ISTANBUL TECHNICAL UNIVERSITY ★ GRADUATE SCHOOL

EVOLUTIONARY ENGINEERING OF FREEZE-THAW STRESS-RESISTANT YEASTS WITHOUT USING CHEMICAL MUTAGENESIS

M.Sc. THESIS

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Thesis Advisor: Prof. Dr. Zeynep Petek ÇAKAR

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İSTANBUL TEKNİK ÜNİVERSİTESİ ★ LİSANSÜSTÜ EĞİTİM ENSTİTÜSÜ

DONMA-ERİME STRESİNE DİRENÇLİ MAYALARIN KİMYASAL MUTAJEN KULLANILMADAN EVRİMSEL MÜHENDİSLİK İLE ELDE EDİLMESİ

YÜKSEK LİSANS TEZİ

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HAZİRAN 2023

İrem BALABAN, a M.Sc. student of ITU Graduate School student ID 521211107, successfully defended the thesis entitled "EVOLUTIONARY ENGINEERING OF FREEZE-THAW STRESS-RESISTANT YEASTS WITHOUT USING CHEMICAL MUTAGENESIS", which she prepared after fulfilling the requirements specified in the associated legislations, before the jury whose signatures are below.

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To my family,

FOREWORD

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TABLE OF CONTENTS

Page

ABBREVIATIONS

ESR	**:** Environmental Stress Response
GSR	**:** General Stress Response
HOG	**:** High- Osmolarity Glycerol
PKC	**:** Protein Kinase C
PKA	**:** cAMP-Protein Kinase A
ROS	**:** Reactive Oxygen Species
OD	**:** Optical Density
YMM	**:** Yeast Minimal Medium
ALE	**:** Adaptive Laboratory Evolution
EMS	**:** Ethyl Methanesulfonate

LIST OF TABLES

Page

LIST OF FIGURES

EVOLUTIONARY ENGINEERING OF FREEZE-THAWSTRESS-RESISTANT YEASTS WITHOUT USING CHEMICAL MUTAGENESIS

SUMMARY

Saccharomyces cerevisiae, also known as budding yeast or baker's yeast is a unicellular microorganism from the fungi kingdom. It has been consistently used in winemaking, brewing and baking bread throughout human history. After 1930s, laboratory studies were conducted to obtain strains with increased product quality. Today, *S. cerevisiae* is the most popular yeast strain due to its good fermentative abilities.

S. cerevisiae with high fermentation performance and tolerance to environmental stresses is preferred for industrial applications. During bread production, yeast cells are exposed to a variety of environmental stresses including freeze–thaw, high sugar concentrations, air-drying and oxidative stress. Stress conditions cause a decline in cell growth rate, product yield and quality. Cells give responses to stress conditions, as environmental stress response (ESR) and stress-specific response. ESR mechanism is not specific to the stress factor and it can be used to explain the cross-resistance of the yeast cells against various stress types. One of the reasons for cross-resistance is the use of the same transcription factors as a response to a variety of different stresses.

S. cerevisiae is exposed to freeze-thaw stress during the cryopreservation and frozen dough process. Freeze-thaw stress causes physiological injuries to cells. At high freezing rates, formation of intracellular ice crystals causes cellular damages; while at low freezing rates formation of extracellular ice crystals causes cellular dehydration. The thawing process causes oxidative stress which leads to oxidative damage on proteins, nucleic acids and other biomolecules inside the cell. Studies conducted in *S. cerevisiaes*' stress-specific response against freeze-thaw stress revealed cells focus on regulating the contents of the cell membrane, protecting cell wall integrity, increasing degradation of damaged proteins from stress and increasing overall protein synthesis under stress conditions.

Cryoprotective agents can be added to decrease ice crystal formation under freezing conditions. Alternatively, yeast levels in the product can be increased to increase product yield. However, these methods can decrease product quality and increase cost. Thus, stress-resistant *S. cerevisiae* strains are preferred for industrial applications. Stress-resistant strains can be obtained by metabolic engineering. Evolutionary engineering is an inverse metabolic engineering method that mimics the natural evolution process. In this approach, the desired phenotype is selected first and the genes responsible for the phenotype are determined later by reverse engineering methods. In this study, freeze-thaw resistant yeast strains were obtained with the evolutionary engineering method. A reference yeast strain was exposed to freeze-thaw stress in the form of pulse stress selection. The evolved strains obtained under stress conditions generally show mutations mainly in their stress-induced genes. This allows ease in reverse engineering studies to determine genes related to the applied stress. Freeze-thaw stress was applied in the form of pulse stress selection to maintain the survival rate of cells with increasing stress levels and to induce selective pressure.

In this study, a *S. cerevisiae* CEN.PK113-7D reference strain was exposed to gradually increasing freeze-thaw stress until the final population was obtained. The final population was obtained after 10 cycles of freeze-thaw stress application. Ten mutant individuals were randomly selected from the final population and their resistance to freeze-thaw stress was tested with the spot assay method. Four evolved strains labeled as FT-1, FT-5, FT-6 and FT-9 that showed the highest freeze-thaw resistance were selected for detailed analysis.

Further physiological characterizations of the evolved strains were made by cross-resistance analysis. FT-1, FT-6 and FT-9 showed cross-resistance to potassium chloride (KCl) and iron stress. KCl, at high concentrations, causes hyperosmotic stress to the cell. This cross-resistance could be the result of a similar response mechanism activated by the cell to protect itself from dehydration caused by freezing stress. Metals such as iron increase generation of ROS in cell and cause oxidative stress. The cross-resistance to iron stress could be the result of activation of similar pathways used by the cell as a response to oxidative stress caused by thawing process. All evolved strains tested showed resistance to boric acid. Boric acid disrupts cell wall synthesis in *S. cerevisiae*. The freezing process also causes cell wall damage in *S. cerevisiae*. Inducing cell wall synthesis due to freezing stress may also result with increased resistance to boric acid.

The aim of this study was to obtain freeze-thaw stress-resistant *S. cerevisiae* strains from a reference laboratory strain, without using chemical mutagenesis, by evolutionary engineering. Physiological characterization of the evolved strains was also performed by determining their cross-resistance to selected stress factors. Further genomic, transcriptomic and proteomic analyses could be performed on the selected FT-9 strain to identify the genes, pathways and molecular mechanisms responsible for resistance against freeze-thaw stress and the pathways that cause cross-resistance to selected stress factors.

DONMA-ERIME STRESİNE DİRENÇLİ MAYALARIN KİMYASAL MUTAJEN KULLANILMADAN EVRİMSEL MÜHENDİSLİK İLE ELDE EDİLMESİ

ÖZET

Ekmek mayası olarak da bilinen *Saccharomyces cerevisiae*, mantar krallığından tek hücreli bir mikroorganizmadır. İnsanlık tarihi boyunca şarap yapımında, bira yapımında ve ekmek pişirmede sürekli olarak kullanılmıştır. 1930'lardan sonra ürün kalitesi yüksek maya suşları elde etmek için laboratuvar çalışmalarında kullanılmıştır. Günümüzde *S. cerevisiae*, yüksek fermentasyon yeteneğinden dolayı en popüler maya türüdür.

Endüstriyel uygulamalarda fermentasyon performansı yüksek ve çevresel streslere karşı toleranslı *S. cerevisiae* suşları tercih edilmektedir. Ekmek üretimi sırasında maya hücreleri, donma-çözülme, yüksek şeker konsantrasyonları, havada kurutma ve oksidatif stres gibi çeşitli çevresel streslere maruz kalır. Stres koşulları; hücre büyüme hızında, ürün veriminde ve kalitesinde düşüşe neden olur. Hücreler, çevresel stres yanıtı (ÇSY) ve strese özgü yanıt olarak stres koşullarına cevap verir. ÇSY mekanizması stres faktörüne özgü değildir ve maya hücrelerinin çeşitli stress faktörlerine karşı gösterdiği çapraz direnci açıklamak için kullanılabilir. Çapraz direncin ortaya çıkmasının nedenlerinden biri, aynı transkripsiyon faktörlerinin farklı streslere yanıt olarak kullanılmasıdır.

S. cerevisiae, kriyoprezervasyon ve dondurulmuş hamur işlemi sırasında donma-çözülme stresine maruz kalmaktadır. Donma-çözülme stresi hücrelerde fizyolojik yaralanmalara neden olur. Yüksek donma hızlarında hücre içi buz kristallerinin oluşumu hücresel hasarlara neden olur; düşük donma hızlarında ise hücre dışı buz kristallerinin oluşumu hücresel dehidrasyona neden olur. Çözdürme işlemi, hücre içindeki proteinler, nükleik asitler ve diğer biyomoleküller üzerinde oksidatif hasarlara yol açar ve oksidatif strese neden olur. *S. cerevisiae*'nın donma çözülme stresine karşı strese özgü yanıtı üzerine yürütülen araştırmalar, hücrelerin stres karşısında hücre zarı içeriğini düzenlemeye, hücre duvarı bütünlüğünü korumaya, stresten zarar görmüş proteinlerin bozulmasını artırmaya ve genel protein sentezini artırmaya odaklandığını ortaya çıkarmıştır.

