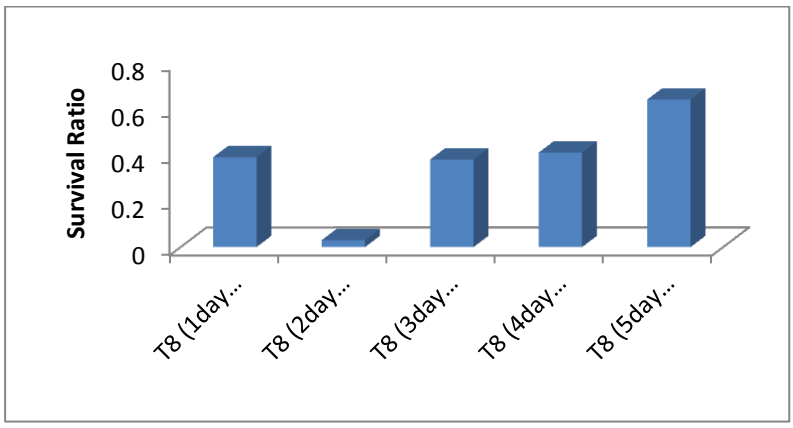


Figure 3.14 : Survival ratios of *T8* individual at each of the 5 successive cultivations in nonselective medium, when exposed to 0.8% (w/v) NaCl stress for MPN assay.

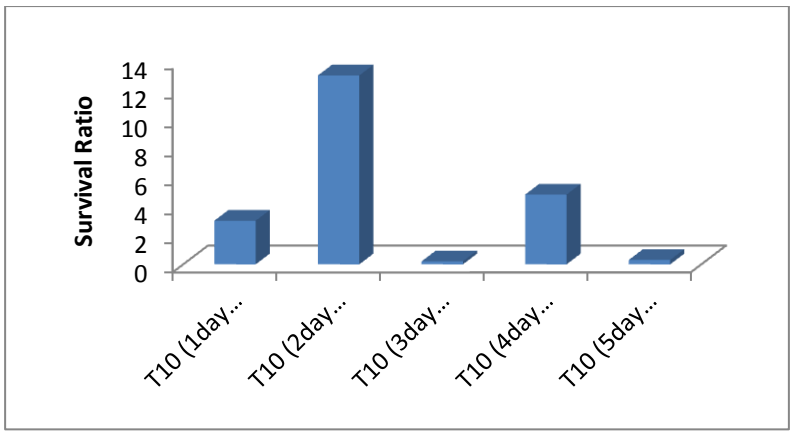


Figure 3.15 : Survival ratios of *T10* individual at each of the 5 successive cultivations in nonselective medium, when exposed to 0.8% (w/v) NaCl stress for MPN assay.

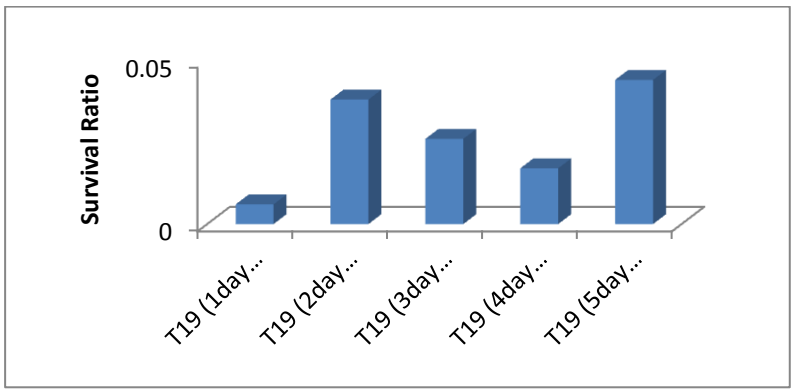


Figure 3.16 : Survival ratios of *T19* individual at each of the 5 successive cultivations in nonselective medium, when exposed to 0.8% (w/v) NaCl stress for MPN assay.

Citric acid individuals were observed to adapt to citric acid stress however, their survival ratios reduced with successive cultivations in nonstressed medium (Figure 3.17, Figure 3.18, Figure 3.19).

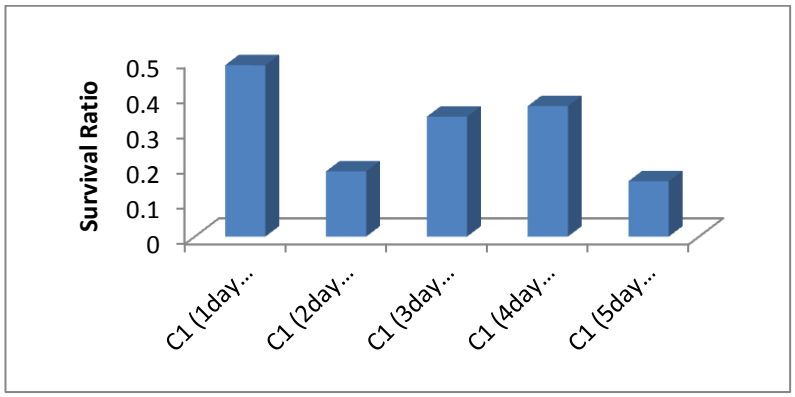


Figure 3.17 : Survival ratios of *C1* individual at each of the 5 successive cultivations in nonselective medium, when exposed to 25mM citric acid stress for MPN assay.

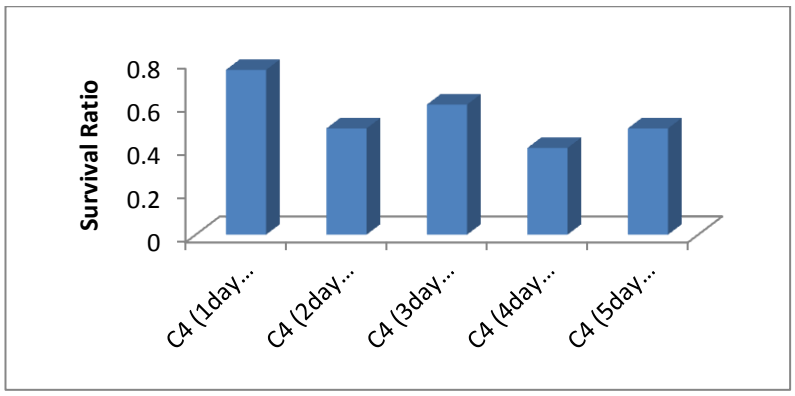


Figure 3.18 : Survival ratios of *C4* individual at each of the 5 successive cultivations in nonselective medium, when exposed to 25mM citric acid stress for MPN assay.

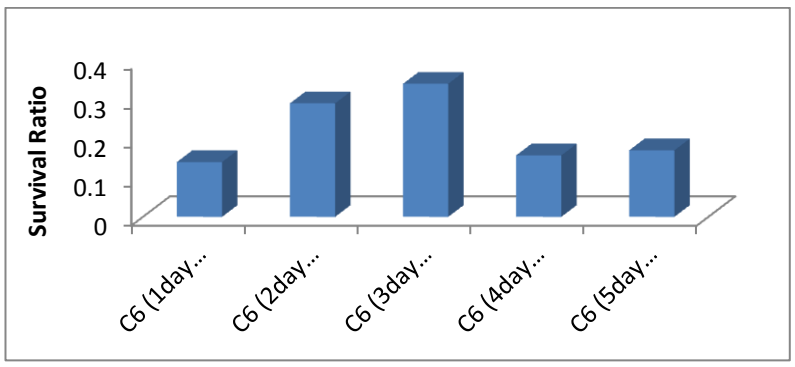


Figure 3.19 : Survival ratios of *C6* individual at each of the 5 successive cultivations in nonselective medium, when exposed to 25mM citric acid stress for MPN assay.

3.5.6 Reverse transcriptase PCR (RT-PCR) studies

3.5.6.1 RNA isolation

Expression levels of *HAL2* and *HOG1* were determined semi-quantitatively by reverse transcription (RT-PCR). For this purpose, T8 and wild type "905" were exposed to 0.7 and 0.9M salt stress for 1.5 h, total RNA was isolated and RNA concentrations were determined as described in section 2.2.11.(Table 3.25).

