İSTANBUL TECHNICAL UNIVERSITY ★ INSTITUTE OF SCIENCE AND TECHNOLOGY

EVOLUTIONARY ENGINEERING OF FREEZE TOLERANCE IN SACCHAROMYCES CEREVISIAE

M.Sc. Thesis by
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Programme : **Molecular Biology Genetics and Biotechnology**

JUNE 2009

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İSTANBUL TEKNİK ÜNİVERSİTESİ ★ FEN BİLİMLERİ ENSTİTÜSÜ

Saccharomyces cerevisiae' da DONMA TOLERANSININ EVRİMSEL MÜHENDİSLİĞİ

YÜKSEK LİSANS TEZİ

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ABBREVIATIONS

EMS : Ethyl Methane Sulfonate
cAMP : Cyclic Adenosine Monophosphate
YMM : Yeast Minimal Medium
MPN : Most Probable Number
GRAS : Generally Regarded as Safe
HPLC : High Performance Liquid Chromatography
YPD : Yeast extract-Peptone-Dextrose (Complex) Medium
ATP : Adenosine Triphosphate
TCA : Tricarboxylic Acid Cycle
FDA : Food and Drug Administration
STRE : Stress Response Elements
HSE : Heat Shock Elements
ROS : Reactive Oxygen Species
SOD : Superoxide Dismutase

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EVOLUTIONARY ENGINEERING OF FREEZE TOLERANCE IN *Saccharomyces cerevisiae*

SUMMARY

Yeast cells are single cell organisms that are living freely in nature. They face large variations in their natural environment and during industrial processes. Declination from the optimal conditions can be a result of physical or chemical changes in the environment. These can alter their metabolism.

The freezing process occurs in most natural habitats where the temperature decreases below 0^0C at night, and also in some regions where the temperature is permanently cold. The mechanism of freeze injury is not well understood yet. As a biochemically and genetically well characterized model organism, *S. cerevisiae* is suitable to study the freeze-thaw injury mechanism.

In this study, the aim was to obtain freeze tolerant *S. cerevisiae* cells by using an inverse metabolic engineering strategy; evolutionary engineering. By designing and using evolutionary engineering strategies, freeze-tolerant individual cells were successfully obtained with concomitant cross-resistances towards other stress conditions. Survival values of cells after exposure to stress conditions were determined by most probable number (MPN) method and specific catalase activity by enzymatic analysis. Most of the mutant cells were found to be multi-stress resistant. Moreover, experimental results showed that the specific catalase activity of the mutant cells were significantly higher than those of the wild type even in the absence of any stress conditions. The transcriptomic and proteomic analysis and further investigations may help to better understand the mechanism of stress tolerance and ultimately exploit it for cryopreservation applications and frozen dough technology.

EVRİMSEL MÜHENDİSLİK YÖNTEMİ İLE DONMAYA TOLERANSLI *Saccharomyces cerevisiae* GELİŞTİRİLMESİ

ÖZET

Maya hücreleri doğada serbest halde yaşayan tek hücreli organizmalardır. Doğal çevrelerinde ve endüstriyel prosesler süresince büyük ölçüde değişen ortam koşulları ile karşılaşırlar. Çevre şartlarındaki fiziksel veya kimyasal değişimler ideal yaşam koşullarından sapmaya neden olur. Bu da metabolizmalarının değişmesine neden olabilir.

Donma prosesi bir çok doğal habitatta, geceleri sıcaklığın 0^0C altına düştüğü yerlerde ve sıcaklığın sürekli olarak düşük olduğu yerlerde görülür. Donma hasarı mekanizması henüz tam olarak açıklanamamıştır. *S. cerevisiae*,biyokimyası ve genetiği iyi bilinen bir model organizma olarak, donma-erime hasar mekanizmasını incelemek için uygun bir mikroorganizmadır.

Bu çalışmada amaç, bir tersine metabolik mühendislik stratejisi olan evrimsel mühendislik yöntemi ile donmaya toleranslı *Saccharomyces cerevisiae* hücreleri elde etmektir. Evrimsel mühendislik yöntemi başarı ile uygulanmış ve donmaya toleranslı ve buna paralel olarak farklı stres koşullarına karşı da çapraz direnç geliştirmiş bireyler elde edilmiştir. Hücrelerin stres koşullarına maruz kaldıktan sonraki hayatta kalma oranları en muhtemel sayı (most probable number, MPN) yöntemi ile, spesifik katalaz aktivitesi ise enzimatik analizlerle belirlenmiştir. Elde edilen mutant hücrelerin çoğunun birden fazla strese karşı direnç kazandıkları görülmüştür. Ayrıca stressiz koşullarda yapılan deneylerde dahi mutant hücrelerin spesifik katalaz aktivitelerinin yabanıl tipe oranla çok daha yüksek olduğu belirlenmiştir. Transkriptomik ve proteomik analizler ve yapılacak araştırmalar, canlılarda strese karşı direnç mekanizmalarının daha iyi anlaşılmasında ve hücrelerin dondurularak saklanması ve donmuş hamur teknolojisi gibi uygulamalarda kullanımında faydalı olacaktır.

1. INTRODUCTION

1.1 General information About *Saccharomyces cerevisiae*

Yeasts are classified, and identified by their morphological, physiological, molecular and sexual characteristics**.** *Saccharomyces cerevisiae* belongs to the kingdom Fungi, phylum Ascomycota, class Hemiascomycetales, order Saccharomycetales, family Saccharomycetacea and genus Saccharomyces. The state of Saccharomyces cerevisiae in yeast phylogeni is depicted in Figure 1.1.

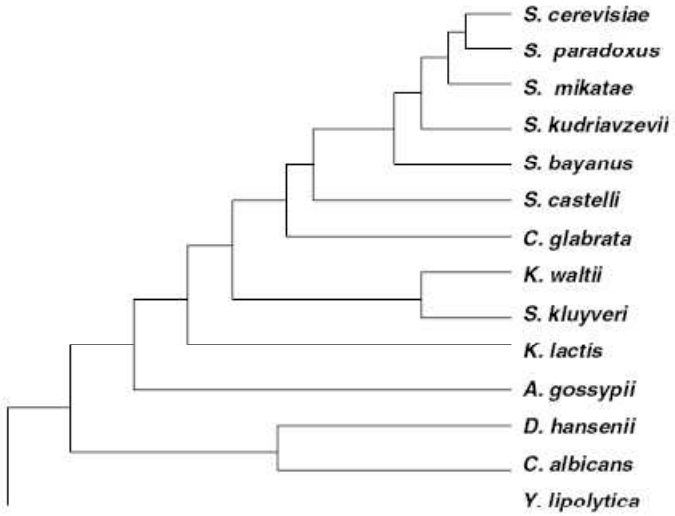

Figure 1. 1: Schematic phylogenetic relationships of the genome-sequenced yeasts [1].

S. cerevisiae cells are eukaryotic, round cells with 5-10 µm in diameter. They have thick, tough cell walls like other fungi [2].

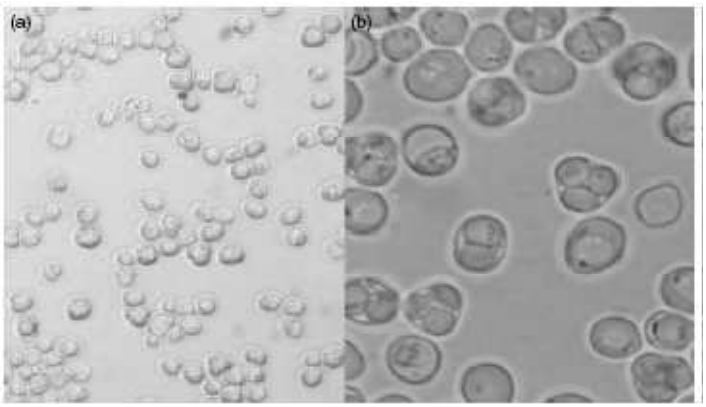

Figure 1. 2: Cells and asci of *Saccharomyces cerevisiae*. Bright field microscopy, (a) magnification: ×400; (b) magnification: ×2000 [3].

S. cerevisiae, also called baker's yeast, can do aerobic respiration in aerobic conditions, and can ferment sugars to produce ethanol and carbon dioxide. This feature is very important for the fundamental processes to produce alcoholic beverages and bakery products [3].

S. cerevisiae can use a wide range of substrates under different conditions. The main energy and carbon source is sugar. The catabolic product of the mono-, oligo- and polysaccharides is glucose. The first step of sugar catabolism, the uptake stage, occures by facilitated diffusion and active transport [3]. Other nutritional requirements are a nitrogen source, such as, ammonium sulfate, urea and/or various amino acids, biotin, salts and trace elements [2].

Table 1.1 displays some carbon assimilation characteristics of *S. cerevisiae*. These characteristics are important to distinguish *S. cerevisiae* from other yeast species.

Table 1. 1: Carbon assimilation characteristics of *S. cerevisiae* [1]. The symbols "+", "-" and "v" indicate "positive", "negative" and "variable", respectively.

	Fermentation						Assimilation reactions and other characteristics																											
	Glucose	Galactose	Sucrose	Maltose	Lactose	Raffinose	Trehalose	Glucose	Galactose	L-Sorbose	Sucrose	Maltose	Cellobiose	Trehalose	Lactose	Melibiose	Raffinose	Melezitose	Inulin	Soluble starch	D-Xylose	L-Arabinose	D-Arabinose	D-Ribose	L-Rhamnose	D-Glucosamine	N-Acetyl-D-glucosamine	Methanol	Ethanol	Glycerol	Erythritol	Ribitol	Galactitol	D-Mannitol	D-Glucitol
S. cerevisiae	+	v	+	v	−	+	−	+	v	−	+	+	−	+	−	v	+	v	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−

As yeasts commonly do, *S. cerevisiae* uses Embden-Meyerhoff pathway, namely glycolysis, to convert glucose into pyruvate. Ten enzymatic steps occur, and the glycolytic pathway yields ATP molecules per glucose as a net yield. Provising intermediates for biosynthesis is the second importantrole of glycolysis. Under aerobic conditions, citric acid cycle and oxidative phosphorilation will follow, under anaerobic conditions, however, fermentative pathway will be followed by *S. cerevisiae*, which will produce ethanol and carbondioxide [3].

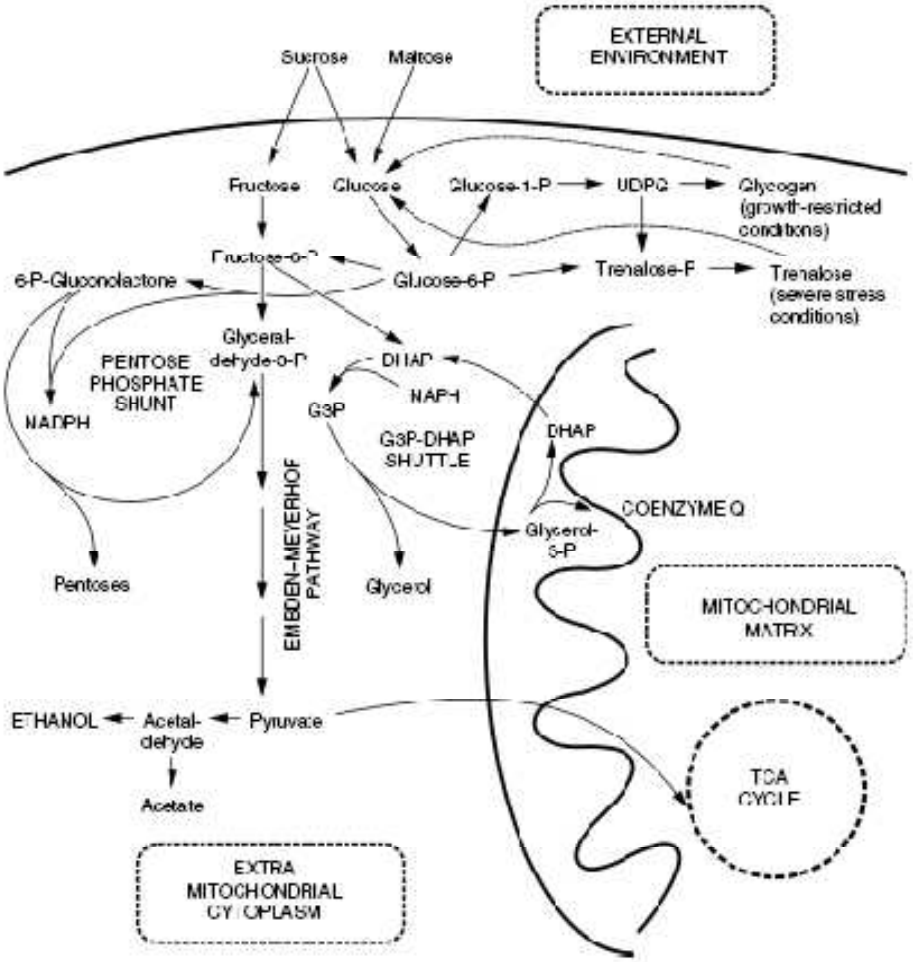

Figure 1. 3: An outline of the sugar utilization pathways in yeasts [3].

The metabolic pathways are strictly controlled. Most of the glycolytic enzymes are synthesized constitutively. Availability of oxygen is one of the major criteria for yeast cells to decide to undergo oxidative phosphorylation or ethanol fermentation, where as the other criterion is the glucose concentration [3].

Growth rate (μ) or generation time (t_g) terms are used to determine the increase in cell number or cell mass with time. By monitoring the increase in the cell number, characteristic growth phases, lag, exponential and stationary, can be observed [3].

Yeasts can grow vegetatively, by mitosis, as either haploid or diploid cells [4]. They can proliferate asexually either by mitosis or by budding. During budding, a small bud is formed from the mother cell and called as the daughter cell. Mother cell's nucleus is divided to generate a new nucleus which will be transported to the daughter cell. After separation from the mother cell, the daughter cell begins to grow. Haploid cells grow and proliferate by mitosis. Yeast can go on to proliferate by mitosis limitlessly, but in appropriate nutritional conditions two cells of opposite mating types, as *a* and *α* (alpha), fuse and form a diploid cell. Diploid cells then undergo meiosis to make four haploid spores (two *a* and two *α*), besides growing throughout mitosis indefinitely. The new generated *a* and *α* can proliferate and grow as before [1, 4]. However, haploid cells will generally die under high stress conditions, diploid cells can endure to stress conditions and go towards meiosis to produce haploid spores [5].

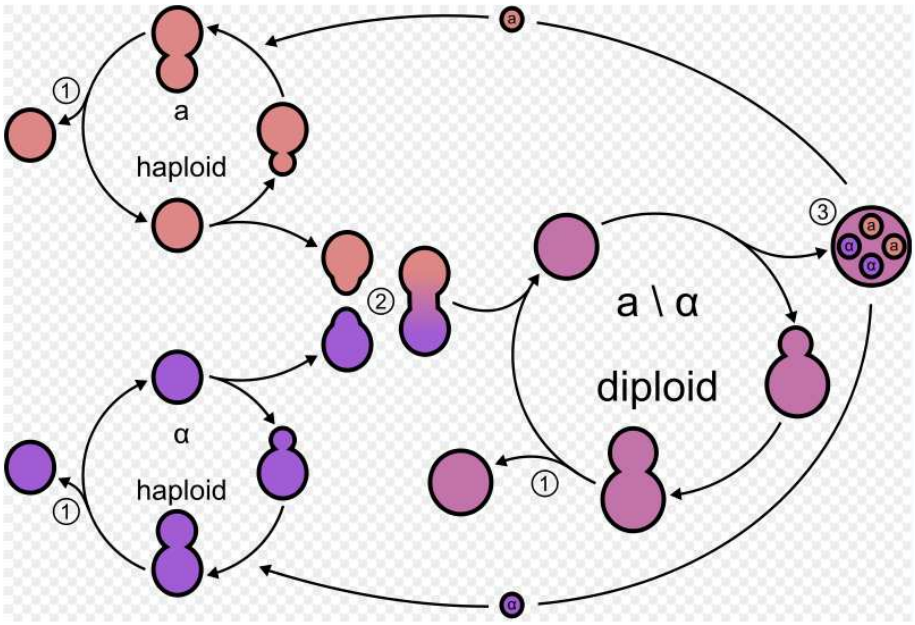

Figure 1. 4: A schematic illustration of yeast cell cycle. Here 1 displays budding process, 2 displays conjugation , and 3 displays spore [6]

Saccharomyces cerevisiae's genome was completely sequenced and published in 1997. It is the first eukaryote the genome of which has been completely sequenced [1] and it is the most investigated eukaryotic microorganism. It is a model organism that helps to understand other eukaryotic cells and also human biology. Many important human proteins like signaling proteins, cycle proteins were firstly discovered by studying their homologs in yeast. For thousands years, it has been used in baking and brewing. As its fermentation characteristics are well established its usefulness has been increasing, thus it became an important microorganism which is used in different processes in food industry, alcoholic beverage industry and pharmaceutical industry. *S. cerevisiae* is a very attractive microorganism to use since it is nonpathogenic and classified as a GRAS (Generally Regard As Safe) organism. It has a short generation time and its cultuvation is relatively easy. Also its genetic properties are well known and it is a suitable microorganism for genetic modifications [7]

1.2 Industrial Application Of *S. cerevisiae*

Baker's yeast has an important market which valued billion dollars and produces million tons of yeast per year. As a result of the increase in population, industrialization, and dietary changes the consumption of it is expanding approximately 4% per year. The cream and compressed yeast forms constitute the 80% portion of the total consumption; the rest is sold in form of dry yeast. The consumption of the dried yeast is thought to increase in the following years correlated with the expected progresses in storage, transport, and enhanced self life [8, 9].

Rapid growth, short generation times, inexpensive and relatively easy cultivation are the benefits of using *S. cerevisiae* for large scale products. Moreover, the fact that it has been classified as a GRAS organism by FDA (Food and Drug Administration) isan additional advantage for the industrial use of *S. cerevisiae*.

Beer and wine are the two alcoholic beverages that have the largest production volume and their commercial value is more than the sum of the other biotechnological products, such as antibiotics, drugs, enzymes, etc. Thus, beer and wine industries are important industries that use *S. cerevisiae* [3].

During wine fermentations, grapes provide a highly selective environment because of the high sugar content (15-20%) and low pH (2.9-3.7). *S. cerevisiae* survives at these conditions better than other microorganisms (Figure 1.5) [3].

