ISTANBUL TECHNICAL UNIVERSITY ★ GRADUATE SCHOOL

INVERSE METABOLIC ENGINEERING OF KCl-RESISTANT *Saccharomyces cerevisiae*

M.Sc. THESIS

Ogün MORKOÇ

Department of Molecular Biology - Genetics and Biotechnology

Molecular Biology - Genetics and Biotechnology Programme

JUNE 2022

ISTANBUL TECHNICAL UNIVERSITY ★ GRADUATE SCHOOL

INVERSE METABOLIC ENGINEERING OF KCl-RESISTANT *Saccharomyces cerevisiae*

M.Sc. THESIS

Ogün MORKOÇ
(521181116)

Department of Molecular Biology - Genetics and Biotechnology

Molecular Biology - Genetics and Biotechnology Programme

Thesis Advisor: Prof. Dr. Zeynep Petek ÇAKAR

JUNE 2022

İSTANBUL TEKNİK ÜNİVERSİTESİ ★ LİSANSÜSTÜ EĞİTİM ENSTİTÜSÜ

TERSİNE METABOLİK MÜHENDİSLİK YÖNTEMİ İLE KCl TUZUNA DİRENÇLİ *Saccharomyces cerevisiae* ELDESİ

YÜKSEK LİSANS TEZİ

Ogün MORKOÇ
(521181116)

Moleküler Biyoloji – Genetik ve Biyoteknoloji Anabilim Dalı

Moleküler Biyoloji – Genetik ve Biyoteknoloji Programı

Tez Danışmanı: Prof. Dr. Zeynep Petek ÇAKAR

HAZİRAN 2022

Ogün MORKOÇ, a M.Sc. student of ITU Graduate School student ID 521181116, successfully defended the thesis/dissertation entitled "INVERSE METABOLIC ENGINEERING OF KCl-RESISTANT *Saccharomyces cerevisiae*", which he prepared after fulfilling the requirements specified in the associated legislations, before the jury whose signatures are below.

Thesis Advisor :	**Prof. Dr. Zeynep Petek ÇAKAR** Istanbul Technical University	.....
Jury Members :	**Prof. Dr. Ayten KARATAŞ** Istanbul Technical University	.....
	Prof. Dr. Berna SARIYAR AKBULUT Marmara University	.....

Date of Submission : 27.05.2022
Date of Defense : 20.06.2022

To my family,

FOREWORD

I would like to thank my supervisor Prof. Dr. Zeynep Petek ÇAKAR for her excellent guidance and support. She always showed me patience and I have learned a lot from her.

I also would like to thank Alican TOPALOĞLU for always helping me and being so kind. I also thank my lab-mates Ömer ESEN, Yıldız ERDENER, Enes Fahri TEZCAN, İrem BALABAN for their assistance in the experimental part.

Finally, I would like to thank my family for their endless support.

May 2022

Ogün MORKOÇ

(Bioengineer)

TABLE OF CONTENTS

Page

ABBREVIATIONS

ACU : Alkali cation uptake
CDW : Cell dry weight
HAK : High-affinity K^+ uptake
HPLC : High-performance liquid chromatography
HOG : High-osmolarity glycerol
MAP : Mitogen-activated protein
MAPK : Mitogen-activated protein kinase
MAPKK : Mitogen-activated protein kinase kinase
MAPKKK : Mitogen-activated protein kinase kinase kinase
OD : Optical density
TMA : Tetramethylammonium
TRK : Transport of K^+
YMM : Yeast minimal medium
YPD : Yeast extract-peptone-dextrose

LIST OF TABLES

Page

LIST OF FIGURES

Page

INVERSE METABOLIC ENGINEERING OF KCl-RESISTANT *Saccharomyces cerevisiae*

SUMMARY

The yeast Saccharomyces *cerevisiae* is a well-known model organism, which has a variety of applications in biotechnology. *S. cerevisiae* is considered a Generally Recognized as Safe (GRAS) organism. The resilience of *S. cerevisiae* against environmental stress factors such as osmotic, oxidative, freezing-thawing, high temperature, and ethanol stress makes *S. cerevisiae* an advantageous cell factory for biotechnology.