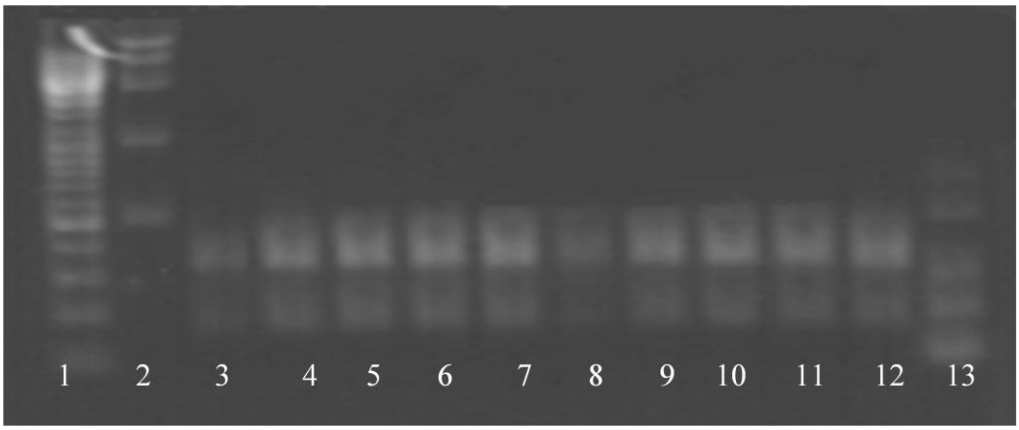
Table 3.25: Isolated RNA concentrations

Strain	Control (μg/ml)	0.7M NaCl (μg/ml)	0.9M NaCl (μg/ml)
905	384.4	351.9	332.3
T8	325.8	203.9	254.8

3.5.6.2 RT-PCR for cycle determination

In order to determine the most efficient cycle number for analysis of *HOG1* (342bp) and *HAL2* (176bp) gene expression levels, RT-PCR was applied. For this purpose *ACT1* was used as the internal control gene. Reaction components and conditions were described previously (Table 2.10).

All RT-PCR reactions started with 100 ng RNA for each sample. To determine the saturation cycle of the PCR product, PCR was run for 36 cycles and 10 μl of samples at 24, 27, 30, 33, 36 cycles were taken. Each sample was transferred to a 1% agarose gel and amplification bands were observed following electrophoresis. The optimal cycle number for RT-PCR protocol was determined as the 30^{th} cycle. The bands are shown in Fig. 3.20.

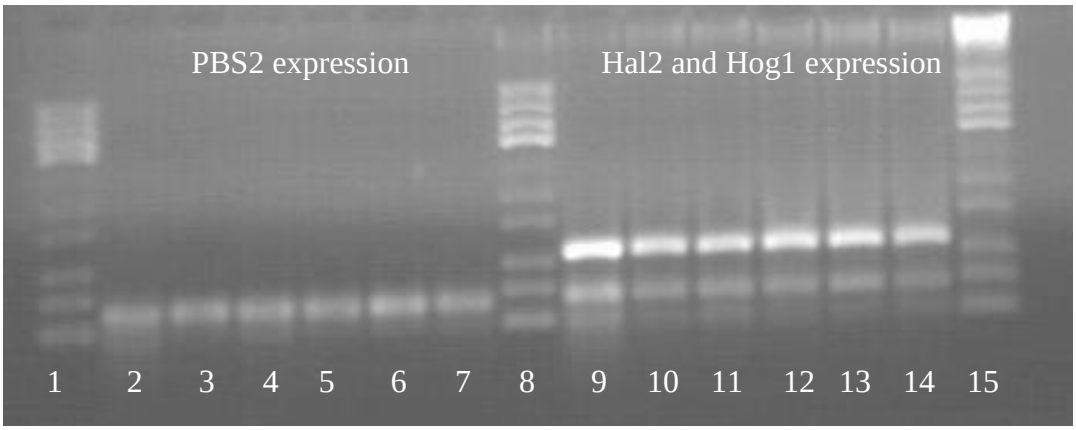


well	Cycle & sample HOG1(342bp)	well	Cycle & sample HAL2 (176bp)
1	Fermentas # SM0338 DNA	**8**	24. cycle mutant T8 control
2	Fermentas # SM1123 DNA	**9**	27. cycle mutant T8 control
3	24. d WT(905) control	**10**	30. cycle mutant T8 control
4	27. cycle WT(905) control	**11**	33. cycle mutant T8 control
5	30. cycle WT(905) control	**12**	36. cycle mutant T8 control
6	33. cycle WT(905) control	**13**	Fermentas # SM1293 DNA
7	36. cycle WT(905) control		

Figure 3.20 : Cycle determination with RT-PCR for *HAL2* and *HOG1* genes.

3.5.6.3 Multiplex-PCR

Multiplex-PCR was carried out for *PBS2/ACT1* and *HAL2/HOG1/ACT1* primer sets for 30 cycles. There were 6 different RNA samples which were isolated from wild type and *T8* incubated on YMM, 0.7M NaCl stress and 0.9M NaCl stress for 1 h. *ACT1* (beta actin) primers were used as the internal control and multiplex RT-PCR protocol conditions were adopted for both *PBS2, HAL2* and *HOG1* genes. Samples loaded on agarose gel corresponded to the 30^{th} cycle. Results are shown in Fig. 3.21



well	Cycle & sample	well	Cycle & sample
1	Fermentas # SM0338 DNA	**9**	30. cycle mutant T8 control
2	30. cycle mutant T8 control	**10**	30. cycle mutant T8 0.7M NaCl
3	30. cycle mutant T8 0.7M NaCl	**11**	30. cycle mutant T8 0.9M NaCl
4	30. cycle mutant T8 0.9M NaCl	**12**	30. cycle WT (905) control
5	30. cycle WT (905) control	**13**	30. cycle WT (905) 0.7M NaCl
6	30. cycle WT (905) 0.7M NaCl	**14**	30. cycle WT (905) 0.9M NaCl
7	30. cycle WT (905) 0.9M NaCl	**15**	Fermentas # SM1293 DNA standard
8	fermentas # SM0338 DNA		

Figure 3.21 : *PBS2*, *HAL2* and *HOG1* expression levels. (30^{th} RT-PCR cycle samples)

Images were investigated via 'Bio-Capt' quantification programme. (Results not shown)

3.5.7 Real time PCR (Q-PCR) studies

3.5.7.1 Standard curves

Standard curves for each gene were obtained by serial dilutions and drawn by LightCycler 480II Software. The results with error values below 0.2 and the slopes in the acceptable ranges according to Dorak (2010) were selected. Relative values to standard gene (β-actin) were calculated for each gene. Figures 3.22-33 show the standard curves for β-actin and the other genes of interest.

In Figure 3.22, 23, 24, standard curves for *β-ACT* , *HAL2*, *HAL3* can be observed respectively.

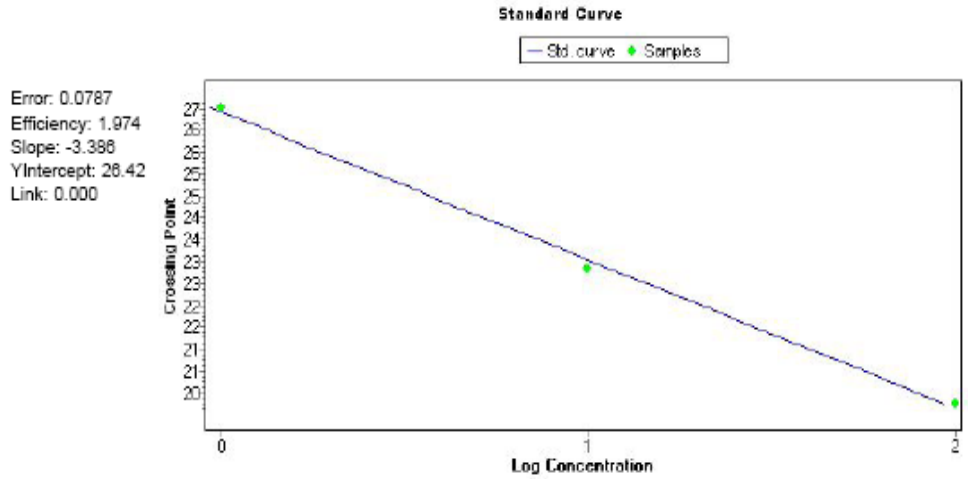


Figure 3.22 : Standard curve obtained for *β-ACT*.

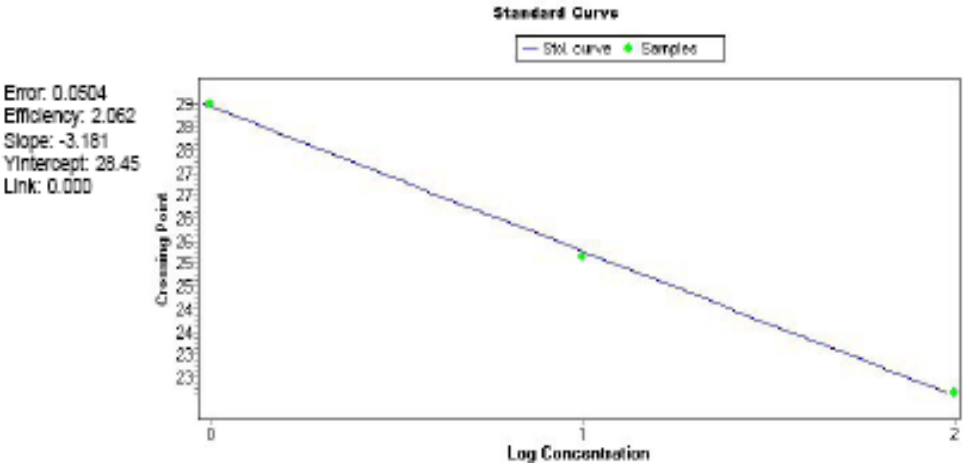


Figure 3.23 : Standard curve obtained for *HAL2*.