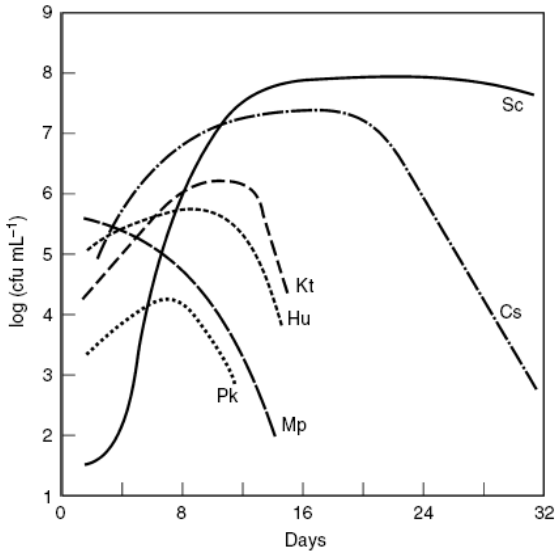

Figure 1. 5: Composite picture in population changes of various yeast species during spontaneous fermentation of wine. Sc: *Saccharomyces cerevisiae*; Cs: *Candida stellata*; Kt: *Kluyveromyces thermotolerans*; Hu: *Hanseniaspora uvarum*; Mp: *Metchnikowia pulcherrima*; Pk: *Pichia kluyveri* [3].

S. cerevisiae is also used in production of beer-type beverages and wine-type beverages like sake and cider, respectively. Distilled spirit production is another application area of *S. cerevisiae* in alcoholic beverage production. Whisky, rum, brandy production can be given as examples to distilled spirits [3].

Bread making is the other oldest and important food-manufacturing process that uses fermentative capacity of *S. cerevisiae* [10]. Requirements of making bread are: flour, water, and yeast [3]. Yeast gives rise to dough by producing gas in leavening stage of the bread.

S. cerevisiae is also used in dairy industry. It fulfills the process requirements in dairy industry, such as, fermentation and assimilation of lactose, proteolytic and lipolytic activity, assimilation of lactic acid and citric acid, growth at low temperature, and salt tolerance. It is mainly used in yoghurt, kefyr, cheese, and ice cream production [3].

1.3 Stress And Stress Responses Of *S. cerevisiae*

Both individual cells and multi-cellular organisms have the ability to respond to changes in environmental conditions by the complex network of sensing and signal transduction. This enables cells to adjust their growth and proliferation, and gene expression program, metabolic activities, and other properties. Environmental conditions that threaten the survival of a cell, or at least prevent it from performing optimally, are commonly referred as cell stress [11].

Because of being genetically and biochemically well characterized euakryote , *S. cerevisiae* can easily be manipulated genetically to construct defined mutants, reporter genes etc. It can also be easily manipulated physiologically through changes in growth and other environmental conditions. Thus, with its relatively rapid generation time, *S. cerevisiae* is also an important organism for stress studies and stress responses. [9].

Optimal physiological conditions for yeast can be described in general terms as incubation with shaking in complex medium that provides abundant easily fermentable sugar, at approximately pH 5 and about 25^0C. When the optimal conditions are not provided yeast strains may display complex stress responses [9].

Yeast cells are single cell organisms that are living freely in nature, and face large variations in their natural environment and during industrial processes [9, 11]. Declination from the optimal conditions can be a result of physical or chemical changes in the environment like, temperature, pressure, radiation, concentration of solutes and water, presence of certain ions, toxic chemical agents, pH and nutrient availability. These can prevent enzyme activities, destabilize cellular structures, disrupt metabolic fluxes, perturb chemical gradients etc., leading to overall instability [11]. Table 1.2 displays the stress types and their consequences during industrial application of baker's yeast, *S. cerevisiae*.

Table 1. 2: Physiological consequences of key stresses encountered by baker's yeast during industrial applications [9].

Stress type	Major occurrence	Damaging consequences
Supraoptimal temperatures	During biomass production, drying of yeast, early stages of baking	Leakiness of membranes, loss of internal solutes, ionic imbalance, internal acidification, generation of free radicals, loss of mitochondrial function, damage to proteins and enzyme activities
Oxidation	During biomass production, during drying of yeast	Formation of free radicals; damage to proteins, lipids, and nucleic acids; damage to mitochondria; membrane leakage
Hyperosmolarity	During biomass production, downstream processing, drying, inoculation into doughs, freezing/thawing of yeast and doughs	Reduction of cell volume and loss of turgor, growth inhibition, disturbance of metabolite concentrations, reduction of fermentative activity
Desiccation/ rehydration	During dewatering of yeast in downstream processing, production of dried yeast and reconstitution in doughs, freezing/thawing of yeast and doughs	Similar to hyperosmotic pressure but with extreme concentration of internal solutes, disruption of macromolecular structure including membranes, loss of internal solutes upon rehydration, breakdown of vacuolar structure and release of degradative enzymes
Freezing/thawing	In storage of yeast block in some bakeries but especially in frozen dough technology	Low internal pH, imbalance of metabolites, dehydration, ionic toxicity, damage to essential membrane processes

The aim of the cellular stress response is to protect the cell from detrimental effects of stress and repair possible damages [11]. Cell survival critically depends on celular ability to sense alterations in the environment and to respond to new situations through the induction of protective stress responses [12]. Rapidly acting mechanisms and powerful adaptation mechanisms are crucial for the survival and for maintaining the proliferation capacity toward a sudden environmental change [11]. Yeast is known to be one of the most suitable systems to study stress tolerance [12].

S. cerevisiae can suffer from sublethal injury, and can repair the damages to continue its growth under normal conditions. But if the stress parameter is too high it will die [3].

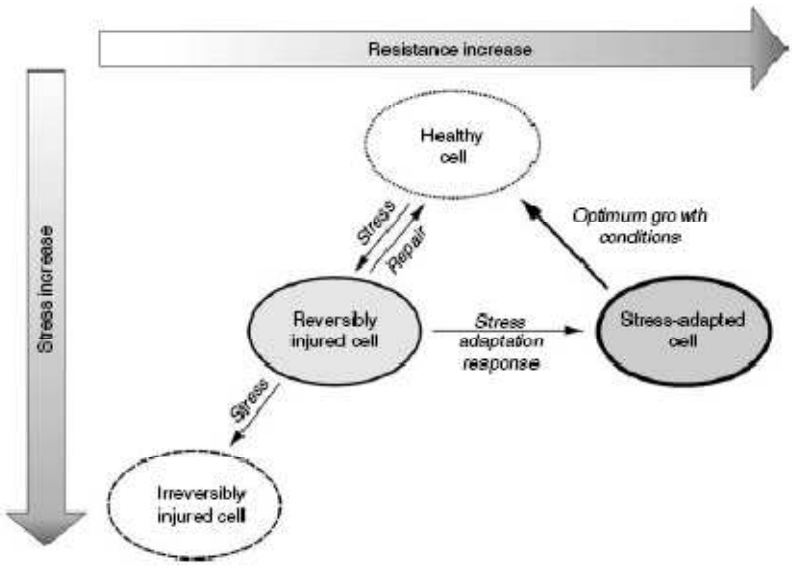

Figure 1. 6: Microbial stress, injury, adaptation and resistance to processing [13].

Despite being exposing to several stresses in industrial practices, yeasts are expected to yield biomass economically and to leaven doughs in a variety of baking processes. Thus, robustness is crucial for yeasts used in the baking industry [9].

Stress conditions studied on yeast cells include heat stress, ethanol stress, oxidative stress, rapid freezing stress, slow freezing stress, salt stress, acetic acid stress, etc [12]. Tolerance to some stresses is found as related, and this relationship is attributed to a possibility of a mechanistic similarity between these stresses, either in the type of injuries, protecting mechanisms, or damage repair mechanisms [11, 12]. In other words, multiple stress tolerance is controlled by a common mechanism, and this control provided by multiple loci is broadly distributed throughout the genome of a yeast cell [11, 14, 15]. Genome wide gene expression analysis shows that about 9-14% of all genes in *S. cerevisiae* are involved in general stress response [16]. These genes are not well characterized and their function in stress tolerance has not been clearly understood [11, 17]. Thus, rational approaches based on modifications in one or a few genes are generally not sufficient to improve complex cellular properties such as multiple stress tolerance [15]. Usually evolutionary engineering methods based on random mutation and selection are required to obtain "robust" phenotypes [15].

A variety of stress conditions trigger cellular stress response mechanisms which begin at the transcriptional level. In general, Msn2 and Msn4 are transcription factors transported to the nucleus, to bind to stress-response elements (STRE) and induce

transcription at target genes. In some stress response mechanisms, heat-shock factor HSF1 activates the transcription of heat shock elements (HSE). On the other hand, in osmostress response, Hog1 protein goes to nucleus, interacts with some transcription factors and activates osmostress-induced gene transcription. Crz1 transcription factor's migration to nucleus is triggered in salt stress, and there it reacts with calcineurin-dependent-response element (CDRE) in target gene promoters. In oxidative stress, Yap1 transcription factor accumulates in the nucleus and interacts with Yap1-response elements (YRE) in order to induce transcription of antioxidant genes. Gis1 transcription factor becomes activated in starvation stress, and induces stationary phase genes by interacting with post-diauxic shift element (PDS) [16].

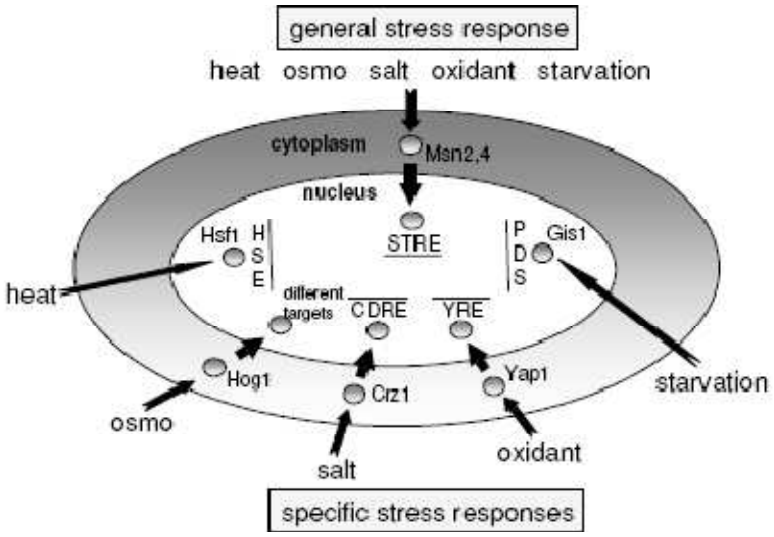

Figure 1. 7: A general representation of stress response mechanism in *Saccharomyces cerevisiae* [16].

Cellular response to stress results in a control on growth, cell sensing, signal transduction, transcription, and posttranslational activities. There are some important signalling pathways that affect the physiological status of yeast cells during growth. *RAS*-cyclic AMP (cAMP) signal transduction pathway is one of them which is fully operative under optimum fermentation conditions, and leads to transcription and posttranslational events that provokes growth and proliferation. It also downregulates stress tolerance factors. Under stress conditions, cAMP-dependent protein kinase A activity is reduced [9, 18].

Stress conditions activate some transcriptional elements, like heat-shock elements (HSEs), stress-response elements (STREs), and AP-1 responsive elements (AREs); lead to accumulation of some proteins and other molecules that provide maintenance and restoration [18].

Table 1. 3: Some key factors and their roles in stress protection or repair in *S. cerevisiae.*

Factor	Function
Hsp 104	Disaggregation of denatured proteins
Hsp 70 representatives	Disassembly and refolding of denatured proteins
Ubiquitin	Tagging of damaged and potentially toxic proteins for degradation
Catalase A (peroxisome)	Detoxification of reactive oxygen species (ROS)
Catalase T (cytosol)	Detoxification of ROS
Cytochrome C peroxidase	Detoxification of ROS
Cu/Zn SOD (cytoplasm)	Detoxification of ROS
MnSOD (mitochondria)	Detoxification of ROS
Tsap	Protecting against oxidation involving thiol groups
Atxp	Heavy metal (and associated ROS) detoxification
Metallothioneins	Heavy metal (and associated ROS) detoxification
H-ATPase	Efflux of protons and regulation of internal pH
Other membrane ATPases	Efflux of ions
Glutathione	Detoxification of ROS
Trehalose	Protection of proteins and lipids-compatible solute
Glycerol	Osmotic equilibration and retention of turgor pressure in hyperosmotic conditions-compatible solute

Recently, some different technologies that have been exploited to improve stress tolerance of yeast strains [14, 15]. By exposing UV-mutagenized *S. cerevisiae* to 200 freeze-thaw cycles, highly freeze-tolerant mutant strains were obtained [19], and mutant strains with multiple-stress tolerance were obtained by subjecting EMS-mutagenized *S. cerevisiae* to freeze-thaw cycles [20].

1.4 Freeze-Thaw

The freezing process occurs in most natural habitats where temperature decreases below 0^0C at night, also in some regions where the temperature is permanently cold. Thus, like many other microorganisms, yeasts also encounter freezing [21].

Freezing is a preservation method that keeps microorganisms without undergoing genetic mutations or loss of function [22, 23]. With decreases in the temperature, the enzymatic reaction rates are also decrease, making freezing a useful method for extending ordinarily short-lived substance life [24]. Thus, freezing has a wide range of applications in industry, medical and food technology, in agriculture and in scientific research. Preservation of microbial strains, even tissues and organs,

cryosurgery [18, 23], and frozen dough technology are important application areas of freezing process.

The mechanism of freeze injury is not well understood yet. As a biochemically and genetically well characterized organism, *S. cerevisiae* is suitable to study on the freeze-thaw injury mechanism [18, 22].

1.4.1 Freeze-thaw stress

When cooled to subzero temperatures, cells and medium initially supercool [21], and during this stage, the cell is not injured [24]. The cell content is more concentrated than the growth medium; therefore, external water freezes and forms ice crystals before intracellular freezing, and causes cell death [21, 22, 24]. After the ice crystal formation in medium, the amounth of water decreases, and leads to an increase in osmotic pressure of the medium. As the outside of the cell becomes concentrated, the intracellular water begins to diffuse out of the cell [21, 22, 24]. Shape, structure, surface area to volume ratio and cell permeability are important cell characteristics to determine the mass transfer and the freezing rate of the cell [18, 22]. Therefore, cells are expected to have a specific freezing rate under the same conditions [18].

During freezing ice crystals forms both in extracellular and intracellular environment. These ice crystals damage the cell in different ways. Extracellular ice crystals entrapt cells and create mechanic and adhesion stress on the cell. Intracellular ice crystals initially damages the cell membrane and this damage is the most detrimental one. It also damages internal membranes, vacuole and leads to cell wall disruption, release of cytochrome c from mitochondria [21]. With DNA microarray analysis freeze-thaw damage on cell wall structure and on cellular organelles have also been showed [25]. Other damage factors are electric fields and gas bubbles, which are thought to cause mechanical damage to organelles, upon association with ice fronts [21].

There are at least two injury mechanisms that occur during freeze-thaw process: oxidative stress and defect in cell wall assembly [17, 21]. As after ischemia and reoxygenation, reactive oxygen species (ROS) is activated by provoking a mechanism that results in excess O_2 in cell cytoplasm, it is thought that freeze-thaw process forms similiar conditions where cells can't access O_2 throughout freezing

and are re-oxygenated during thawing [23, 26]. The leakage of oxygen and electrons from the mitochondrial electron transport chain is thought to form superoxide radicals which are ROS. ROS causes oxidative stress during thawing state, and results in cellular damage, such as protein inactivation, DNA and membrane damage due to lipid peroxidation [21, 23]. It is reported that only a fraction of genes required for oxidative stress tolerance are essential for freeze-thaw tolerance [27].

The cooling rate is a very important parameter that influences cell injury throughout freezing [22]. At low cooling rates, ice crystals form in the extracellular environment which provides sufficient time for intracellular water to flow out besides forming ice crystals inside the cell [22, 24, 28]. On the other hand, at high cooling rates, intracellular water forms ice crystals, which is also lethal for the cell [28].

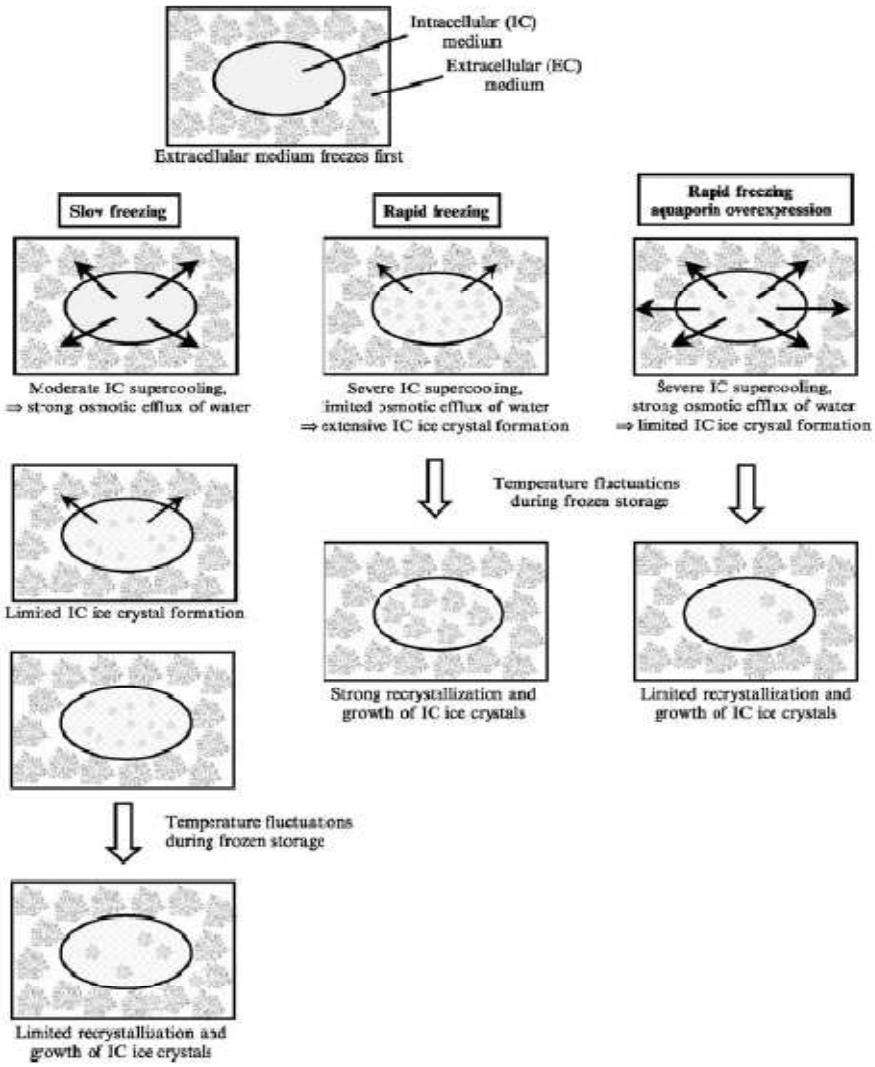

Figure 1. 8: Schematic illustration of the status and the movement of extracellular (EC) and intracellular (IC) water in the cell suspension during slow and rapid freezing as well as frozen preservation [21].

