ISTANBUL TECHNICAL UNIVERSITY ★ GRADUATE SCHOOL

GENOMIC ANALYSIS OF FREEZE-THAW STRESS-RESISTANT *Saccharomyces cerevisiae*

M.Sc. THESIS

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Department of Molecular Biology-Genetics and Biotechnology

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İSTANBUL TEKNİK ÜNİVERSİTESİ ★ LİSANSÜSTÜ EĞİTİM ENSTİTÜSÜ

DONMA-ERİME STRESİNE DİRENÇLİ *Saccharomyces cerevisiae'NİN* GENOMİK ANALİZİ

YÜKSEK LİSANS TEZİ

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Çağla GÜNEY, a M.Sc. student of ITU Graduate School student ID 521211104, successfully defended the thesis/dissertation entitled "GENOMIC ANALYSIS OF FREEZE-THAW STRESS-RESISTANT *Saccharomyces cerevisiae*", which she prepared after fulfilling the requirements specified in the associated legislations, before the jury whose signatures are below.

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Date of Submission : 24 May 2024
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To my beloved ones,

FOREWORD

As I begin my words, I would like to express my gratitude to my thesis advisor Prof. Dr. Zeynep Petek ÇAKAR. Thank you for accepting me to study in yeast laboratory and your guidance during my M.Sc. study.

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May 2024

Çağla GÜNEY
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ABBREVIATIONS

°C	: Degree Celsius
µl	: Microliter
µM	: Micromolar
cAMP	: Cyclic adenosine monophosphate
ddH_2O	: Double-distilled water
EMS	: Ethyl methane sulfonate
ESR	: Environmental stress response
GEF	: Guanine nucleotide exchange factor
h	: Hour
ml	: Milliliter
mM	: Millimolar
ng	: Nanogram
OD	: Optical density
OD_{600}	: Optical density at 600 nm
OXPHOS	: Oxidative Phosphorylation
PKA	: Protein Kinase A
ROS	: Reactive oxygen species
rpm	: Revolutions per minute
SNV	: Single nucleotide variation
U	: Unit
YMM	: Yeast minimal medium
YPD	: Yeast extract-peptone-dextrose

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GENOMIC ANALYSIS OF FREEZE-THAW STRESS-RESISTANT *Saccharomyces cerevisiae*

SUMMARY

The budding yeast *Saccharomyces cerevisiae* is a well-known unicellular eukaryotic organism widely used in industry for making bread and for ethanol production. In addition, it is highly used as a eukaryotic model organism for research purposes in molecular biology and genetics. Since its genome sequence is well-known for several years and it has homologous genes with other eukaryotes including humans, *S. cerevisiae* is used as model organism for studying higher eukaryotic organisms. Additional advantages of *S. cerevisiae* are its easy manipulation and well-known growth conditions.

Freeze-thaw stress is a physical stress type, and freeze-thaw stress resistance is highly desired in *S. cerevisiae*. Freeze-thaw stress causes physiological damage to cell. Freezing step causes ice crystal formation and cellular dehydration and damages the cell. Thawing step creates damage as a result of ROS formation. *S. cerevisiae* is faced with this stress type while used in bakery industry. First, the dough prepared with yeast is frozen and stored. Then, the dough is thawed for the baking process. This would cause the drop of gassing power of the yeast. The drop in rising power is due to cyclic Adenosine Monophosphate (cAMP)-Protein Kinase A (PKA) pathway. There is a glucose-induced increase of cAMP which results in the activation of PKA. Then, trehalase breaks down the trehalose, which is an important component in yeast stress resistance, including resistant to freeze- thaw stress. Thus, freeze-thaw stress resistance is important for improving the efficiency of bread making process and other yeast bioprocesses.

In this study, genomic and physiological analysis of previously obtained freeze-thaw stress-resistant *S. cerevisiae* strains were performed. The freeze-thaw stress-resistant mutant strains were obtained by an inverse metabolic engineering strategy, evolutionary engineering, without using any chemical mutagenesis. After batch selection, genetic stability of the mutants was determined to understand if the resistance to freeze-thaw stress was an adaptation, or it was caused by a permanent genomic change. The selected FT1, FT5, FT6 and FT9 mutant strains were found to be genetically stable.

Determination of the cell wall integrity was done using lyticase susceptibility assay. The assay was performed with the genetically stable, freeze-thaw stress-resistant mutant FT9. Results of the lyticase susceptibility assay demonstrated that the freeze-thaw stress-resistant mutant FT9 was resistant to lyticase degradation more than the reference strain (905), under both stress and nonstress conditions.

Comparative whole genome sequencing analysis of the freeze-thaw stress-resistant mutant (FT9) revealed only one single nucleotide variation (SNV). The SNV was located on *CDC25* gene, and it was a missense SNV. As a result of the missense SNV

on this gene encoding a cell division cycle (Cdc) protein, threonine was replaced by lysine (T1415K).

In conclusion, a previously obtained freeze-thaw stress-resistant *S. cerevisiae* evolved strain was characterized in this study at genomic and physiological levels. The results revealed that cAMP/PKA pathway-related changes occurred in the freeze-thaw stress-resistant mutant strain in order to protect it against damage. However, in order to fully comprehend the molecular basis of freeze-thaw stress resistance of this strain, its comparative transcriptomic and metabolic analyses would be necessary as future studies.

DONMA-ERİME STRESİNE DİRENÇLİ *Saccharomyces cerevisiae*'NİN GENOMİK ANALİZİ

ÖZET

Saccharomyces cerevisiae mayası, tek hücreli, yuvarlak ve tomurcuklanarak üreyen ökaryotik bir mikroorganizmadır. Bu mayanın yaşam döngüsü; eşeyli üreme, eşeysiz üreme ve dinlenme evrelerinden oluşur. Genomu, 1996 yılında tamamen dizilenmiştir ve dizilenme sonucunda 16 kromozom ve yaklaşık 6000 gen belirlenmiştir. *S. cerevisiae* mayasının ana karbon kaynağı glikozdur. Glikozu, hem fermantasyon hem de oksijenli solunumda kullanmaktadır, fakat oksijenli ortamda glikoz düzeyi yeterli ise fermantasyon da yapmaktadır.

S. cerevisiae, endüstride genellikle ekmek mayalamak ve etanol üretmek için kullanıldığından, ekmek mayası ve bira mayası olarak da bilinir. Bu mayaya ait enzimler de biyoyakıt dahil birçok endüstriyel uygulamada sıklıkla kullanılmaktadır. Endüstriyel kullanımının dışında *S. cerevisiae*, moleküler biyoloji ve genetik alanlarındaki araştırmalarda model organizma olarak da yaygın olarak kullanılır. Genom dizisi uzun süredir bilindiği için ve insan gibi diğer ökaryotlarla homolog genleri olduğu için *S. cerevisiae,* daha gelişmiş ökaryotları araştırmak için tercih edilen bir model organizmadır. Bu maya türünün diğer avantajları da genetik açıdan kolay manipüle edilebilmesi ve gelişme koşullarının iyi bilinmesidir.

S. cerevisiae, genel stres tepkisi veya çevresel stres tepkisi olarak da bilinen bir yolak ile birçok stres türüne karşı hızlı bir cevap oluşturmasıyla da bilinmektedir. Çevresel stres faktörleri ilk uygulandığında, hücre duvarı ve hücre zarına zarar verir. Daha sonra denatüre olmuş proteinler hücre içerisinde yığın oluşturur ve hücrede reaktif oksijen türlerinin arttığı görülür. Bu olaylar sonucunda, hücre organelleri, özellikle endoplazmik retikulum stresi oluşur. Bütün bu hasara karşı, stres sensörleri, önemli sinyal yolaklarını aktive etmek için uyarılar iletir ve stres tepkisi verilir. En sonunda bu stres türlerine karşı direnç kazanmak için stres genlerinin ekspresyonu aktive edilir. *S. cerevisiae* mayasında yaklaşık 900 genin stres tepkisi ile ilişkili olduğu görülmüştür.

Donma-erime stresine karşı direnç, *S. cerevisiae* mayasında istenilen bir fiziksel stres direncidir. Donma-erime stresi, hücrelerde fizyolojik hasara neden olur. Donma aşaması, buz kristalleri ve hücresel dehidrasyona neden olarak hücreye zarar verir. Erime aşaması da reaktif oksijen türleri üreterek hücreye zarar verir. *S. cerevisiae,* fırıncılık endüstrisinde bu stres tipine maruz kalır. İlk olarak, hamur, maya ile hazırlanıp dondurularak depo edilir. Daha sonra, donmuş hamur erime aşamasına maruz kalarak pişirilir. Bu aşamalar, mayanın kabarma kapasitesinde düşüşe sebep olur. Bu düşüş cAMP (siklik adenozin monofosfat)-PKA (protein kinaz A) yolağına bağlı olarak gerçekleşir. Glikoz kaynaklı cAMP artışı, PKA'yı da aktive eder. Buna bağlı olarak trehalaz enzimi, mayada stres faktörlerine, özellikle de donma-erime stresine karşı koruyucu olan trehalozu parçalar. Bu nedenle, ekmek üretiminde ve diğer maya biyoproseslerinde verimi arttırmak için donma-erime stresi direncine sahip mayaların geliştirilmesi önemlidir.

Metabolik mühendislik, ilk olarak James Bailey tarafından 1991 yılında, "rekombinant DNA teknolojisi kullanılarak; hücrenin enzimatik, taşıma ve düzenleyici fonksiyonlarının manipülasyonuyla hücresel aktivitelerin iyileştirilmesi" olarak tanımlanmıştır. Bu yaklaşım, değiştirilmesi istenen hücresel fonksiyonun analiz edilmesi ve bu analiz sonucunda genetik mühendisliği yöntemleri kullanılarak istenilen hücresel fonksiyona sahip bir mikrobiyal suşun geliştirilmesini içerir. Daha sonra, bu suşun istenilen fenotipte olup olmadığına orijinal suş ile karşılaştırılarak karar verilir. Bu klasik ya rasyonel metabolik mühendislik yaklaşımının bazı sınırlamaları vardır. Örneğin, modifiye edilmesi istenen metabolik yolak veya hücresel aktivitelere ait detaylı bilgi gerekmektedir. Bu sınırlamaların sonucu olarak tersine metabolik mühendislik yaklaşımı geliştirilmiştir.