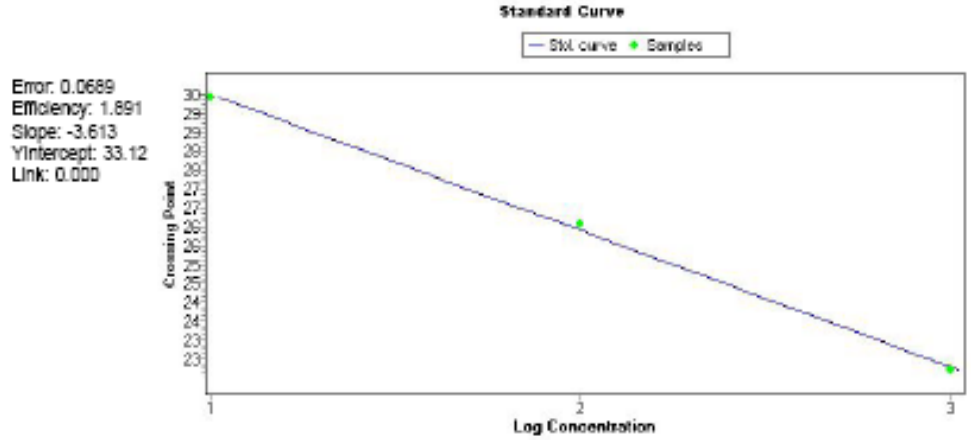


Figure 3.24 : Standard curve obtained for *HAL3*.

In Figure 3.25, 26, 27, standard curves for *HOG1*, *PBS2* and *CTT1* can be observed respectively. It can be seen that error values below 0.2 and the slopes in the acceptable ranges.

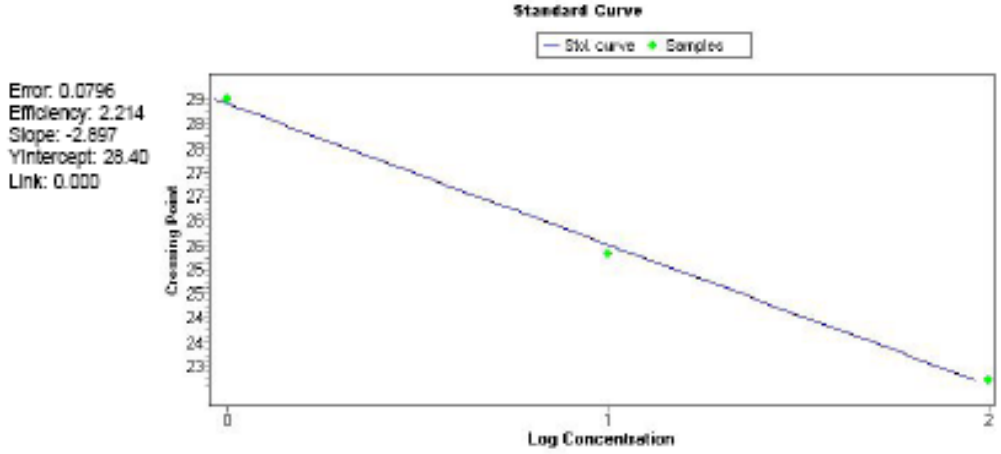


Figure 3.25 : Standard curve obtained for *HOG1*.

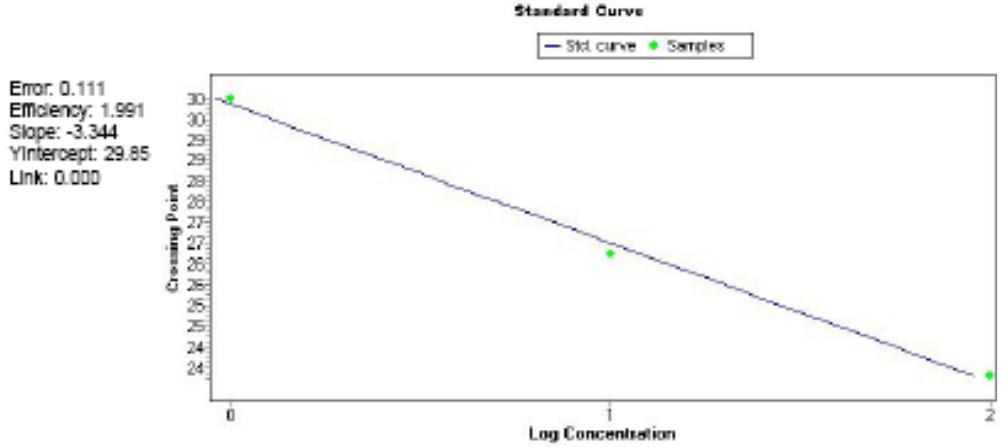


Figure 3.26 : Standard curve obtained for *PBS2*.

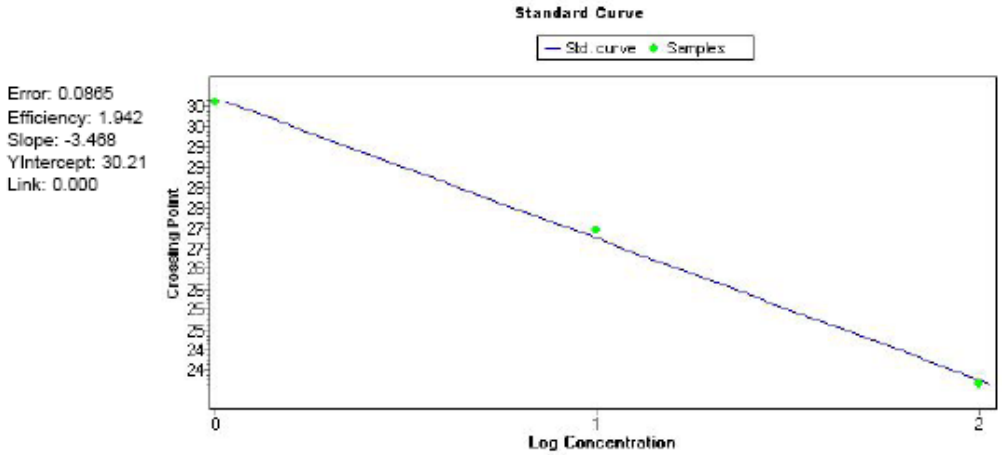


Figure 3.27 : Standard curve obtained for *CTT1*.

In Figure 3.28, 29, 30, standard curves for *ALR1*, *ALR2* and *NHA1* can be observed respectively.

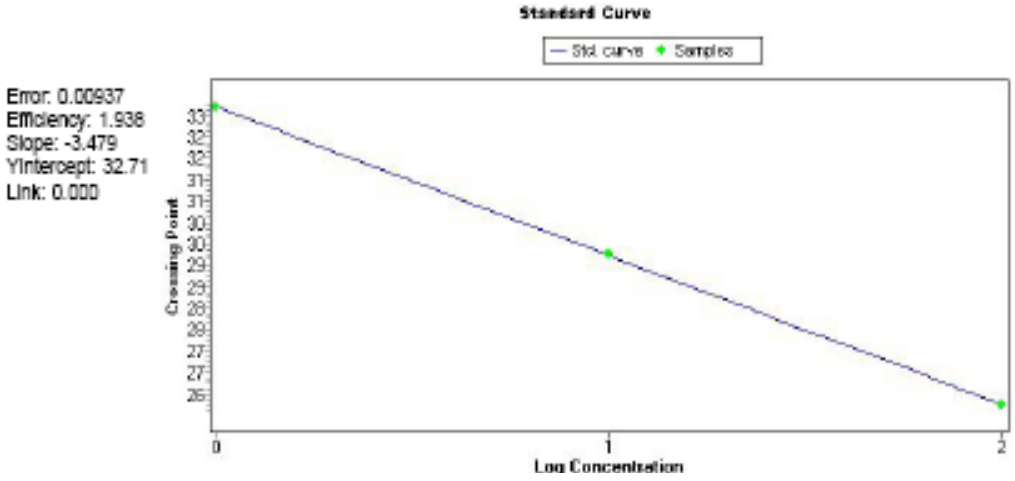


Figure 3.28 : Standard curve obtained for *ALR1*.