Dumont et al. (2003), studied the relation between cooling rate during freezing and cell mortality using four different cooling rates. They concluded that very low cooling rates and high cooling rates result in high mortality. On the other hand, slow and very high cooling rates did not damage cells as much as other conditions did. In low cooling rates, the inlet water can diffuse out of the cell slowly, and this does not disrupt the cell. According to the high viability results upon very high cooling rates, it was assumed by the authors that the inlet water vitrificates or crystallizes before any water loss which provides high cell viability [22]. The inlet crystal formation is known as a major factor for cell damage.It was suprising to observe that very high cooling rates have minor damaging effects on the cells and it remains to be investigated how the intracellular crystal formation does not destroy the cells under these conditions.

The freezing rate has an important effect on cell survival after freezing. Besides freezing rate, the physiological conditions, such as the nutritional condition and growth phase, and the genetic background of the cell are also important variables that contribute to cell survival during the freezing process [18, 27, 29].

Cell tolerance to freeze-thaw stress seems to be the highest during the diauxic shift and lag phases, and lowest during early exponential phase. The freeze-tolerance at stationary phase is also relatively high. It is suggested that the accumulation of glycerol and trehalose in stationary phase may form thick cell walls and protect the cell from stress [18].

It was also observed that growing on nonfermentable carbon source was favorable than growing on fermentable carbon source to display freeze tolerance. In nutrient deficient media, which leads to change in intracellular concentration of cAMP, cells tolerate the freezing stress better. Thus, RAS-cAMP signal transduction pathway apparently has an effect on freeze-thaw tolerans. However, when starved cells continue growing under optimal conditions, they lose their freeze tolerance ability. This is thought to be the result of catabolism of the storage carbohydrates, glycerol and trehalose, which provide a protecting barrier for cell during freeze stress [18].

Ando et al (2006) identified and classified the genes required for freeze-thaw stress tolerance revealed by genome-wide screening of *S. cerevisiae* deletion strains. They found that genes required for freeze tolerance belonged to one of the three functional groups: interaction with the cellular environment, protein fate, and protein synthesis.

[27]. They reported those genes as *VMA* genes, structural components of vacuolar H^+-ATPase, which is responsible in the regulation of pH homeostasis by vacuolar acidification; glycosylation genes, such as *ANP1*, *MNN10*, and *MNN11* that are required for cell integrity; and also genes for ribosomal and mitochondrial ribosomal proteins, such as *RPL27A* and *MRPL32*. Those genes seem to play critical roles in freeze tolerance. These results showed that the regulation of pH homeostasis by vacuolar acidification, cell integrity, and unimpaired functions of ribosome are required for freeze tolerance [27].

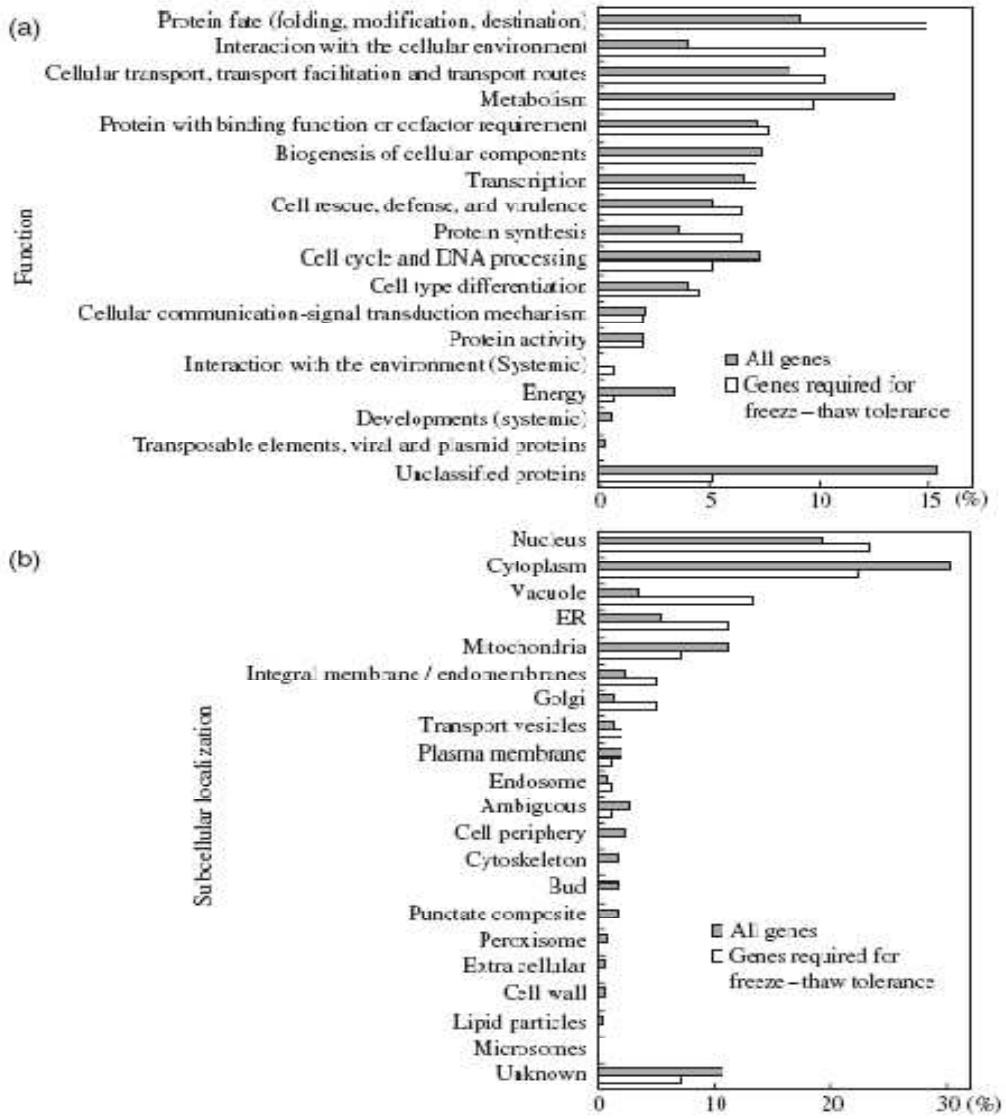

Figure 1. 9: Function (a) and subcellular product localization (b) of genes required for tolerance (58 genes) to freeze-thaw stress and of all organelles deleted in the complete set of strains [27].

According to Figure 1.9, most of the gene products required for freeze-thaw tolerance are localized in vacuole. ER and Golgi apparatus are also necessary for vacuolar and cell wall biogenesis [27].

Unlike other stress types, it was reported in some studies that the cells cannot gain resistance against freezing stress. According to these studies, when cells allowed to grow under optimal conditions, repeated freezing and thawing did not induce tolerance [18, 21]. This has to be overcame for improvements in frozen dough technology [9, 21, 30]

1.5 Freeze Tolerance Mechanisms

In general, yeast stress response is related with a strictly controlled metabolism via growth control, signal transduction, transcription, translation, and posttranslational control. Nevertheless, the components of the response mechanism are known, and recent research also provides new data to highlight the mechanism. However, the stress response mechanism is still too complicated to understand.

Trehalose is an important storage carbohydrate and stress protectant. Trehalose protects cells during stress conditions by preventing protein aggregation and denaturation [31], and stabilizing the membrane by replacing water [21]. Trehalose production increses at late exponential phase [31].

Although Lewis et al. (1996) reported that there was no correlation between trehalose content and tolerance to freezing and other stress types, except acetic acid stress [12], there are evidences that trehalose content may be correlated with the freeze tolerance. Research with genetically modified organisms showed that trehalose has an important role to protect the cell from freezing stress damage. It was also shown that the amount trehalose affects cell viability against freezing stress [8, 32, 33]

Glycerol is a well known commercial cryoprotectant, and used to minimize freezing damage. Being a colorless, odorless, nontoxic, and relatively cheap water soluble chemical, it is advantageous for practical applications. Glycerol also plays an important role in lipid structure [34], which is related to membrane characteristics and fluidity, both important in freeze tolerance.

With deletions of genes related with glycerol metabolism, glycerol transformation was prevented and intracellular-glycerol- enriched cells were obtained. The resulting

cells could live well in glycerol medium after freeze-thaw treatment, and were used for dough-making. It was also reported that glycerol was used as a cryoprotectant inside the cell. The precise role of glycerol in protecting cells from the freezing stress is not clear yet but at least it can be said that glycerol is a useful cryoprotectant. Beside its cryoprotectant effect, glycerol is thought to contribute the protection of the cell indirectly from freezing stress effects [35]. The speculations about the indirect protection mechanisms of glycerol are: affecting the lipid composition on the membrane and provide cell protection [36]; and impacting on the signal transduction pathway to trigger the stress response [37].

Oxidative stress that appears during freezing stress is judged as one of the most important reasons for cll injury. Living cells that use oxygen as the terminal electron acceptor, produce ROS during respiration reactions unavoidably. There are enzymatic and non-enzymatic reactions in cells to detoxify ROS. The important enzymes on the detoxification mechanism are superoxide dismutase (SOD), catalase, and glutathione peroxidase. The enzymes and reactions they catalyze are illustrated in Figure 1.10.

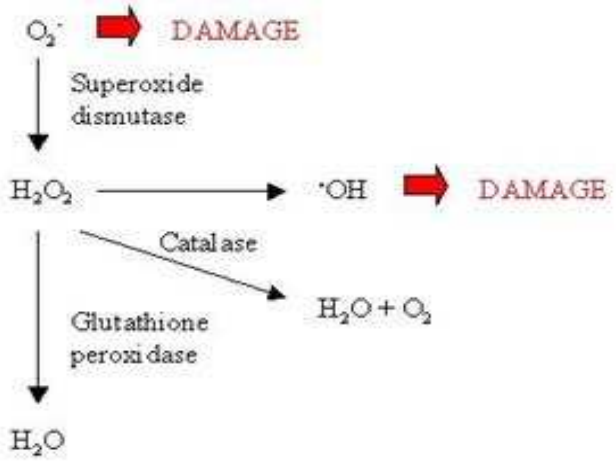

Figure 1. 10: The enzymatic reaction chain of detoxifying ROS, and functionary enzymes [38].

Yeast has two types of SOD and two types of catalase. The Cu,Zn-SOD is located in the cytoplasm and encoded by *SOD1*, the Mn-SOD is located in mitochondrial matrix and encoded by *SOD2,* whereas, catalase is encoded by *CTT1* located in cytoplasm, and *CTA1* encoded catalase is located in peroxisome [39].

Park et al. (1998) reported that both types of SOD play an important role in cell defense during freeze-thaw stress. On the other hand, as the absence of catalase was

compensated by the cells, neither catalase, nor glutathione peroxidase had a significant effect on cell survival during freeze-thaw stress. This result indicated that the most important stage in oxidative defense just after the freeze-thaw stress is the removal of O_2^- radical. Working with gene deletion mutants it was discussed that the main lethal effect may occur in the cytoplasm and Cu,Zn-SOD may be more important in cell defense than Mn-SOD [23].

In another study, it was found that N-acetyltransferase Mrp1 may protect the cells from freeze-thaw injury, by reducing the intracellular oxidation level in an enidentified way [40].

Aquaporins are involved in transporting water and small solutes through the membrane. Microarray analysis showed that the *AQY2*, aquaporin gene expression was higher in freeze tolerant strains, which may also be an evidence for the influence of aquaporins on freeze tolerance mechanism [10]. This finding highlights the membrane role in freeze tolerance by water transport.

Molecular chaperones are a class of proteins which bind to incompletely folded or assembled proteins in order to assist their folding or prevent them from aggregation [41]. Although protein aggregation is not the major injury throughout freeze-thaw stress [28], a bacterial chaperon was reported with high affinity to frozen denatured proteins, which also refolds these proteins [42].

1.6 Importance Of Freeze Tolerance

Understanding microbial freeze tolerance mechanism is important both for basic research and commercial applications. There is a large potential for commercial applications with freeze tolerant microorganisms, freeze-stress related biomolecules, and microbial cryoprotectants [21].

The use and production of frozen doughs is continously increasing in all industrialized countries because of the great convenience in separation and use of dough, economy of scale to manufacturers, saving in labor time and maintenance costs for bakers, and to provide fresh baked goods to customers [10, 21, 27]. However, there are significant drawbacks of this technology, such as significant reduction of the leavening capacity during freeze storage, reduced bread volume, diminished bread quality after freezing , freeze storage, and thawing [21]. These

problems are mostly attributed to two major bottlenecks: one is the dramatic change in stress tolerance throughout the onset of the fermentation, possibly due to cAMP activation [18], and the other is the aggregation and damage of gluten proteins. Ribotta et al. (2001) also reported that, storage in frozen conditions causes depolymerization in bread protein matrix [43]. The schematic outline depicted in Figure summarizes these major problems and their results.

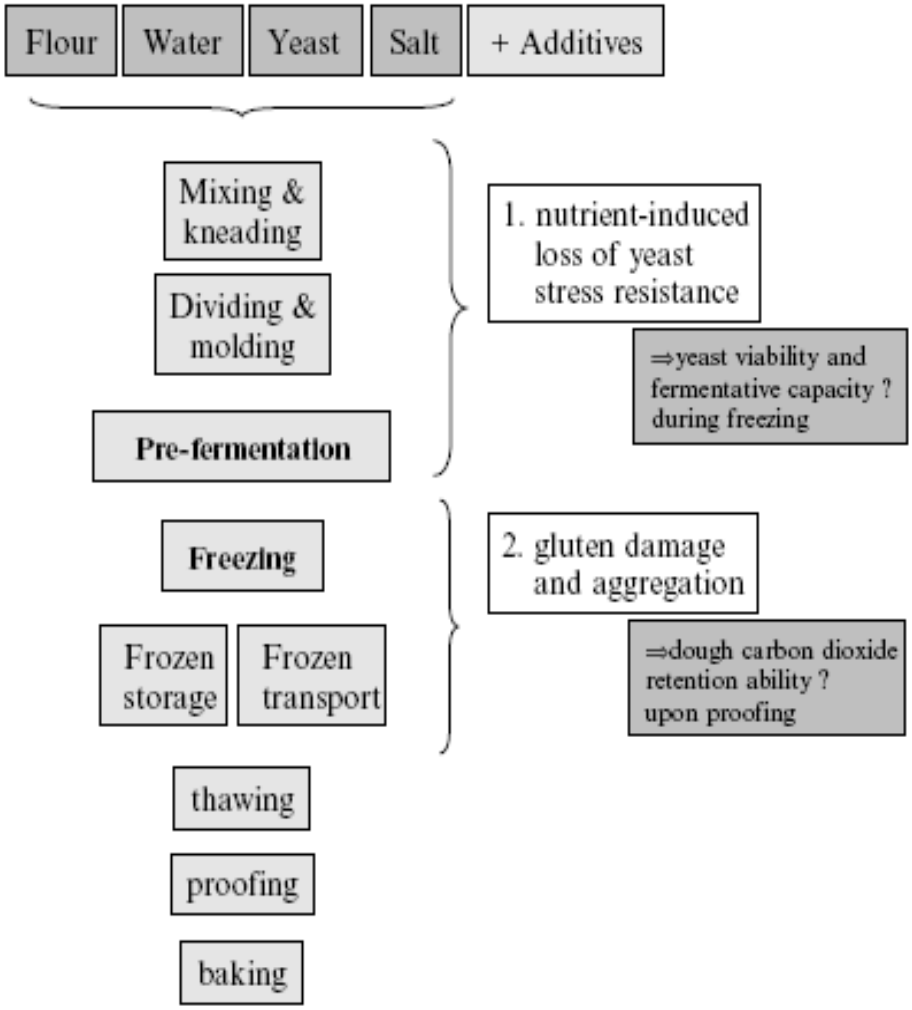

Figure 1. 11: Schematic outline of the frozen dough process [21].

Many studies have been reported on handling the problems in frozen dough technology but no significant results could be obtained yet. The expected solution to the problems seems as the use of a robust microbial strain that is suitable for this technology [30].

1.7 How To Obtain Freeze Tolerant *S. cerevisiae*

Different methods can be used to obtain genetically improved, mutant organisms with complex, desirable phenotypes. Random mutagenesis, metabolic engineering, evolutionary engineering, sexual recombination, metagenomic strategy, and genome shuffling are some of these methods [15].

One of the recent attempts for obtaining genetically modified microorganisms with improved industrial properties is metabolic engineering. Metabolic engineering was defined by Stephanopoulos et al. (1998) as the direct improvement of product formation or cellular properties through the modification of specific biochemical reactions or the introduction of new ones with the use of recombinant DNA technology [44]. Metabolic engineering seems to be a suitable method for obtaining freeze-tolerant microorganisms.

1.7.1 Metabolic and inverse metabolic engineering

As DNA technology develops, it enables manipulations in metabolic pathways by direct approaches. Metabolic engineering allows modification of gene promoter strength or type, deletion of genes or introduction of new genes into the cell [15, 44, 45].

The application potential of this method covers a wide range of areas in biotechnology. The aim of metabolic engineering is to improve new processes and new products, and enhance existing processes, in order to create biologically derived processes as a favorable alternative to chemical processes [45, 46]. It is a multidisciplinary method between chemical engineering, biochemistry, molecular and cell biology, and computational science [46].

The metabolic engineering consists of two general stages: analysis and synthesis, as shown in Figure 1.12 [7].