Tersine metabolik mühendislik yaklaşımında, rasyonel metabolik mühendisliğin aksine ilk adım istenilen fenotipin elde edilmesidir. İkinci adımda, bu fenotipe yol açan genetik yapı ve çevresel faktörler tespit edilir. Belirlenen genetik değişiklik, diğer organizmalarda da bu fenotipi elde etmek için kullanılabilir. Evrimsel mühendislik ise, selektif prosedürler uygulanarak istenilen mikrobiyal fenotiplerin elde edilmesinde kullanılan bir tersine metabolik mühendislik yöntemidir. Mikrobiyal popülasyon, stres gibi bir selektif baskıya maruz bırakıldıktan sonra, popülasyonda doğal yolla mutasyonlar oluşur ve bu popülasyon uygulanan stres gibi selektif koşullara dayanıklı hale gelir. İstenilen fenotipe sahip mikrobiyal suşlar, uygun koşullarda seçilir ve bu fenotipe yol açan mutasyonlar tespit edilir.

Genom dizilemesi, canlıların genomunu oluşturan dört nükleotit baz dizisinin belirlenmesi yöntemidir. Yeni nesil dizileme yöntemleri de araştırma, tarım ve sağlık hizmetleri gibi alanlarda sıklıkla kullanılan yöntemlerdir. Sanger dizileme gibi klasik yöntemlere göre daha hızlı olmasıyla bilinen yeni nesil dizileme, çok sayıda örneği aynı anda paralel olarak dizilediği için paralel dizileme/sekanslama olarak da bilinmektedir. Bu dizilemede ilk adım, dizilenecek nükleik asidin organizmadan izole edilmesidir. İzole edilen nükleik asit, ikinci adımda parçalara ayrılarak bir kütüphane oluşturulur. Daha sonra, primerler ve adaptörler eklenir ve farklı platformlar kullanılarak paralel dizileme gerçekleşir. Dizilemesi biten genom, referans genom ile karşılaştırılır ve biyoinformatik analiz araçları yardımıyla mutasyonlar tespit edilir.

Bu çalışmada, daha önce elde edilmiş olan, donma-erime stresine dirençli *S. cerevisiae* mutant suşlarının genomik ve fizyolojik analizi yapıldı. Donma-erime stresine dirençli mutant suşlar, bir tersine metabolik mühendislik yöntemi olan evrimsel mühendislik yöntemi ile kimyasal mutajenez kullanmadan elde edilmiştir. Seleksiyon sonrasında, mutant suşların donma-erime stres direncinin adaptasyon mu yoksa kalıcı bir genomik değişiklikten mi kaynaklandığını anlamak için genetik kararlılık testi yapıldı. Seçilen FT1, FT5, FT6 ve FT9 mutant suşları, genetik olarak kararlı bulundu.

Hücre duvarı bütünlüğü, genetik olarak kararlı, donma-erime stresine dirençli FT9 suşuna litikaz duyarlılık testi yapılarak incelendi. Litikaz duyarlılık testinin sonuçları, donma-erime stresine dirençli FT9 mutantının referans suşa (905) göre litikaz ile parçalanmaya, hem stresli hem de stressiz koşullarda daha dirençli olduğunu gösterdi.

Donma-erime stresine dirençli FT9 mutantının karşılaştırmalı tüm genom dizileme sonuçları, sadece bir tek nükleotit varyasyonu (SNV) olduğunu gösterdi. Bu varyasyon *CDC25* geninde yer alan bir yanlış anlamlı (missense) mutasyondur. Hücre bölünmesi döngüsü proteini kodlayan bu gendeki yanlış anlamlı varyasyon sonucunda, treonin lizin ile yer değiştirmiştir (T1415K). *CDC25* geninden üretilen Cdc25 proteini, Ras/cAMP/PKA yolağında Ras2GTP'yi aktive ederek PKA üretimine yol açar.

Normal koşullarda, PKA, Msn2 ve Msn 4 gibi stres regülatörlerinin hücre çekirdeğine lokalizasyonunu engellemektedir. *CDC25* geninde bulunan ve Ras2GTP aktivasyonuna engel olmaya yol açacak bir bölgede bulunan bu yanlış anlamlı mutasyonun PKA üretimine de engel olması beklenmektedir. Bu nedenle, stres regülatörleri hücre çekirdeğine lokalize olabilecektir. Msn2 ve Msn4, stres koşullarında çekirdeğe lokalize olduklarında, stres genlerinin promotor bölgesinde bulunan stres tepki elementlerine bağlanırlar ve bu genlerin aktivasyonunu sağlarlar. Sonuçta, stres tepkisi verilir ve donma-erime stresine karşı direnç kazanılmış olur.

Bugüne dek literatürde, *S. cerevisiae* mayasının donma-erime stresine karşı direnciyle ilişkili olabilecek birçok gen bulunmuştur, fakat bu dirençle ilgili tüm genler henüz belirlenememiştir. Bu çalışma, *CDC25* gen mutasyonunun da *S. cerevisiae* mayasında donma-erime stres direnci ile ilgili olabileceğini ortaya koymuştur.

Sonuç olarak, daha önce evrimsel mühendislik ile kimyasal mutajenez kullanılmadan elde edilmiş olan bir *S. cerevisiae* mutant suşu, bu çalışmada genomik ve fizyolojik olarak karakterize edilmiştir. Bu analizin sonucunda, donma-erime stresine dirençli mutantta, hücreyi hasara karşı korumak için cAMP/PKA yolağına bağlı değişiklikler gözlemlenmiştir. Donma-erime stresine dirençli FT9 mutant suşunun donma-erime stresine direncinin moleküler yapısını tam olarak anlayabilmek için, gelecek çalışmalar olarak karşılaştırmalı transkriptomik ve metabolik analizlerin de yapılması uygun olacaktır.

1. INTRODUCTION

1.1 General Information about the Model Organism *Saccharomyces cerevisiae*

Saccharomyces cerevisiae is a unicellular eukaryotic organism which belongs to the kingdom Fungi. Its morphological shape is round, and it is usually 5-10 μm in diameter. It has a cell wall made from β-glucan and/or chitin (Knop, 2011). Figure 1.1 shows *S. cerevisiae* cell structure.

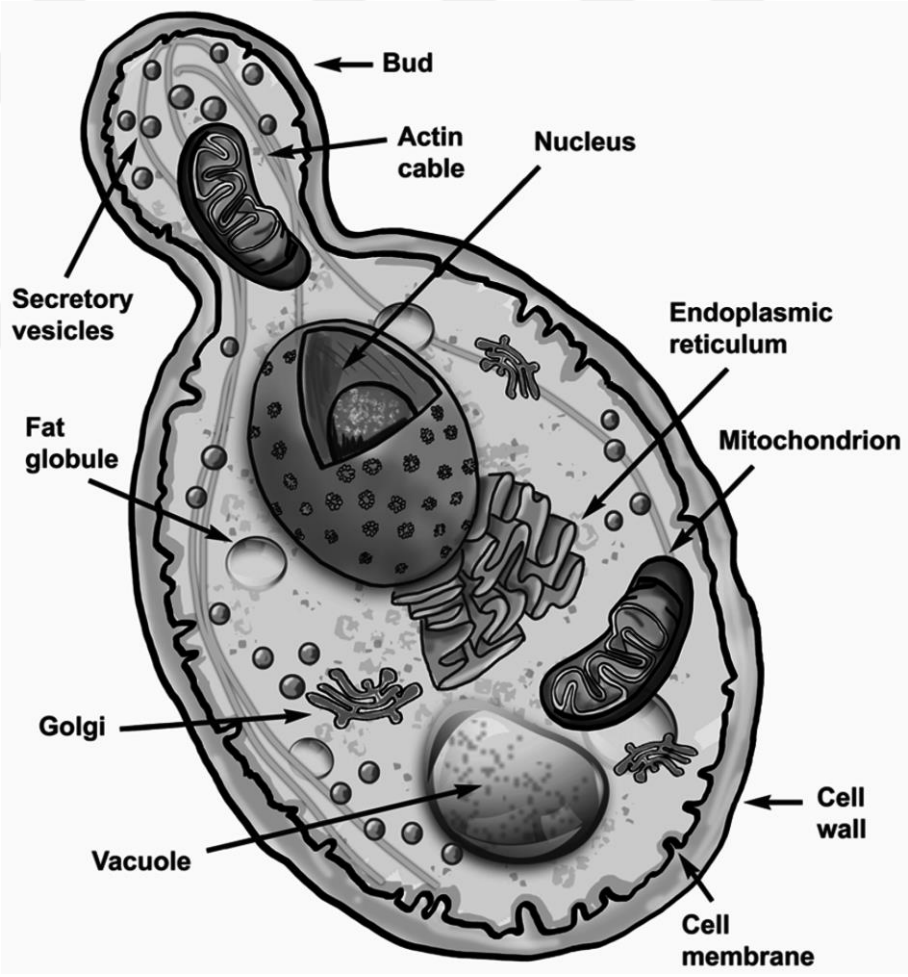

Figure 1.1 : *S. cerevisiae* cell structure (Walker & Stewart, 2016).

S. cerevisiae genome was sequenced and published in 1996. Sequencing results revealed that 16 chromosomes of *S. cerevisiae* had a nuclear genomic DNA of 12068 kilobases (kb) and approximately 6000 genes (Goffeau et al., 1996). 5570 genes of

these genes encode proteins (Wood et al., 2001). Table 1.1 shows *S. cerevisiae* genome sequencing results as a summary.

Table 1.1 : *S. cerevisiae* genome sequencing results (Engels et al., 2014).

Chromosome	Length (bp)	Sequencing Methodology
I	230,218	Manual, automated
II	813,184	Diverse methods in collaborating laboratories
III	316,620	Diverse methods in collaborating laboratories
IV	1,531,933	Automated
V	576,874	Automated
VI	270,161	Automated shotgun, primer walking
VII	1,090,940	Diverse methods in collaborating laboratories
VIII	562,643	Diverse methods in collaborating laboratories
IX	439,888	Shotgun, primer walking
X	745,751	Diverse methods in collaborating laboratories
XI	666,816	Diverse methods in collaborating laboratories
XII	1,078,177	Diverse methods in collaborating laboratories
XIII	924,431	Automated shotgun, primer walking
XIV	784,333	Diverse methods in collaborating laboratories
XV	1,091,291	Manual, automated, shotgun, primer walking
XVI	948,066	Automated shotgun, primer walking

Life cycle of *S. cerevisiae* involves three phases: asexual expansion, sexual reproduction, and quiescence. Figure 1.2 illustrates the life cycle of budding yeast.