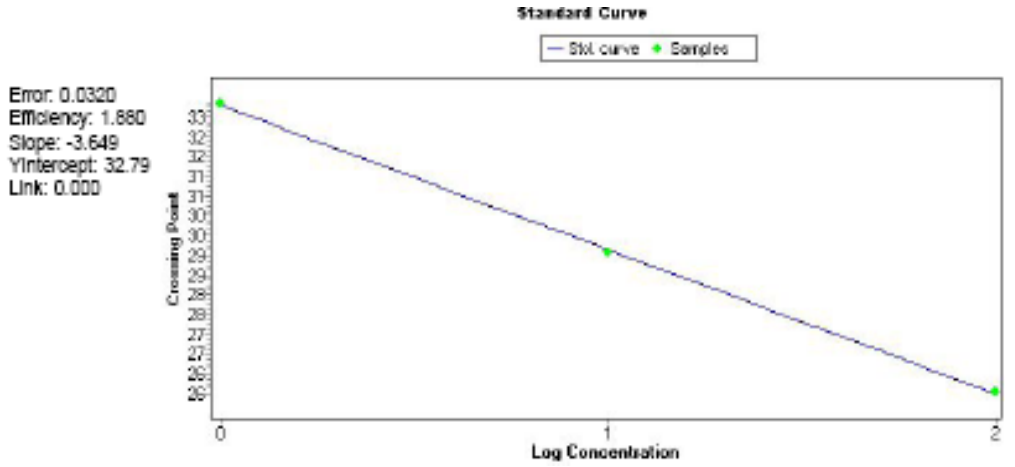


Figure 3.29 : Standard curve obtained for *ALR2*.

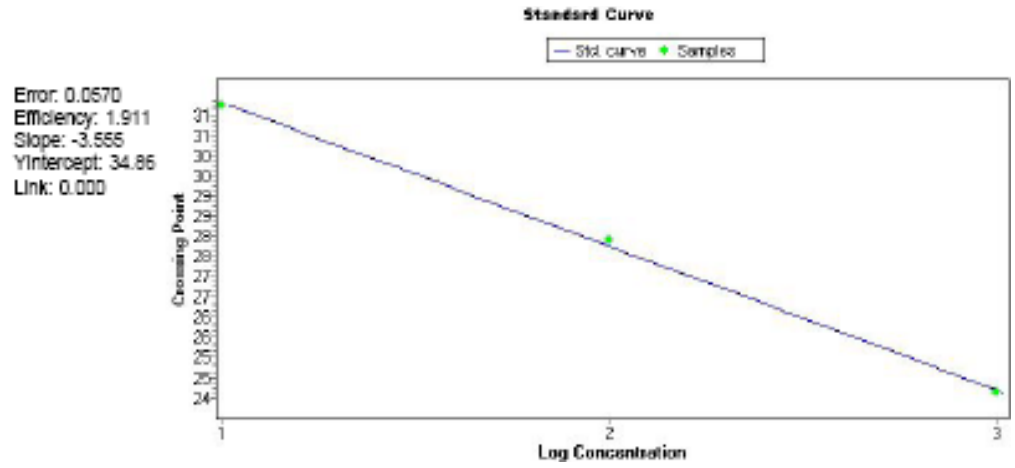


Figure 3.30 : Standard curve obtained for *NHA1*.

In Figure 3.31, 32, 33, standard curves for *AQY1*, *AQY2* and *ENA1* can be observed respectively.

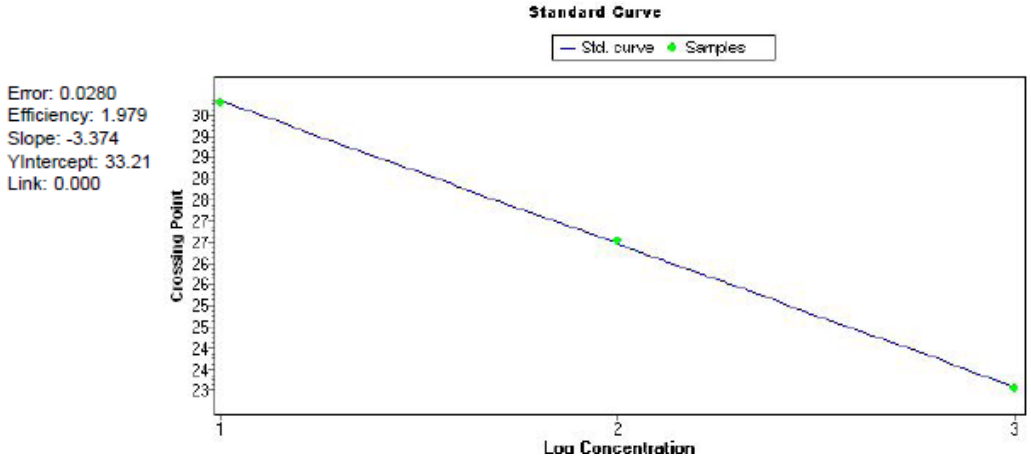


Figure 3.31 : Standard curve obtained for *AQY1*.

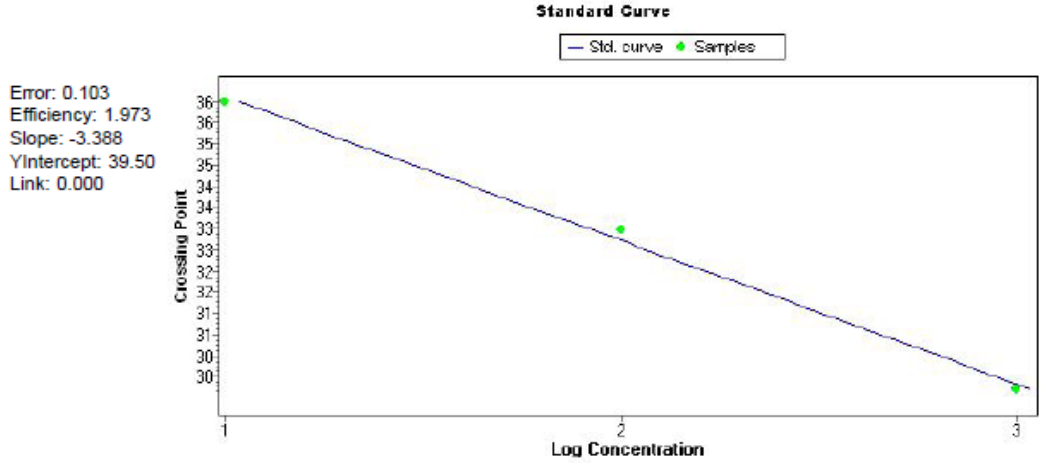


Figure 3.32 : Standard curve obtained for *AQY2*.

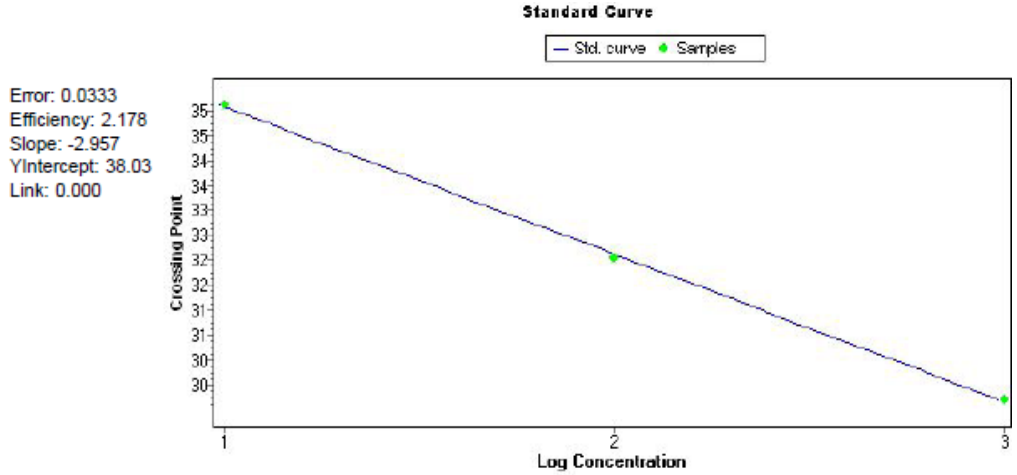


Figure 3.33 : Standard curve obtained for *ENA1*.

3.5.6.2 C_T values

The mean C_T values were determined both for the mutant and the wild type, for each gene, including the reference gene (Table 3.26, Table 3.27) Then, using the mean C_T, (reference-target) values were calculated. ΔC_T values were calculated assuming the efficiency was 2 for all genes, the fold increases at threshold cycles were determined by 2 (reference-target) (Fig. 3.31).

Table 3.26 : C_T values of wild type and mutant T8.