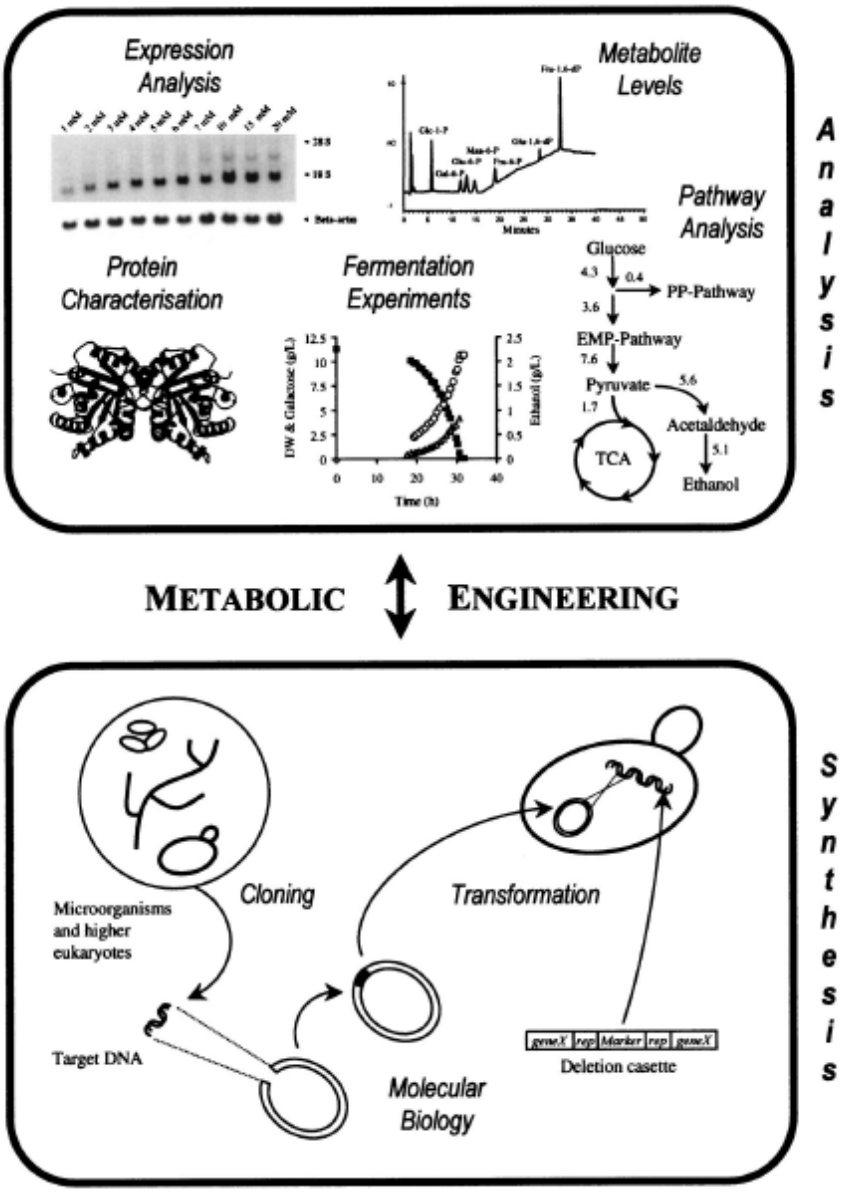

Figure 1. 12: A schematic illustration of metabolic engineering pathway on two parts [7].

The major requirement for successful application of metabolic engineering is that the cellular system and the genetic target must be well-known, and powerful algorithms must be used. [15, 44, 45]. Such difficulties in metabolic engineering applications lead to development of an alternative approach called inverse metabolic engineering.

Inverse metabolic engineering, in contrast to metabolic engineering, starts with the organism that has the desired phenotype and determines the genetic or environmental basis of that phenotype. Consequently, this genetic basis is used to improve the same organism or different organisms [45, 46].

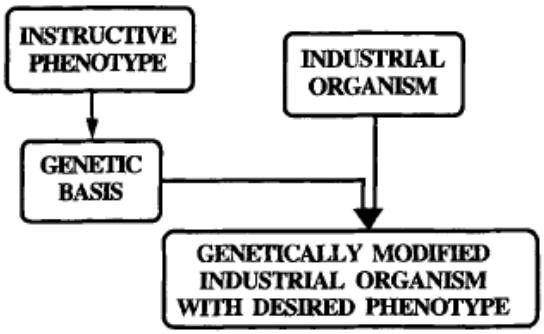

Figure 1. 13: Schematic diagram of information flow in inverse metabolic engineering [45].

1.7.2 An inverse metabolic engineering strategy: evolutionary engineering

In evolutionary engineering, the first step is to increase the genetic diversity of the population by using chemical or physical mutagenesis. Thus, genetic variants with desired characteristics are isolated from this large, diverse population. Evolutionary engineering is based on random mutagenesis and selection [15].

Ethylmethane sulphonate (EMS) is a well-known mutagenic organic compound with formula $C_3H_8O_3S_6$. It gives alkylation reaction and generates G/C to A/T point mutations in DNA. These mutations can cause loss of function or alteration of the normal function of a gene. These mutations are not directed, besides randomly distributed in the genome [41].

The recombinant DNA technology is a relatively new technology and its long term effects on health are still unknown. Thus, there are generally public reactions against goods produced by the use of recombinant DNA technology. As compared to recombinant DNA technology, the use of evolutionary engineering is publically more acceptable as a more “natural” strategy [20].

1.7.3 The aim of this study

The aim of this study was to obtain freeze tolerant yeast cells by developing and using evolutionary engineering strategies. For this purpose, selection at two different freezing temperatures were employed and the efficiency of two different selection procedures were compared according to the freeze-tolerance levels of the selected mutants.

Basic characterization of the mutants was performed by quantitatively determining their survival under the stress conditions at which they had been selected and also by

determining their cross-resistance against other stresses. The mutants and the information to be obtained from their further characterization could be exploited in industrial applications, such as frozen dough technology.

2. MATERIALS AND METHODS

2.1 Materials

2.1.1 Yeast strain

Saccharomyces cerevisiae CEN.PK113-7D was kindly provided by Dr.Peter Kötter from Johann Wolfgang Goethe University, Frankfurt, Germany Internet Server-Client Technology

2.1.2 Yeast culture media

Yeast minimal medium (YMM)

Yeast Nitrogen Base without amino acids	6.7 g
Dextrose	20 g
Agar (for solid media)	20 g

in 1 liter of distilled water.

Xylose medium

Yeast Nitrogen Base without amino acids	6.7 g
Xylose	20 g
Agar (for solid media)	20 g

per liter of distilled water.

YMM without dextrose

Yeast Nitrogen Base without amino acids	6.7 g
Agar (for solid media)	20 g

per liter of distilled water.

2.1.3 Chemicals

Hydrogen peroxide (35%, v/v) was obtained from Merck (Germany).

D(+) - Trehalose dihydrate was obtained from Riedel-de Haën (Germany).

2.1.4 Laboratory equipment

Thermomixer	Eppendorf, Thermomixer Comfort 1.5-2 ml, (Germany)
Microfuge	Beckman®Coulter Microfuge (USA)
Rotor	Beckman Coulter JA-30.50i (USA)
UV-Visible Spectrophotometer	Shimadzu UV-1700 (Japan),
	Perkin Elmer 25 UV/VIS (USA)
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 (Italy)
Autoclaves	Tuttnauer Stystec Autoclave 2540 ml (Switzerland)
	NüveOT 4060 Steam Sterilizer (Turkey)

Deep Freezes and Refrigerators	80˚C Heto Ultrafreeze 4410 (Denmark),
	-20˚C Arçelik (Turkey)
	+4˚C Arçelik (Turkey)
Orbital Shaker Incubators	Certomat S II Sartorius (Germany)
Incubators	Nüve EN400 (Turkey)
	Nüve EN500 (Turkey)
Light Microscope	Olympus CH30 (USA)

HPLC (High Performance Liquid Chromatography) System

- Refractive Index Detector	Shimadzu RID10A (Japan)
- System Controller	Shimadzu SCL10A) (Japan)
- Liquid Chromatography	Shimadzu LC-10AD (Japan)
- Degasser	Shimadzu ss DGU-14A (Japan)
- Column Oven	Shimadzu CTO-10AC (Japan)

2.2 Methods

2.2.1 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 in order to have the 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.

300 µl of EMS was added into each 10 ml of cell suspension in a screw-cap glass tube. The tube was vortexed and then incubated for 30 minutes at 30º C. In order 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 30.50 i 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 201. The original wild-type cells were named as 200.

2.2.2 Application of freeze-thaw stress

500 µl inoculum from the frozen stocks kept at -80^{0}C in 30% (v/v) glycerol was inoculated into 10 ml of YMM, and the culture was incubated overnight. The cell culture growth was monitored by optical density measurements at 600 nm wavelength (OD_{600}).

Freezing stress was applied during the late exponential growth phase when OD_{600} was between 4.5 and 6.0. When the cell cultures reached this OD range, the culture was harvested by centrifugation at 14000 rpm for 5 min using a Beckman Coulter JA50 i type rotor. The supernatant was discarded and the remaining cell pellet was resuspended in yeast minimal medium without dextrose and shaken vigorously by a vortex. This step was repeated twice. After the washing step, the cells were ready for stress application.

To freeze cells at different rates during selections, two different freezing temperatures were used: -80^{0}C and -196^{0}C (freezing at liquid nitrogen).

During selections, the initial stress level was expodure to one cycle of freezeing and thawing. At each succesive generation, the number of cycles (repetitions) was increased up to 10 and 16 cycles for the last population of -196^{0}C and -80^{0}C freezing selection, respectively.

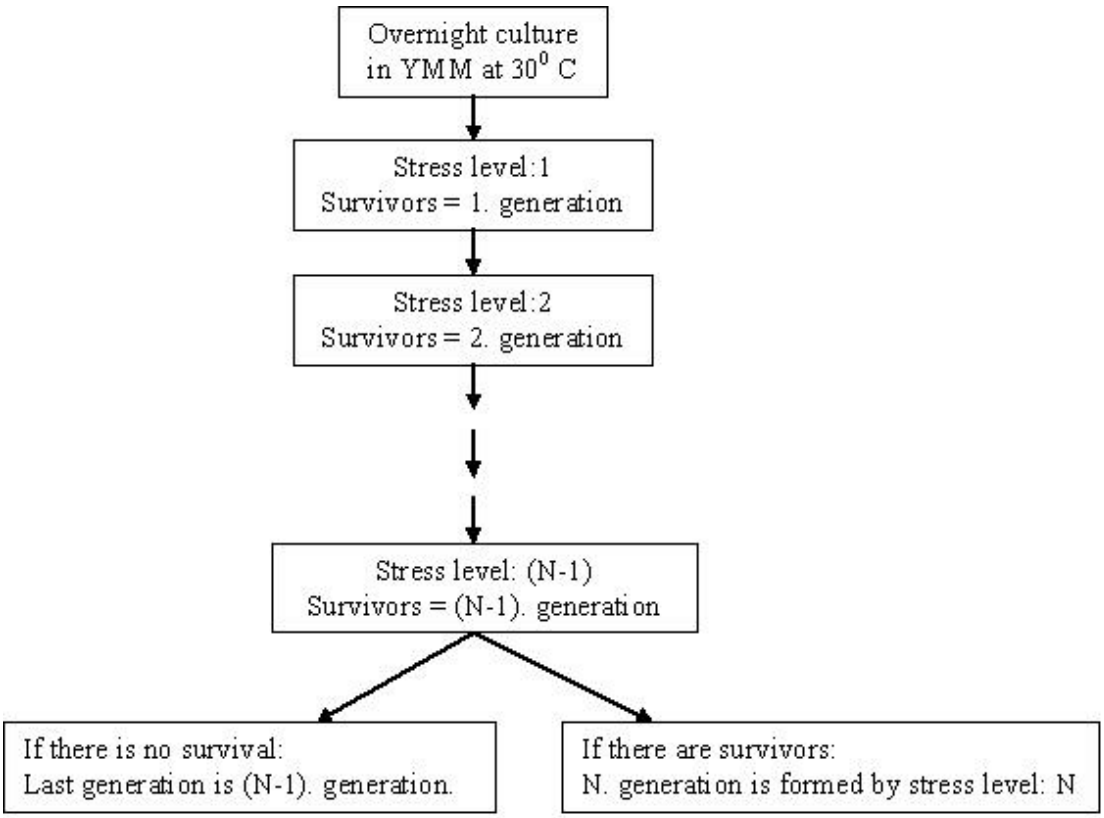

Figure 2. 1: The scheme of the pathway of obtaining generations freeze-tolerant yeast mutants [47].

2.2.2.1 Freezing at -80^{0}C

Cell cultures wereincubated until the late exponential phase. After reaching desired OD_{600} values (4.5 – 5.5), one ml volume was transferred to 1.5 ml microfuge tubes. The samples in microfuge tubes were then washed twice with glucose-free yeast minimal medium (YMM). For this purpose, the tubes were centrifuged at 14000 rpm for 5 min in a benctop centrifuge at each washing step. The supernatant was discarded and cells were resuspended with dextrose free-YMM each time.

To apply freezing stress at -80^{0}C, microfuge tubes that contained cell suspensions were left in the deep-freezer at -80^{0}C for 45 min and thawed at 30^{0}C for 10 min using a thermomixer. Cell viabilities of both the control and the experimental group were determined by appliying five tube Most Probable Number (MPN) Method in microtiter plates (section 2.2.2.3).

After the stress application, the cell suspension in the microfuge tube was regarded as a new generation that survived the freezing stress step. After thawing the cell suspension, 100 µl was withdrawn for inoculating the next selection generation, and eventually 500 µl was withdrawn to prepare frozen stocks of this new generation.

2.2.2.2 Freezing at -196^{0}C in liquid nitrogen

Cell suspension was prepared for stress application as described in 2.2.2.1.

To apply freezing stress in liquid nitrogen microfuge tubes were submerged into the liquid nitrogen (at -196^{0}C) for 25 min. After freezing, they were thawed at 30^{0}C for 15 minutes in a thermomixer. Control groups were not exposed to freezing stress. Cell viability of the control group and the experimental group was determined by five tube MPN method (section 2.2.2.3).

2.2.2.3 Most probable number (MPN) method

The **M**ost **P**robable **N**umber (MPN) method was used to determine the cell viability. This method is also known as the method of Poisson zeroes. It is based on serial dilution strategy, and used to get quantitative data about concentrations of discrete items from positive/negative data [20, 48].

According to the MPN method, the initial culture was diluted 10 fold in YMM at each dilution step. This method was performed in microtiter plates firstly by filling 40 wells (5 wells in horizontal line x 8 wells in vertical line) with 180 µl sterile YMM. Twenty µl culture was then added to each of the five wells in the first horizontal row (wells A1, A2, A3, A4, A5, in Figure 2.2). Diluted cell suspension in the first row was mixed well by multichannel micropipette and 20 µl of this suspension was transferred to the 2nd row (B wells in Figure 2.2). In this way, the initial culture was diluted at each step upt to 10^{-8} by 8 rows, and in 5 parallel columns, representing a “five-tube” experiment, i.e.

After incubation, wells were observed for the presence or absence of growth. Theoretically, if at least one viable yeast cell were present in the well, visible growth would be observed in that well. The cell viability results are evaluated using statistical, standard 5 tube MPN tables [20].

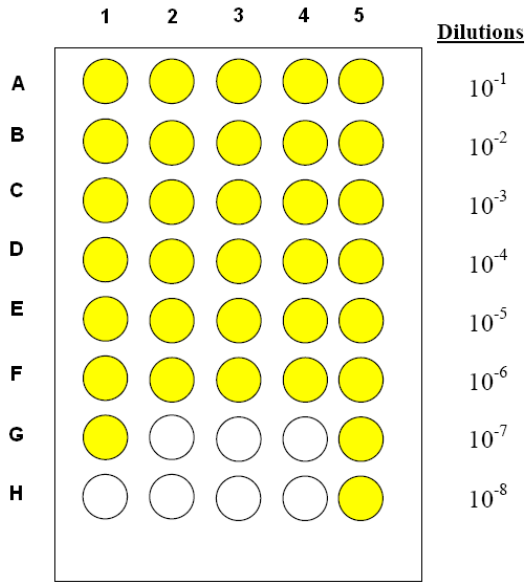

Figure 2. 2: A schematic visualization of a 96-well microtiter plate used for MPN analysis. White circles indicate the wells with no growth and shaded ones the wells with growth.

Figure 2.2 shows the design of a multi-well plate for MPN analysis. The shaded wells represent wells with growth and the white ones show well without any growth. Each row represents a ten-fold dilution of the previous row of wells. The last three rows where cell growth was observed were considered for determining the cell viability (e.g. rows F,G and H in Figure 2.2).

Here is an example of how to estimate MPN based on the growth data given in Figure 2.2.

10^{-6} dilution: 5 positive tubes with growth

10^{-7}dilution: 2 positive tubes with growth

10^{-8}dilution: 1 positive tube with growth

This result entitled as 521G, and the viable cell number that counterparts thecorresponds to this coding is found from the standard 5 tube MPN table [49].

MPN results were obtained at the 24^{th}, 48^{th} and 72^{th} hour incubation of cultures and the corresponding viable cell numbers were obtained from standard 5 tube MPN table.

2.2.2.4 Calculating cell viability

The cell viability result is the most important data to predict how resistant the obtained mutant cell culture as compared with the wild type. This data acquired by

calculating the ratio of the living cell number after stress to living cell number before stress. The equation is given below:

$$Cell\ Viability = \frac{t_1}{t_0} \quad \textbf{(2.1)}$$

Where t_0 is the number of living cells that were not exposed to stress conditions, and t_1 is the number of living cells that were exposed to the stress conditions. These two parallel cultures were left to incubation simultanously and under exactly the same conditions.

2.2.2.5 Preparing stock cultures

Stock cultures were preserved at -80^{0}C using a deep-freezer. 500µl cell suspensions in YMM were mixed with 500 µl 60% (v/v) glycerol in sterile 1.5 ml microfuge tubes. The final glycerol concentraiton was 30%. The suspension was vortexed and transferred to -80^{0}C for long-term storage.

2.2.2.6 Obtaining and storing individual mutant cells

To obtain individual mutantsfrom final mutant populations, final populations were plated on YMM-agar plates using dilutions to reveal individual colonies. Colonies were then picked from the plates randomly, using sterile tootpicks and transferred to culture tubes containing 10 ml YMM for overnight incubation at 30^{0}C 200 rpm. Frozen stocks were prepared form these liquid cultures.

2.2.3 Determination of cross-resistances to other stress types

After obtaining freeze tolerant mutant individuals, they were exposed to different stress types to determine potential cross-resistances. Cell viability was determined using MPN method as described in section 2.2.2.3.

2.2.3.1 Freezing at -196^{0}C

Cells were incubated in YMM at 30^{0}C 200 rpm until late exponential phase of growth. After reaching desired OD_{600} values (4.5-5.5), one ml culture was transferred to 1.5 ml microfuge tubes. The samples were washed twice with glucose-free YMM centrifuged at 14000 rpm for 5 min in a benchtop centrifuge to remove supernetant after each washing steps.

Next, cells in microfuge tubes were submerged into a liquid nitrogen container (at -196^{0}C) for 25 min. After this rapid freezing step, they were left for thawing at 30^{0}C

for 10 min. Control groups were not exposed to freezing stress. Cell viability of the control group and the experimental group were determined by five-tube MPN method (section 2.2.2.3).

2.2.3.2 Freezing at -20^{0}C

This stress test was applied similar to the -196^{0}C freezing stress test. The difference was the freezing temperature. For -20^{0}C freezing stress, the cells were kept at -20^{0}C deep-freezer for hour and thawed at 30^{0}C for 15 min.

2.2.3.3 Pulse hydrogen peroxide (H_2O_2) stress

Cells were incubated until they reached OD_{600} values between 0.5 and 0.6. one ml fresh cultures were centrifugated at 14000 rpm for 5 min to obtain 0.3 M final H_2O_2 concentration in a final volume 1 ml, 300 µl was used from 1 M stock H_2O_2 solution.