Donma koşulları altında buz kristali oluşumunu azaltmak için kriyoprotektif maddeler eklenebilir. Alternatif olarak, ürün verimini artırmak için üründeki maya miktarı artırılabilir. Ancak bu yöntemler ürün kalitesini düşürebilir ve maliyeti artırabilir. Bu nedenle endüstriyel uygulamalarda strese dayanıklı *S. cerevisiae* suşları tercih edilmektedir. Strese dayanıklı suşlar, metabolik mühendislik ile elde edilebilir. Evrimsel mühendislik, doğal evrim sürecini taklit eden bir tersine metabolik mühendislik yöntemidir. Bu yaklaşımda önce istenen fenotip seçilir ve fenotipten sorumlu genler daha sonra tersine mühendislik yöntemleriyle belirlenir. Bu çalışmada, donma-erime stresine dirençli maya suşları, evrimsel mühendislik yöntemiyle elde

edildi. Referans laboratuvar suşu, kısa süreli şok (pulse) stres seçilimi şeklinde donma-çözülme stresine maruz bırakıldı. Stres koşulları altında elde edilen dirençli suşlar, genel olarak stresle indüklenen genlerinde mutasyonlar gösterir. Bu durum, uygulanan stresle ilgili genlerin belirlenmesi için tersine mühendislik çalışmalarında kolaylık sağlar. Artan stres seviyeleri ile hücrelerin hayatta kalma oranını korumak ve stresin seçici etkisini arttırmak için donma-çözülme stresi kısa süreli şok (pulse) stres seçilimi şeklinde uygulandı.

Bu çalışmada, bir *Saccharomyces cerevisiae* CEN.PK113-7D referans suşu, son poülasyon elde edilene kadar kademeli olarak artan donma-çözülme stresine maruz bırakıldı. 10 döngü donma-çözülme stresi uygulandıktan sonra son popülasyon elde edildi. Son popülasyondan rastgele 10 mutant birey seçildi ve donma-çözülme stresine karşı dirençleri nokta tahlili (spot assay) yöntemi ile test edildi. En yüksek düzeyde donma-çözülme direnci gösteren FT-1, FT-5, FT-6 ve FT-9 adlı dört suş kapsamlı analizler için seçildi.

Dirençli suşların diğer fizyolojik karakterizasyonları, çapraz direnç analizi ile yapılmıştır. FT-1, FT-6 ve FT-9 suşları, potasyum klorür (KCl) ve demir stresine karşı çapraz direnç gösterdi. KCl, yüksek konsantrasyonlarda hücrede hiperozmotik strese neden olur. Bu çapraz direnç, donma stresinin neden olduğu dehidrasyondan kendini korumak için hücre tarafından etkinleştirilen benzer bir tepki mekanizmasının sonucu olabilir. Demir ve benzeri metaller hücrede ROS üretimini arttırır ve oksidatif strese neden olur. Demire karşı çapraz direnç, hücre tarafından çözülme işleminin neden olduğu oksidatif strese bir yanıt olarak kullanılan benzer yolakların aktivasyonunun bir sonucu olabilir. Test edilen tüm dirençli suşlar borik aside direnç göstermiştir. Borik asit, S. *cerevisiae*'de hücre duvarı sentezini bozar. Dondurma işlemi ayrıca *S. cerevisiae*'de hücre duvarı hasarına neden olur. Donma stresi nedeniyle hücre duvarı sentezinin indüklenmesi, borik aside karşı direncin artmasıyla da sonuçlanabilir.

Bu çalışmanın amacı, bir referans laboratuvar suşundan, donma-çözülme stresine dirençli *S. cerevisiae* suşlarının, kimyasal mutajenez uygulamadan, evrimsel mühendislik ile elde edilmesidir. Seçilen stres faktörlerine karşı çapraz dirençlerini belirleyerek dirençli suşların fizyolojik karakterizasyonu da gerçekleştirilmiştir. Donma-çözülme stresine karşı dirençten sorumlu genleri, yolakları, moleküler mekanizmaları ve seçilen kimyasallara karşı çapraz dirence neden olan yolakları belirlemek için, seçilen FT-9 suşu üzerinde kapsamlı genomik, transkriptomik ve proteomik analizler yapılabilir.

1. INTRODUCTION

1.1 Characteristics of *Saccharomyces cerevisiae*

Yeasts are eukaryotic unicellular microorganisms from the fungi kingdom that have been used by humans for leavening, brewing, and winemaking since 4 to 5 millennium BCE. Today, yeast production for commercial purposes is over 1.8 million tons per year (Richard and Bachhawat, 2014). Yeasts are a highly diverse community distributed in a variety of environments. They can be found in water, soil, plants, animals, and insects. Although their preferred habitats are leaves and fruits of plants; some species can be found in animal intestines and other extreme environments with low temperature and low accessibility to water and oxygen (Walker, 2009). The species can be characterized and identified by their cellular differences in morphology, physiology immunology and molecular biology. Currently, there are around 1500 identified yeast species; with the unidentified species the total number is estimated to be around 670,500 (Kurtzman and Fell, 2006; Verstrepen et al., 2006). Yeast from genus Saccharomyces are the most valued species in the industry due to their remarkable fermentation abilities. *Saccharomyces cerevisiae* also known as budding yeast or baker's yeast is from the phylum Ascomycota, class Hemiascomycetes, order Saccharomycetales and family Saccharomycetacae (Table 1.1) (Shaban-Nejad and Haarslev, 2008).

Table 1.1: Classification of *S. cerevisiae* (Shaban-Nejad, 2008).

Domain	**Eukaryota**
Kingdom	Fungi
Phylum	Ascomycota
Class	Hemiascomycetes
Order	Saccharomycetales
Family	Saccharomycetaceae
Genus	Saccharomyces
Species	*Saccharomyces cerevisiae*

Yeast cells can be found in a globular, ovoidal, or cylindrical shape with a smooth and flat surface (Stewart 2014). The eukaryotic cells possess a tough cell wall, a nucleus and mitochondria (Figure 1.1). They can reproduce asexually by multilateral budding or sexually by mating with another yeast cell (Alberts et al., 2015). *S. cerevisiae* role in fermentation was discovered by Louis Pasteur in 1857. After 1930s it was studied in laboratories to improve the brewing quality of strains (Liti, 2015). Currently, it is commercially the most exploited yeast species in the industry. It is used in brewing, winemaking, baking bread, distilling, flavoring food and for therapeutic purposes (Stewart, 2014).

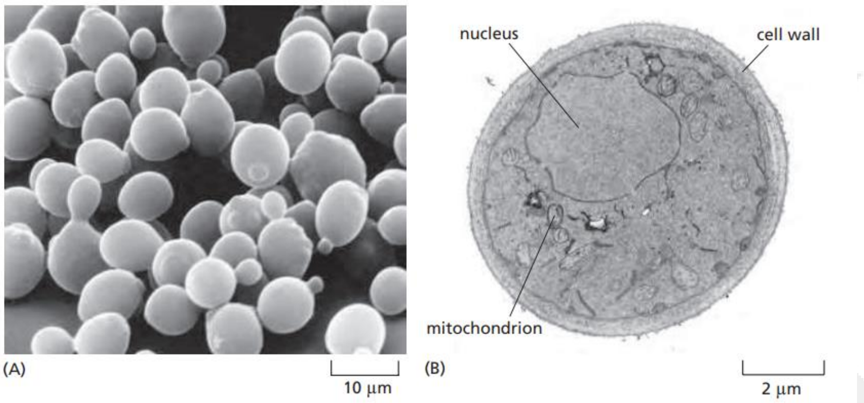

Figure 1.1: (A) A scanning electron micrograph of *S. cerevisiae* cells. (B) A transmission electron micrograph of a yeast cells' cross section with its nucleus, mitochondrion, and cell wall displayed (Alberts et al., 2015)

1.2. *S. cerevisiae* as a Model Organism

Model organisms are used in researches to obtain knowledge about a target organism and to provide optimization and standardization for analytical approaches. They can be considered as representative for large groups of organisms for specific biological processes (Karathia et al., 2011). Several genetic studies were performed with *S. cerevisiae* cells through time to gain insight into their cellular physiology to improve their fermentation abilities. These studies led *S. cerevisiae* to become the first eukaryotic organism with a completely sequenced genome and a model organism for eukaryotes (Burgess et al., 2017). *S. cerevisiae,* compared to higher eukaryotes, is a simple organism with a significantly smaller genome. Proteins encoded by *S. cerevisiae* show significant similarity based on their primary sequence and functional homology to higher eukaryotic organisms; which allows complex eukaryotic cellular pathways to be examined with ease and physiological processes of eukaryotic organisms to be understood better (Alberts et al., 2015; Pausch et al., 2005). Another advantage of *S. cerevisiae* is that it can be genetically manipulated by exogenous DNA by using recombinant DNA technology for gene addition/deletion. The cells cultured as haploids allow ease in isolation of mutants during experimental studies and examination of the relationship between phenotype and genotype by genome-wide analysis. It also has a rapid doubling time and can be cultured easily at low costs. Therefore, *S. cerevisiae* is accepted as a good model organism for animals and plants with similar eukaryotic cellular structures (Burgess et al., 2017; Pausch et al., 2005).