	WT-905 / Control			**T8**		
	0M NaCl	0.7M NaCl	0.9M NaCl	0M NaCl	0.7M NaCl	0.9M NaCl
B-ACT	18.5	21.93	20.78	24.28	19.78	20.65
B-ACT	19.9	20.9	20.92	22.71	20.11	21.33
HAL2	21.8	24.06	23.54	25.1	21.9	22.83
HAL2	22	24.28	23.68	24.34	22.13	23.01
HOG1	21.65	25.62	24.51	24.61	22.67	23.44
HOG1	21.8	25.75	25.82	25.92	23.92	24.7
PBS2	22.94	25.23	24.55	25.07	23.33	23.67
PBS2	23.15	25.45	24.8	25.33	23.41	23.92
HAL3	22.81	22.18	22.5	21.81	21.25	22.3
HAL3	23.1	23.52	23.81	24.22	20.33	21.2
CTT1	22.99	24.16	26.08	21.21	22.63	23.83
CTT1	23.12	24.23	26.7	23.55	20.1	23.96
ALR1	25.69	25.73	25.7	25.28	24.96	25.7
ALR1	25.78	25.84	25.84	25.41	25.11	25.84
ALR2	25.86	24.7	25.1	25.65	23.95	24.57
ALR2	26.08	25.6	26.2	26.18	24.18	25.38
NHA1	24.77	23.93	23.98	24.07	23.09	23.74
NHA1	26.16	24.26	24.04	24.8	23.51	24.22

Table 3.26: C_T values of wild type and mutant T8 (continued).

	WT-905 / Control			T8		
	0M NaCl	0.7M NaCl	0.9M NaCl	0M NaCl	0.7M NaCl	0.9M NaCl
B-ACT	23.05	19.5	19.13	19.31	19.29	21.2
B-ACT	23.06	19.19	18.83	19.96	19.13	21.21
ENA1	29.2	25.08	23.92	25.15	24.7	24.27
ENA1	29.14	25.03	23.94	24.99	25.04	24.44
AQY1	29.21	25.49	25.29	25.19	24.21	25.6
AQY1	29.23	25.72	24.25	24.99	24.13	25.74
AQY2	28.03	24.96	23.85	24.53	25.08	25.8
AQY2	27.91	24.87	23.96	24.62	24.97	25.74

Normalized ΔC_T values of mutant which refer to gene expression levels indicate that genes that are related or involved in HOG pathway such as *PBS2* and *CTT1* were up-regulated remarkably in mutant *T8* in control conditions. On the other hand *HOG1* expression levels did not have a significant difference compared to wt (Figure 3.34).

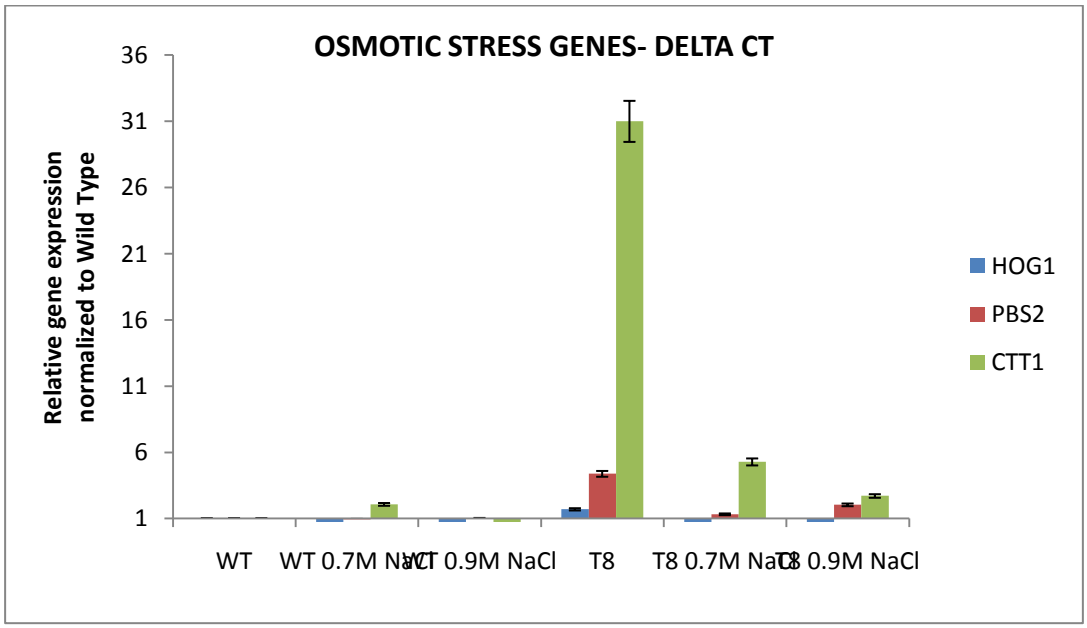


Figure 3.34 : Gene expression levels in wild type '*905*' and mutant '*T8*' with respect to ΔC_T

HAL3, ALR1, ALR2 and *NHA1* genes were significantly higher than wild type especially in nonstressed condition (Figure 3.35).

In 0.9M NaCl stress conditions *ENA1* (which encodes transmembrane Na^+/K^+ ATPase) and *AQY1* expresssion was observed to increase significantly in mutant '*T8'* (Figure 3.36).

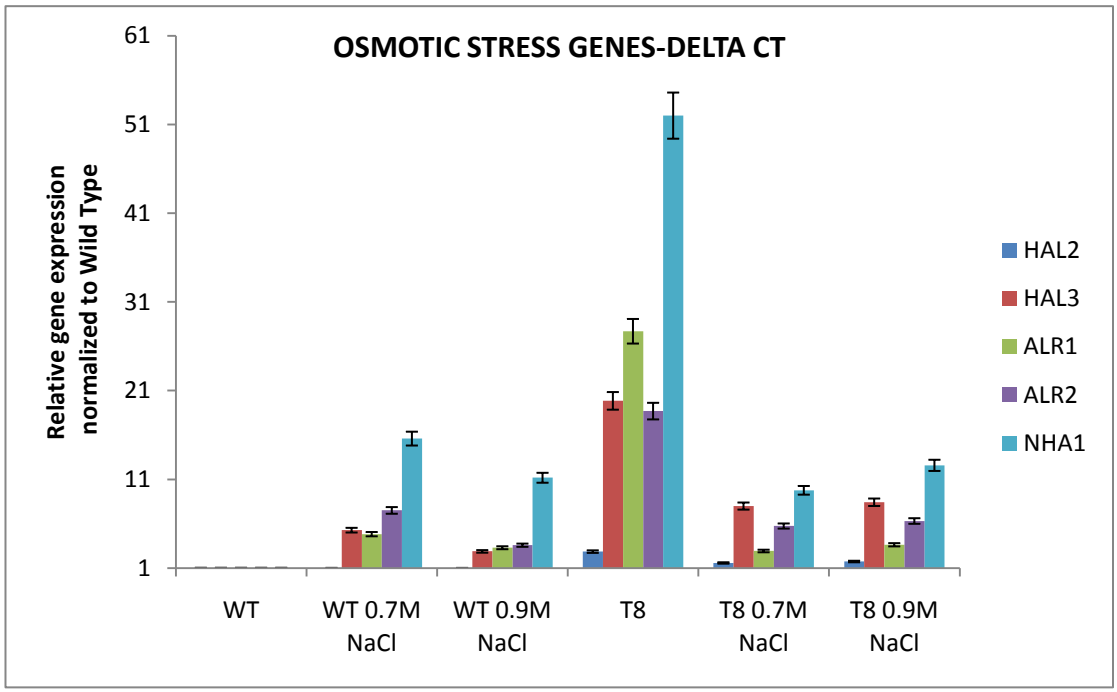


Figure 3.35 : Gene expression levels in wild type '*905*' and mutant '*T8*' with respect to ΔC_T.

Expression of aquaporin encoding gene *AQY1* was observed to increase in mutant *'T8'* in high osmolarity, however *AQY2* expression did not change significantly. In 0.9 M NaCl stress, *ENA1* which encodes major transmembrane Na^+/K^+ ATPase is expressed in mutant '*T8*'approximately 8 fold of wild type (Figure 3.36).

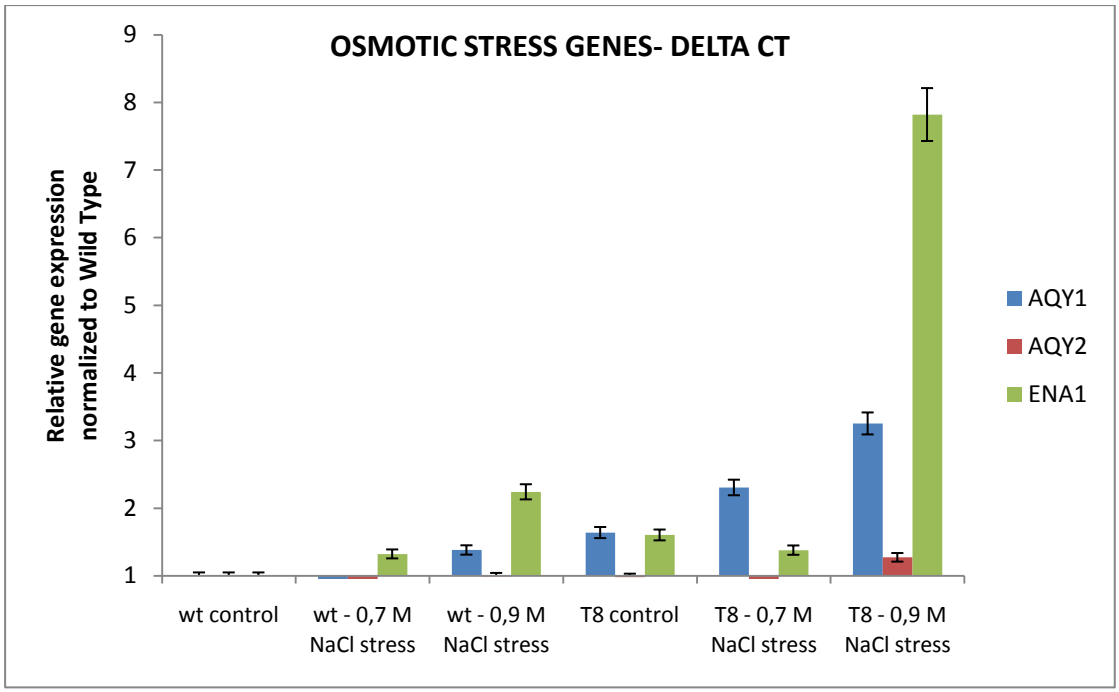


Figure 3.36 : Gene expression levels in wild type '*905*' and mutant '*T8*' with respect to ΔC_T.