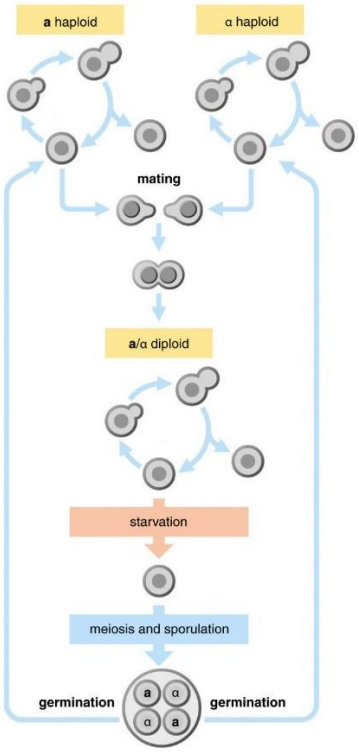

Figure 1.2 : Life cycle of budding yeast (Sun & Heitman, 2011).

This cycle can change between haploid and diploid phases (Herskowitz, 1988). When the nutrients are sufficient, diploid cells proliferate mitotically (Pringle and Hartwell, 1981). When the nutrients are limiting, diploid cells undergo meiosis and four haploid spores are produced (Mitchell, 1988). Mating types of haploid *S. cerevisiae* are determined by *MAT* locus and can be either MATa or MATα (Strathern et al., 1981). The "a factor" attracts "α factor" for mating (Fischer et al., 2021).

Recent studies revealed that *S. cerevisiae* isolates used in industrial processes can also be triploids, tetraploids and polyploids. This can be caused by unprogrammed mating type switching of diploids. After that, diploid cells can mate with haploid cells or diploid cells to produce triploids and tetraploids, respectively. Additionally, failure in meiosis can cause multinucleated polyploid cells which have 2-3 nuclei; therefore, 4N-6N DNA content (Al Safadi et al., 2010).

Major carbon source for *S. cerevisiae* is glucose. There are two pathways where glucose is utilized: aerobic respiration and anaerobic fermentation. Figure 1.3 shows the energy metabolism of *S. cerevisiae*.

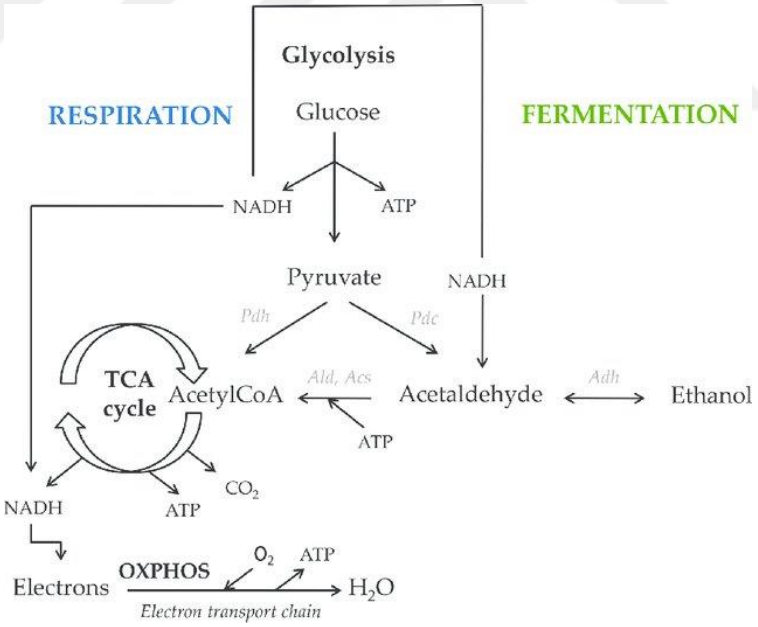

Figure 1.3 : Energy metabolism of *S. cerevisiae* (García et al., 2016).

Starting from glycolysis, pyruvate produced from glucose is converted into carbon dioxide, ethanol, and ATP via fermentation. Via aerobic respiration, pyruvate from glycolysis is oxidized to carbon dioxide and ATP through the tricarboxylic acid (TCA) cycle and oxidative phosphorylation (OXPHOS). Through fermentation, less ATP is produced than OXPHOS (Pfeiffer & Morley, 2014). Even under aerobic conditions,

fermentation is observed in *S. cerevisiae*. Ethanol produced from the fermentation can be used as a carbon source in low glucose environment. Therefore, energy production is shifted from fermentation to respiration. Additionally, lactate, acetate and glycerol can be used as a carbon source (Gasmi et al., 2014).

1.2 Importance of *Saccharomyces cerevisiae* in Research and Industry

S. cerevisiae has been used as a model organism for many years. It was the first eukaryotic organism with its genome sequenced in 1996 (Galao et al., 2007). Since it has ortholog genes with human genome and its genome is easy to manipulate, it can be used for studying higher eukaryotic biological processes such as metabolism, cell division, stress resistance and cell responses. Additionally, it is easy and cheap to grow in laboratories because of its small size and rapid growth. These properties make it a valuable model organism (Karathia et al., 2011). For pharmaceutical applications, *S. cerevisae* is used for vaccine preparation, and production of secreted proteins such as monoclonal antibodies and cytokines.

Except from research purposes, *S. cerevisiae* has also been used in large scale industrial applications. *S. cerevisiae* is named as baker's yeast since it has been used for many centuries for leavening bread dough and it is known as brewer's yeast for fermenting alcohol in industry. The fermentation ability of *S. cerevisiae* makes it also a prominent microorganism for industrial biofuel production (Duina et al., 2014). In addition, many *S. cerevisiae* enzymes can be used in diverse industrial applications as shown in Figure 1.4.

Figure 1.4 : Utilization of *S. cerevisiae* enzymes in different fields (Vieira and Delerue-Matos, 2020).

1.3 General Stress Response in *S. cerevisiae*

All organisms need to maintain homeostasis for optimal growth and becoming resistant against several stress factors such as starvation, freeze-thaw stress, osmotic stress, oxidative stress, or toxic chemicals. *S. cerevisiae* has a rapid molecular response that is activated against stress factors. Repair of cellular damage caused by stress factors is done by environmental stress response (ESR) and tolerance against these stress factors is gained (Park et al., 1998).

Environmental stresses damage the cell wall and cell membrane of the *S. cerevisiae.* After that, denatured proteins aggregate and reactive oxygen species (ROS) level increases which ends up in endoplasmic reticulum (ER) stress. ESR process is initiated because of all these damages. Firstly, for stimulating key signalling pathways, membrane stress sensors pass the stress signals. As a result of the working of these functional pathways together, *S. cerevisiae* survives (Lin et al., 2022). Environmental stress response of *S. cerevisiae* is illustrated in Figure 1.5.

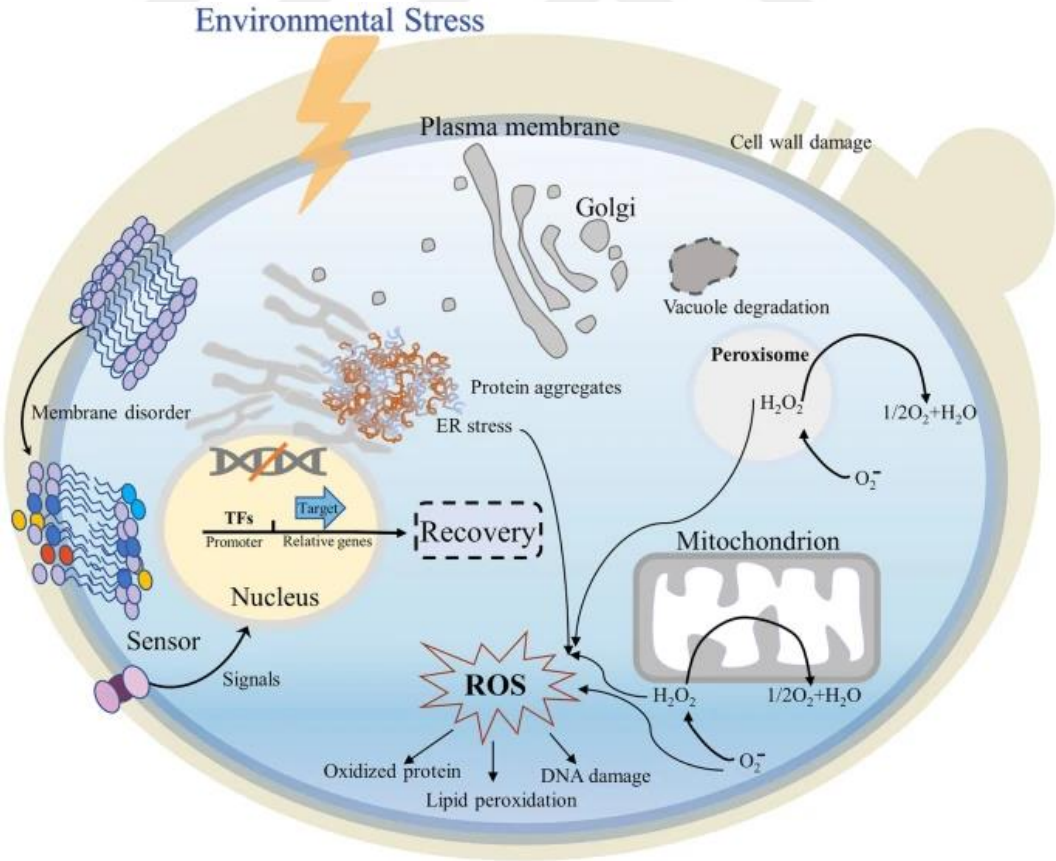

Figure 1.5 : Environmental stress response of *S. cerevisiae* (Lin et al., 2022).

Signalling pathways affect the gene expression of certain genes. Transcription of the stress-induced genes is altered, and these gene products manage a cross-protective effect to not only the exposed stress factor, but also to other stress factors. For example, applying different stress types such as hydrogen peroxide, cold-heat shock, or NaCl to yeast make it also resistant to freeze-thaw stress. Generally, hydrogen peroxide makes yeast tolerant to H_2O_2 or oxidative stress, as expected, but also to high glucose concentrations, ethanol, freeze–thaw stress, and chronological aging (Nakagawa et al., 2013).