1.3. Industrial Importance of *S. cerevisiae*

S. cerevisiae is favored in industrial applications due to its tolerance to high sugar concentrations and ability to produce several aromatic compounds, most importantly ethanol. In the fermentation process, *S. cerevisiae* converts hexose and disaccharide sugars into CO_2, ethanol and other organic compounds. In alcohol fermentation and bread production, *S. cerevisiae* improves the flavor and quality of the product (Stewart, 2014). It is used in the alcoholic beverage industry to produce fermented beverages: beer, wine and cider and of distilled beverages: vodka, rum, whisky and brandy. In bioethanol production, sugars from different sources are converted into ethanol and the produced ethanol, sometimes mixed with gasoline, is used as a biofuel (Parapouli et al., 2020).

In bread making; flour, sourdough and water get mixed to produce the bread dough. Sugars in this dough are used for fermentation with *S. cerevisiae* acting as a leavening agent. Initially, *S. cerevisiae* prefer monosaccharides such as glucose and fructose for sugar fermentation. However, the dough mostly contains more complex sugars. Thus, after consumption of monosaccharides, *S. cerevisiae* uses other di- and polysaccharides in dough (Parapouli et a, 2020; Bell et al., 2001). At the beginning of the process, *S. cerevisiae* uses oxygen trapped in the dough during mixing for respiration and reproduction. After consumption of excessive oxygen, fermentation occurs under almost anaerobic conditions. For fermentation, the optimum temperature is in between 34–38 °C and the optimum pH is in between 4.0–5.2 (Hidalgo and Brandolini, 2014). The CO_2 produced by the cells during aerobic respiration and fermentation causes the volume of bread dough to increase and dough texture to change. At the same time, the organic acids and volatile products synthesized during fermentation adds flavor to the bread. The produced ethanol, on the other hand, is vaporized during the baking of the bread. Strains with good fermentation performance are preferred in the baking industry. Strains fermentation performance can be measured by their CO_2, biomass and ethanol production rate, cell growth rate and by the volume, texture, aroma and flavor of the dough (Parapouli et al., 2020).

Another industrial selection trait for *S. cerevisiae* is its resistance to environmental stresses. In the industrial scale, *S. cerevisiae* is faced with a variety of environmental stresses. Under stress conditions cells' growth rate, product yield and the quality of the product decline. The stress can be caused by alterations in temperature, pH, pressure, osmosis, oxidation rate and from different chemicals in the environment (Dangi et al., 2017). *S. cerevisiae* cells are mainly exposed to freeze–thaw, high sugar concentrations, air-drying and oxidative stresses during bread production (Figure 1.2). Stress conditions can cause macromolecules such as cellular organelles, membranes, proteins and nucleic acids to get damaged, which could lead up to inhibition in cell growth and cell death. Certain strains can overcome those stress conditions with a proper stress-tolerance mechanism. Under stress conditions, yeast cells induce production of stress proteins, repress translation, change membrane composition and induce stress-triggered signaling pathways. Due to these reasons, a variety of studies have been focused on developing strains with tolerance to environmental stress (Shima, and Takagi 2009; Dangi et al., 2017).

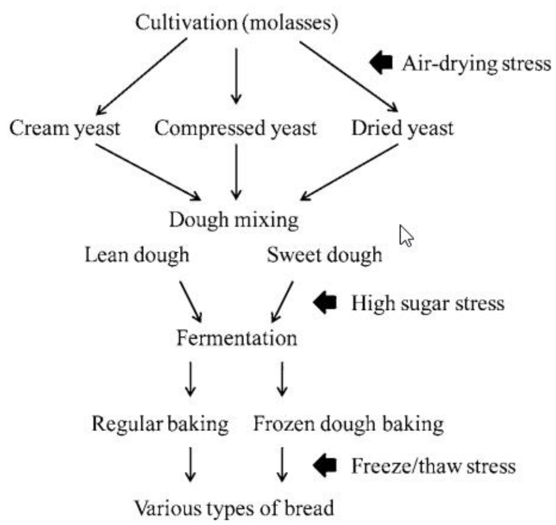

Figure 1.2: Environmental stresses *S. cerevisiae* is exposed to during industrial scale bread production (Shima and Takagi, 2009)

1.4 Environmental Stress Response of *S. cerevisiae*

S. cerevisiae stress response mechanisms can be divided into two categories environmental stress response (ESR) also known as general stress response (GSR), and stress-specific response. The environmental stress response is not specific to the stimulus. It can be triggered by any of the environmental stresses such as heat shock, oxidative stress, osmotic shock, starvation and more. (Święciło, 2015; Bose et al., 2005). Cells can initiate ESR rapidly after being introduced to a new environment. Under stress conditions, ESR gene expression levels are induced, while growth-related gene expression levels are reduced. This allows cells to adapt into their new environment rather quickly and after adaptation, cells continue their growth. ESR genes mainly regulate cAMP/PKA, TOR, SNF1/AMPK, and HOG MAPK pathways and transcriptional factors Msn2p and Msn4p related to cAMP/PKA pathway, as a response to stress (Gutin et al., 2019). Even though cells give a common response to stress through certain stress pathways, expression levels of ESR genes and the production of stress proteins differ under different stress conditions (Gasch, 2003).

For instance, under osmotic stress, cells induce Protein Kinase C (PKC) pathway and the high osmolarity glycerol (HOG) pathway. PKC pathway is responsible for regulating the cell wall integrity; while HOG pathway is responsible for accumulating glycerol inside the cell to prevent dehydration under hyperosmotic stress (Saxena and Sitaraman, 2016; Dihazi et al., 2004). Another well-known pathway cAMP-protein kinase A (PKA) has a role in the regulation of various cellular processes including inhibition of stress-induced genes. Stress conditions can cause the PKA pathway to be downregulated which leads to growth arrest and accumulation of Msn2p and Msn4p transcription factors. Msn2p and Msn4p recognize and bind to a common cis element in promoters of ESR genes. Under stress conditions, they accumulate in the nucleus and induce ESR genes. Stress proteins synthesized by this mechanism include heat shock proteins, proteases and enzymes involved in DNA repair, homeostasis, cell wall modification, carbohydrate and fatty acid metabolism and intracellular signal transduction (Estruch, 2000; Święciło, 2015). Other examples for the ESR mechanism can be given for heat shock and oxidative stress. During heat shock stress, proteins in cells get unfolded and damaged; as a response, cells induce heat shock proteins to restore denatured proteins to their native structure and ubiquitin–proteasome system to degrade proteins that cannot be restored. Oxidative stress can cause damages in

DNA and induce apoptosis. Cells respond to this stress by inducing antioxidant production to eliminate reactive oxygen species (ROS) (Lin et al., 2021).

The ESR mechanism can also be used to explain the cross resistance of the yeast cells (Figure 1.3). Cross-resistance is often a result of the same transcription factors being used as a response to a variety of different stress factors. Other reasons could be cells defense and repair mechanisms being less selective for their activating molecules and activity of different defense mechanisms leading to same biochemical results (Święciło, 2015).

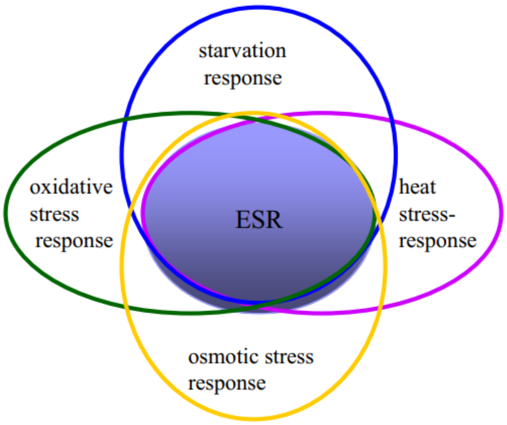

Figure 1.3: The connecting role of environmental stress response (ESR) in cross-resistance mechanism of cells (Święciło, 2015).

1.5 Freeze-Thaw Stress

The yeast cells ability to survive under freeze-thaw stress is important for industrial and commercial applications. Under industrial conditions, yeast cells are exposed to freeze-thaw stress during cryopreservation and frozen dough process. Cryopreservation allows yeast cells to be stored frozen for long periods of time. In cryopreservation, yeast cells are grown in growth rich medium and frozen with a cryoprotective agent, at temperatures -60^{o}C and lower (Amberg, 2005). Industrial bakeries and bakers prefer frozen-dough for baking due to its ease in labor work and storage. Besides, pre-fermented frozen dough has a desirable texture and flavor. The frozen dough is usually stored around -20^{o}C with a 16-week shelf-life. When needed, the dough can be thawed and used which allows fresh baked products to be available at all times (Ando, et al. 2006; Phimolsiripol, 2009).