3.5.7 Tetrad analysis of salt- and sorbitol-resistant mutants

Osmotic stress resistant mutants *S18, S19* and salt resistant mutant *T8* were backcrossed to *934* which is the wild type strain with the opposite mating type (Mat α). *T8, S18, S19* and zygotes obtained were tested on 2M sorbitol, 0.7M NaCl and 0.9M NaCl on YPD plates via spotting assay. In bothe concentrations of salt stress, mutant *'T8'* had high resistance, however its zygote with wild type (*T8*934*) seemed to be sensitive. On the other hand in sorbitol stress, neither *'T8'* nor sorbitol resistant mutants had a significant difference compared to wild type. 48th hour results are shown in Figure 3.37.

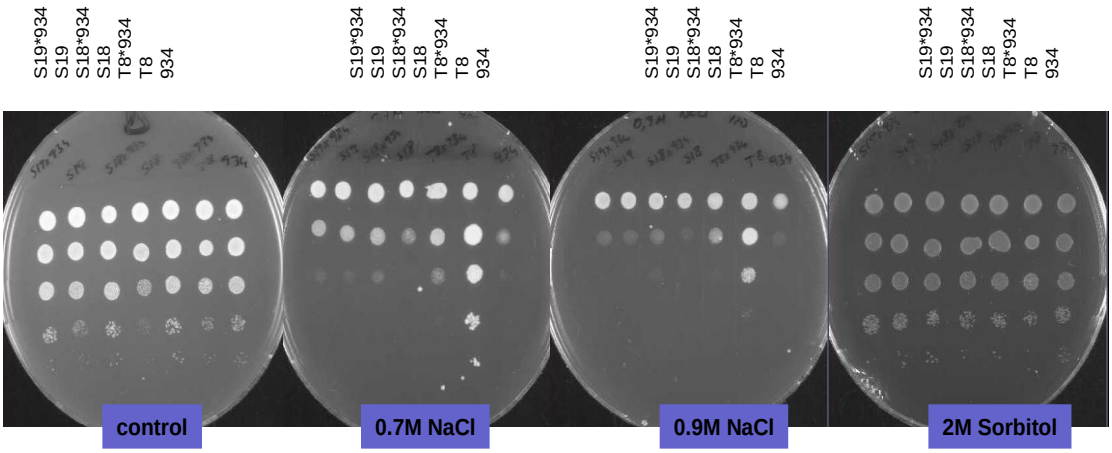


Figure 3.37 : Resistance levels of *T8*934, S19*934, S19*934* zygottes, when exposed to NaCl and sorbitol stress on YPD plates.

Tetrads of *T8*934* were dissected with micromanipulator and segregants from 8 tetrads were replica-plated to YPD plates containing 0.9 NaCl and 7.5mM $AlCl_3$ (Figure 3.38).

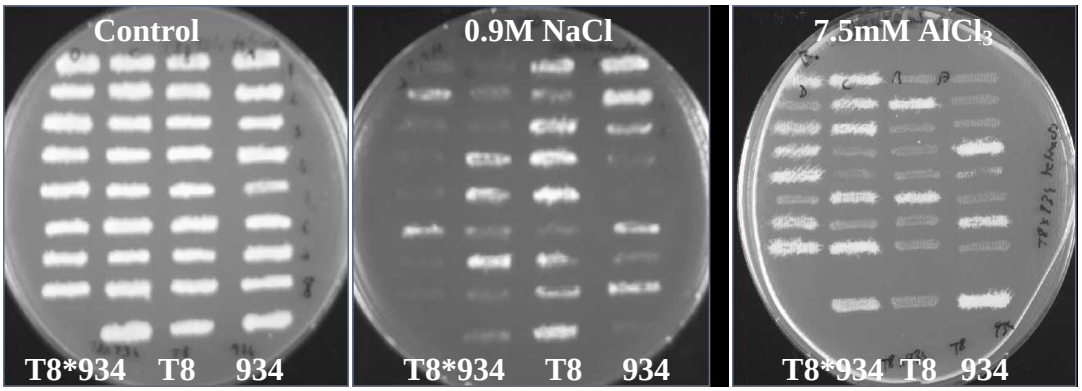


Figure 3.38 : Resistance levels of *T8*934* segregants on YPD plates containing 0.9M NaCl and 7.5mM$AlCl_3$.

All segregants in NaCl stress was observed to have 2(+):2(-) segregation and the zygote *(T8*934)* was observed to be sensitive to NaCl stress. On the other hand, they had opposite segregation in 7.5mM $AlCl_3$ stress. 24 hour cultivation results of these segregants are shown in Figure 3.38.

4. DISCUSSION AND CONCLUSIONS

Yeast cells in industry are often exposed to rapid alterations in water activity and especially to high osmolarity. This caused the necessity of improvement of yeast strains and their performance under these stress conditions. Additionally to improve food preservation technologies, clarification of molecular mechanisms of low water activity is necessary in relationship with other stress factors such as freezing-thawing, acidity, oxidative stress or heat. In this respect, obtaining multiple stress resistant yeast is one of the most important requirement of industry. However, a multiple stress resistant phenotype appears to have multi-factorial genetic alterations. Empirical procedures based on random mutation and selection are useful approaches to optimize robustness of yeast strains (Çakar et al., 2005).

The primary aim of this study was to obtain *S. cerevisiae* individuals resistant to osmotic stress, salt stress and acid stress. For this purpose,'evolutionary engineering', an inverse metabolic engineering strategy was applied. First step was to increase the genetic diversity of the initial *S. cerevisiae* population by exposure to the mutagen ethyl methane sulphonate. Based on evolutionary engineering methodology, following an initial screening, this diverse population was exposed to increasing concentrations of each stress factor. By passing the survivors of a stress level to a higher stress level, several generations of populations were obtained and then individuals were selected randomly from the final populations of each stress.

4.1 Investigation of Salt Stress Resistant Individuals

In order to determine the minimum inhibitory concentration for salt stress and the initial level of salt stress selection, wild type strain and the mutagenized initial population were grown in various concentrations of of sodium chloride in the medium. Generally *Saccharomyces cerevisiae* strains are known to have tolerance to NaCl stress in a range between 1-2M concentration. However screening studies

showed that both wild type of CEN.PK 113-7D strain and EMS mutagenised population could not tolerate over 0.85 M NaCl concentration. According to this result, 1% sodium chloride was selected as initial stress concentration for evolutionary engineering selection. Starting with the initial stress concentration, this genetically diverse population was then exposed to batch continuously increasing stress conditions in batch selection.

By passing the survivors of each stress step to the following step, 40 generations were obtained up to a final population which survived 8.5% NaCl (1.45M) concentration. This final population (MO40) was spread on YMM agar plates and 23 colonies were selected randomly.

Individuals selected from the final population were investigated via serial dilution method on Petri plates for tolerance to NaCl stress. Among the 23 individuals, ten most resistant colonies were selected. These best individuals of NaCl stress selection were coded as *T12, T23, T4, T13, T19, T20, T8, T15, T10, T11* and they were tested via 5-tube MPN method for 2 and 2.5 M sorbitol, 5 and 8% NaCl stress. NaCl stress displays features in common with hyperosmotic stress, but also evokes physiological systems commonly engaged in ion homeostasis. Thus, sorbitol stress was applied to test whether NaCl selection resulted with a cross-resistance to hyperosmotic stress.

T8, T10, T13 and *T19* were observed to be the most resistant individuals in salt stress. *T12, T23* and *T11* were more resistant than other individuals in 2M sorbitol stress, but in 2.5 M sorbitol stress, they were not more resistant than the wild type. It can be concluded that, most of the salt stress-resistant individuals were not cross-resistant to sorbitol stress. Thus, mutations of these individuals might have been probably on response pathways that are related to ionic toxicity rather than its osmotic stress effect. In order to maintain high internal K^+/Na^+ ratio despite high extracellular Na^+ concentrations, they might have mutations that effect the action of plasma membrane transport systems or the intracellular compartmentalization of Na^+ ions usually in vacuoles.