In *S. cerevisiae*, 900 genes are related with stress response. These genes are induced by various stress types through the general stress response element (STRE) which are found in the promoter region of stress genes (Gasch et al., 2000). Stress-responsive transcription factors Msn2 and Msn4 control the increase of tolerance to many types of cellular stress by upregulating genes during the periods of oxidative stress. They bind to stress genes by recognizing them in the STRE promoter region. Protein Kinase A (PKA) and the Snf1 which senses glucose are the two kinases that phosphorylate Msn2 and Msn4 stress factors. This blocks the nuclear import of Msn2 and Msn 4 (Görner et al., 1998). *ATH1* and *TPS1* are also the additional genes having a role in generalized stress response, including freeze-thaw stress. When the Ath1 enzyme is absent in the cell, trehalose is accumulated. Therefore, under freezing conditions, dehydration and ethanol toxicity, cell survives. When Tps1 is deficient in the cell, trehalose cannot be synthesized, and the cell shows less tolerance to temperature-related stresses (Kim et al., 1996). Despite this valuable information, however, stress response in *S. cerevisiae* is a complex phenomenon which is yet to be understood. There are still various studies in progress.

1.4 Freeze-Thaw Stress

Freeze-thaw stress resistance is an important characteristic that is desired to be acquired in industrial microorganisms (Lorenz, 1984). *S. cerevisiae* is used in baking industry for making dough for many decades. These doughs are prepared and frozen to be used later. After initial fermentation of the yeast in the dough, dough is frozen. However, this would end up in loss of resistance to many stress types, including freeze-thaw stress. As a result, dough-rising capacity drops gradually. This problem is overcomed by making yeasts resistant to freeze-thaw stress (Hsu et al., 1979). The

drop in rising power is due to cyclic AMP-PKA pathway. There is a glucose-induced increase in cAMP which eventually results in PKA activation. Then, trehalase breaks down the trehalose which is an important component in yeast resistance against stress factors, especially freeze- thaw stress (Park et al., 1998; Teunissen et al., 2002).

Freeze-thaw stress is linked to other stress types such as oxidative stress and cold -heat shock stress. Genes and pathways participating in freeze-thaw stress resistance are also observed in other pathways like cell wall integrity pathway, nutrient sensing signaling and proteosome activity. Factors playing roles in freeze-thaw stress resistance can be lipid components which make cell membrane flexible to become resistant and some metabolites preventing ice crystal formation (Cabrera et al., 2020).

Physiological damage can occur to cells by freeze-thaw stress. During freezing step, mostly ice crystal formation and cellular dehydration cause the damage. Although most of the damage is caused by the freezing step, thawing step also creates damage because of ROS formation (Mazur et al., 1972). Freezing rate influences the cell survival; as the freezing rate is slow, extracellular ice crystals are formed mostly instead of intracellular ice crystals which are more dangerous to the cell. Generation of less intracellular ice crystals would result in decrease in cell damage and increase in cell viability. However, thawing rate is not as much important as freezing rate (Mazur, 1977). Freeze-thaw stress response of *S. cerevisiae* is shown in Figure 1.6.

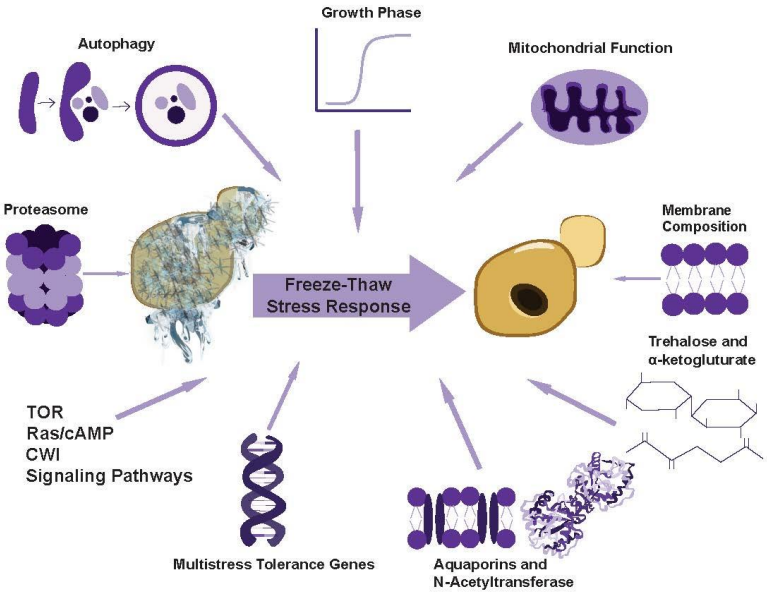

Figure 1.6: Freeze-thaw stress response of *S. cerevisiae* (Cabrera, 2020).

1.5 Metabolic Engineering

In 1991, James Bailey defined metabolic engineering for the first time as "the improvement of the cellular activities by manipulation of enzymatic, transport and regulatory functions of the cell with the use of recombinant DNA technology" (Bailey, 1991). Since then, metabolic engineering has been successfully used and improved in the fields of biotechnology, genomics, and bioinformatics (Çakar et al., 2012). Rational metabolic engineering enables scientists to introduce genetic changes using recombinant DNA technology. First step of a successful metabolic engineering is making analysis of the cellular function to be changed and construction of improved strain by genetic engineering, according to analysis results. Second step is analysis of performance of recombinant strain compared to original strain. Last step is designing next target for genetic engineering (Bailey et al., 1996). However, rational metabolic engineering has some limitations. First of all, it requires background information about the pathways or cell functions that are desired to be modified. Even though the background information is acquired, the obtained results after modification can be different from the expected results. Since rational metabolic engineering requires extensive information about the chemical networks and cellular systems, it can be difficult to acquire all the information, and failures may happen (Bailey, 1991; Bailey et al., 1996). Thus, a bottom-up approach which is called as inverse metabolic engineering was developed to overcome the limitations of rational metabolic engineering (Bailey et al., 1996).

1.6 Inverse Metabolic Engineering

Inverse metabolic engineering was introduced by Bailey and co-workers as a solution to the limitations of rational metabolic engineering (Bailey et al., 1996). With this method, genetic manipulations are done without the need for any background information. In this bottom-up approach, identification of the desired phenotype is the first step. The second step is the elucidation of the genetic basis or environmental factors leading to this phenotype. Lastly, the identified genetic basis is transferred to an industrial organism in order to get the desired phenotype. In the first step, some mutagenesis techniques to create genetic diversity can be used. After acquiring the desired phenotype, omics techniques are used to understand the genetic basis. After determination of the genetic basis and getting the information about the metabolic

pathways, desired phenotypes can be achieved in other organisms by introducing the determined changes (Bailey et al., 1996). One of the strategies used in the first step of inverse metabolic engineering is evolutionary engineering (Butler et al., 1996; Sauer, 2001).

1.6.1 Evolutionary engineering

Evolutionary engineering which is an inverse metabolic engineering strategy is used for obtaining desired microbial phenotypes by applying selective procedures (Çakar et al., 2012). Microbial population is kept under a selective pressure in evolutionary engineering. The mutations will then naturally arise and make the population fitter to growth conditions. Strains having desired properties can be selected/screened under suitable conditions that favor the desired properties. Also, mutagenesis prior to selection can also be applied like Ethyl methane sulfonate (EMS) mutagenesis to increase genetic diversity. Thus, strains having the mutations that made them fitter to growth conditions are selected (Winkler and Kao, 2014). Steps of evolutionary engineering is depicted in Figure 1.7.

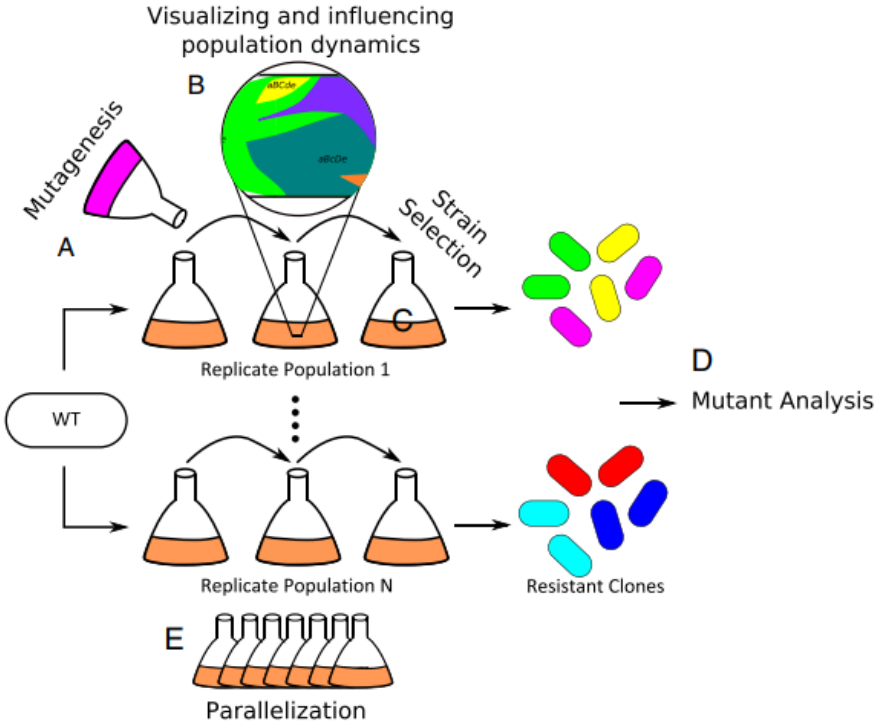

Figure 1.7 : Steps of evolutionary engineering (Winkler and Kao, 2014).

Selection can be done in either batch or continuous (chemostat) culture. In batch culture, the culture environment continuously changes; nutrients would be depleted and, growth, substrate utilization, and chemical production can be ended. However, in

continuous culture, fresh nutrient medium is continually added to the culture. In these cultures, stress conditions can be applied as a pulse or continuously for selection. Stress is applied either constantly or increasingly in each type of selection (Alkım et al., 2014). Batch and chemostat cultures selection procedures are shown in Figure 1.8. In addition, selection procedures under pulse stress and continuous stress conditions are shown in Figures 1.9 and 1.10, respectively. Under pulse stress conditions, stress is applied for a specific time to the strains, it is then removed from the environment until it is applied again. Between the times stress is applied, passages are done in stress-free conditions. However, under continuous stress condition, stress is continuously applied, and passages are done in stress conditions and also samples are taken for frozen stocks (Alkım et al., 2014).