The stress from freezing and thawing can cause physiological injuries to the yeast cells. This stress can cause damages in cell wall and cell membrane and degradation of DNA and proteins. The yeast cells physiological conditions, its genetic background and freezing conditions all affect the formation of freeze-thaw injuries (Ando et al., 2006). Freezing conditions such as freezing rate can cause ice crystals formation and cellular dehydration. While at high freezing rates, formation of intracellular ice crystals leads to cellular damages; at low freezing rates, the formation of extracellular ice crystals causes cellular dehydration (Figure 1.4) (Mazur et al., 1972; Mazur 1977). Different strategies to prevent ice crystals are used in the industry. One strategy for yeast cells to have higher cell viability is to freeze slowly at 7^{o}C and lower temperatures (Park et al., 1997). Another method can be the addition of cryoprotective agents such as glycerol to decrease the formation rate of ice crystals and minimize the damage (Izawa et al., 2004).

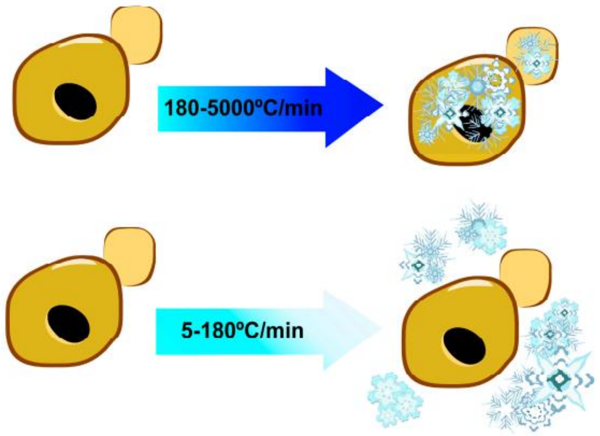

Figure 1.4: High freezing rates (180-5000^{o}C/min) can cause intracellular ice formation, while low freezing rates (5-180^{o}C/min) can cause extracellular ice formation (Cabrera et al., 2020).

Physiological conditions such as membrane composition, cells' growth phase, mitochondrial function, trehalose accumulation ect. affect cellular resistance to stress. Cell membranes containing high numbers of lipids tend to be more fluid and flexible, harder to rupture and can protect their membrane integrity under freeze-thaw stress (Cabrera et al., 2020). Yeast cells' resistance against freeze-thaw stress also varies during their growth phases. Cells show the highest sensitivity to stress during the logarithmic phase, where cell numbers increase exponentially by cell division. Furthermore, they show the highest resistance during the stationary phase of growth where the viable cell number stays the same (Park et al., 1997). *S. cerevisiae* resistance to stress increases with increasing trehalose concentration in cells. Trehalose is a disaccharide produced by *S. cerevisiae* cells. It protects the cells against dehydration.

It stabilizes dry membranes and proteins. Cells prefer accumulating trehalose compared to other disaccharides, due to the fact that trehalose is less likely to form crystals. Therefore, trehalose is accepted as a good cryoprotectant (Crowe et al., 1992).

The thawing process can be damaging for cells, as well. It can cause oxidative damage in biomolecules such as proteins, nucleic acids and lipids inside the cell. It can cause accumulation of ROS which leads to oxidative stress. ROS generation usually occurs by electron leakages originated in the mitochondrial transport chain where respiration process takes place (Park et al., 1997; Farrugia and Balzan, 2012).

1.6 Freeze-Thaw Stress Response of *S. cerevisiae*

S. cerevisiae cells respond to freeze-thaw stress through ESR mechanisms and freeze-thaw stress response mechanisms. Some of the ESR triggered by freeze-thaw stress can be listed as the regulation of cAMP signaling pathway, increase in production of proteasomes and activation of autophagy (Cabrera et al., 2020). The cAMP regulates various stress-induced genes. It also regulates an early autophagy step in *S. cerevisiae*. The autophagy process has a role in the degredation of proteins and other cytoplasmic components by being taken into vacuolar compartments to recycle nitrogen and carbon sources. The activation of autophagy mechanism may lead to an increase in freeze-thaw tolerance in *S. cerevisiae* (Budovskaya et al., 2004; Park et al., 1997).

While stress-specific response of the *S. cerevisiae* is not understood completely, studies in the literature enlightened parts of the response mechanism. A previous study identified and classified some of the genes involved in freeze-thaw stress response mechanism, by using *S. cerevisiae* deletion strains. In that study, following the genes *VMA2* and *VMA8* involved in producing structural components of vacuolar H+-ATPase and regulating pH homeostasis; *ANP1, MNN10,* and *MNN11* involved in protein glycosylation and maintaining cell wall integrity; *RPL27A* and *MRPL32* involved in protein synthesis and synthesizing ribosomal and mitochondrial ribosomal proteins were also found to be important for freeze-thaw tolerance (Ando et al., 2006).

Cells response mechanism to freeze-thaw stress correlates with its response mechanism to cold-heat shock and oxidative stress. Transcription factors Yap1p, Skn7p, Msn2p, and Msn4p in *S. cerevisiae* are involved in cellular defense mechanism against the oxidative stress. They are used to upregulate the transcription of genes associated with antioxidant defenses. In the presence of oxidative agents such as H_2O_2; Yap1p and Skn7 get activated and promote transcription of genes involved in encoding antioxidant agents and proteins such as catalases, thioredoxins superoxide dismutase enzymes and heat shock proteins (Farrugia and Balzan, 2012). Another study identified cold shock activated genes; *TPI1*, *IMH1* and *ERG10*. *S. cerevisiae* under freezing conditions induced the expression of these genes. Over-expression of *ERG10* which allows optimization in sterol content of the membrane was found to increase freeze-thaw tolerance of cells (Rodriguez-Vargas et al., 2002). *S. cerevisiae* defense mechanism against freeze-thaw stress can be summarized as to protect cell wall integrity, regulate cellular membrane content and increase degradation of damaged proteins, while at the same time trying to increase protein synthesis.

1.7 Metabolic and Evolutionary Engineering

Stress conditions decrease yeast growth and product yield in the industry. This problem can be overcome by increasing the yeast amounts 2-8% above the regular levels in the products. However, this application results with a decrease in the quality of product taste while increasing the production cost. Therefore in industrial applications, strains with high tolerance to environmental stresses are desired. For this purpose, recombinant DNA technology is being used as an important tool to manipulate genes and pathways to regulate cell metabolism (Randez-Gil et al., 2003). The recombinant DNA technology studies conducted by Cohen and Bailey in 1973 were the pioneering studies for metabolic engineering. They developed *in vitro* plasmid constructs which were used for ethanol and insulin production by introducing the target genes into *Escherichia coli*. By the 1980s, engineered microorganisms were being used for the synthesis of various chemicals, pharmaceuticals and proteins (Volk et al., 2022).

The definition of metabolic engineering was made by James Bailey in 1991 as “the improvement of cellular activities by manipulation of enzymatic, transport, and regulatory functions of the cell with the use of recombinant DNA technology.” (Bailey, 1991). The metabolic engineering methodologies can be divided as the classical (rational) metabolic engineering and inverse metabolic engineering. In the classical approach, recombinant DNA technology is used for making selected genetic modifications to alter cell metabolism and pathways (Yang et al., 2007). There are several limitations to this method. It requires prior knowledge of the biochemical pathways, regulatory factors and kinetics of enzymes involved in the desired outcome. Due to the complexity of cellular physiology, alterations in certain pathways can cause unexpected activation or inhibition of other pathways in cell. Finally, the genetic complexity of strains can cause difficulties during the cloning of industrial strains (Çakar et al., 2012).

In situations where there is a lack of information about biochemical pathways, inverse metabolic engineering strategy can be applied. Inverse metabolic engineering is a "bottom up" approach where a desired phenotype is selected first. In this approach, a genetically diverse library can be formed by random mutagenesis and then screened for mutants with the desired phenotype. Mutants with the desired phenotype are identified and their genotype is identified by genetic analysis. The alterations in the genome allow a greater understanding of the biochemical pathways responsible for the phenotype (Bailey et al., 1996; Skretas and Kolisis, 2012).

Evolutionary engineering is an inverse metabolic engineering strategy. It mimics the natural evolution process. The strains evolve to desired metabolic phenotypes under constant or increasing selection pressure over time. Evolutionary engineering is also known as adaptive laboratory evolution (ALE). There; after selective pressure applied to cells for generations, cells that develop beneficial mutations outgrow others in the culture. The evolved strains are then isolated and their genome is sequenced. The genome sequencing is used for reverse engineering to determine mutations in the genome responsible for desired phenotype (Figure 1.5) (Ko et al., 2020).