Five of the highly salt stress resistant individuals (*T8, T10, T13, T15, T19*) were selected for cross-resistance studies for 6% (v/v) ethanol, 0.8 mM H_2O_2, -196^0C freezing-thawing and 60^0C heat stress via MPN method. *T8, T10, T13* and *T19* had a higher resistance to 6% (v/v) ethanol compared to wild type. *T8* and *T13*, however,

have shown the highest resistance levels. On the other hand, at 72 h, 6% (v/v) ethanol cross-resistance results showed that the differences in resistances became less significant between the mutants and the wild type. Especially, although *T8* and *T13* have higher resistance compared to wild type (905) at 48 hour, survival of 905 has almost reached the same levels as those of *T8* and *T13* within 72 h. This might indicate that the individuals adapt to ethanol stress more rapidly than the wild type. However, the wild type strain tolerated ethanol within 72 hour and no significant resistance difference of salt resistant individuals was observed for ethanol. This early adaptation may be related to overlapping cellular responses of ethanol and osmotic stress which are usually found in turgor changes. High ethanol concentrations are known to cause water stress due to dehydration of the cell however, there is no known relation between ionic toxicity and ethanol stress. This supports the idea that although these individuals seem to have earlier adaptation to ethanol stress, their mutations are likely to be more related to ionic toxicity of Na^+, rather than its osmotic stress.

The response of yeast to oxidative stress is based on protection from oxidative damage, which is normally active in yeast cells performing oxidative ATP by respiration. Protection from oxidative damage encompasses, in conjunction with the action of reducing compounds produced by the such as glutathione and thioredoxin and enzymatic activities such as superoxide dismutase or catalase. Here we have investigated cytoplasmic catalase activities. In order to investigate resistance mechanisms of individuals, total catalase activity was determined in both control conditions and upon 1mM H_2O_2 continuous stress application. In both control and stress conditions, all individuals and the final population had significantly higher catalase activity than that of the wild type. Generally, the most resistant individuals of salt stress selection had highest catalase activity, even when no oxidative stress (H_2O_2) was applied to the culture. In 0.8mM H_2O_2, *MO40* final population of salt stress had 19.4 fold survival of the wild type. Especially in stress conditions, catalase activity increased up to 3 fold of the values at control conditions, except for *T15*.

The best individual with the highest resistance under 8% NaCl stress condition was '*T8*', but it was one of the least resistant mutants under 2.5mM sorbitol stress. Both in the presence and absence of H_2O_2, it has significantly higher catalase activity than the wild type. This result indicates a relationship between salt resistance and catalase

activity. Hyperosmotic stress has been shown to induce oxidative stress response. For example expression of the cytosolic catalase encoding gene '*CTT1*' is increased with the Msn2/4p and Msn1p transcription factors upon exposure to osmotic shock. *MSN2* and *MSN4* are known to be general stress transcription factors activated in various stress conditions (Kobayashi and McEntee, 1990; Gasch et al., 2000). In response to osmotic stress, Msn2 and Msn4 enter the nucleus and bind the *CTT1* promoter. Through interaction with Msn2 and Msn4, Hog1 binds to DNA and activates expression of *CTT1* (O'Rourke et al., 2002). Thus eventhough the mutants did not gain an osmo-resistance, the main response pathway to osmotic stress, named as HOG pathway might also have been activated in these mutants.

Freezing-thawing can be regarded as an extreme form of osmotic stress. In this study freezing stress was applied by pulse exposure to liquid nitrogen which is known to be at about -196^0C. Final population of salt stress application (*MO40*) had 4-fold of survival in freezing-thawing stress than the wild type, however, all individuals had lower survival than the wild type. This result also seems to support the hypothesis that their mutations might be related especially to ionic toxicity of NaCl rather than its osmotic effect. The difference between the individuals and the final mutant population again indicated the heterogenity of the final mutant population, as observed in previous studies (Çakar et al., 2005; Çakar et al., 2009).

Upon 60^0C heat stress application, neither the final population nor the individuals had higher survival than the wild type. Heat stress affects cellular systems via denaturation and aggregation of proteins, destruction of protein complexes and other cellular structures and increases cell membrane fluidity (Hohmann, 2002). Formation of heat-denatured signal proteins evokes the response systems, which induces thermo protective mechanisms. Changes in plasma membrane fluidity and structure of transport proteins might be the reason of the sensitivity of salt stress resistant individuals to heat stress. Also ion leakage (Ca^+, Na^+, and K^+) after the heat shock stress is possibly affect the ion homeostasis of mutant individuals (Guyot et. al., 2005).

Salt-resistant mutants were also tested for heavy metal resistance in 2.5mM $CrCl_3$, 0.1mM $CuCl_2$, 0.3 mM $NiCl_2$, 5mM $ZnCl_2$, 9mM $ZnCl_2$, 15mM $MnCl_2$, 4mM $CoCl_2$, 5mM $CoCl_2$ and 13mM $AlCl_3$. Salt resistant individuals were observed to

have ten fold higher resistance in $CuCl_2$, and up to 10^4 fold resistance in $CoCl_2$. However, in $AlCl_3$, they have approximately 100 fold lower resistance than that of the wild type, indicating a sensitivity to aluminum.

Among these five salt-resistant individuals, *T8, T10, T19* were tested for genetic stability. Survival ratios of *T8*, and *T19* did not decrease throughout five successive cultivations in nonselective medium. Thus, they were assumed as genetically stable mutants.

T8 which was the highest salt resistant and genetically stable individual, was selected to be the appropriate individual for genetic analysis of osmotic stress and salt stress resistance. Although it had the highest resistance in 8% NaCl, it was one of the most sensitive mutants in 2.5mM sorbitol stress.

T8 was crossed with the opposite mating type strain (934) and their tetrads were dissected. Segregants from 8 tetrads were replica plated to 0.9M NaCl stresss on Petri plates. All segregants had 2+:2- segregation upon NaCl stress. This proved that the mutation of T8 which resulted in NaCl resistance was on a single gene. Sensitivity of the zygote *(T8*934)* to NaCl stress indicated that this mutant gene has recessive characteristics.

These 2+:2- segregation in NaCl stress was also observed in 1mM H_2O_2 and 500mM $LiCl_2$ stress. On the other hand, in 100mM $AlCl_3$ stress, opposite segregation of resistance was observed. These results indicated that this single gene mutation might have caused aluminium sensitivity, and resistance to oxidative and lithium stress. *ALR1* and *ALR2* are known to encode the main transporter proteins for Mg^+ uptake and their overexpression protects cells against Al^{3+} toxicity (MacDiarmid and Gardner, 1998).

Reverse transcription PCR (RT-PCR) method was used to investigate the expression levels of genes that are known to be involved in salt stress related pathways. *HOG1* was selected to be investigated since it encodes a mitogen-activated protein kinase involved in HOG transduction pathway and it has a role as transcriptional activator of stress response elements. *PBS2* also has role in HOG pathway, encoding the MAP kinase kinase which is known to have role in phosphorylating *HOG1*. *HAL2* gene was selected since its expression is known to increase upon NaCl and LiCl stresses. *HAL2* is identical to *MET22* which is involved in methionine biosynthesis. And

methionine supplementation is known to improve sodium and lithium tolerance (Glaser et al., 1993). Gene expression levels were determined for *T8* and wild type (905) in 0.7M NaCl, 0.9M NaCl and control medium without NaCl. Multiplex PCR reactions were set and *ACT1* (Beta actin) housekeeping gene was used as the internal control in all reactions. The gel images were examined using Bio-Capt programme (data not shown), however, this analysis was semiquantitative. Thus, Real Time PCR (qRT-PCR) was preferred for a better quantitative expression analysis of gene expression.

Expression levels of particular genes in control and stress conditions were investigated via qRT-PCR. Expression data calculated via ΔC_T method indicated that *CTT1* which encodes cytoplasmic catalase, were up-regulated remarkably in mutant *T8* especially in nonstressed conditions. It is known from studies of O'Rourke et. al. that, *CTT1* gene expression is regulated by Hog1p which is the MAPK in HOG pathway (O'Rourke et al., 2002). Thus, the genetic mutation on *T8* mutant is possibly responsible for the activation of HOG pathway. On the other hand, expression of *PBS2* which is MAPKK in HOG pathway was observed to be higher than its expression in wild type (Figure 3.34).

HAL3 gene also improves resistance of yeast cells to toxic concentrations of sodium and lithium (Ferrando et al., 1995). It has a possible role in increasing cytoplasmic K^+ and decreasing Na^+ and Li^+ in the cell. Increase in *HAL3* expression levels confirmed our previous results that, higher lithium tolerance was observed in *T8*934* segregants. *HAL3* gene is also known to be necessary for the activation of *ENA1*, transmembrane ATPase which is the major responsible for Na^+/ K^+ efflux (Ferrando et. al., 1995). Significant increase in *ENA1* expression is in correlation with the increase in HAL3 expression in mutant 'T8' (Figure 3.36).