Supernetants of centrifuged cells were discarded and cell pellets were resuspended in microfuge tubes with 700 µl YMM. To this suspension, 300 µl of 1 M H_2O_2 was added to have a final H_2O_2 concentration of 0.3 M.

Cells were incubated in 0.3 M H_2O_2-YMM medium for 1.5 h at 30^{0}C. they were then centrifuged and washed twice as described previously. After removal of H_2O_2, the MPN method was applied to the cultures and their controls to determine the viability as described before.

2.2.3.4 Pulse ethanol (EtOH) stress

Cells were incubated until their OD_{600} values reach between 1.0-1.5. one ml of culture was placed into 1.5 ml microfuge tube and washed twice with glucose-free medium. Cells were then incubated for h in YMM broth containing 20% (v/v) EtOH, at 30^{0}C and 200 rpm.

After incubation, cells were centrifuged at 14000 rpm for 5 min in a benchtop centrifuge, and washed twice with glucose-free YMM to remove ethanol. The cells that have been exposed to pulse stress and the control group were then transferred to 96-well plates containing YMM to determine cell viabilities according to 5-tube MPN method.

Table 2. 1:. Composition EtOH-YMM broth (20% (v/v) EtOH) at final volume of 20 ml.

Content	Amounts
2X YMM*	8ml
EtOH	4ml
dH_2O	8ml

(*1xYMM: 6.7 g yeast nitrogen base without amino acids, 20 g dextrose in 1 liter distilled water)

2.2.3.5 Continuous EtOH stress

Cells were incubated in YMM at 30^0C and 200 rpm until their OD_{600} values were between 1-1.5. 20 µl of culture was then transferred to 180 µl YMM in 96-well plates containing 7% (v/v) ethanol, and incubated at 30^0C for 72 hours. In control groups with YMM were also inoculated under the same condiitons in 96-well plates.

In parallel with 96-well-plate tests, cells were also cultivated on solid YMM plates containing varying concentraitons of ethanol.

Table 2. 2: Composition of solid YMM plates with varying ethanol concentrations.

Content	8% (v/v) EtOH	10% (v/v) EtOH	12% (v/v) EtOH
2X YMM-agar*	15 ml	15 ml	15 ml
EtOH	2.4 ml	3 ml	3.6 ml
dH_2O	12.6 ml	1 2ml	11.4 ml

(*1xYMM-agar: 6.7 g yeast nitrogen base without amino acids, 20 g dextrose, 20 g agar in 1 liter distilled water.)

2X YMM-agar was melted in microvave oven and cooled down to approximately 45-50^0C. The appropriate volume (Table 2.2) was then mixed with warm distelled water and ethanol, shaken vigorously, poured in petri plates, and left for solidification.

Cells were cultivated overnight in YMM until OD_{600} values reached between 1.2 and 1.6. They were then diluted 10^1, 10^2, 10^3, 10^4, 10^5, 10^6, 10^7 fold on the 96-well plate, and from each diluted cell suspension 1.5 µl were withdrawn and inoculated on petri plates containing EtOH. The control plates without EtOH were also incubated together with EtOH-plates at 30^0C for 72 hour.

2.2.3.6 Continuous NaCl stress

Cells were incubated untl they reached OD_{600} values between 1.2 and 1.6. The 96-well plates were filled with 180 µl YMM contain 4%(w/v) NaCl per well.cells were inoculated and incubated in these plates, as described previously.

Control groups were incubated in YMM-containing 96-well plates.

2.2.3.7 Continuous sorbitol stress

Cells were incubated untill they reached OD_{600} values between 1.2 and 1.6. the 96-well plates were filled with 180 µl YMM contain 1.6 M sorbitol per well. Cells were inoculated as described previously and incubated as serial dilutions as before. Control groups were incubated in YMM-contaning 96-well plates.

2.2.4 Genetic stability tests for mutant individuals

Mutant individuals obtained from selections were grown for 5 succesive cultivations under nonselective condiitons, i.e. without being exposed to freezing stress. During each cultivation, freeze-tolerance of the culture was determined by MPN assay, as described previously. The survival rates of each succesive culture were determined and compared to each other to determine genetic stability.

2.2.5 Cell disruption method

One ml cell suspension was transferred to 1.5 ml microfuge tube, and centrifuged at 14000 rpm for 5 min in a benchtop centrifuge. The supernatant was discarded and the pellet was washed with 1 ml distilled water, and centrifuged again. The supernatant discarded, and pellets resuspended with 250 µl phosphate buffer (50 mM, pH 7.2). Sterile glass beads with 0.5 mm diameter were added to tubes until the total volume reached at 1 ml. Then, the microfuge tubes were kept at -80^0C in deep-freezer for 1 min and vortexed for 1 min. This vortex-freeze cycle was repeated ten times. The cell extracts were taken by syringe and transported to new microfuge tubes. These tubes centrifuged at 12000 rpm for 10 minutes in a benchtop centrifuge. The supernatant was used for enzymatic analysis, and determination of trehalose and glycerol concentrations.

2.2.6 Catalse activity test for mutant individuls

680 µl phosphate buffer (pH 7.2) and 480 µl 40 mM H_2O_2 were mixed, and incubated at 30˚C for 2.5 min. 40 µl cell extracts (obtained by cell disruption method, as described in section 2.2.5) were mixed with this mixture. The decrease at OD_{240} was measured by using quartz-cuvettes.

The catalase activity determined as "Δ absorbance of H_2O_2/second" at 240 nm wavelength.

2.2.7 Determining total protein amounts of mutant individuals

The Bradford Method was used to determine the total protein amount. The standard bovine serum albumin (BSA) solutions prepared at 0, 125, 250, 500, 750, 1000, 1500, and 2000 µg/ml protein concentrations.

5 µl of the standard/ sample solution was mixed with 200 µl Bradford reagent at a 96-well plate. Twice of each standard and triple of each sample were prepared for the measurements.

The composition of each loaded well was introduced to the microplate-reader system. After 5 min of incubation, the absorbance was measured at 595 nm.

2.2.8 Calculating specific catalase activity

Specific catalase activity was determined by using the equation (2.2).

$$specific\ activity = \frac{total\ units\ of\ enzyme\ in\ fraction}{total\ amount\ of\ protein\ in\ fraction} \quad \textbf{(2.2)}$$

In our experiment the unit of enzyme determined as "Δ absorbance of H_2O_2/second" at 245nm

3. RESULTS

3.1 Determination Of Freeze-Tolerance Of The Wild-Type And EMS-Mutagenized Initial Cultures At -80^{0}C And -196^{0}C Freezing Stress

Before selections under freezing stress, the wild type (200) and EMS-mutagenized (201) cultures were screened to determine their resistance against consecutive freeze-thaw applications at -80^{0}C and -196^{0}C, using MPN analysis. The viability results were obtained at 24th, 48th and 72nd hour. The lowest stress level applied was freezing only once at either -80^{0}C or in liquid nitrogen (-196^{0}C), and the highest level was 20 times successive freezing-thawing cycles for -80^{0}C freezing (Table 3.1) and 15 times successive freezing-thawing cycles for -196^{0}C freezing (Table 3.2), respectively.

The results generally showed that freezing in liquid nitrogen seemed to decrease viability more than freezing at -80^{0}C, particularly during very early cycles. However, even in -80^{0}C freezing tests, cell viability dramatically decreased from the fourth cycle on, both for 200 and 201 (Table 3.1). Generally, for both screening tests of -80^{0}C and -196^{0}C, the wild type (200) seemed to have higher viability than the EMS-mutagenized mutant (201) during early cycles of screening. However, in later cycles, no detectable difference in viability was observed between 200 and 201.

Table 3. 1: Cell viability of wild type and EMS-mutagenized initial culture during -80^{0}C freezing stress screening, based on MPN analysis.

Number of cycles*	Cell viability at 24th h		Cell viability at 48th h		Cell viability at 72nd h	
	200	201	200	201	200	201
1	0.034	0.011	0.046	0.085	0.046	0.071
2	0.138	0.009	0.270	0.055	0.175	0.071
3	0.088	0.005	0.165	0.027	0.165	0.023
4	0.038	0.002	0.027	0.027	0.027	0.023
5	0.022	0.002	0.027	0.035	0.027	0.029
6	0.044	0.001	0.030	0.025	0.033	0.021
7	0.007	0.001	0.046	0.018	0.046	0.015
8	0.100	0.001	0.120	0.018	0.120	0.015
9	0.015	0.000	0.027	0.027	0.027	0.015
10	0,003	0.000	0.085	0.008	0.085	0.007
11	0.175	0.011	0.055	0.004	0.055	0.003
12	0.029	0.000	0.047	0.003	0.047	0.002
13	0.100	0.001	0.080	0.005	0.080	0.004
14	0.015	0.000	0.008	0.008	0.008	0.007
15	0.022	0.000	0.018	0.004	0.018	0.003
16	0.002	0.000	0.001	0.001	0.012	0.001
17	0.022	0.000	0.027	0.002	0.027	0.001
18	0.034	0.000	0.027	0.003	0.027	0.002
19	0.138	0.001	0.032	0.003	0.018	0.002
20	0.022	0.003	0.009	0.001	0.009	0.001

(*Successive stress repetitions)

3.2 Obtaining Mutant Generations And Determination Of Freeze Tolerance

Mutant generations were obtained by successive cultivation of surviving cells of the previous stress application. The stress levels were gradually until no survivors were observed upon overnight cultivation. Initially, the EMS-mutagenized culture 201 was used as the starting culture for selection experiments, to increase genetic diversity. The survivors of the stress step constituted the mutant generation and used to prepare stock cultures, and inoculate the next culture with an increased stress level, to obtain the next mutant generation. To gain insight into the freeze-tolerance characteristics of mutant generations, cell viabilities of each generation were determined. At each step of increasing stress application, number viable stressed cells and normal cells were described previously. Figures 3.1 and 3.2 indicate the chances in cell viability

among mutant generations in -80^{0}C and -196^{0}C selections, respectively, based on the particular stress level applied for the selection of each particular generation.

Cell viabilities of each generation in -80^{0}C freezing selection are shown in Table 3.3. The generation number also indicates how many times the -80^{0}C freezing stress was repeated for that particular generations. Thus, the generation number also indicates the stress level.

Table 3. 2: Cell viability of wild type and EMS-mutagenized initial culture during -196^{0}C freezing stress screening, based on MPN analysis.

Number of cycles*	Cell viability at 24th h		Cell viability at 48th h		Cell viability at 72nd h	
	200	201	200	201	200	201
1	0.012	2E-09	0.048	1E-08	0.060	2E-08
2	0.012	2E-09	0.048	1E-08	0.060	2E-08
3	0.009	2E-09	0.011	2E-09	0.014	3E-09
4	0.018	3E-09	0.000	1E-10	0.115	3E-08
5	0.000	5E-11	0.022	5E-09	0.003	7E-10
6	0.001	2E-10	0.004	9E-10	0.005	1E-09
7	0.000	5E-11	0.005	1E-09	0.006	2E-09
8	0.000	4E-11	0.001	3E-10	0.002	4E-10
9	0.000	5E-11	0.002	4E-10	0.002	6E-10
10	0.000	5E-11	0.005	1E-09	0.006	2E-09
11	0.000	5E-11	0.002	4E-10	0.002	6E-10
12	0.000	3E-11	0.001	2E-10	0.009	2E-09
13	0.000	2E-11	0.003	9E-10	0.004	1E-09
14	0.000	3E-11	0.002	5E-10	0.002	6E-10
15	0.000	0.000	0.002	9E-10	0.002	9E-10

Table 3. 3: Cell viability of mutant generaitons of -80^{0}C selections based on MPN analysis.

Generation Number	Cell viability at 24th h	Cell viability at 48th h	Cell viability at 72nd h
1	0.06	0.06	0.06
2	0.11	0.11	0.12
3	0.17	0.43	0.67
4	0.27	0.27	0.27
5	0.10	0.10	0.15
6	0.06	0.38	0.38
7	0.17	0.12	0.12
8	0.05	0.05	0.05
9	0.06	0.22	0.22
10	0.10	0.10	0.11
11	0.65	0.65	0.65
12	0.57	0.57	0.59
13	0.15	0.15	0.15
14	0.04	0.35	0.35
15	0.02	0.02	0.02
16	0.10	0.02	0.02

The cell viability of the16th mutant generation after being exposed to 16 successive freeze-thaw cycles was 0.02 at 72nd h of incubation (Table 3.3). On the other hand, the initial culture had a viability value 201 survival ratio is of 0.001 after 16 cycles of freezing and thawing at -80^{0}C (Table 3.1).

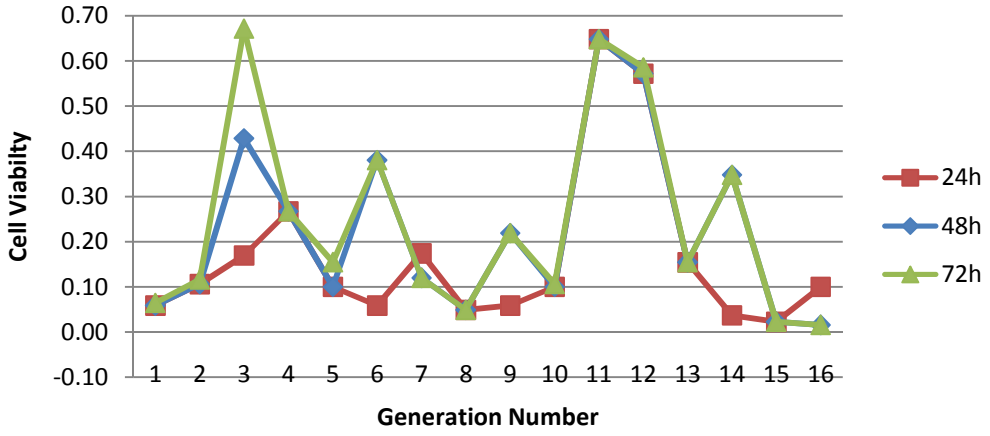

Figure 3. 1: The cell viability changes among mutant generations of -80^{0}C freezing selection.

Cell viabilities of each generation in -196^{0}C freezing selection are shown in Table 3.4. The generation number also indicates how many times the -196^{0}C freezing stress was repeated for that particular generation. Thus, the generation number also indicates the stress level.

Table 3. 4: Cell viability of mutant generations of -196^{0}C selection, based on MPN analysis.

Generation Number	Cell viability at 24th h	Cell viability at 48th h	Cell viability at 72nd h
1	0.0004	0.0006	0.0017
2	0.0013	0.0036	0.0056
3	0.0038	0.0338	0.0338
4	0.0003	0.0032	0.0054
5	0.0170	0.0170	0.0296
6	0.0383	0.0631	0.0383
7	0.0170	0.0170	0.0170
8	0.0631	0.00631	0.0631
9	0.0015	0.0020	0.0042
10	0.0002	0.0000	0.0000

The cell viabilities of generations 9 and 10 after being exposed to 9 and 10 succissive freeze-thaw cycles, respectively, were higher than the viability of 201 based on screening results, at -196^{0}C (Tables 3.2 and 3.4).

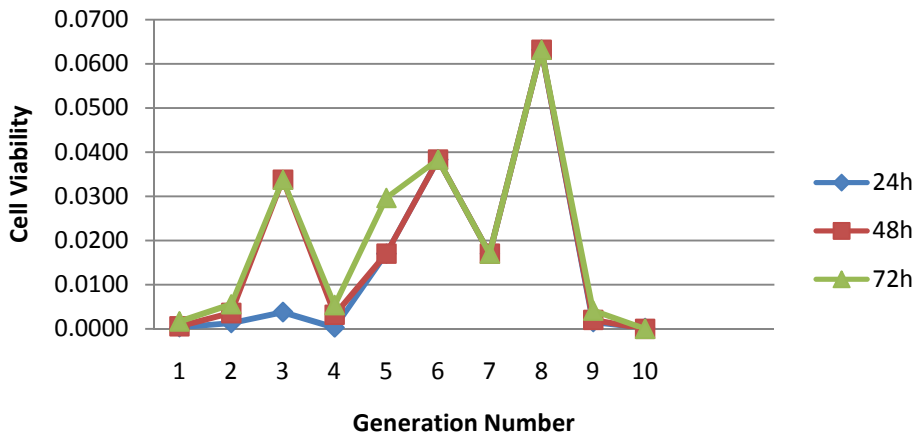

Figure 3. 2: The cell viability changes among mutant generations of -196^{0}C freezing selection.

The cell viabilities decreased dramatically at the final generations and because of that it is adjudged to cut down carying on obtaining generations. In both selections, cell viabilities decreased dramatically in the last few generations (Figures 3.1 and 3.2). Thus, the selection procedures were stopped and individual mutants were selected from final mutant generations.

3.3 Selection Of Individual Mutants From Final Mutant Populations

The final generations of both selection strategies were inoculated onto solid YMM plates by spreading and incubated at 30^0C for 48 h. Twenty five colonies from each plate were chosen randomly, and these individulas were inoculated onto YMM plates which either contained 2% xylose or 2% glucose as the sole carbon source, incubated for 48 h. As *S. cerevisiae* cannot utulize xylose, xylose plates were used to test any possible contamination by other wild yeasts. All colonies were xylose negative. Thus, no contamination was observed (Figures 3.3 and 3.4). Ten selected colonies among the initial twenty five colonies were then inoculated into liquid YMM and left for overnight growth at 30^0C for further tests. Nomenclature of the mutant individuals given in Table 3.5.

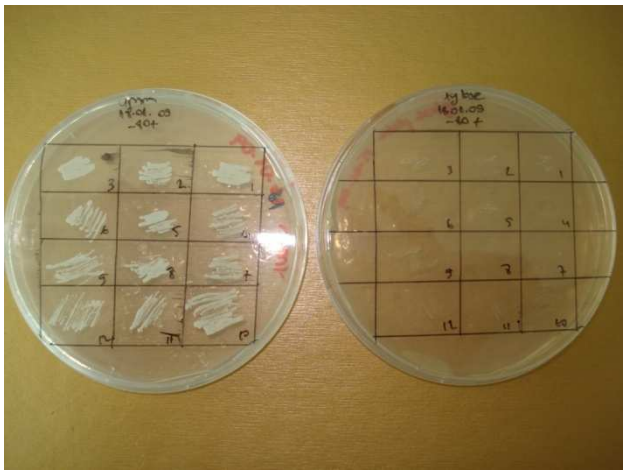

Figure 3. 3: YMM solid cultures of mutant individual colonies of -80^0C freezing selection procedure after 48 hour incubation at 30^0C. Left: YMM plate with 2% dextrose as the sole carbon source.

Figure 3. 4: YMM solid cultures of mutant individual colonies of -196^0C freezing selection procedure after 48 hour incubation at 30^0C. Left: YMM plate with 2% dextrose as the sole carbon source.