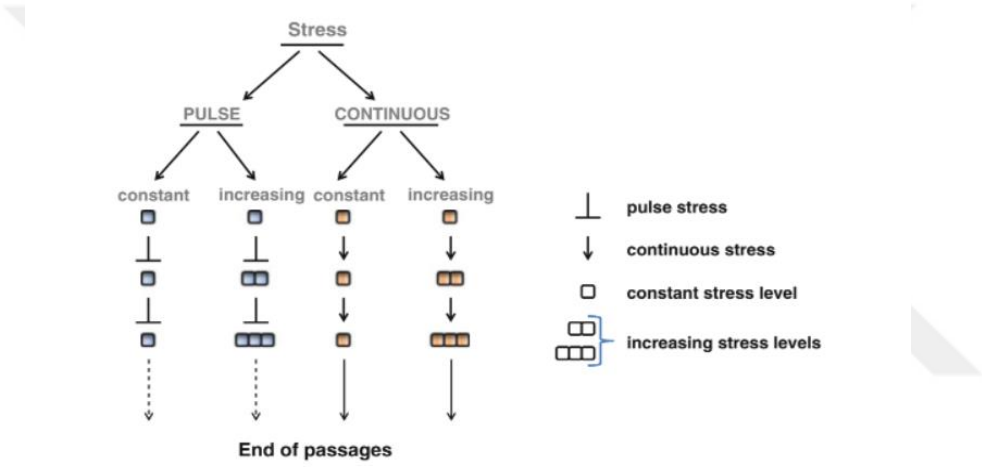

Figure 1.8 : Batch and chemostat culture selection procedures (Alkım et al., 2014).

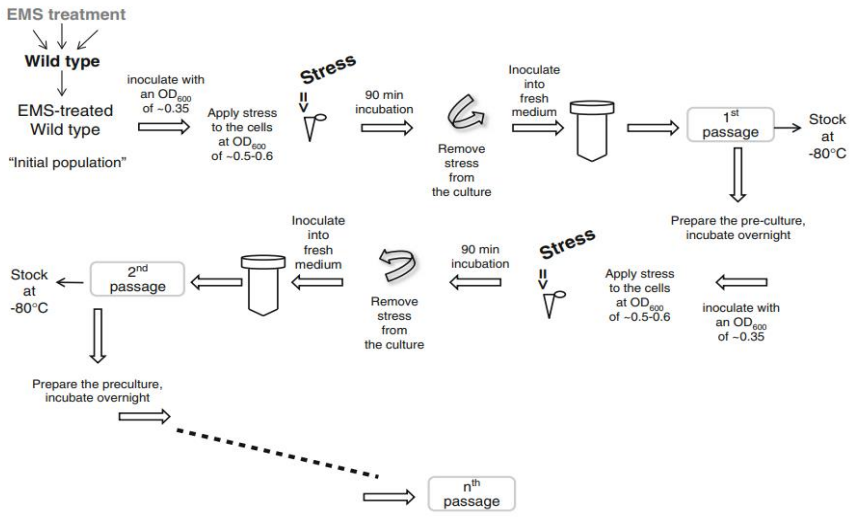

Figure 1.9 : Selection procedure under pulse stress conditions (Alkım et al., 2014).

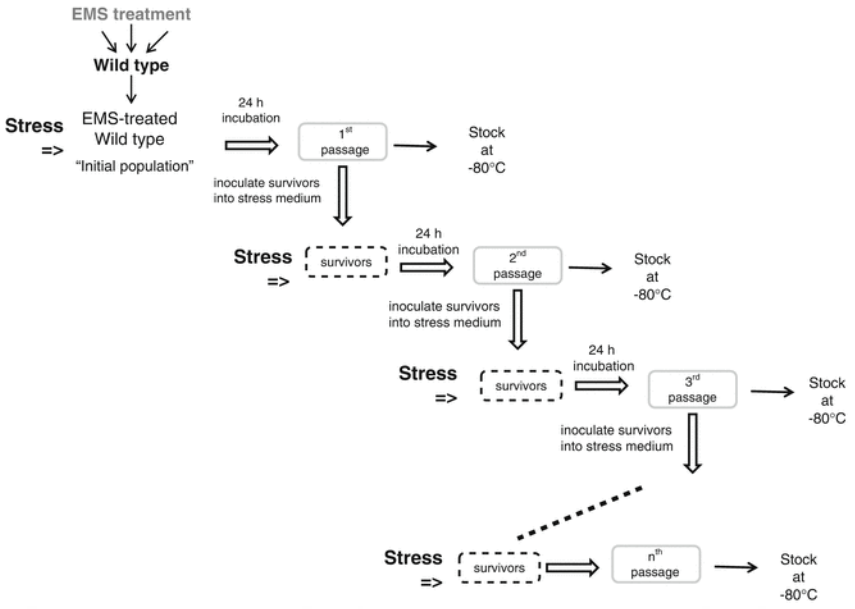

Figure 1.10 : Selection procedure under continuous stress conditions (Alkım et al., 2014).

1.7 Next Generation Sequencing

Order of the four nucleotides within the certain area of the genome is determined by sequencing process. After completion of Human Genome Project which utilized Sanger Sequencing, interest on Sequencing Technologies has increased. New technologies for DNA sequencing has been improved and at this point, Next Generation Sequencing Technologies have emerged. Next Generation Sequencing (NGS) which is also known as massively parallel or deep sequencing is the new technology for sequencing DNA or RNA and finding the variants and/or mutations (Behjati & Tarpey, 2013).

Basic difference between NGS and Sanger Sequencing is that Sanger Sequencing sequences the DNA/RNA fragments by chain termination, then the capillary electrophoresis is utilized for reading. On the other hand, NGS sequences the DNA/RNA by synthesis. NGS has many advantages over the Sanger Sequencing. One example is that Sanger Sequencing has limitations in reading the substitutions, small insertions, and deletions. However, NGS can discover these small mutations easily. Another important advantage is that NGS can read whole genome in a shorter period of time than Sanger Sequencing. Whole human genome is sequenced in a single day by NGS, while Sanger Sequencing required over a decade (Qin, 2019).

Sequencing in NGS platforms work as sequencing the bunch of parallel small fragments. These fragments are then joined to each other by mapping the separate

reads to the reference genome. The first part of NGS sequencing is DNA fragmentation. In this part, targeted DNA is broken into many small fragments. These fragments should be 100-300 bases long generally. For breaking the fragments, mechanical methods, enzymatic digestion, and polymerase chain reaction are the examples of the utilized methods (Knierim et al., 2011). The second part of the massively parallel sequencing is library preparation. In this part, sample specific index is added to DNA fragments. It is important to distinguish the samples between each other. Additionally, adaptors are added to the DNA fragments in this part so that primers for sequencing can bind to them (Hess et al., 2020). The third part is sequencing in which the prepared library is uploaded to a matrix in a specific sequencer. Different matrices are used in different NGS sequencers. For example, flow cells and sequencing chips are used for Illumina NGS sequencer and Ion Torrent NGS sequencer, respectively. The goal is massively sequencing all fragments at the same time (Qin, 2019). Lastly, the information obtained from sequencing is analyzed by bioinformatic tools and the data are interpreted. The sample genome is compared to a reference genome. Thus, variants/mutations can be targeted. As a result, this methodology is utilized in many areas such as research, agriculture, and health care applications for the diagnosis of diseases, finding genetic mutations, and identifying organisms (Gargis et al., 2015). The steps of next generation sequencing are summarized in Figure 1.11.

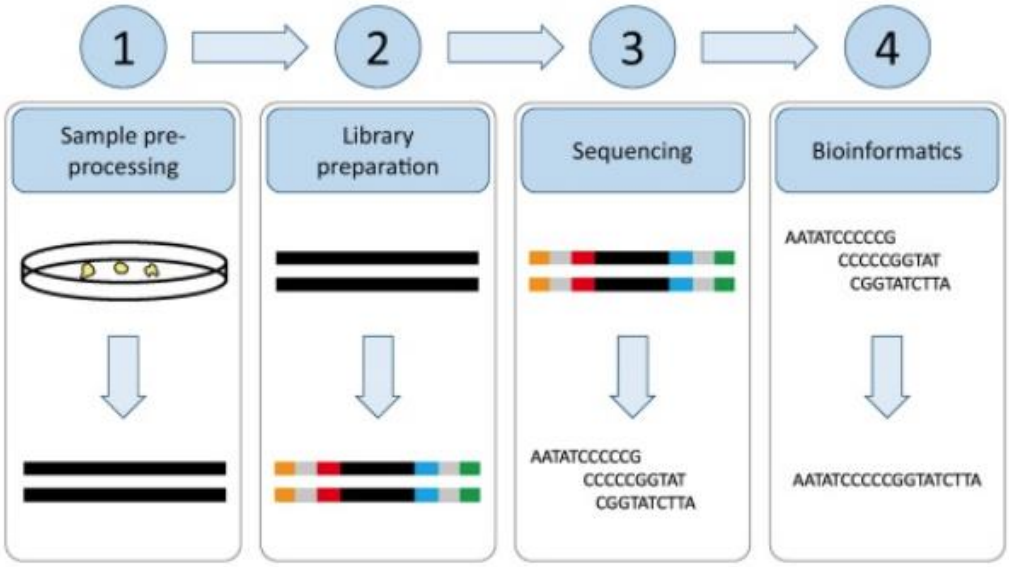

Figure 1.11 : Steps of next generation sequencing (Hess et al., 2020).

1.8 Aim of the Study

S. cerevisiae is exposed to various types of stress conditions during its growth and particularly in industrial processes. In bakery, harsh conditions like storage at freezing temperatures and thawing the yeast dough after freezing reduce the yeast cell viability and its leavening capacity. These conditions have negative economic and technological outcomes. Thus, improving freeze-thaw stress tolerance of *S. cerevisiae* is an important research purpose in biotechnology (Attfield, 1997).

S. cerevisiae freeze-thaw stress response has not been completely understood yet. Generally, this stress response is linked to respiratory metabolism, growth phase, lipid composition of the membrane and accumulation of trehalose (Rodrigues et al., 2002). There have been some genes studied related to freeze-thaw stress. However, not all of the genes related to freeze-thaw stress resistance in *S. cerevisiae* have been studied.

To this date, *TIP1* (temperature-inducible protein) which is a cold shock-activated gene and two homologous genes, *TIR1* and *TIR2* were found as related to freeze-thaw stress in *S. cerevisiae*. *SSD1, TPI1, YNL278w, MMS2, ERG10, PAK1, SEC11, IMH1,* and *YFL030w* are the other genes that are activated in cold shock stress (Schade et al., 2004). Cryo-resistance in *S. cerevisiae* is positively correlated with the activation of *TIP1* and *ERG10*. Specifically, *ERG10* is overexpressed for increasing the freeze-thaw stress tolerance in *S. cerevisiae* (Rodrigues et al., 2002).