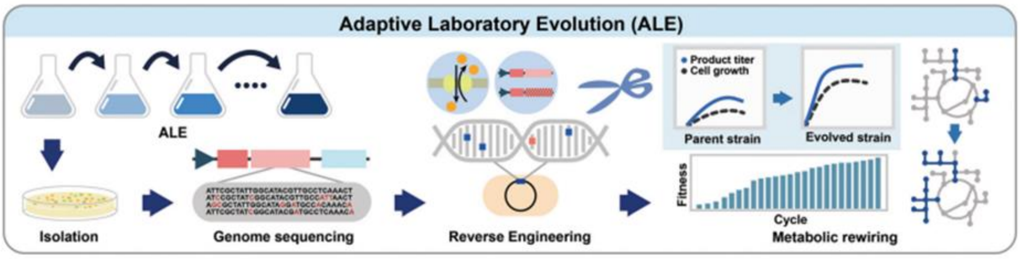

Figure 1.5: Steps of adaptive laboratory evolution (ALE) (Ko et al., 2020)

In evolutionary engineering, wild-type cells or mutant cells (wild-type cells treated with mutagens) can be used as the starting culture. Mutagens are chemical or physical agents that are responsible for increasing the frequency of mutations; hence increasing the genetic diversity of the starting culture (Sikora et al., 2012). A genetically diverse starting culture can be advantageous for evolutionary engineering studies. However, chemical mutagens cause random mutations unrelated to the applied stress. Therefore, a wild-type strain treated with ALE strategy is more beneficial to specifically map stress-induced genes (Brown, 2002; Trovao et al., 2022).

The stress applied to the culture for the selection of the stress-resistant mutant strains can be applied in the form of continuous stress or pulse stress. In the continuous stress, stress is applied uninterrupted. The culture is incubated in a stress medium and at the end of the incubation it is passaged to a new medium with constant or increasing stress conditions. Whereas in pulse stress selection, the stress is applied for a short period of time, as a pulse or shock. For the culture, incubation under stress conditions is followed by incubation under no stress. For both methods, stress is applied at each passage until final population is obtained. Pulse stress selection is preferred for stress types that are not convenient for continuous application such as freeze-thaw and temperature stress (Alkım et al., 2014).

The advantage of evolutionary engineering is that it does not require foreign/recombinant genes to be introduced to the microorganism of interest; with this method, the already existing cellular characteristics are evolved. *S. cerevisiae* cells engineered with this method are not considered as genetically modified organisms (GMOs) and their usage in the industry is accepted by the public at higher rates (Çakar et al., 2012). The strategy is used in a variety of biotechnological applications including; increasing the tolerance to stress conditions and certain metabolites, increasing product yield, improvement of carbon source utilization of organisms and identification of unidentified mechanisms of organisms (Ko et al., 2020).

1.8 Aim of the Study

The purpose of this study was the selection and identification of a highly freeze-thaw stress-resistant *S. cerevisiae* strain derived from a haploid laboratory strain *S. cerevisiae* CEN.PK113-7D, using evolutionary engineering without chemical mutagenesis, to eliminate unrelated mutations in the evolved strain. Cross-resistance analysis of the evolved strain against selected stress factors were performed for further physiological characterizations. Future studies focused on genomic and physiological characterization of the freeze-thaw resistant *S. cerevisiae* evolved strain can help enlighten the pathways affected by freeze-thaw stress and identify the genes responsible for freeze-thaw resistance of the evolved strain.

2. MATERIALS AND METHODS

2.1. Materials

2.1.1 Yeast strain

Saccharomyces cerevisiae CEN.PK113-7D (MATa, MAL2-8c, SUC2) strain, kindly provided by Prof Dr. Jean Marie François and Dr. Laurent Benbadis (University of Toulouse, France), was used as the reference strain. FT strains selected from the reference strain using evolutionary engineering were also used in this study.

2.1.2 Culture media

S. cerevisiae strains from the stock were revived, precultured and cultivated in yeast minimal medium (YMM). YMM was prepared by dissolving the ingredients in 1L distilled water (Table 2.1). The prepared media were sterilized at 121ºC for 15 min using an autoclave. Agar was added to the liquid media to obtain solid YMM media.

Table 2.1: Composition of Yeast Minimal Medium (YMM)

Chemicals	Concentration (g/L)
Yeast Nitrogen Base without Amino Acids	6.7
Dextrose	20
Agar (for solid media)	20

2.1.3 Chemicals

The chemicals used in this study are listed in Table 2.2.

Table 2.2: The list of chemicals

Chemical	Supplier
Agar	Difco
Dextrose	Riedel-de-Haen
Yeast Nitrogen Base without Amino Acids	Difco
Ammonium iron (II) sulfate hexahydrate ($(NH_4)_2Fe(SO_4)_2 \cdot 6H_2O$)	Merck
Boric Acid ($B(OH)_3$)	Fluka
Chrome chloride ($CrCl_3$)	Acros Organics
Potassium Chloride (KCl)	Merck

2.1.4: Laboratory equipment

Laboratory equipment used in this study are listed in Table 2.3.

Table 2.3: The list of laboratory equipment

Laboratory Equipment	Supplier
Autoclave	Zealway GR110DF
Laminar Flow Cabinet	Faster BH-EN 2003
Deep freezer	-80ºC SANYO UltraLow
Refrigerators	+4ºC Arçelik
Ultrapure water systems	USF-Elga UHQ
Balance	Precisa BJ 610 C
Orbital Shaker	Certomat S II Sartorius
Microcentrifuge	Eppendorf Microcentrifuge 5424
Vortex mixer	Nuve NM 110
Thermomixer	Eppendorf
Micropipettes	Eppendorf

2.2 Methods

2.2.1 Yeast culture cultivation

S. cerevisiae CEN.PK 113-7D cells from the glycerol stocks (30 %; v/v) were revived in 50 ml culture tubes containing 10 ml YMM, at 30ºC and 150 rpm, for 24 h. For the preculture, spectrophotometric measurements at 600 nm optical density (OD_{600}) were made to inoculate the cells at an OD_{600} of 0.2 (approximately $2.8x10^6$ cells/ml) into 10 ml YMM in 50 ml culture tubes. The cells were incubated in shake flasks overnight (for 16h) at 30 ˚C, 150 rpm until the OD_{600} value reached 4.5-5.5 (approximately $6.3x10^7$-$7.7x10^7$ cells/ml).

2.2.2 Pulse stress selection strategy

The gradually increasing stress levels method was applied to the reference strain during pulse stress selection. Cells were revived and precultured into 50 ml culture tubes containing 10 ml YMM. One ml from the preculture was transfered into two sets of microfuge tubes. The tubes were centrifuged at 12000 rpm for 5 min and the cell pellets were washed with YMM w/o glucose twice after discarding supernatants. Later, cells were resuspended in YMM. The first tube was used as a control and the Second tube was exposed to freeze-thaw stress. No stress was applied to the control. The control was inoculated into 96 well-plates containing YMM for Five tube-MPN (Most Probable Number) analysis. The culture in the second tube was exposed to freeze-thaw stress by freezing for 120 min at -20ºC and thawing for 10 min at 30ºC. The culture obtained from this procedure was accepted as the first population. The first population was also inoculated into 96 well-plates containing YMM for five tube-MPN analysis. Later, 1 ml from the first population was inoculated into 10 ml YMM for the preparation of the next population. The next populations were obtained by repeating the same procedure with increasing freeze-thaw cycles until the 10^{th} population (final population) was obtained. For the second population, freeze-thaw cycle was applied twice, and this was increased until 10 freeze-thaw cycles in the last or 10^{th} population. Stocks were prepared for each population by taking 1ml from the cultures into two sets of microfuge tubes and storing them in 30% glycerol at -80 ˚C.

MPN analysis was performed for each population of selection to determine their survival rate after the application of stress. For this purpose serially diluted cells before (as control) and after application of stress were inoculated into 96 well-plates containing YMM. The growth pattern on the plates were used to determine the survival rate of each evolved population.

2.2.3 Selection of Mutant Individuals from the Final Population

After the application of 10^{th} freeze-thaw cycle, individual mutants with the highest freeze-thaw resistance were selected from the final population obtained. The final population was inoculated into 50 ml culture tubes containing 10 mL YMM and grown overnight. Grown cells were diluted to 10^{-5} and spread onto the YMM agar plate. The plate was incubated at 30^{o}C for 48h. 10 colonies were picked from the plate randomly by using a micropipette tip and inoculated individually into 5 ml YMM. The chosen mutant colonies were incubated in YMM at 30^{o}C overnight. One mL from each culture was taken as stocks.

Selected mutants and the reference strain were revived and precultured in 50 ml culture tubes containing 10 mL YMM. Spot assay method was applied to determine the mutants with the highest resistance. For this, 1 mL from each grown individual mutant and reference strain were taken into two sets of microfuge tubes. The cells in microfuge tubes were washed twice with YMM without glucose. Three cycles of freeze-thaw stress in the form of freezing for 120 min at -20^{o}C and then thawing for 10 min at 30^{o}C was applied to the first set of microfuge tubes while no stress was applied to the cells in the second set of tubes used as control. The control and stress-applied cells were diluted from 10^{0} to 10^{-5} and inoculated to the YMM agar plate using spot assay method. The plate was incubated at 30^{o}C for 24h. After incubation, four mutants that showed the best growth under freeze-thaw stress compared to the reference strain were chosen as the highly freeze-thaw resistant mutant individuals, or evolved strains.