Table 3. 5: Nomenclature for mutant individuals selected from final populations of -80^{0}C and -196^{0}C stress selections.

Individual mutant	Code
-80 freezing stress individual mutant 1	*2*
-80 freezing stress individual mutant 2	*3*
-80 freezing stress individual mutant 3	*4*
-80 freezing stress individual mutant 4	*5*
-80 freezing stress individual mutant 5	*11*
-80 freezing stress individual mutant 6	*13*
-80 freezing stress individual mutant 7	*14*
-80 freezing stress individual mutant 8	*16*
-80 freezing stress individual mutant 9	*19*
-80 freezing stress individual mutant 10	*20*
-196 freezing stress individual mutant 1	*3f*
-196 freezing stress individual mutant 2	*5f*
-196 freezing stress individual mutant 3	*6f*
-196 freezing stress individual mutant 4	*7f*
-196 freezing stress individual mutant 5	*8f*
-196 freezing stress individual mutant 6	*11f*
-196 freezing stress individual mutant 7	*12f*
-196 freezing stress individual mutant 8	*14f*
-196 freezing stress individual mutant 9	*17f*
-196 freezing stress individual mutant 10	*23f*

3.4 Determination Of Freeze Tolerance

Freeze tolerance of the individual mutants selected were tsted at the freezing temperature applied during their selection. Tables 3.6 and 3.7 indicate the viabilities of freeze tolerant mutant individuals selected at -80^{0}C, upon exposure to -80^{0}C freezing stress. The showed that individuals with the codes 5, 13, 20 had the highest freeze-tolerance at -80^{0}C, ranging approximately between 7 and 23-fold of that of the wild-type (Tables 3.6 and 3.7).

Table 3. 6: Viabilities of freeze tolerant mutants selected at -80^{0}C upon exposure to -80^{0}C freezing stress. Viabilities were determined by MPN method at 24th, 48th and 72nd h of cultivation in YMM following freezing stress.

	Cell Viabilities		
Mutant code	24th h	48th h	72nd h
2	0.59	0.38	0.38
3	0.22	0.58	0.58
4	4.0	1.54	1.54
5	10.0	10.0	10.0
11	1.54	2.63	2.63
13	7.27	4.44	4.44
14	0.38	0.50	0.50
16	2.25	4.58	4.58
19	2.45	2.45	3.18
20	22.5	2.25	1.52
Wild-type	1.00	0.65	0.65

Table 3. 7: Viabilities of freeze tolerant mutants selected at -80^{0}C upon exposure to -80^{0}C freezing stress normalized to wild-type viability.

	Cell Viability (normalized to wild type)		
Mutant code	24th h	48th h	72nd h
2	0.59	0.59	0.59
3	0.22	0.88	0.88
4	4.0	2.37	2.37
5	10.0	15.4	15.4
11	1.54	4.04	4.04
13	7.27	6.84	6.84
14	0.38	0.77	0.77
16	2.25	7.05	7.05
19	2.45	3.78	4.89
20	22.5	3.46	2.34

Similarly, viability was also determined for freeze tolerant mutant selected at -196^{0}C, upon exposure to -196^{0}C freezing stress. The results are shown in Tables 3.8 and 3.9.

Table 3. 8: Viabilities of freeze tolerant mutants selected at -196^{0}C upon exposure to -196^{0}C freezing stress. Viabilities were determined by MPN method at 24th, 48th and 72nd h of cultivation in YMM following freezing stress.

	Cell Viabilities		
Mutant code	24th h	48th h	72nd h
3f	0.686	0.255	0.255
5f	4.571	0.218	0.218
6f	0.100	0.491	0.491
7f	0.023	0.225	0.225
8f	0.034	0.159	0.159
11f	0.263	0.383	0.383
12f	0.059	0.541	0.541
14f	0.100	0.315	0.315
17f	0.174	0.587	0.587
23f	0.100	0.218	0.141
Wild-type	0.046	0.017	0.017

Table 3. 9: Viabilities of freeze tolerant mutants selected at -196^{0}C upon exposure to -196^{0}C freezing stress normalized to wild-type viability.

	Cell Viability (normalized to wild type)		
Mutant code	24th h	48th h	72nd h
3f	15.0	15.0	15.0
5f	100	12.8	12.8
6f	2.19	28.9	28.9
7f	0.49	13.2	13.2
8f	0.74	9.36	9.36
11f	5.75	22.6	22.6
12f	1.28	31.8	31.8
14f	2.19	18.5	18.5
17f	3.80	34.5	34.5
23f	2.19	12.8	8.30

Although the absolute values for viability were not high (Table3.8), the viability of all mutant individuals selected at -196^{0}C freezing stress were significantly higher than those of the wild type, considering 48 and 72 h data (Table 3.9).

3.5 Determination Of Cross-Resistance Of Selected Mutant Individuals To Other Stress Types

3.5.1 Freezing at -196^{0}C

To determine the resistance of mutants selected at -80^{0}C against -196^{0}C freesizng stress, those mutants were exposed to -196^{0}C freezing stress in liquid nitrogen. The viability results are shown in Tables 3.10 and 3.11.

Table 3. 10: Viabilities of freeze tolerant mutants selected at -80^{0}C upon exposure to -196^{0}C freezing stress. Viabilities were determined by MPN method at 24th, 48th and 72nd h of cultivation in YMM following freezing stress.

	Viability		
Mutant code	24th h	48th h	72nd h
1	0,49	1,70	1,70
2	0,01	0,01	0,01
3	0,10	0,17	0,17
4	0,18	0,23	0,23
5	0,10	0,10	0,10
11	0,10	0,12	0,12
13	0,01	0,02	0,02
14	1,00	1,70	1,70
19	0,00	0,00	0,00
20	0,00	0,02	0,02
Wild-type	0,05	0,02	0,02

Table 3. 11: Viabilities of freeze tolerant mutants selected at -80^{0}C upon exposure to -20^{0}C freezing stress normalized to wild-type viability.

	Viability (normolized wild type value)		
Mutant code	24th h	48th h	72th h
1	10	100	100
2	0,1	0,5	0,6
3	2,2	10	10
4	4,0	13	13
5	2,2	5,9	5,9
11	2,2	7,1	7,1
13	0,3	1,3	1,3
14	22	100	100
19	0,0	0,1	0,3
20	0,0	1,0	1,0

3.5.2 Freezing stress at -20^{0}C

As the freezing at -20^{0}C is a very common procedure applied in industry, the resistance of the obtained mutants upon -20^{0}C freezing stress was also determined. Tables 3.12 and 3.13 indicate the viabilities of -80^{0}C selection mutants upon -20^{0}C freezing stress.

Table 3. 12: Viabilities of freeze tolerant mutants selected at -80^{0}C upon exposure to -20^{0}C freezing stress. Viabilities were determined by MPN method at 24th, 48th and 72nd h of cultivation in YMM following freezing stress.

	Viability		
Mutant code	24th h	48th h	72th h
2	0.38	0.38	0.38
3	0.15	0.15	0.15
4	1.54	1.54	1.54
5	10.0	5.87	10.0
11	0.69	0.69	0.69
13	1.59	0.65	0.65
14	0.26	0.26	0.26
16	0.10	0.15	0.15
19	1.59	1.59	3.18
20	6.67	6.67	3.48
Wild-type	0.10	0.13	0.13

Table 3. 13: Viabilities of freeze tolerant mutants selected at -80^{0}C upon exposure to -20^{0}C freezing stress normalized to wild-type viability.

	Viability (normalized to wild type)		
Mutant code	24th h	48th h	72nd h
2	4	3	3
3	2	1	1
4	15	12	12
5	100	45	77
11	7	5	5
13	16	5	5
14	3	2	2
16	1	1	1
19	16	12	24
20	67	51	27

Similarly, viabilities of mutants selected from -196^{0}C were also determined upon -20^{0}C freezing stress. The results are shown in Tables 3.14 and 3.15.

Table 3. 14: Viabilities of freeze tolerant mutants selected at -196^{0}C upon exposure to -20^{0}C freezing stress. Viabilities were determined by MPN method at 24th, 48th and 72nd h of cultivation in YMM following freezing stress.

	Viability		
Mutant code	24th h	48th h	72nd h
3f	0.648	1.000	1.000
5f	0.444	0.343	0.343
6f	1.000	0.575	0.575
7f	3.375	1.543	1.543
8f	1.543	0.500	0.500
11f	0.648	0.338	0.338
12f	1.543	1.000	1.000
14f	0.380	0.380	0.380
17f	0.146	0.225	0.225
23f	1.000	0.688	0.688
Wild-type	0.263	0.315	0.407
FREEZE 9E*	0.26	0.40	0.40

(* FREEZE 9E is a freeze-tolerant *S. cerevisiae* mutant previously obtained from batch selection in liquid nitrogen (-196^{0}C) [20].)

Table 3. 15: Viabilities of freeze tolerant mutants selected at -196^{0}C upon exposure to -20^{0}C freezing stress normalized to wild-type viability.

	Viability (normalized to wild-type value)		
Mutant code	24th h	48th h	72nd h
3f	2.4	3.2	2.5
5f	1.6	1.1	0.8
6f	3.8	1.8	1.4
7f	13	4.9	3.8
8f	5.8	1.6	1.2
11f	2.4	1.1	0.8
12f	5.8	3.1	2.5
14f	1.4	1.2	0.9
17f	0.5	0.7	0.6
23f	3.8	2.2	1.7
FREEZE 9 E	2.6	3.1	3.1

3.5.3 Pulse oxidative (H_2O_2) stress

Pulse oxidative (H_2O_2) stress test was applied to mutant individuals of two selections, as described in section 2.2.3.3. The viability results of -80^{0}C selection mutants based on MPN analysis are given in Tables 3.16 and 3.17.

Table 3. 16: Viabilities of freeze tolerant mutants selected at -80^{0}C upon exposure to pulse H_2O_2freezing stress. Viabilities were determined by MPN method at 24th, 48th and 72nd h of cultivation in YMM following.

	Viability		
Mutant codes	24th h	48th h	72nd h
2	0.00000	0.00017	0.0004
3	0.00000	0.00000	0.0000
4	0.01000	0.02629	0.0100
5	0.02629	0.00761	0.0076
11	0.00010	0.00049	0.0010
13	0.00245	0.00648	0.0065
14	0.00010	0.00059	0.0006
16	0.00001	0.00046	0.0006
19	0.00016	0.00500	0.0206
20	0.00071	0.00383	0.0020
Wild-type	0.00000	0.00000	0.0000

Table 3. 17: Viabilities of freeze tolerant mutants selected at -80^{0}C upon exposure to pulse H_2O_2 stress normalized to wild-type viability.

	Viability (normalized to wild type value)		
Mutant code	24th h	48th h	72nd h
2	3	190	410
3	2	5	16
4	10800	28390	10800
5	28390	8220	8217
11	108	525	1080
13	2650	7000	7000
14	108	635	635
16	10	495	630.0
19	170	5400	22235
20	765	4140	2160

Similarly, pulse H_2O_2 stress was also applied to test individual mutants selected at -196^{0}C. The viability results are shown in Tables 3.18 and 3.19.

Table 3. 18: Viabilities of freeze tolerant mutants selected at -196^{0}C upon exposure to pulse H_2O_2 stress. Viabilities were determined by MPN method at 24th, 48th and 72nd h of cultivation in YMM following stress.

	Viability		
Mutant Code	24th h	48th h	72nd h
3f	0.069	0.015	0.015
5f	0.001	0.004	0.004
6f	0.225	0.073	0.073
7f	0.001	0.004	0.004
8f	0.002	0.001	0.001
11f	0.009	0.032	0.032
12f	0.002	0.007	0.007
14f	0.013	0.015	0.015
17f	0.026	0.018	0.018
23f	0.010	0.006	0.006
Wild-type	0.000	0.000	0.000
FREEZE 9E	0.001	0.003	0.003

Table 3. 19: Viabilities of freeze tolerant mutants selected at -196^{0}C upon exposure to pulse H_2O_2 stress normalized to wild-type viability.

	Viability (normalized to wild-type value)		
Mutant code	24th h	48th h	72nd h
3f	10435	3424	3424
5f	88	1027	1027
6f	34525	17075	17075
7f	211	900	900
8f	335	331	331
11f	1444	7458	7458
12f	230	1565	1565
14f	2017	3628	3628
17f	4033	4338	4338
23f	1534	1522	1522
FREEZE 9E	153	400	400

All individuals selected at -80^{0}C and -196^{0}C freezing stress displayed significantly resistanceto oxidative stress, applied by H_2O_2 pulse.

3.5.4 Pulse ethanol stress

Pulse ethanol stress was applied to mutant individuals of two selections, as described in section 2.2.3.4. the viability results of -80^{0}C selection mutants based on MPN analysis are given in Tables 3.20 and 3.21.

Table 3. 20: Viabilities of freeze tolerant mutants selected at -80^{0}C upon exposure to pulse ethanol stress. Viabilities were determined by MPN method at 24th, 48th and 72nd h of cultivation in YMM following stress.

	Viability		
Mutant code	24th h	48th h	72nd h
2	0.00006	0.00001	0.00001
3	0.00000	0.00000	0.00000
4	0.00026	0.00154	0.00154
5	0.00457	0.00380	0.00380
11	0.00002	0.00002	0.00002
13	0.00016	0.00130	0.00130
14	0.00001	0.00001	0.00001
16	0.00002	0.00001	0.00001
19	0.00002	0.00002	0.00002
20	0.00146	0.07083	0.07083
Wild-type	0.00001	0.00000	0.00000

Table 3. 21: Viabilities of freeze tolerant mutants selected at -80^{0}C upon exposure to pulse ethanol stress normalized to wild-type viability.

	Viability (normalized to wild type value)		
Mutant code	24th h	48th h	72nd h
2	10	32	105
3	1	8	8
4	43	4165	4165
5	750	10270	10270
11	4	60	7
13	26	3500	5500
14	1	32	135
16	3	19	55
19	3	40	52
20	240	191250	99782

Similarly, viabilities of -196^{0}C selection mutants were also determined upon exposure pulse ethanol stress. The results are given in Tables 3.22 and 3.23.

Table 3. 22: Viabilities of freeze tolerant mutants selected at -196^{0}C upon exposure to pulse ethanol stress. Viabilities were determined by MPN method at 24th, 48th and 72nd h of cultivation in YMM following stress.

	Viability		
Mutant code	24th h	48th h	72nd h
3f	0.005	0.174	0.174
5f	0.030	0.200	0.200
6f	0.100	1.000	1.000
7f	0.150	0.667	0.667
8f	0.046	1.000	1.000
11f	0.010	0.407	0.407
12f	0.015	0.500	0.500
14f	0.296	0.239	0.239
17f	0.069	0.315	0.315
23f	0.010	0.636	0.636
Wild-type	0.007	0.010	0.010
FREEZE 9E	0.00015	0.00003	0.00003

Table 3. 23: Viabilities of freeze tolerant mutants selected at -196^{0}C upon exposure to pulse ethanol stress normalized to wild-type viability.

	Viability (normalized to wild-type value)		
Mutant code	24th h	48th h	72nd h
3f	0.7	17	17
5f	4	20	20
6f	15	100	100
7f	22	67	67
8f	7	100	100
11f	1.5	41	41
12f	2.2	50	50
14f	43	24	24
17f	10	31	31
23f	1.5	64	64
FREEZE 9E	25	75	75

The viability results given in Tables 3.20-3.23 indicated taht resistance to pulse EtOH stress was at different levels between -80^{0}C and -196^{0}C selection individulas. All individuals of -196^{0}C selection were cross-resistant to pulse ethanol stress, compared with wild-type (Tables 3.22 and 3.23). However, the resistant levels of individuals of -80^{0}C selection to pulse ethanol stress were not high (Tables 3.20 and 3.21).

3.5.5 Continuous ethanol stress

Cross-resistance of mutant individuals of both selection strategies against continuously applied ethanol stress was also determined by MPN method, as described in section 2.2.4.3.

Table 3. 24: Viabilities of freeze tolerant mutants selected at -80^{0}C upon exposure to continuous ethanol stress. Viabilities were determined by MPN method at 24th, 48th and 72nd h of cultivation in YMM following stress.

	Viability		
Mutant code	24th h	48th h	72nd h
2	0.0001	0.23	3.83
3	0.0001	0.06	0.44
4	0.0002	0.05	0.05
5	0.0001	0.26	1.33
11	0.0001	0.01	1.46
13	0.0001	0.06	0.30
14	0.0000	0.01	0.22
16	0.0000	0.05	0.63
19	0.0000	0.00	0.10
20	0.0001	0.15	2.25
Wild-type	0.0000	0.06	1.20

Table 3. 25: Viabilities of freeze tolerant mutants selected at -80^{0}C upon exposure to continuous ethanol stress normalized to wild-type viability.

	Viability (normalized to wild-type value)		
Mutant code	24th h	48th h	72nd h
2	3.85	3.83	3.21
3	2.64	1.10	0.37
4	7.10	0.85	0.04
5	5.43	4.38	1.12
11	3.85	0.25	1.22
13	2.64	1.10	0.25
14	0.58	0.17	0.18
16	1.71	0.78	0.53
19	1.71	0.06	0.08
20	2.64	2.48	1.88
905	1.00	1.00	1.00

Similarly, viability of mutant individuals selected at -196^{0}C were also determiend upon continuous ethanol stress. Results are shown in Tables 3.26 and 3.27.

Table 3. 26: Viabilities of freeze tolerant mutants selected at -196^{0}C upon exposure to continuous ethanol stress. Viabilities were determined by MPN method at 24th, 48th and 72nd h of cultivation in YMM following stress.

	Viability		
Mutant code	24th h	48th h	72nd h
3f	0.00004	0.15	2.96
5f	0.00007	0.17	2.04
6f	0.00004	0.10	0.59
7f	0.00010	0.67	4.58
8f	0.00010	0.26	1.54
11f	0.00004	0.04	1.65
12f	0.00010	0.15	2.96
14f	0.00010	0.26	2.63
17f	0.00007	0.32	5.41
23f	0.00007	0.44	3.15
Wild-type	0.00007	0.15	0.26
FREEZE 9E	0.0001	0.04	0.65

Table 3. 27: Viabilities of freeze tolerant mutants selected at -196^{0}C upon exposure to continuous ethanol stress normalized to wild-type viability.

	Viability (normalized to wild-type value)		
Mutant code	24th h	48th h	72nd h
3f	0.65	1.00	11.35
5f	1.00	1.11	7.80
6f	0.65	0.65	2.25
7f	1.46	4.33	17.56
8f	1.46	1.71	5.91
11f	0.65	0.28	6.31
12f	1.46	1.00	11.35
14f	1.46	1.71	10.07
17f	1.00	2.06	20.73
23f	1.00	2.89	12.06
FREEZE 9E	2.64	0.76	0.54

Neither the mutant individuals of -80^{0}C selection, nor -196^{0}C selection were significantly more resistant to continuous ethanol stress, as compared to wild type (Tables 3.24-3.27). the same behaviour was observed when continuous sthanol stress was applied on solid YMM with 8, 10 and 12% (v/v) ethanol (data not shown).