The first aim of this study was to determine if a freeze-thaw stress-resistant *S. cerevisiae* strain acquired by using evolutionary engineering without any chemical mutagenesis was genetically stable. The second aim of this study was the genomic characterization of the freeze-thaw stress-resistant strain, to understand the genomic changes and potential metabolic and molecular factors contributing to freeze-thaw stress-resistance.

For this purpose, first, *S. cerevisiae* strains obtained previously by evolutionary engineering in our laboratory (Balaban, 2023) were tested for genetic stability. These freeze-thaw stress-resistant strains were obtained without any chemical mutagenesis. Comparative whole genome re-sequencing was then performed on a selected freeze-thaw stress-resistant yeast strain and the reference strain to determine the genetic factors responsible for freeze-thaw stress resistance. Lastly, to test the cell wall integrity of the freeze-thaw stress-resistant yeast strain and compare its cell wall with

that of the reference strain, lyticase susceptibility assay was performed as a physiological analysis.

2. MATERIALS AND METHODS

2.1 Materials

2.1.1 Yeast Strains and Media Preparation

The yeast strain used as a reference in this thesis study was *Saccharomyces cerevisiae* CEN.PK113-7D *(MATa, MAL2-8c, SUC2)*. It was kindly provided by Prof. Dr. Jean Marie François and Dr. Laurent Benbadis (University of Toulouse, France). The reference strain was not subjected to ethyl methane sulfonate (EMS) mutagenesis, and it was referred as "905" in this study.

For preculturing and cultivations, yeast minimal medium (YMM) which contained 2% (w/v) dextrose, and 6.7 g yeast nitrogen base without amino acids in 1 L of distilled water were used. Preculturing was done by incubation at 30°C and 150 rpm, approximately for 16 h. For solid media, 2% (w/v) agar was added to the prepared YMM. Components of YMM are shown in Table 2.1.

Table 2.1 : Components of Yeast Minimal Medium (YMM).

Component	Amount (g)
Yeast Nitrogen base without amino acids	6.7
Dextrose	20
Agar (for solid media)	20
ddH_2O	up to 1 L

For revival of the cells, Yeast Extract-Peptone-Dextrose (YPD) medium was used. Reviving of the cells was done by incubation at 30°C and 150 rpm, for 24 h. Components of YPD are shown in the Table 2.2.

Table 2.2: Components of Yeast Extract-Peptone-Dextrose (YPD) Medium

Component	Amount (g)
Bacto yeast extract	10
Dextrose	20
Bacto peptone	20
Agar (for solid media)	20
ddH_2O	up to 1L

2.1.2 Chemicals

Chemicals that were used in this study are listed in the Table 2.3.

Table 2.3 : Chemicals that were used in this study.

Chemical	Company	Country
Agar	BD DifcoTM	USA
Yeast nitrogen base without amino acids	BD DifcoTM	USA
Yeast Extract	Merck	Germany
Dextrose	Riedel-de-Haen	Germany
Peptone	Difco	USA
Tris HCl	Merck	Germany
β-Mercaptoethanol	Merck	Germany
Lyticase	Merck	Germany

2.1.3 Laboratory equipment

Laboratory equipment used in this study are listed in Table 2.4.

Table 2.4: Laboratory equipment used in this study.

Equipment	Company
Autoclave	GR 110 GF-Zealway
Balance	Precisa XB220A
Deep freezer (-80℃)	SANYO UltraLow
Laminar Flow Cabinet	Faster BH-EN 2003
Microcentrifuge	Eppendorf Microcentrifuge 5424
Orbital Shaker	Certomat S II Sartorius
UV-Visible Spectrophotometer	Shimadzu UV-1700
Vortex Mixer	Nüve NM 100
Refrigerator (+4℃)	Arçelik
Micropipettes	Eppendorf
Nanodrop 2000	Thermo Fisher Scientific

2.1.4 Kits

Kit that was used in this study is listed in Table 2.5.

Table 2.5 : Kit used in this study.

Kit Name	Company	Country
MasterPure DNA Purification Kit	Epicentre	USA

2.2 Methods

2.2.1 Genetic stability analysis

To test whether the freeze-thaw stress-resistant mutants were genetically stable or not, four most resistant mutant strains and the reference strain (905) were revived in YPD medium from their -80° C glycerol stock (30 % v/v). Then, cells were precultured in 10 ml YMM in 50 ml culture tubes at 30˚C, 150 rpm overnight, without any stress application. At 0.2 $OD_{600,}$ cells were inoculated into fresh YMM and successive 10 passages were done for 10 days without any stress application. After each passage, samples were taken from these cultures as 1 ml and kept in 30% (v/v) glycerol solution at -80 ˚C as frozen stocks.

Stocks from 0th, 2nd, 4th, 6th, 8th and 10th days were revived in YPD medium and precultured by inoculating the cells in 10 ml YMM. After incubated at 30˚C, 150 rpm overnight, OD_{600} values were set to 0.2 and they were incubated for 5 h until OD_{600} values of the cells reached 4.5. One ml of cells from the reference strain and each day of passage of the mutant strain cultures were then put into two sets of microfuge tubes. The first set was used as control and no stress was applied to this set of tubes. The second set of tubes was exposed to freeze-thaw stress for 5 cycles by freezing at -20˚C for 2 h and thawing at 30˚C for 10 min. Control and mutant cultures were serially diluted up to 10^{-5} with ddH_2O. They were then inoculated onto YMM-agar plates as 5 µL by spot assay. Agar plates were incubated at 30˚C for 48 h. Finally, the plates were photographed and stress resistance of each mutant strain on each day (or each passage) was compared with 0th day and the reference strain, based on their growth on plates.

2.2.2 Whole genome re-sequencing of the evolved strain

A highly freeze-thaw stress-resistant mutant (evolved) strain was revived in 100 mL YPD medium in 500 mL flasks, by incubating at 150 rpm and 30°C overnight. Genomic DNA of the mutant strain was isolated with MasterPure DNA Purification Kit (Epicentre) according to the kit manual. For measuring DNA concentration and quality, Nanodrop spectrophotometer was used. Comparative whole genome re-sequencing of the freeze-thaw stress-resistant evolved strain was performed by outsourcing (Gen Era Diagnostik A.Ş.), using Illumina NextSeq 550 next-generation sequencing platform. Results of re-sequencing was subjected to quality control performed by FastQC software (Babraham Bioinformatics). *S. cerevisiae*

CEN.PK113-7D reference genome was aligned by Burrows-Wheeler alignment software MEM (Li & Durbin, 2009). Genome Analysis Toolkit (DePristo et al., 2011) was used for variant search and Genome Browser (Golden Helix) software was used for nucleotide variations. High-quality point mutations were filtered using R software (Team, 2019).

2.2.3 Lyticase susceptibility assay

Lyticase susceptibility assay was applied to freeze-thaw stress-resistant mutant strain to test the cell wall integrity of the mutant. It was performed as described previously (Kuranda et al., 2006). For this purpose, the reference strain (905) and the mutant strain were revived. They were then inoculated into 100 mL YMM in 500 mL flasks and incubated at 30° C and 150 rpm. After cultivation for 24 h, OD_{600} values of the cultures were set to 0.2 and two sets of cells (one set without stress and one set after freeze-thaw stress was applied as 5 cycles) were grown until they reached the stationary phase of growth. Centrifugation was applied to cultures at 5500 g for 10 min. The pellets were resuspended in 10 ml of 10 mM Tris/HCl buffer (pH 7.4) including 40 mM β-mercaptoethanol, by setting at 0.9 OD_{600} per ml of culture. Cultures were then incubated for 30 min at 25 °C. Later, 2 U/ml of lyticase (β-1,3 glucanase from *Arthrobacter luteus*) was added to each sample along with the blank solution (10 mM Tris/HCl buffer (pH 7.4)). Finally, all the samples (prepared as triplicate) were incubated at 30 °C 300 rpm. During incubation, the decrease in OD_{600} value of the samples was recorded every 20 min.

3. RESULTS

3.1 Genetic Stability of Freeze-Thaw Stress-Resistant Evolved Strains

Selected freeze-thaw stress-resistant mutant strains named as FT1, FT5, FT6 and FT9 were tested for their genetic stability. While mutant strains were passaged daily for 10 days without any freeze-thaw stress, samples were taken, and frozen stocks were prepared. The spot assay was then applied to 0th, 2nd, 4th, 6th, 8th and 10th day of these stock cultures under non-stress and freeze-thaw stress conditions. Spot assay results demonstrated that all mutant strains tested maintained their freeze-thaw stress resistance through passages under non-selective conditions (the absence of freeze-thaw stress). Thus, they were genetically stable (Figures 3.1 and 3.2).

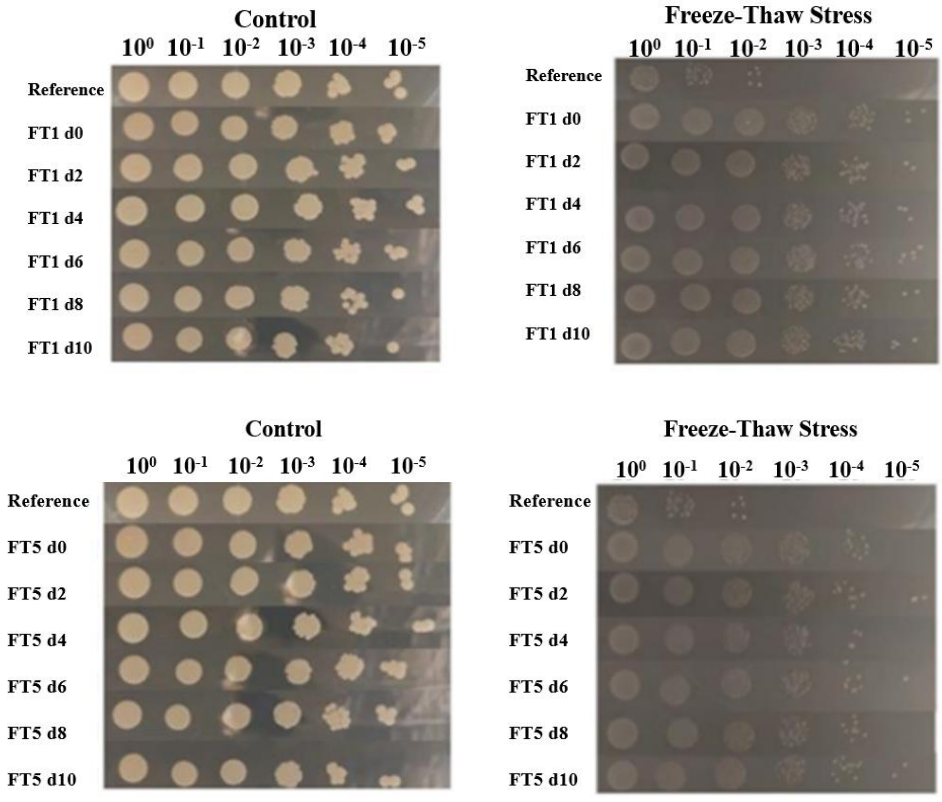

Figure 3.1: Genetic stability test results of FT1 and FT5 mutants. Spot assay photos were taken at 48 h of incubation. d0 to d10 indicate the day of passage in YMM medium without selective pressure. Cultures were serially diluted up to 10^{-5} dilution.