2.2.4 Cross-resistance analysis of the evolved strains

Cross-resistance analysis was performed to selected freeze-thaw stress resistant, evolved strains to determine their potential resistance to different stress types. Continuous stress selection method was applied to the cells regarding stress factors, such as, potassium chloride, iron, boric acid and chromium by using spot assay analysis. Evolved and reference strains were revived and precultured in 10 ml YMM in 50 ml culture tubes, as described previously. Cells that reached their mid-exponential phase were collected at 4.0 OD_{600}. The cells were serially diluted from 10^{-1} to 10^{-5}. The diluted cultures were spotted onto YMM agar plates containing different stress factors. Cells were also spotted on YMM agar plates containing no stress factors as controls. The cultures were incubated at 30 ºC for 72 h. Cross-resistance of the evolved strains was determined by comparing their growth under stress conditions to control conditions and to that of the reference strain. The stress conditions applied on plates were as follows: 1.75 M KCl, 50mM $(NH_4)_2Fe(SO_4)_2 \cdot 6H_2O$, 50 mM $B(OH)_3$ and 2.5 mM $CrCl_3$.

3. RESULTS

3.1 Evolutionary Engineering Strategy

Pulse stress selection in the form of gradually increasing freeze-thaw stress was applied to the *S. cerevisiae* cells to obtain freeze-thaw stress-resistant, evolved strains. The stress was gradually increased through populations based on the survival rates of the cultures. The MPN analysis results showed the viable cell number of the reference strain and the stress-applied populations in 96 well-plates. The survival rate of populations were calculated by dividing the number of cells in freeze-thaw stress applied populations to the number of cells when they were grown under non-stress (control) conditions (Table 3.1).

Table 3.1: Number of cells in 10 freeze-thaw stress-applied populations and their controls. Their survival rates are also indicated.

Control Conditions				**Freeze-Thaw Stress Conditions**				
Cycle Number	**MPN Result**	**Dilution Rate**	**Number of Cells**	**Cycle Number**	**MPN Result**	**Dilution Rate**	**Number of Cells**	**%Survival Rate**
C-1	0.79	10000000	$7.9x10^6$	**FT-1**	0.33	10000000	$3.3x10^6$	41.8
C-2	2.4	10000000	$2.4x10^7$	**FT-2**	2.4	1000000	$2.4x10^6$	10.0
C-3	0.79	10000000	$7.9x10^6$	**FT-3**	0.79	1000000	$7.9x10^5$	10.0
C-4	0.79	10000000	$7.9x10^6$	**FT-4**	0.33	1000000	$3.3x10^5$	4.2
C-5	2.4	1000000	$2.4x10^6$	**FT-5**	0.49	100000	$4.9x10^4$	2.0
C-6	1.3	1000000	$1.3x10^6$	**FT-6**	1.3	100000	$1.3x10^5$	10.0
C-7	2.4	10000000	$2.4x10^7$	**FT-7**	1.3	1000000	$1.3x10^6$	5.4
C-8	2.4	10000000	$2.4x10^7$	**FT-8**	0.33	1000000	$3.3x10^6$	13.7
C-9	2.4	10000000	$2.4x10^7$	**FT-9**	2.4	10000000	$2.4x10^6$	10.0
C-10	2.4	1000000	$2.4x10^6$	**FT-10**	0.49	1000000	$4.9x10^5$	20.4

Ten stress-applied mutant populations were obtained. After the first stress application (FT-1) the survival rate dropped from 41% to 10%. The survival rate fluctuated between 2 to 14%; until it reached 20% in the final population, FT-10 (Figure 3.1). This population was obtained by 10 successive freeze-thaw cycles.

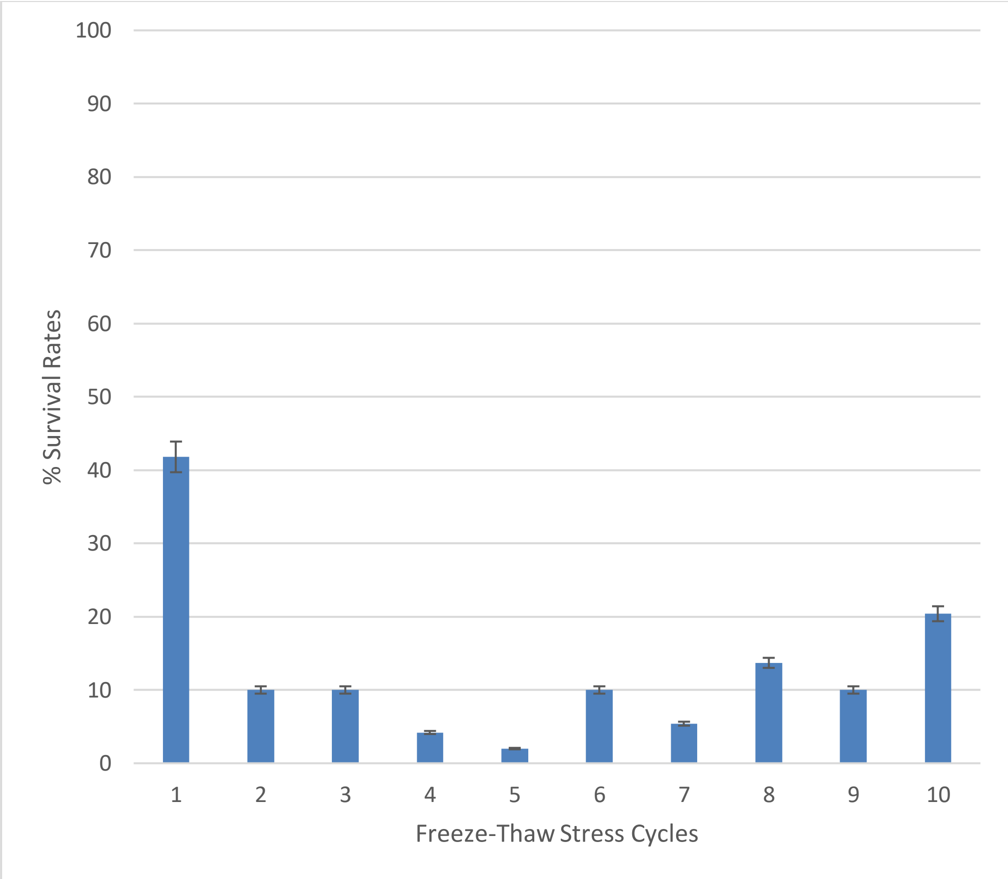

Figure 3.1: Percent survival rates of mutant populations after each stress cycle of selection

3.2 Determination of Individual Mutants' Freeze-Thaw Stress-Resistance

Ten mutant individuals were randomly selected in two sets from the final population spread on to the YMM agar plate. No stress was applied to the first set of FT mutants and the reference strain, while three cycles of freeze-thaw stress was applied to the second set. The serially diluted cells were spotted onto YMM agar plates. The spot assay results showed the growth of reference and 10 FT mutants on the YMM agar plate with and without freeze-thaw stress (Figure 3.2). It was observed that under control conditions, all FT mutant cells' growth levels were similar to that of the reference strain. However, under freeze-thaw stress conditions, FT-1, FT-5, FT-6 and FT-9 mutant strains had better growth than the reference strain, due to their improved resistance to freeze-thaw stress. Thus, these mutant individuals were chosen for further analyses, such as physiological characterization.