3.5.6 Continuous NaCl stress

Continous NaCl stress test was applied to mutant individuals of two selections, as described in section 2.2.3.6.The viability results of -80^{0}C selection mutants based on MPN analysis are given in Tables 3.30 and 3.31.

Table 3. 28: Viabilities of freeze tolerant mutants selected at -80^{0}C upon continuous NaCl stress. Viabilities were determined by MPN method at 24th, 48th and 72nd h of cultivation in YMM following stress.

	Cell Viabilities		
Mutant code	24th h	48th h	72nd h
2	0.0000	0.38	0.92
3	0.0001	0.17	0.30
4	0.0018	0.03	0.08
5	0.0014	0.08	0.44
11	0.0010	3.83	3.83
13	0.0007	0.03	0.41
14	0.0002	0.10	0.34
16	0.0004	0.15	1.00
19	0.0004	0.06	0.17
20	0.0007	0.67	1.17
Wild-type	0.0000	0.04	0.18

Table 3. 29: Viabilities of freeze tolerant mutants selected at -80^{0}C upon exposure to continuous NaCl stress normalized to wild-type viability.

	Viability (normalized to wild-type value)		
Mutant code	24th h	48th h	72nd h
2	0.4	10.1	4.9
3	2.6	4.5	1.6
4	70.8	0.9	0.4
5	54.1	2.0	2.4
11	38.3	100	20.8
13	26.3	0.8	2.2
14	5.8	2.6	1.8
16	17.0	4.1	5.4
19	17.0	1.5	0.9
20	26.3	17.5	6.3

Similiarly, viabilities of mutants selected at -196^{0}C were also determined under continuous NaCl stress conditions. The results are shown in Tables 3.30 and 3.31.

Table 3. 30: Viabilities of freeze tolerant mutants selected at -196^{0}C upon exposure to continuous NaCl stress. Viabilities were determined by MPN method at 24th, 48th and 72nd h of cultivation in YMM following stress.

	Cell Viabilities	
Mutant code	48th h	72nd h
3f	0.007	0.146
5f	0.006	0.030
6f	0.010	0.065
7f	0.038	0.146
8f	0.038	0.100
11f	0.100	0.219
12f	0.046	0.131
14f	0.059	0.261
17f	0.049	0.049
23f	0.100	0.444
Wild-type	0.067	0.225
FREEZE 9E	0.02	0.17

Table 3. 31: Viabilities of freeze tolerant mutants selected at -196^{0}C upon exposure to continuous NaCl stress normalized to wild-type viability.

	Viability (normalized to wild-type value)	
Mutant code	48th h	72nd h
3f	0.10	0.65
5f	0.09	0.14
6f	0.15	0.29
7f	0.57	0.65
8f	0.57	0.44
11f	1.49	0.97
12f	0.68	0.58
14f	0.88	1.16
17f	0.72	0.22
23f	1.49	1.98
9 freeze E	0.45	0.93

None of the -196^{0}C mutants could grow at 24th h of cultivation in continuous NaCl stress. Thus Tables 3.30 and 3.31 do not have 24th h viability data.

Geenerally, mutants of -80^{0}C selection were more resistant to continuous NaCl stress. The mutants of -196^{0}C selection, however, did not show cross-resistance against salt stress.

3.5.7 Continuous sorbitol stress

The resistance of the obtained mutants against continuous sorbitol stress was also determined, as described in section 2.2.3.7. The viability of -196^{0}C selection mutants upon continous sorbitol stress are shown in Tables 3.32 and 3.33.

Table 3. 32: Viabilities of freeze tolerant mutants selected at -196^{0}C upon exposure continuous sorbitol stress. Viabilities were determined by MPN method at 24th, 48th and 72nd h of cultivation in YMM following stress.

	Viability		
Mutant code	24th h	48th h	72nd h
3f	0.010	0.383	0.708
5f	0.015	0.380	0.587
6f	0.010	0.185	0.315
7f	0.023	0.667	0.667
8f	0.009	1.000	0.587
11f	0.225	2.188	2.188
12f	0.046	4.000	4.000
14f	0.418	0.380	0.261
17f	0.017	4.857	4.857
23f	0.159	1.000	0.648
Wild-type	0.044	0.667	0.667

Table 3. 33: Viabilities of freeze tolerant mutants selected at -196^{0}C upon exposure to continuous sorbitol stress normalized to wild-type viability.

	Viability (normalized to wild-type value)		
Mutant code	24th h	48th h	72nd h
3f	0.22	0.57	1.06
5f	0.35	0.57	0.88
6f	0.22	0.28	0.47
7f	0.51	1.00	1.00
8f	0.21	1.50	0.88
11f	5.06	3.28	3.28
12f	1.03	6.00	6.00
14f	9.40	0.57	0.39
17f	0.39	7.28	7.28
23f	3.58	1.50	0.97

Generally, mutants of -196^{0}C selection were not cross-resistant to continuous sorbitol stress applied by sorbitol treatment.

3.5.8 Summary of resistance ofmutants

The summary of results of resistance tests upon a variety of stresses are shown for -80^{0}C selection mutants in Table 3.34.

Table 3. 34: Viabilities of mutant individuals selected at -80^{0}C upon a variety of stresses, normalized to wild-type.

Mutant code	-80^{0}C freezing	-20^{0}C freezing	-196^{0}C freezing	H_2O_2 pulse	EtOH pulse	EtOH continuous	NaCl continuous
2	1	3	100	188	32	4	10
3	1	1	1	5	8	1	5
4	2	12	10	28389	4166	1	1
5	15	45	13	8217	10272	4	2
11	4	5	6	525	61	0	101
13	7	5	7	7000	3500	1	1
14	1	2	1	634	32	0	3
16	7	1	100	495	19	1	4
19	4	12	0	5400	41	0	2
20	4	51	1	4140	191250	3	18

Accroding to the cross-resistance tests, some ofthe mutants displayed cross-resistance to several stresses, some to a few different, and the rest did not show significant cross-resistance to other stresses. Individuals coded as 5 and 20 from the -80^{0}C selection showed high cross-resistance. Mutant coded 4 and 13 diplayed multi-stress resistance to the sama stress types (Table 3.34). Those four individuals were identified as the important mutants obtained from -80^{0}C. Almost all of the mutants of -80^{0}C selection had significantly high hydrogen peroxide resistance. Regarding ethanol resistance, it is important to note that mutants 5 and 20 of -80^{0}C selection had very high levels of resistance against ethanol stress applied as pulse.

Table 3. 35: Viabilities of mutant individuals selectes at -196^{0}C upon a variety of stresses, normalized to wild-type.

Mutant code	-196^{0}C freezing	-20^{0}C freezing	EtOH continuous	EtOH pulse	H_2O_2 pulse	Sorbitol continuous	NaCl continuous
3f	15	3	1	17	10435	1	0
5f	13	1	1	20	88	1	0
6f	29	2	1	100	3453	0	0
7f	13	5	4	67	211	1	1
8f	9	2	2	100	335	2	1
11f	23	1	0	40	1444	3	1
12f	32	3	1	50	230	6	1
14f	19	1	2	24	2017	1	1
17f	35	1	2	31	4033	7	1
23f	13	2	3	64	1535	2	1
FREEZE 9E	-	3	1	75	400	-	0

The viabilities of mutant individuals selected at -196^{0}C upon exposure to different stresses are shown in Table 3.35. These mutants generally had no cross-resistance to osmotic stress as indicated by NaCl and sorbitol test results. Mutants 7f and 23f were the most multi-resistant individuals. 5f, 6f, 8f and 14f were also multi-resistant, with resistance to the same types of stresses.

The FREEZE 9E mutant, obtained from previous research (Cakar et al., 2005) also displayed cross resistance to most of the stresses, except osmotic stress and continuous ethanol stress, as mutants 7f and 23f in this study. When FREEZE 9E and the new mutants 7f and 23f were compared, they both showed similiar types of cross-resistance at similiar levels.

Generally, high resistance to H_2O_2 stress was observed in mutants of both selections at -80^{0}C and -196^{0}C (Tables 3.34 and 3.35).

3.6 Genetic stability of mutant individuals

To determine the genetic stability of the mutant individuals, they were cultured overnight five successive times. Under non-selective conditions, and after each cultivation, their resistance to freezing stress was determined. Individuals that had been selected at -80^{0}C were exposed to -80^{0}C stress, and those selected at -196^{0}C were exposed to -196^{0}C stress. The resistances were determined for each succissive cultivation and changes in resistance level was evaluated.

For genetic stability tests, the multi-stress resistnt mutants 20 and 5f were chosen. The results showed that there were fluctuations in freezing resistance among successive cultivations (Table 3.36).

Table 3. 36: Genetic stability test results of mutant individuals based on viability upon -80^{0}C and -196^{0}C freezing stress, respectively **.**

Freeze-thaw	Cell viability of 20 based on MPN data			Cell viability of 5f based on MPN data		
	24th h	48th h	72th h	24th h	48th h	72th h
1st cycle	1.00	1.00	1.00	0.74	2.00	2.00
2nd cycle	1.50	2.25	2.25	0.30	0.77	0.77
3th cycle	0.13	1.00	1.00	2.96	2.96	2.96
4th cylce	2.25	1.17	1.17	1.00	0.34	0.44
5th cycle	0.70	0.74	1.00	0.17	1.00	1.00

3.7 Evolutionary Engineering Of Freezing Stress Resistance

To determine the evolution of the freeze tolerance, the generations of each selection strategy were exposed to either one time freezing at -196^{0}C or at -80^{0}C, depending on the type of selection. Living cell numbers were determined by using MPN method before and after the stress application. The cell viability ratio results at 48th hour of cultivation folloving stress shown in Tables 3.37 and 3.38 for -80^{0}C and -196^{0}C mutants, respectively.

Table 3. 37: Viability results of -80^{0}C selection generations after one cycle of freeze-thaw application at -80^{0}C, based on MPN analysis.

Generation number of -80^{0}C selection	Cell viability
2	0.26
4	0.38
6	0.03
8	0.34
10	0.15
12	0.58
14	0.42
16	1

Table 3. 38: Viability results of -196^{0}C selection generations after one cycle of freeze-thaw application at -196^{0}C, based on MPN analysis.

Generation number of -196^{0}C selection	Cell viability
1	0.49
2	0.26
3	0.22
4	0.31
5	0.46
6	0.10
7	0.03
8	0.19
9	0.15
10	0.07

3.8 Some Characteristics Of Mutants 5 And 23f

Individual mutant 5 from -80^{0}C selection and 23f from -196^{0}C selection were selected to be further characterized. For this purpose, the mutants growth curve was obtained (Figure 3), and catalase activity, trehalose and glycerol concentrations were determined under non-stress conditions.

3.8.1 The growth curves of mutants 5 and 23f

To obtain growth curves, the absorbance of mutant 5 was measured at 600 nm in spechtropohometer at some time intervals along the 34 h cultivation. The growth curve of mutant 5 shown in Figure 3.5.

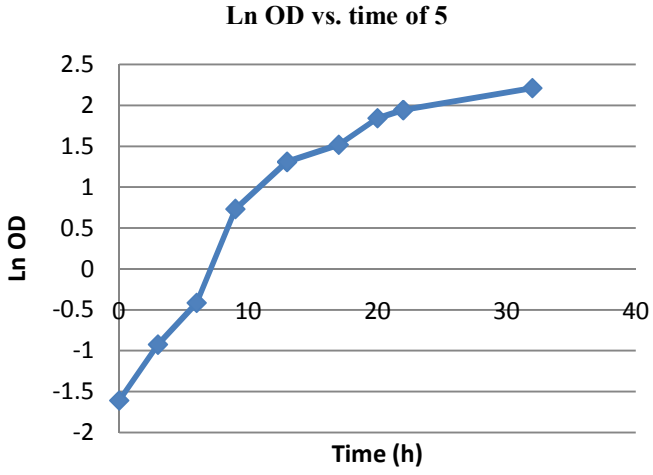

Figure 3. 5: Growth curve of mutant 5.

Similiarly, growth curve of mutant 23f was also determined by measuring OD_{600} values in spechtrophotometer.

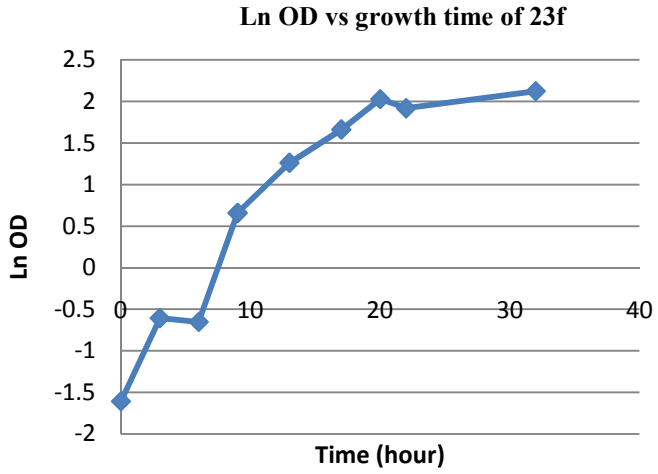

Figure 3. 6: Growth curve of mutant 23f.

The specific growth rate (μ) of wild type was compared with those of the mutant individuals. The μ values of the mutant individuals 5 and 23f and wild type were 0.405, 0.432 and 0.58, respectively. The μ values of the mutant individuals seems to be lower than the wild type, as expected.

3.8.2 The catalase activity of mutants 5 and 23f in the absence of stress

The catalase activity measurement was done in non-stress conditions; in other words, at optimum growth conditions at 9^{th}, 13^{th}, 20^{th}, 22^{nd} and 32^{nd} hour of cultivation. The specific catalase activity results of wild type, mutants 23f and 5 are given in Figure 3.7.

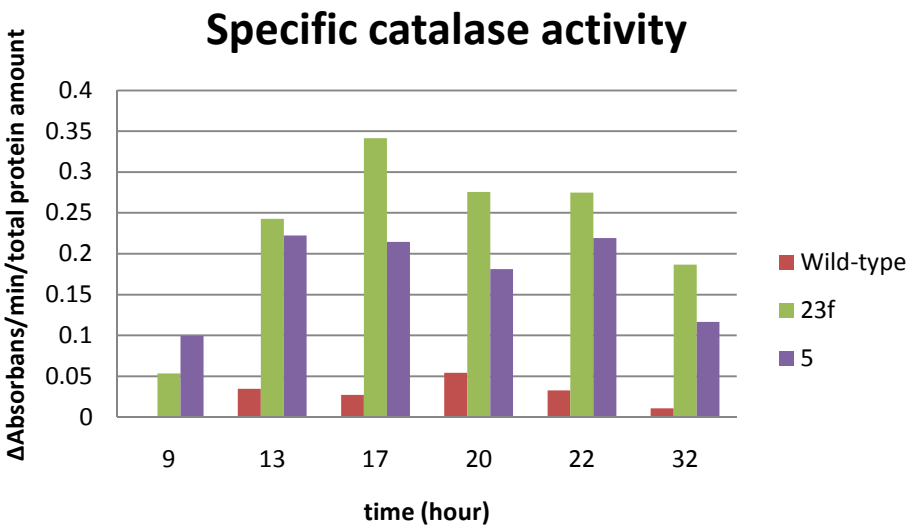

Figure 3. 7: The ΔAbsorbans/min/total protein amounts of the 5, 23f and 9wild type.

The specific catalse activity of wild type and mutant individuals, in the absence of any stress conditions, shown in Figure 3.7. The specific catalase activities of mutant individulas 5 and 23f are also given in Table 3.39, as fold of wild type value.

Table 3. 39: Specific catalse activities of mutants 5 and 23f were determined by enzymatic activities normalized to wild-type value.

Time (h)	Fold of wild type values	
	5	23f
13	6.5	7.1
17	7.9	12.6
20	3.3	5.1
22	6.7	8.4
32	11.1	17.8

At the 9th h of cultivation catalase activity of wild-type was zero. Thus, 9th h data could not given in Table 3.39.

The specific catalase activity was significantly higher in mutant individuals than wild type at any cultivation time. Nevertheless, the specific catalase activity seem to be increase in the stationary phase in mutant individuals, and the fold of wild type value were the highest at this stage in both 5 and 23f.

4. DISCUSSION

In this research, the primary aim was to obtain freeze-tolerant *Saccharomyces cerevisiae* cells. Evolutionary engineering strategies were designed and applied as a tool to obtain cells with that desired phenotype. Initially, the genetic diversity in the population was increased by EMS mutagenesis on the initial culture, as applied previously [20]. The EMS mutation caused randomly distributed point mutations in the genom, which might have altered some gene functions in the cells [41]. The freezing stress was used as the selective pressure in this study to obtain the freeze-tolerant individuals.

At first, the mutagenized culture and the wild type were screened at two different freezing temperatures, -80^0C and -196^0C. For this purpose, the cultures were exposed to sequential freeze-thaw treatment at those temperatures. The aim was to determine the freeze-tolerance of the EMS mutagenized and wild-type cultures at both freezing temperatures. Screening results at -80^0C screening results showed that the cell viabilities of the cultures decreased dramatically after 13^{th} and 9^{th} freeze-thaw cycle in wild type and mutagenized culture, respectively, based on 72 h viability data. Screening the cultures at -196^0C showed that the cell viabilities decreased sharply just after the 4^{th} and already at the first freeze-thaw cycle, in wild type and mutagenized culture, respectively. The screening results indicated that the freezing stress was detrimental for cells and especially the freezing stress applied at -196^0C seemed to have a more severe effect on cell viability than freezing at -80^0C.

The mutant generations were obtained by transferring the survivors of a stress step to the next stress step. Two different selection strategies were adopted during obtaining the generations: freezing at -80^0C and freezing at -196^0C. The initial stress level was exposure to one freeze-thaw cycle. As the generation number increased, the stress level was also increased by increasing the number of freeze-thaw cycles by one at each successive generation. Thus, ten and sixteen generations were obtained from -196^0C and -80^0C freezing selections, respectively. The individual mutants were selected randomly from the final populations of each selection strategy.

The freeze-tolerance of the wild type and the mutant generations were compared. For evaluating the freeze tolerance, -80^{0}C generations were exposed to freezing stress at -80^{0}C, and -196^{0}C mutant generations were exposed to freezing stress at -196^{0}C. The wild type was exposed to freezing stress at both freezing temperatures, to serve as a reference. The wild type's viability after the freezing stress at -80^{0}C was 0.046 at 48 h (Table 3.1) where the 16th generation's cell viability ratio was 1 (Table 3.37) which indicates that the freeze tolerance increased approximately 22- fold of the wild type after the evolutionary engineering application. On the countrary, the wild type's viability was 0.048 at 48 h after one cycle freezing stress at -196^{0}C (Table 3.2), where: the 9th mutant generation viability was 0.15 (Table 3.38) which approximately 3-fold of the wild type. These results generally indicated that the freezing stress resistnce was improved compared to the wild-type- and evolutionary engineering was applied successfully.