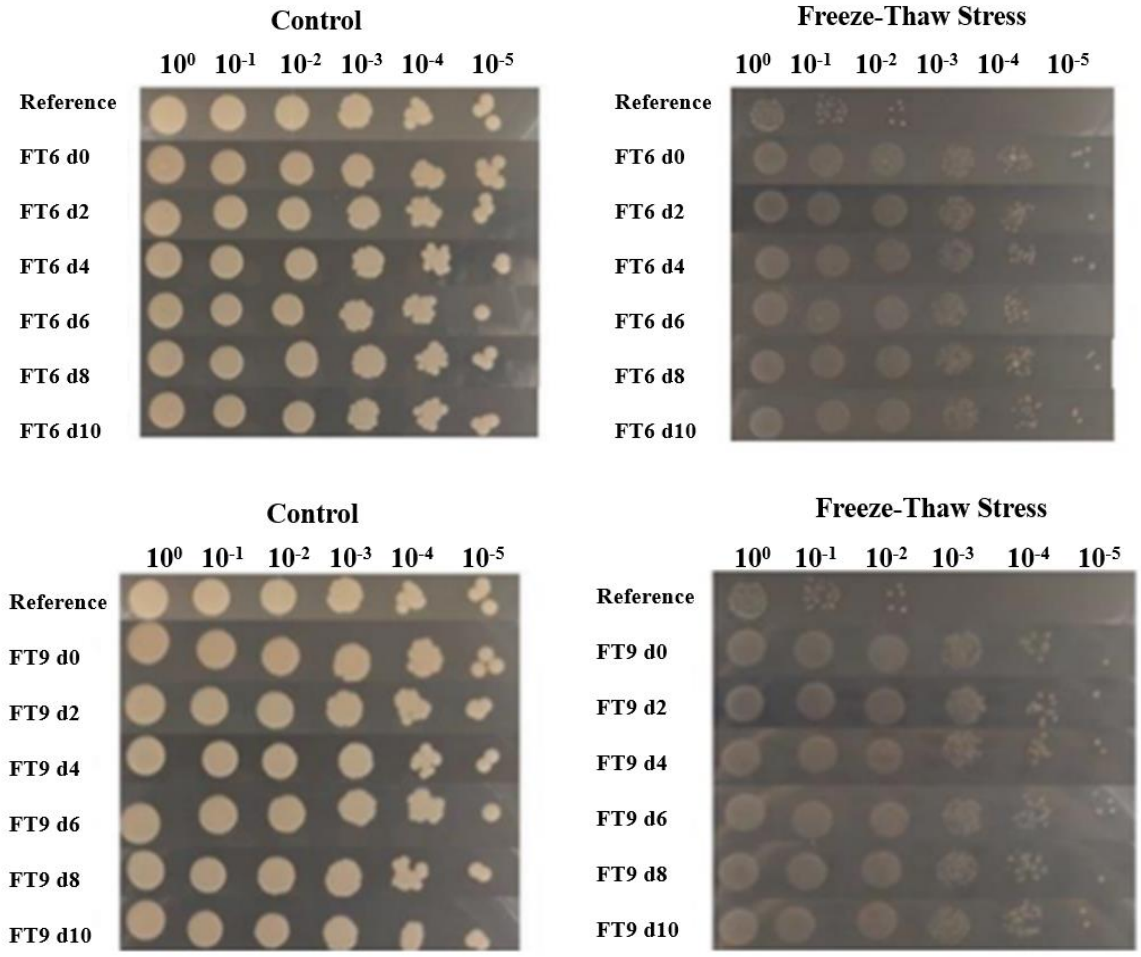

Figure 3.2: Genetic stability test results of FT6 and FT9 mutants. Spot assay photos were taken at 48 h of incubation. d0 to d10 indicate the day of passage in YMM medium without selective pressure. Cultures were serially diluted up to 10^{-5} dilution.

3.2. Whole Genome Re-sequencing of the Freeze-Thaw Stress-Resistant Evolved Strain

Comparative whole genome re-sequencing analysis of the highly freeze-thaw stress-resistant, and genetically stable mutant strain (FT9) revealed only one single nucleotide variation (SNV). The SNV was located on *CDC25* gene, and it was a missense SNV. As a result of the missense SNV on *CDC25* gene encoding a cell division cycle protein, replacement of threonine by lysine (T1415K) was expected as an amino acid change (Table 3.1).

Table 3.1: The single nucleotide variation (SNV) identified in FT9 as compared to the reference strain.

Chromosome	cDNA Position	Gene	Codon Change	Amino Acid Change	Description
XII	4244	*CDC25*	aCa/aAa	T1415K	Membrane bound guanine nucleotide exchange factor; also known as a GEF or GDP-release factor (yeastgenome.org).

CDC25 is located on chromosome XII of *S. cerevisiae* genome, as shown in Figure 3.3.

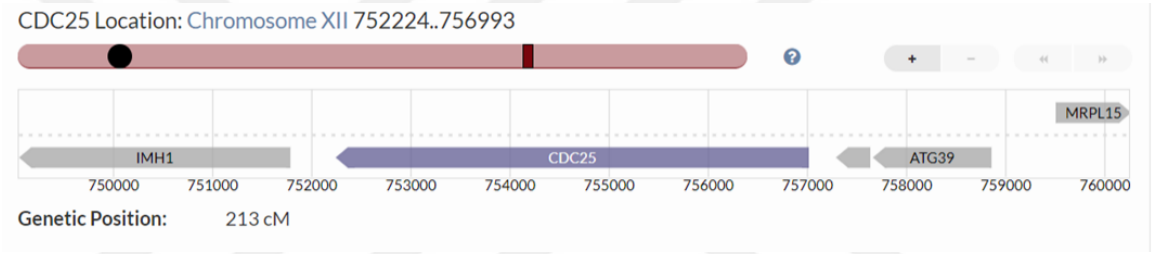

Figure 3.3: Location of *CDC25* gene (yeastgenome.org).

3.3 Lyticase Susceptibility Assay

To determine the cell wall integrity of the highly freeze-thaw stress-resistant and genetically stable mutant strain (FT9), lyticase susceptibility assay was performed. After using cell wall β-1,3-glucan degrading enzymes, the decrease in OD_{600} was monitored for 150 min to evaluate the resistance of the selected strain against cell lysis spectrophotometrically. Results of lyticase susceptibility assay demonstrated that the highly freeze-thaw stress-resistant mutant strain FT9 had a higher cell wall integrity than the reference strain (905), under both stress and non-stress conditions (Figure 3.4).

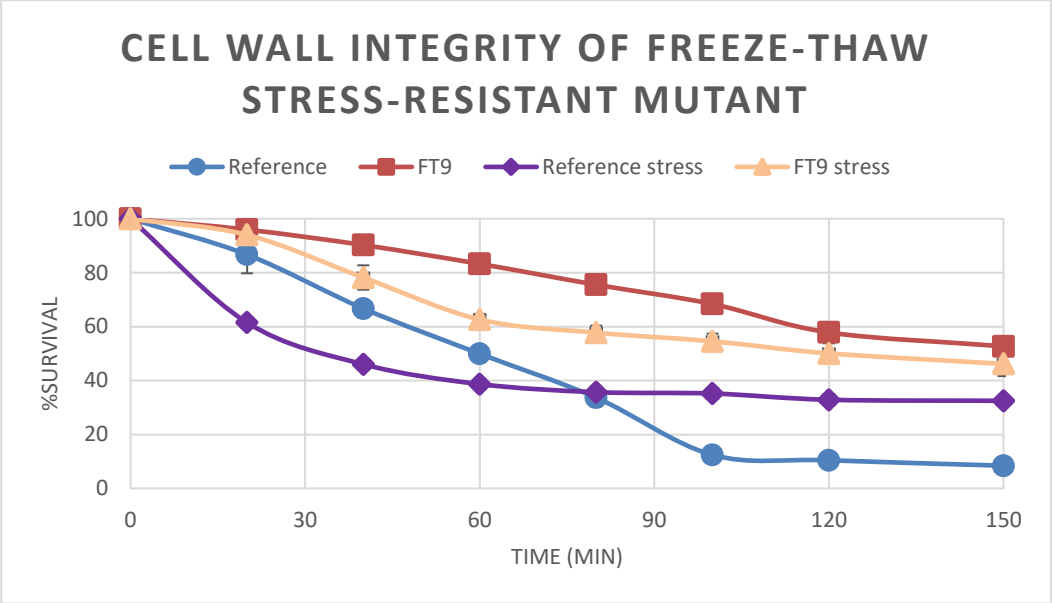

Figure 3.4: Lyticase susceptibility assay results of the highly freeze-thaw stress-resistant mutant (FT9) and the reference strain (905), in the presence and absence of freeze-thaw stress. Lyticase susceptibility was determined as the decrease in % survival upon freeze-thaw stress. 100% is the initial % survival before lyticase treatment.

4. DISCUSSION

Saccharomyces cerevisiae is an important microorganism for both industrial and research applications because of its genetic and biochemical characteristics. In diverse industrial applications, *S. cerevisiae* is cryopreserved in large quantities to be used later. However, repetetive freezing and thawing can result in stress conditions for yeast cells. Freeze-thaw stress is a physical stress type that causes physiological damage to cells. From the freezing step, mostly ice crystal formation and cellular dehydration cause the damage. Although most of the damage is caused by freezing step, thawing step also results in damage because of the formation of reactive oxygen species (ROS) (Mazur et al., 1972). In this study, a freeze-thaw stress-resistant *S. cerevisiae* mutant strain (FT9) previously developed in a former study (Balaban, 2023) by evolutionary engineering without any chemical mutagenesis was examined with the purpose of determination of its genomic and physiological characteristics. For this purpose, genetic stability test, whole genome re-sequencing and lyticase susceptibility assays were applied to the mutant strain.