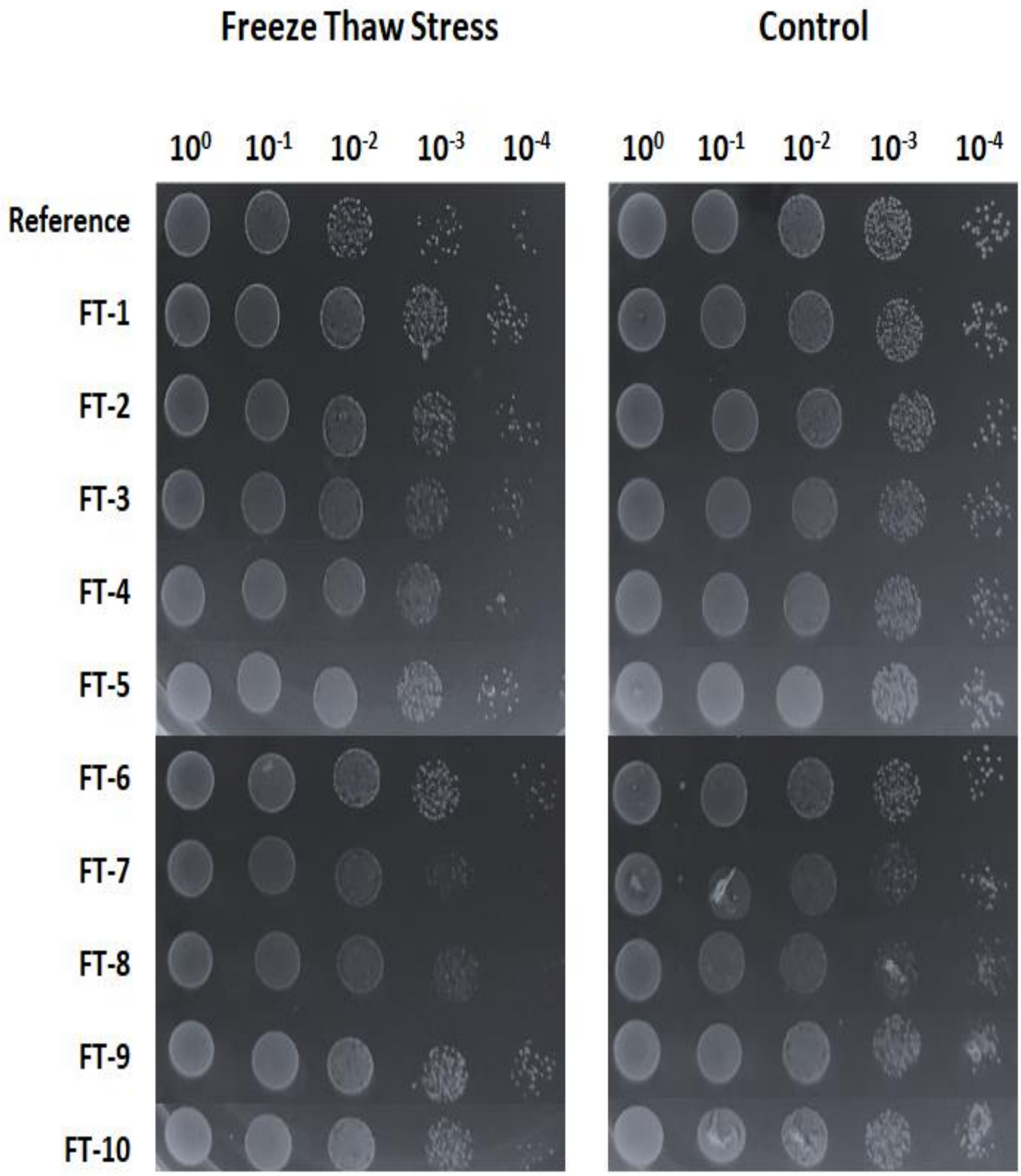

Figure 3.2: Spot assay analysis results of the reference strain and 10 individual FT evolved strains grown on YMM agar plates with and without (control) freeze-thaw stress. Cells were grown at 30^{o}C for 48h.

3.3 Cross Resistance of Selected FT Evolved Strains

Cross resistances of the evolved strains to other stress types were determined by using spot assay method. The reference strain and evolved strains FT-1, FT-5, FT-6 and FT-9 were inoculated into YMM agar plates containing chromium, iron, boric acid and potassium chloride each. Four individual plates were prepared with 1.75M KCl, 50mM $(NH_4)_2Fe(SO_4)_2 \cdot 6H_2O$, 50mM $B(OH)_3$ and 2.5mM $CrCl_3$ chemicals.

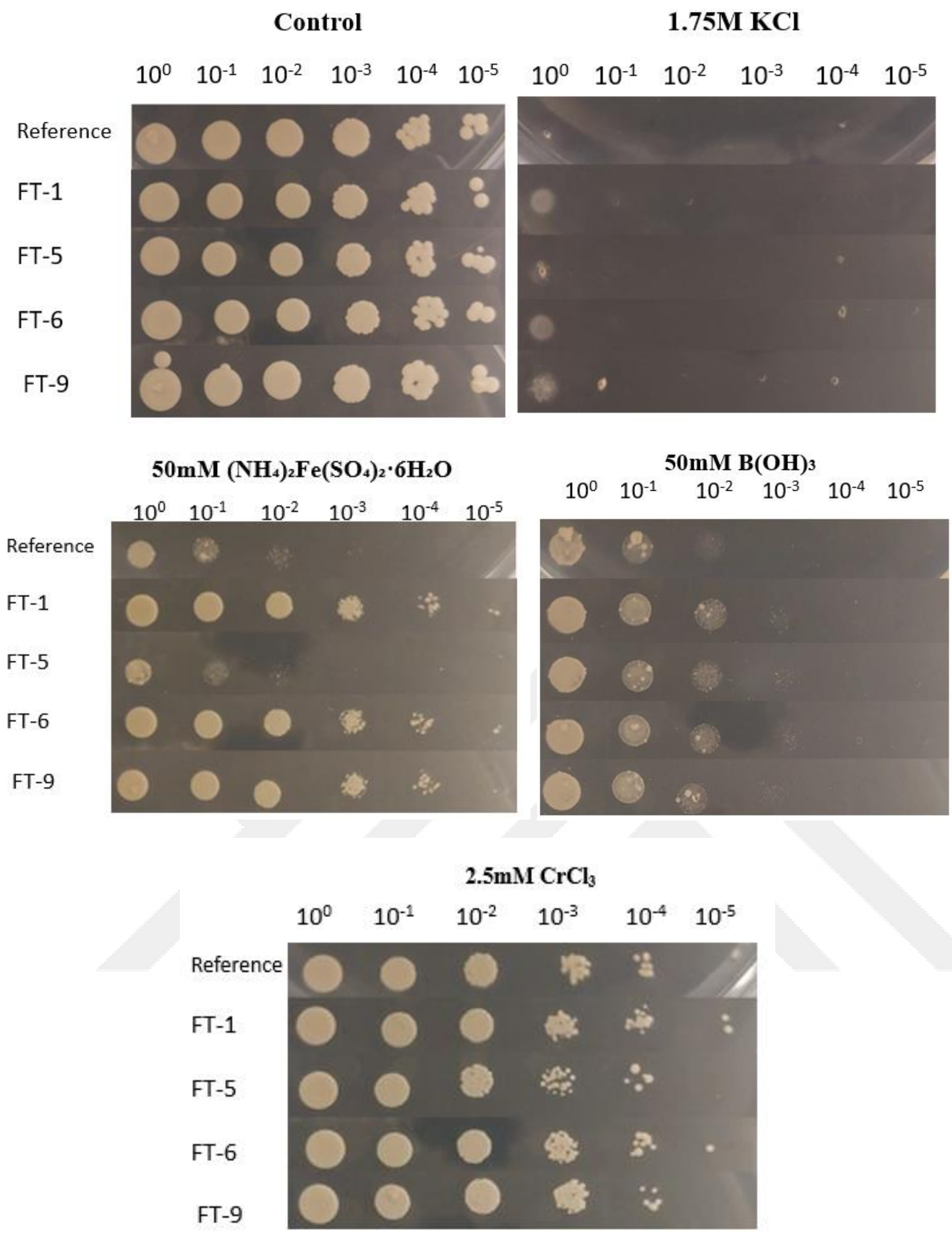

Figure 3.3: Cross-resistance analysis results of FT-1, FT-1, FT-5, FT-6 and FT-9 evolved strains against potassium chloride, iron, boron and chromium stress. Cells were grown on YMM plates for 48 h.

Cross-resistance analysis showed that all strains had similar growth rates under control conditions when no stress was applied. FT-1, FT-6 and FT-9 strains showed resistance against KCl and iron stress. However, FT-5 strain did not show any resistance or sensitivity towards KCl and iron. All evolved strains showed resistance against boric acid ($B(OH)_3$) compared to the reference strain. However, they showed no resistance or sensitivity to chromium ($CrCl_3$) stress.

4. DISCUSSION

S. cerevisiae is an industrially favorable microorganism used in a variety of processes including bread production. Industrial *S. cerevisiae* strains are selected based on their high production rate and resistance to environmental stresses. *S. cerevisiae* cells are exposed to freeze–thaw, high sugar concentration and air drying stresses during the bread production process. Stress conditions can cause cells' growth rate, product yield and the quality of the product to decline (Dangi, 2017). Thus *S. cerevisiae* strains with high tolerance to stress are desired for industrial applications.

The purpose of this study was to obtain a highly freeze-thaw resistant *S. cerevisiae* from a reference strain, using evolutionary engineering. Evolutionary engineering is an inverse metabolic engineering approach, in which the desired phenotype is determined first and the genotype responsible for that phenotype is determined later through genetic studies (Bailey et al., 1996). Evolutionary engineering takes advantage of evolution where strains evolve under constant or increasing selection pressure to desired phenotype (Ko et al., 2020). The desired phenotype was obtained by applying pulse stress selection to the reference, in the form of freezing and thawing in repetitive cycles. The evolved individuals that showed growth after stress application were selected and studied thoroughly. Although this study focuses on obtaining highly freeze-thaw resistant *S. cerevisiae* strains; obtaining evolved strains resistant to high sugar concentration and air drying would also be beneficial for industrial applications. An evolved strain with increased tolerance to high sugar concentrations would be preferable for sweet dough production and an air-drying stress resistant strain for dried yeast production.