The freeze-tolerance of mutant individuals was tested, as well. The mutants obtained from -80^{0}C freeze-tolerant generation were exposed to -80^{0}C; the mutants obtained from -196^{0}C freeze-tolerant generation to -196^{0}C. The freeze-tolerance of the mutant individuals were generally lower than that of the generation. For instance, the highest tolerance to -80^{0}C freezing stress belonged to the individual number 5, which was approximately 15- fold of that of the wild type (Table 3.34) where as the population tolerance was 30- fold of the wild type (Tables 3.1 and 3.37). On the other hand, some of the mutants selected from the -196^{0}C freezing stress displayed higher tolerance to this stress than the population. The population tolerance was 22-fold of the wild type, where there were some mutants that could tolerate this stress up to 35-fold of the wild type (Table 3.9). These results indicated that the stress resistance of the mutant population and individuals can significantly differ from each other, and the populations are generally heterogeneous, as shown prevously (Çakar et al., 2005).

The individuals were exposed to different stresses to evaluate cross-resistance of the mutants to different types of stresses commonly encountered in yeast bioprocesses. The results of cross-resistance experiments were summarized in Tables 3.34 and 3.35.

All -80^{0}C and -196^{0}C mutants survived under 3M pulse H_2O_2 stress conditions with significantly higher survival values than the wild type. The cross-resistance levels of -80^{0}C mutants as fold of wild type were approximately two fold of -196^{0}C mutant

levels. These individuals seemed to have gained cross-resistance to oxidative strss induced by hydrogen peroxide.

All mutants obtained from the two selection strategies also gained cross-resistance to ethanol pulse stress. Some of the mutants of -80^{0}C selection had survival values in the range of thousands-fold of the wild type under pulse ethanol stress. The average cross-resistance of -80^{0}C mutants to pulse ethanol stress as fold of wild type was generally higher than those of -196^{0}C mutants. However, most of the mutants did not show significant cross-resistance to continuous ethanol stress.

The -196^{0}C mutants generally did not display cross-resistance to sorbitol and sodium chloride continuous stresses. Some of the -80^{0}C mutants were cross-resistant to sodium chloride stress up to 100-fold of wild type value.

Another interesting cross-stress resistance result was obtained against the -20^{0}C freezing stress. Almost none of the -196^{0}C mutants displayed cross-resistance to this stress; however, some of the -80^{0}C mutants were cross-resistant. Freezing at -20^{0}C is a widely used freezing method in industrial applications; and, tolerance to freezing stress at this temperature is very important feature.

The aim of the cross-stress experiments was to determine the cross-resistances of the mutants and to compare the cross-stress resistances of the mutants that were obtained from two different selective strategies. The cross-resistance results showedthat most of the mutants gained cross-resistance to several stresses. The resistance mechanisms and the genes involved in these mechanisms may have common factors for these stresses. The mutants 5, 20, 7f and 23f displayed high levels of multi-stress resistance. In general, the -80^{0}C mutants gained cross-resistant to -20^{0}C freezing stress and sodium chloride stress; but the -196^{0}C mutants generally did not. Also the total average cross-resistance levels of the -80^{0}C mutants were higher than the average levels of the -196^{0}C mutants.

FREEZE 9E. the multi-stress resistant mutant individual obtained previously (Çakar et al. 2005) was included in the stress tests in this study to compare its cross-resistance against various stresses with those the mutants obtained in this study. In general,its resistance levels were similar to those of 23f. On the other hand, it was obvious that the mutants 5 and 20 gained cross-resistance to more stress types.

The differences in resistance levels between the mutants of -80^{0}C and-196^{0}C selection indicate the different effects of those two freezing temperatures on the cells. The final freezing temperature and the cooling rate were different in these two freezing conditions. These parameters have important influence on the cell destruction during freezing [22, 24, 30]. The significant differences in cross-stress resistances implied that the two selection strategies may have improved different cellular features during evolutionary engineering.

Previous research on evolutionary engineering on multi-stress resistance showed that other selective pressure treatments did not resulted in multi-stress resistant mutants, except for selection at freezing stress conditions [20]. The result of our research also verifies the previous research. Additionally, this study showed for the first tme the differences in stress-resistance of the mutants when the freezing stress was applied at different temperatures: -80^{0}C and -196^{0}C. Thus, the freezing temperature is important in selection.

In most of the bioprocesses, cells are being exposed to many stresses simultaneously or consecutively because of the deviation from the optimal conditions in bioreactor. The resistance to these stresses, or “robustness”, is very important to achieve the desired productivity and product quality. This research is a further evidence for the multi-stress-resistance of the mutants selected under freezing stress conditions. In future studies, the resistance mechanism and the relevant genes should be determined to transfer these phenotypic features to industrial strains.

Reactive oxygen species (ROS) are formed during respiration reactions of the cell, and freeze-thaw stress triggers their formation [23, 27, 50]. The mutants obtained in this study were both freeze-tolerant and highly cross-resistant to oxidative stress (H_2O_2). According to these data, the mutants may have gained a stronger ROS defense system. To understand if there was an increase in enzymatic protection against ROS, the specific catalase activity of the multi-stress resistant mutants 5 and 23 were determined in the absence of stress conditions. It has been shown that the specific catalase activity of the mutants was significantly higher than those of the wild type , as depicted in Figure 3.7 and Table 3.39.

Although the exact molecular mechanism underlying freeze-thaw damage or freeze-thaw tolerance has not been fully understood yet, a recent using yeast deletion mutants showed that a high number of genes was re-regulated for freeze-tolerance

[27]. Thus, several metabolic pathways seem to be included in this defense system. The RAS-cyclic AMP signal transduction pathway [18], aquaporins expression [51], intracellular glycerol levels [35], trehalose level [33], N-acetyltransferase Mpr1 level [40], cytoplasmic Cu,Zn SOD [23], Fe SOD and catalase [50] are some of the factors which were found to be related with the freeze tolerance.

The inverse metabolic engineering approach could provide us the genetic basis of the complex, desired phenotype of freeze tolerance and multi-stress resistance.

To summarize, freeze-tolerant populations and individuals were obtained by evolutionary engineering strategies. Most of the freeze tolerant individuals obtained also had multi-stress resistance characteristics. By further investigations of the selected individuals, freeze-tolerance mechanism in *S. cerevisiae* could be studied in detail and the common pathways in resistance mechanisms towards other stress conditions could be illuminated. Transcriptomic and proteomic analysis could provide the necessary information for understanding the stress-resistance mechanisms in detail. The information about freeze-tolerance mechanism could be exploited in various applications such as cryoprotectants and frozen dough technology.

REFERENCES

[1] **Bolotin-Fukuhara, M.**, Genomics and Biodiversity in Yeasts, in Biodiversity and Ecophysiology of Yeasts. 2006. p. 45-66.

[2] **Alberts, B., A. Johnson, J. Lewis, M. Raff, K. Roberts, and P. Walter**, *Molecular Biology of The Cell*, 4th ed. 2002: Nex York, Garland Science.

[3] **Deak, T.**, Handbook of Food Spoilage Yeast*, CRC press*, 2007.

[4] **Guttman, B.S.**, *Biology*, 1st ed. 1999: McGraw-Hill.

[5] **Neiman, A.M.**, Ascospore formation in the yeast *Saccharomyces cerevisiae, Microbiol. Mol. Biol. Rev.*, 2005. 69(4): p. 565-584.

[6] *http://en.wikipedia.org/wiki/File:Yeast_lifecycle.svg,* Date [cited 09.04.2009] URL-1. Update; Available from.

[7] **Ostergaard, S., L. Olsson, and J. Nielsen**, Metabolic Engineering of Saccharomyces cerevisiae*, Microbiology and Molecular Biology Reviews*, 2000. 64(1): p. 34-50.

[8] **Attfield, P.V., A. Raman, and C.J. Northcott**, Construction of *Saccharomyces cerevisiae* strains that accumulate relatively low concentrations of trehalose, and their application in testing the contribution of the disaccharide to stress tolerance*, FEMS Microbiology Letters*, 1992. 94(3): p. 271-276.

[9] **Attfield, P.V.**, Stress tolerance: The key to effective strains of industrial baker's yeast*, Nature Biotech*, 1997. 15(13): p. 1351-1357.

[10] **Tanghe, A., V.P. Dijck, F. Dumortier, A. Teunissen, S. Hohmann, and J.M. Thevelein**, Aquaporin expression correlates with freeze tolerance in Baker's Yeast, and overexpression improves freeze tolerance in industrial strains*, Applied and Environmental Microbiology*, 2002. 68(12): p. 5981-5989.

[11] **Hohmann, S. and P.W.H. Mager**, Yeast Stress Responses*, Springer*, 2003. 1.

[12] **Lewis, J.G., R.P. Learmonth, P.V. Attfield, and K. Watson**, Stress co-tolerance and trehalose content in baking strains of *Saccharomyces cerevisiae, Journal of Industrial Microbiology and Biotechnology*, 1997. 18(1): p. 30-36.

[13] **Lado, B.H. and A.E. Yousef**, Alternative food-preservation technologies: efficacy and mechanisms*, Microbes and Infection*, 2002. 4(4): p. 433-440.

[14] **Wei, P., Z. Li, Y. Lin, P. He, and N. Jiang**, Improvement of the multiple-stress tolerance of an ethanologenic *Saccharomyces cerevisiae* strain by freeze-thaw treatment*, Biotechnology Letters*, 2007. 29(10): p. 1501-1508.

[15] **Giudici, P., L. Solieri, A.M. Pulvirenti, and S. Cassanelli**, Strategies and perspectives for genetic improvement of wine yeasts*, Applied Microbiology and Biotechnology*, 2005. 66(6): p. 622-628.

[16] **Tanghe, A., B. Prior, and J. Thevelein**, Yeast responses to stresses, in Biodiversity and Ecophysiology of Yeasts. 2006. p. 175-195.

[17] **Ando, A., F. Tanaka, Y. Murata, H. Takagi, and J. Shima**, Identification and classification of genes required for tolerance to high-sucrose stress revealed by genome-wide screening of *Saccharomyces cerevisiae, FEMS Yeast Research*, 2006. 6(2): p. 249-267.

[18] **Park, J.I., C.M. Grant, P.V. Attfield, and I.W. Dawes**, The freeze-thaw stress response of the yeast Saccharomyces cerevisiae is growth phase specific and is controlled by nutritional state via the RAS-cyclic AMP signal transduction pathway*, Applied and Environmental Microbiology*, 1997. 63(10): p. 3818-3824.

[19] **Teunissen, A., F. Dumortier, M.F. Gorwa, M. Bauer, A. Tanghe, ., A. Loiez, P. Smet, P. Van Dijck, and J.M. Thevelein**, Isolation and characterization of a freeze-tolerant diploid derivative of an industrial baker's yeast strain and its use in frozen doughs*, Applied and Environmental Microbiology*, 2002. 68(10): p. 4780-4787.

[20] **Cakar, Z.P., U.O.S. Seker, C. Tamerler, M. Sonderegger, and U. Sauer**, Evolutionary engineering of multiple-stress resistant *Saccharomyces cerevisiae, FEMS Yeast Research*, 2005. 5(6-7): p. 569-578.

[21] **Tanghe, A., P. Van Dijck, and J.M. Thevelein**, Determinants of freeze tolerance in microorganisms, physiological importance, and biotechnological applications, in Advances in Applied Microbiology, Vol 53. 2003, *Academic Press Inc*: San Diego. p. 129-176.

[22] **Dumont, F., P.A. Marechal, and P. Gervais**, Influence of cooling rate on *Saccharomyces cerevisiae* destruction during freezing: unexpected viability at ultra-rapid cooling rates*, Cryobiology*, 2003. 46(1): p. 33-42.

[23] **Park, J.I., C.M. Grant, M.J. Davies, and I.W. Dawes**, The cytoplasmic Cu,Zn superoxide dismutase of *Saccharomyces cerevisiae* is required for resistance to freeze-thaw stress. Generation of free radicals during freezing and thawing*, Journal of Biological Chemistry*, 1998. 273(36): p. 22921-22928.

[24] **Mazur, P.**, Physical and Temporal Factors Involved in the Death of Yeast at Subzero Temperatures*, Biophysical Journal*, 1961. 1(3): p. 247-264.

[25] **Odani, M., Y. Komatsu, S. Oka, and I. H.**, Screening of genes that respond to cryopreservation stress using yeast DNA microarray*., Cryobiology*, 2003. 47: p. 155-164.

[26] **Roy, R.S. and J.C. McCord**, Oxyradicals and their scavenging*, Elsevier Science Publishers*, 1983. 2: p. 145-153.

[27] **Ando, A., T. Nakamura, Y. Murata, H. Takagi, and J. Shima**, Identification and classification of genes required for tolerance to freeze-thaw stress revealed by genome-wide screening of Saccharomyces cerevisiae deletion strains*, FEMS Yeast Research*, 2007. 7(2): p. 244-253.

[28] **Mazur, P.**, Cryobiology- freezing of biological systems*, Science*, 1970. 168(3934): p. 939-&.

[29] **Sleight, S.C., N.S. Wigginton, and R.E. Lenski**, Increased susceptibility to repeated freeze-thaw cycles in *Escherichia coli* following long-term evolution in a benign environment*, Bmc Evolutionary Biology*, 2006. 6: p. 8.

[30] **Tanghe, A., P. Van Dijck, and J.M. Thevelein**, Determinants of freeze tolerance in microorganisms, physiological importance, and biotechnological applications, in Advances in Applied Microbiology. 2003, *Academic Press*. p. 129-176.

[31] **Novo, M.T., G. Beltran, N. Rozès, J.M. Guillamón, and A. Mas**, Effect of nitrogen limitation and surplus upon trehalose metabolism in wine yeast*, Applied Microbiology and Biotechnology*, 2005. 66(5): p. 560-566.

[32] **Kim, J., P. Alizadeh, T. Harding, A. Hefner-Gravink, and D.J. Klionsky**, Disruption of the yeast ATH1 gene confers better survival after dehydration, freezing, and ethanol shock: potential commercial applications*, Applied and Environmental Microbiology*, 1996. 62(5): p. 1563-1569.

[33] **Soto, T., J. Fernandez, J. Vicente-Soler, J. Cansado, and M. Gacto**, Accumulation of trehalose by overexpression of tps1, coding for trehalose-6-phosphate synthase, causes increased resistance to multiple stresses in the fission yeast *Schizosaccharomyces pombe, Applied and Environmental Microbiology*, 1999. 65(5): p. 2020-2024.

[34] *http://en.wikipedia.org/wiki/Glycerol*, Glycerol*,* 10.04.2009.

[35] **Izawa, S., M. Sato, K. Yokoigawa, and Y. Inoue**, Intracellular glycerol influences resistance to freeze stress in *Saccharomyces cerevisiae*: analysis of a quadruple mutant in glycerol dehydrogenase genes and glycerol-enriched cells*, Applied Microbiology and Biotechnology*, 2004. 66: p. 108-114.

[36] **Murakami, Y., K. Yokoigawa, F. Kawai, and H. Kawai**, Lipid composition of commercial bakers' yeasts having different freeze-tolerance in frozen dough*, Bioscience, Biotechnology, and Biochemistry* 1996. 60: p. 1874-1876.

[37] **Siderius, M., Wuytswinkel O.V., Reijenga K.A., M. Kelders, and W.H. Mager**, The control of intracellular glycerol in *Saccharomyces cerevisiae* influences osmotic stress response and resistance to increased temperature*, Molecular Microbiology*, 2000. 36(6): p. 1381-1390.

[38]*http://student.biology.arizona.edu/honors2005/group07/Experiments_clip_image005.jpg,* 10. 04. 2009

[39] **Gralla, E.B.**, Oxidative stress and the molecular biology of antioxidant defences, J.G. Scandalios, Editor. 1997, *Cold Spring Harbor Laboratory, Cold Spring Harbor*. p. 309-343.

[40] **Du, X. and H. Takagi**, N-Acetyltransferase Mpr1 Confers Freeze Tolerance on *Saccharomyces cerevisiae* by Reducing Reactive Oxygen Species*, J Biochem*, 2005. 138(4): p. 391-397.

[41] **Lewin, B.**, *Genes VIII*, International ed. 2004: Pearson Prentice Hall.

[42] **Kawahara, H., N. Koda, M. Oshio, and H. Obata**, A cold acclimation protein with refolding activity on frozen denatured enzymes*,*

Bioscience, Biotechnology, and Biochemistry, 2000. 64(12): p. 2668-2674.

[43] **Ribotta, P.D., A.E. Leon, and M.C. Anon**, Effect of freezing and frozen storage of doughs on bread quality, *Journal of Agricultural and Food Chemistry*, 2001. 49(2): p. 913-918.

[44] **Stephanopoulos, G.N., A.A. Aristidou, and A. Nielsen**, Metabolic Engineering, in Principles and Methodologies. 1998, *Elsevier*. p. 1-20.

[45] **Bailey, J.E., A. Sburlati, V. Hatzimanikatis, K. Lee, W.A. Renner, and P.S. Tsai**, Inverse metabolic engineering: A strategy for directed genetic engineering of useful phenotypes, *Biotechnology and Bioengineering*, 2002. 79(5): p. 568-579.

[46] **Yang, Y.T., G.N. Bennett, and K.Y. San**, Genetic and metabolic engineering, *EJB Electronic Journal of Biotechnology* 2003.

[47] **Turanlı, B.**, Evolutionary engineering of metal-binding/metal-resistant yeast for biomimetics, in *Molecular Biology Genetics and Biotechnology*. 2006, *Istanbul Technical University*.

[48] **Estelle Russek and R.R. Colwell**, Computation of most probable numbers, *Applied and Environmental Microbiology*, 1983. 45 (5): p. 1646-1650.

[49] *http://www.jlindquist.net/generalmicro/102dil3a.html,* 08. 2008

[50] **Stead, D. and S.F. Park**, Roles of Fe superoxide dismutase and catalase in resistance of *Campylobacter coli* to freeze-thaw stress, *Applied and Environmental Microbiology*, 2000. 66(7): p. 3110-3112.

[51] **Tanghe, A., P. Van Dijck, D. Colavizza, and J.M. Thevelein**, Aquaporin-mediated improvement of freeze tolerance of *Saccharomyces cerevisiae* is restricted to rapid freezing conditions, *Applied and Environmental Microbiology*, 2004. 70(6): p. 3377-3382.

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