The previously obtained freeze-thaw stress-resistant evolved strains FT1, FT5, FT6 and FT9 (Balaban, 2023) were selected for genetic stability analysis which revealed that all four strains were genetically stable. FT9 was chosen to be used in further analyses.

After genetic stability test, comparative whole genome re-sequencing was applied to FT9 and the reference strain. According to re-sequencing results, only one missense mutation was identified in FT9 genome on *CDC25* gene which is located on chromosome XII.

CDC25 gene is involved in *S. cerevisiae* cell division cycle by controlling Ras/cyclic AMP (cAMP) pathway. This pathway regulates growth and metabolism according to nutrients; especially, glucose. Elevated extracellular glucose levels signal for cAMP production through Cdc25p. Gene product of *CDC25* which is Cdc25p guanine nucleotide-exchange factor (GEF) stimulates Ras2p for conversion of inactive Ras-

GDP to active Ras-GTP. Then, Ras2GTP activates Cyr1p adenylate cyclase which sythesizes cAMP. At the end, activation of Protein Kinase A (PKA) occurs (Figure 4.1). With the activation of PKA, yeast cells can grow and give response to other metabolic effects. The guanine nucleotide exchange factors (GEF) Cdc25p and Sdc25p positively control Ras2p (Van Aelts et al., 1990). Ras2p is also negatively regulated by Ira2p which is the GTPase activating protein (GAP). Additionally, cAMP levels are decreased by Pde1p and Pde2p after they are phosphorylated by PKA. This process is also negative feedback (Besozzi et al., 2012). In summary, Cdc25p plays a role in fermentative growth, non-fermentative growth, cell size regulation, cell cycling, and sporulation, because of regulating cAMP levels (Van Aelts et al., 1990).

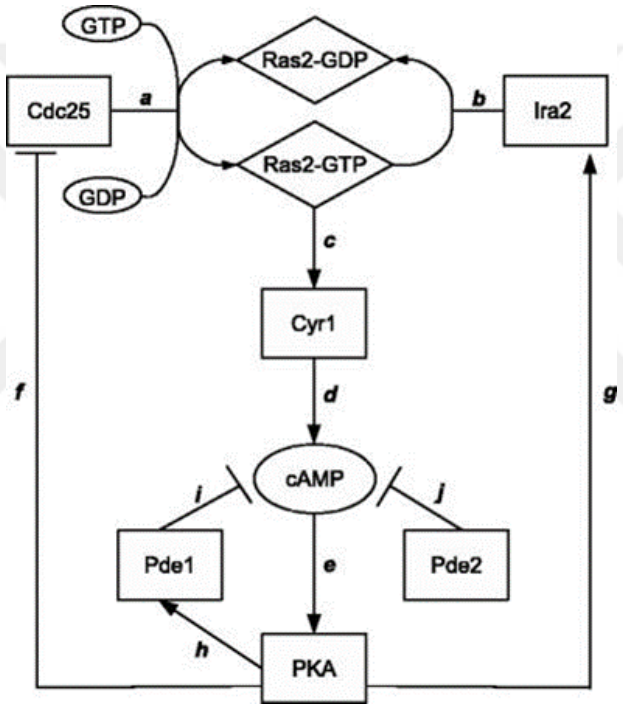

Figure 4.1: Ras/cAMP/PKA pathway (Besozzi et al., 2012).

Cell stress tolerance is downregulated by the cAMP/PKA (protein kinase A) pathway. This pathway controls the yeast cells at the G1 and G0 phase of the cell cycle and decides on the cell size for budding and mitosis. Components of cAMP/PKA signal pathway can be affected by stress factors like freeze-thaw stress and cellular response against these stress factors can be achieved as a result of the contribution of this pathway (Paiardi et al., 2007).

Msn2 and Msn4 (multicopy suppressor of *SNF1* mutation proteins 2 and 4) are the two master regulators in *S. cerevisiae* which regulate gene expression as a stress response.

These proteins bind to stress responsive element (STRE) specifically to upregulate expression of the genes related to a variety of stress types such as osmotic stress, heat stress, and carbon starvation stress. In order to be activated, Msn2/4 should be localized into nucleus from cytosol; however, activation of PKA inhibits their localization to the nucleus upon phosphorylation. When stress is induced, PKA activation can be inhibited so that Msn2/4 can be localized into nucleus to generate stress response (Rajvanshi et al., 2017). Additionally, it has been reported that the destruction of Ras/PKA pathway resulted in freeze-thaw stress tolerance (Cabrera et al., 2020).

Cdc25p is a membrane-bound protein and it is 180 kDa. There are SH3 motif and cyclin destruction box motif in N- terminal domain of Cdc25p. They bind to adenylate cyclase and mediate Cdc25p ubiquitin-dependent degradation respectively. On the other hand, there is a catalytic domain and a membrane localization signal in the C-terminal domain of the Cdc25p. C- terminal domain is very important for the normal growth of the cell and its full biological activity (Paiardi et al., 2007).

The mutation in the *CDC25* gene of FT9 mutant is a missense mutation converting the nucleotide C to A in the 4244^{th} position of the cDNA. This will result in an amino acid change in the 1415^{th} position which is threonine (T) turning into lysine (K). Threonine is a polar uncharged amino acid; however, lysine is a positively charged amino acid. The 1415^{th} position of CDC25p is a RAS-GEF domain. Therefore, the missense mutation in this domain may affect the interactions of the CDC25p with Ras1p and Ras2p. With this mutation, Ras2p-GTP may not be produced from Ras2p-GDP by the GEF domain and consequently, PKA activation may not occur. This could result in nuclear localization of Msn2 and Msn4. Thus, cell stress response to freeze-thaw stress can be accomplished.

There are some studies in the literature revealing the relationship between cAMP signaling pathway and freeze-thaw stress response in *S. cerevisiae*, since the general stress response, also known as environmental stress response (ESR) in *S. cerevisiae* induces this pathway to respond to various type of stresses (Cabrera, 2020). Hence, some genes were found to be related with both general stress response and stress-specific response against freeze-thaw stress. These genes can be listed as *TIR1, TIR2, YNL278w, SEC11, MMS2, PAK1, ERG10, TPI1, SSD1, IMH1, TIP1* (temperature-

inducible protein) (Schade et al., 2004), *ERG10* (Rodrigues et al., 2002), *ANP1*, *VMA2*, *VMA8, MNN10, RPL27A, MNN11*, and *MRPL32* (Ando et al., 2006). However, not all the genes that are related with freeze-thaw stress-response in *S. cerevisiae* have been identified yet. This study has shown the potential role of the *CDC25* gene mutation in freeze-thaw stress resistance of *S. cerevisiae* to further analyze the relationship of this mutation with metabolic and molecular factors contributing to freeze-thaw stress-resistance.

The improvement of cell wall integrity was shown to be associated with increased yeast stress resistance (Terzioğlu et al., 2020; Kocaefe-Özşen et al., 2022). For this purpose, cell wall integrity of the freeze-thaw stress-resistant strain FT9 was tested by lyticase susceptibility assay, following incubation of the reference strain and FT9 in freeze-thaw stress and control conditions. The cell wall of *S. cerevisiae* is composed of an outer layer of mannoproteins, and an inner layer formed by β-glucans and chitin in which glycosylphosphatidylinositol (GPI) connects them. The yeast cell wall is a crucial structure for the survival of yeast which protects the cell, advances it through the cell cycle and maintains cell shape and integrity by the elasticity and tensile strength of coiled spring-like structures of β-glucans. Thus, the cell wall of *S. cerevisiae* is an important component for developing stress tolerance (Sahana et al., 2024). Zymolyase or lyticase which are the cell wall β-1,3-glucan-degrading enzymes can be used to track cell lysis which is done by monitoring the decrease in OD_{600} with time spectrophotometrically (Kuranda et al., 2006). After the cell wall integrity experiment, it was observed that the cell wall of the FT9 was more resistant to lyticase degradation than the reference strain (Figure 3.4). Freeze-thaw stress is a physical stress type which harms the yeast cell membrane. Yeast cells having polyunsaturated fatty acids, ergosterol and phospholipids with linoleyl residues are more resistant against lysis by lyticase since with high proportions of lipids, membrane fluidity increases. Thus, exposure to freeze-thaw stress possibly strenghtened the cell wall of the evolved strain (Cabrera et al., 2020). It remains to be investigated whether the mutation on the *CDC25* gene might have caused the thickening of the cell wall, such that lyticase could not degrade the cell wall easily.

5. CONCLUSION

In this study, a freeze-thaw stress-resistant *S. cerevisiae* strain (FT9) which was previously obtained by evolutionary engineering without using any chemical mutagenesis (Balaban, 2023) was characterized at genomic and physiological levels. The genetic stability test results showed that FT9 was genetically stable, along with other selected mutant strains. According to lyticase susceptibility assay results, freeze-thaw stress-resistant mutant FT9 had higher cell wall integrity than the reference strain (905) under both freeze-thaw stress and control conditions. Results of comparative whole genome re-sequencing analysis demonstrated that the freeze-thaw stress-resistant strain had only one single nucleotide variation (SNV). The SNV was located on *CDC25* gene, and it was a missense SNV. As a result of the missense SNV on *CDC25* which encodes a cell division cycle protein, threonine was expected to be replaced by lysine (T1415K). In conclusion, the physiological and genomic analysis results of this study imply that changes in Ras/cAMP/PKA pathway occurred in freeze-thaw stress- resistant mutant strain to overcome the gradually increased levels of freeze-thaw stress. However, comparative transcriptomic and metabolic analysis of this mutant strain FT9 would be necessary as a future study to fully comprehend the complex molecular basis of freeze-thaw stress-resistance in *S. cerevisiae*.

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- **Güney, Ç., Holyavkin, C., Topaloğlu, A., Çakar, Z. P.,** Genomic Analysis of Freeze-Thaw Stress-Resistant *Saccharomyces cerevisiae,* International Graduate Research Symposium (IGRS'23), 16-18 May 2023, Istanbul, Turkey. (Oral Presentation)

OTHER PUBLICATIONS, PRESENTATIONS AND PATENTS:

- **Güney, Ç.,** Roles Of C-Myc, P53, PRb And Ras in Cell Cycle and Apoptosis**,** Cukurova 11th International Scientific Research Conference 22-24 August 2023, Adana, Turkey. (Oral Presentation)
- **Güney, Ç.,** Metabolic Reprogramming in Cancer, Batumi Annual International Multidisciplinary Conference on Economics, Business, Technology and Social Sciences - 2023, 1-2 July 2023, Batumi, Georgia. (Poster Presentation)