The previous study (Yılmaz, 2013) focuses on the evolutionary engineering of an evolved *S. cerevisiae* strain, by starting the selection with a chemically mutagenized population, to obtain freeze-thaw resistant strain. The chemical mutagen ethyl methanesulfonate (EMS) used in that study is a well-known mutagen that causes random mutations in genetic material by nucleotide substitution. Chemical mutagens can be used to obtain a genetically diverse starting culture with high mutation rates (Sikora et al., 2012). A genetically diverse starting culture is advantageous for evolutionary engineering studies. However, using an EMS-treated starting culture could make it more difficult to identify the mutations caused directly by the stress factors. EMS can cause random mutations in the genome some of which may be unrelated to the stress factors . The mutations unrelated to the stress factor of selection can make the analysis of genomic data more complicated. The identification of stress mechanisms and genes responsible for tolerance to that stress factor can also be more difficult. On the other hand, under stress conditions, mutations occur mostly on the stress-induced genes, because stress-induced genes usually show higher expression levels under stress conditions, compared to the housekeeping genes. Thus, in evolutionary engineering studies, the direct use of a wild-type strain without prior mutagenesis can be more beneficial to map stress-induced genes specifically (Brown, 2002; Trovao et al., 2022). This study specifically focuses on the evolutionary engineering of a *S. cerevisiae* strain without chemical mutagenesis application. The evolved strains obtained from this study can be further analyzed by omics technologies to enlighten the freeze-thaw resistance mechanism and to identify freeze-thaw stress-induced genes of *S. cerevisiae*, easily, without further complications by unrelated mutations caused by EMS mutagenesis.

In this study, evolutionary engineering was applied to the *S. cerevisiae* CEN.PK113-7D strain. *S. cerevisiae* CEN.PK113-7D is a laboratory strain highly preferred for metabolic engineering and systems biology studies in academia and industry. Genome sequencing studies of this strain revealed that its genome shows characteristics of both laboratory strains and industrial strains (Nijkamp et al., 2012). This is also the reference strain that has been used in previous research made by ITU yeast laboratory led by Prof. Çakar. For consistency and comparability with previous studies, this strain was preferred. Yeast minimal medium (YMM) was used as the culture medium through this study. YMM is a commonly used medium for culturing *S. cerevisiae*, and for screening yeast libraries. The YMM content, unlike other nutrient-rich media is precise which allows consistency during experimentation (Treco and Lundblad, 1993). *S. cerevisiae* cells were precultured overnight (16h) until they reached from 0.2 to 4.5-5.5 OD_{600} (approximately $6.3x10^7$-$7.7x10^7$ cells/ml). According to a previous growth physiology study, the reference strain incubated in YMM reaches late-exponential phase at 4.5-5.5 OD_{600} around 16h (Topaloğlu, 2018). Yeast cells show the highest sensitivity to freeze-thaw stress during the exponential phase where cell numbers increase exponentially (Park et al., 1997). Thus, freeze-thaw stress was applied to the cells at the late-exponential phase when they are most sensitive to stress. Stressful environments increase the mutation rates of organisms. The increased sensitivity to stress can result with faster rates of mutation (Lukačišinová et al., 2017).

The methods and stress application patterns used in the previous study (Yılmaz, 2013) were repeated for this study, as well. This allowed comparison of the results of these two studies, where the main difference was the treatment of the starting culture with a mutagen in the previous study. Pulse stress selection was applied to the reference strain in the form of freezing at -20ºC for 120 min and thawing at 30ºC for 10 min. This method is mostly used for stress conditions that cannot be applied continuously to the cells. During freeze-thaw stress, freezing can cause ice crystals formation and cellular dehydration which can lead to damage in cell wall and cell membranes, while thawing can cause oxidative damage to biomolecules which can cause degradation of DNA and proteins (Ando et al., 2006). For these reasons, pulse stress in the form of repeated cycles of freezing and thawing creates high levels of selective pressure on the culture.

Increasing freeze-thaw stress was applied to *S. cerevisiae* cells for 10 successive passages. The survival rate dropped to 41% during the first population. *S. cerevisiae* cells can rapidly regulate their stress response mechanism to tolerate mild levels of stress. The high survival rate of the first generation could be due to one freeze-thaw cycle causing only mild stress to cells. After the second freeze-thaw cycle, the survival rates dropped to 10% and until the final population, the survival rate oscillated between 2-10%. This oscillation could be the results of cells being taken randomly from the previous culture to the next passage. The growth phase of cells taken to the next passage can be effective on their resistance. As it was previously mentioned, yeast cells show the highest sensitivity to freeze-thaw stress during their exponential phase and the highest resistance during their stationary phase of growth (Park et al., 1997). After the fifth population, the survival rates of the cells started to increase and by the final population it reached to 20%. The outgrowing of cells that develop beneficial mutations in the culture due to applied stress could be responsible for the increased survival rates. Ten randomly selected mutant individuals were taken from the final population. Upon plating selected mutant individuals' resistance to freeze-thaw stress were tested with the spot assay method. For this purpose, 5 cycles of freeze-thaw stress were applied to the mutant individuals and the cells were spotted on YMM agar plates. FT-1, FT-5, FT-6 and FT-9 mutants showed the highest resistance to freeze-thaw stress and were selected for further analysis.

S. cerevisiae uses ESR mechanisms and the same transcription factors to respond to different stress factors. The common response allows stress-resistant *S. cerevisiae* cells to show cross-resistance to other types of stresses, as well (Święciło, 2016). As mentioned previously, freeze-thaw stress causes membrane and cell wall damage, cellular dehydration and oxidative damage. A freeze-thaw resistant *S. cerevisiae* strain can show resistance to other types of stress that target the same cellular mechanisms as freeze-thaw stress.

Cross-resistance analysis was performed by using the spot assay method. FT-1, FT-6 and FT-9 strains were found to be resistant to KCl and iron stress. At high concentrations, KCl causes hyperosmotic stress to cells. *S. cerevisiae* adapts to hyperosmotic stress by inducing the HOG pathway. HOG pathway triggers glycerol accumulation inside the cells to prevent dehydration (Lu et al., 2005; Dihazi et al., 2004). Freeze-thaw stress is also known to cause cellular dehydration. The results indicate that the freeze-thaw resistant evolved strains may be using a similar response mechanism against dehydration caused by KCl stress. However, the 1.75M KCl amount was too high for the cells to grow properly on plates. For more accurate results lower KCl concentrations should be used.

Iron is mainly used as a cofactor in a variety of enzymes in cell. Most iron-containing proteins have active roles in redox reactions that take place in oxidative respiration, DNA replication and repair and more. Iron is known to increase the generation of ROS in cells (Matsuo et al., 2017). The thawing process also causes ROS generation and oxidative stress in yeast cells. Thus the evolved strains cross-resistance to iron stress can be resulting from the regulation of oxidative stress-related genes and pathways to increase tolerance to freeze-thaw stress, which is yet to be clarified by further transcriptomic and genomic analyses.

All evolved strains showed slight resistance to $B(OH)_{3.}$ Boric acid causes disruption of cell wall synthesis in *S. cerevisiae*. Boric acid activity causes the septation apparatus to be impaired which results in the disruption of the cytoskeleton. *S. cerevisiae* regulates cell wall composition by increasing chitin synthesis to repair the cell wall damage under excessive boric acid amounts (Schmidt et al., 2010). The freezing part of freeze-thaw stress also causes cell wall damage in *S. cerevisiae*. The freeze-thaw resistant strain's induced activity to repair cell wall damages may have also provided a slight cross-resistance to boric acid.

Physiological studies, such as cross-resistance analysis, give only general information about the evolved strains' physiology. Further investigations at genomic level, guided by the informations obtained from cross-resistance analysis can confirm the alterations in the metabolism caused by the mutations. For instance in this study, evolved strains' resistance to boric acid only indicates their cell wall repairing activity to be induced. Thus, further genomic, transcriptomic and proteomic analyses have to be performed to confirm the induced activity of cell wall repair in evolved strains.

The alterations in metabolism that increase adaptation to certain environments can cause other beneficial traits of the cell to decline, resulting in a trade-off (Çakar et al., 2012). Under stress conditions, ESR mechanism induces expression of ESR-related genes; while reducing expression of growth-related genes. Thus, the gained resistance to a stress condition could result in a decrease in the growth rate in the evolved strains. Growth physiology analysis, that compares the growth rate of the evolved strains to a reference strain could be performed in the future to determine the possible freeze-thaw stress resistance-growth rate trade-off in the evolved strains.

5. CONCLUSION

Evolutionary engineering strategy was applied to *S. cerevisiae* CEN.PK113-7D strain to obtain a highly freeze-thaw resistant *S. cerevisiae* strain. *S. cerevisiae* CEN.PK113-7D, a reference strain was preferred as the starting culture to obtain evolved strains with gene alterations mostly related to freeze-thaw stress. For this purpose, increasing levels of freeze-thaw stress was applied to the reference strain in the form of pulse stress selection. Four evolved strains, FT-1, FT-5, FT-6 and FT-9, that showed the highest resistance to the freeze-thaw stress were selected. Cross-resistance analysis was performed for the physiological characterization of the selected evolved strains. FT-1, FT-6 and FT-9 strains showed resistance against KCl and iron stress. All evolved strains showed resistance against boric acid stress, while none of the strains showed resistance against chromium. In this study, FT-9 evolved strain was selected for further genetic analysis, because of its high resistance to freeze-thaw stress and cross-resistance to multiple stress factors. In the evolutionary engineering strategy, the desired phenotype is obtained first and genetic analysis is performed later to identify alterations in the genome responsible for the desired phenotype. Further genomic, transcriptomic and proteomic analyses should be performed on the evolved strain FT-9 to enlighten the genetic and molecular mechanisms responsible for freeze-thaw stress resistance.

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