ALR1 and *ALR2* genes are the major genes that are known to be responsible for aluminium resistance (Macdiarmid and Gardner, 1998). They encode transmembrane protein involved in transport of mainly Mg^{2+} and other divalent cations. Their study also revealed that increased activity of Mg^{2+} uptake confers resistance to Al^{3+} stress and overcomes uptake of other cations (Graschopf et al., 2001). Tetrat analysis studies have revealed that segregants of *T8*934*, which were resistant to NaCl stress were sensitive to Al^{+3}, thus *ALR1* and *ALR2* expression was investigated in *T8*. qRTPCR

results has indicated that in nonstressed conditions, their expression is higher in *T8* compared to wild type.

Uptake or efflux of water is controlled by water channel proteins that are encoded by *AQY1* and *AQY2* in *Saccharomyces cerevisiae*. *AQY1* encodes a spore-specific water channel that has role in decreasing the spore water content (Pettersson et.al., 2005). It was observed from the qRTPCR studies that in mutant '*T8'*, *AQY1* expression is higher than wild type and increases with the high osmolarity. On the other hand *AQY2* expression is known to be reduced with high osmolarity which is known to be partly depending on the HOG pathway (Hohmann, 2002). In contrast to *AQY1* expression results, *AQY2* expression did not seem to have changed in mutant *'T8'*.

4.2 Investigation of Sorbitol Stress Resistant Individuals

In order to determine the minimum inhibitory concentration for sorbitol stress selection, wild type strain and the genetically diverse initial population were grown in various concentrations of sorbitol in the medium. Screening studies showed that both wild type and the initial mutant population could not survive over 2.75 M sorbitol concentration. Thus the initial stress level for selection was determined as 1.2 M and by sequentially and continuously increasing stress levels, 17 populations were obtained. The final population which had survived 3M sorbitol was coded as *SO17*. Twenty-three colonies were selected randomly from this population. They were screened on Petri plates containing sorbitol and most resistant clones were coded as *S6, S22, S9, S17, S18, S15, S8, S3, S1, S19*. These individuals were tested for survival via 5-tube MPN method in 2 and 2.5 M sorbitol, 5 and 8% NaCl stress.

Among these individuals; *S17, S18, S15* were observed to have high resistance upon 2M sorbitol stress, up to 200-, 225- and 146-fold of the wild type resistance level respectively. Upon 2.5 M sorbitol stress, *S19* was the most resistant individual. In general, sorbitol stress selection individuals were resistant to salt stress conditions. *S3, S6, S9* had high resistance upon both 5 and 8 % NaCl stress. On the other hand, salt stress individuals were not observed to have gained tolerance to sorbitol stress.

S3, S6, S15, S17, S18, S19 were investigated for cross-resistance to 6% (v/v) ethanol, 0.8 mM H_2O_2, -196^{0}C freezing-thawing and 60^{0}C heat stress via MPN method. Sorbitol stress resistant individuals were more resistant to ethanol stress compared to

wild type, except *S6* and *S19*. In wine fermentation, yeast cells are exposed to ethanol concentration of up to 15-20% per volume. This causes water stress because it affects hydration of the cell. The response mechanisms to ethanol stress differ from osmotic stress, however, they are overlapping in those for turgor changes. Besides, a protein kinase A (cyclic AMP [cAMP]-dependent protein kinase) has been shown to mediate a general stress response under essentially all stress conditions, including ethanol stress and osmotic stress (Hohmann 2002).

All of the six sorbitol stress resistant individuals (*S3, S6, S15, S17, S18, S19*) were more resistant than the wild type in 0.8mM H_2O_2 stress. Among them, *S3, S19* and the population *SO17* were the most resistant. Especially, the resistance of *S19* and *SO17* were significantly higher than that of *S3*. These results were in parallel with the catalase activity test results. The catalase activities of sorbitol and salt resistant individuals were determined both in 1mM H_2O_2 containing YMM and in YMM without any stress factor. The activities were calculated as the ratio of absorbance difference to total protein amount which was obtained by Bradford method. Catalase activities of sorbitol-resistant mutants in control medium were approximately two or three fold of the wild type value. *S19* had 4-fold activity of the wild type under 1 mM H_2O_2 and 16-fold activity of the wild type in control medium. Similarly, in control medium, *SO17* population had nearly 77-fold catalase activity of the wild type. However the other mutants tested did not have that much catalase activity which again indicated the high level of heterogeneity of the final mutant population. It is important to note that the final mutant population of the sorbitol selection had much higher cytoplasmic catalase activity than the final mutant population of salt stress selection.

Under -196^0C freeze-thaw stress conditions, sorbitol stress individuals were more resistant than the wild type, except *S6.* Mutant individuals, *S3, S19, S17* and especially *S15* have shown resistance. They were also investigated under -20^0C freeze thaw stress conditions, however, only *S18* had higher resistance than the wild type (905).

S18, which had high resistance at 2M sorbitol stress, was also resistant upon 2.5M sorbitol stress, but was sensitive to NaCl stress. Interestingly, this mutant had significantly high resistance to freeze-thaw stress applied both at -20^oC and -196^oC

(liquid nitrogen). It also has shown significantly higher catalase activity than the wild type, both in the presence and absence of H_2O_2 stress. *S18* and *S19* mutants from sorbitol selection were therefore selected to be investigated for further genetic analysis.

S3, S18 and *S19* mutants were tested for genetic stability however only *S19* was observed to be genetically stable through five successive cultivation in nonselective medium. *S19* was selected as an appropriate mutant for genetic analysis. Further investigations of these mutants on Petri plates showed that their resistance was an adaptation mechanism developed for osmotic stress, rather than a permanent mutation for resistance. This 'reversible adaptation' probably includes activation of HOG pathway which is also responsible in activation of cytoplasmic catalase.

Mutants obtained from citric acid selection were investigated for genetic stability during sequential cultivations in minimal medium without stress. However MPN results indicated that they were not genetically stable and their survival ratio was not significantly higher than the wild type.

To summarize, *S. cerevisiae* individuals resistant to osmotic stress, salt stress and acid stress were obtained via 'Evolutionary Engineering' strategy. *T8* mutant from salt stress application was investigated genetically in detail. Results obtained have shown that this genetic mutation which is responsible for salt stress resistance is a recessive single gene mutation and is responsible for activation of cytoplasmic catalase, possibly via activation of HOG pathway. This mutation also has a possible role in the activation of *HAL3* gene which improves resistance of yeast cells to sodium and lithium. Another interesting finding was that the expression of genes that encode transmembrane proteins such as *NHA1, ALR1, ALR2, ENA1, AQY1* were significantly higher in mutant *T8* than in the wild type especially in control conditions without any stress. As a result, this single mutation activates salt and osmotic stress response through activation of highly complex genetic mechanisms.

In order to create a global picture of cellular function, further investigations of the selected individuals via gene expression profiling methods will be essential. With the improvements in these techniques, it is possible to measure the activity of thousands of genes at once and to understand how genes response to various conditions. So it enables us to clarify these resistance mechanisms in *S. cerevisiae* in detail and

illuminate common response mechanisms with other stress conditions. Also grouping similar genes or samples together with the use of hierarchical clustering will be usefull in gene expression studies.

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APPENDIX

APPENDIX A: MPN Index For Five Test Tubes

No. of Tubes Positive in			MPN in the inoculum of the middle set of tubes
first set	middle set	last set	
0	0	0	‹0.01
0	0	1	0.02
0	1	0	0.02
0	2	0	0.04
1	0	0	0.02
1	0	1	0.04
1	1	0	0.04
1	1	1	0.06
1	2	0	0.06
2	0	0	0.05
2	0	1	0.07
2	1	0	0.07
2	1	1	0.09
2	2	0	0.09
2	3	0	0.12
3	0	0	0.08
3	0	1	0.11
3	1	0	0.11
3	1	1	0.14
3	2	0	0.14
3	2	1	0.17
4	0	0	0.13
4	0	1	0.17
4	1	0	0.17
4	1	1	0.21
4	1	2	0.26
4	2	0	0.22
4	2	1	0.26
4	3	0	0.27
4	3	1	0.33
4	4	0	0.34
5	0	0	0.23
5	0	1	0.31
5	0	2	0.43
5	1	0	0.33
5	1	1	0.46
5	1	2	0.63
5	2	0	0.49
5	2	1	0.7
5	2	2	0.94
5	3	0	0.79
5	3	1	1.1
5	3	2	1.4
5	3	3	1.8
5	4	0	1.3
5	4	1	1.7
5	4	2	2.2
5	4	3	2.8
5	4	4	3.5
5	5	0	2.4
5	5	1	3.5
5	5	2	5.4
5	5	3	9.2
5	5	4	16
5	5	5	›24

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