KCl is an odorless white metal halide salt. When it dissolves in water, K^+ and Cl^- ions are generated. Potassium ion is a vital cation for maintaining membrane potential and a variety of cellular functions such as enzyme activation, osmoregulation, pH regulation, and protein translation. In contrast to Na^+ and Li^+, K^+ is not toxic to the cells. A high ratio of K^+/Na^+ is imperative for normal cellular functions, because the uptake of K^+ reduces the driving force for the uptake of toxic cations to balance membrane potential. Regulation of potassium homeostasis is controlled by complex signaling networks including High Osmolarity Glycerol (HOG), Calcineurin, and Rim pathways. The uptake and efflux of the K^+ are mediated by membrane transporter proteins. Trk1,2 transporters are responsible for K^+ uptake. On the other hand, Tok1, Nha1, and Ena1-5, 6 transporters control the export of redundant K^+.

Under normal growth conditions, *S. cerevisiae'*s internal K^+ concentration is around 200-300 mM. However, an external concentration of more than 400 mM creates hyperosmotic stress. Exposure to hyperosmotic stress results in immediate water loss and cell shrinkage. To cope with the effect of osmotic stress, yeast cells trigger a couple of osmotic stress responses controlled by the HOG pathway. The most effective strategy against hyperosmotic stress is accumulating compatible osmolytes such as glycerol. Activation of the HOG pathway regulates the transcription of more than 500 genes for stress response. Therefore, understanding the cell response against hyperosmotic stress caused by high concentrations of KCl salt is crucial.

In this study, an inverse metabolic engineering strategy, evolutionary engineering was used to obtain KCl-induced hyperosmotic stress-resistant *S. cerevisiae.* The batch selection was conducted under gradually increasing KCl stress. Before selection, a screening experiment was performed. 0.5 M KCl stress was determined as the initial stress level. The stress level was increased by 0.05 M at each passage until the 22^{nd} population. It was then reduced to 0.02 M. The selection was continued up to 2.5 M KCl stress for 72 populations. After obtaining the final population, 12 randomly selected individual colonies were isolated on plates and named K1-K12. Among them, K1, K9, K10, K11, and K12 have shown better performance at 1.75 M KCl stress. Percent survival rate calculations revealed that at 2 M KCl stress, K9 strain showed 1.9-fold higher resistance compared to the reference (905) strain. Subsequently,

genetic stability test was performed, K1, K9, and K10 strains were found to be genetically stable and their resistance to KCl was shown to be permanent.

Cross-resistance tests of the genetically stable, evolved strains were performed with various stress types (0.7 M NaCl, 5 mM LiCl, 0.8 M $CaCl_2$, 0.5 mM $NiCl_2$, 7.5 mM $AlCl_3$, 0.75 mM H_2O_2, 10 % ethanol, 15 mM caffeine, 1.5 M sorbitol, 1 M NH_4Cl, 15 mM $MnCl_2$). The K1 and K10 strains showed similar behavior against many stress types: both of them gained resistance against NaCl, H_2O_2, and slightly LiCl stress. However, the K9 strain showed sensitivity against NaCl, H_2O_2, LiCl, $AlCl_3$, and caffeine stress.

Apart from these chemicals, resistance against cationic drugs (0.5, 0.8, and 1 M TMA, 1.2, 2.5 mM Spermine, 125 µg/mL Hygromycin B) were tested. Tolerance to these toxic cations indicates a reduction in membrane potential. Furthermore, at different pH (3.5, 5.5, and 7.5) values cross-resistance of the evolved strains was tested. The results showed that at low pH evolved strains showed no resistance or sensitivity. However, at high pH evolved strains showed slight sensitivity. Unlike to the other chemicals tested, the resistance results of cationic drugs and different pH seem to be correlated between K9, K1, and K10 strains.

Growth profiles, metabolite (glucose, acetate, ethanol, glycerol), and storage carbohydrates (trehalose and glycogen) analyses of K1 and K9 strains were also performed. Strain K9 was chosen because its cross-resistance results differed from the other evolved strains. Strain K1 was chosen because its percent survival rate was higher than that of strain K10 at 1.75 M and 2 M KCl stress levels.

The results showed that under KCl stress, the evolved strains produced a high amount of glycerol, and acetate, and they stored trehalose. In the absence of stress, however, acetate was continued to be produced by evolved K1, and K9 strains. K1 strain produced more glycerol than K9 and the reference strain, both in the presence and absence of stress: This may indicate that high KCl concentration causes hyperosmotic stress and possible favorable mutations during evolutionary engineering might have caused the activation of the HOG pathway, which leading to the production of glycerol and acetate.

In conclusion, KCl resistant, two distinct strains (K1 and K9) were successfully obtained in this study by evolutionary engineering. The two evolved strains showed different cross-resistance behaviour and metabolite profiles. This might be due to diverse mutations in different metabolic pathways. Further genomics and transcriptomics analyses would be necessary to reveal the complex molecular network that regulates hyperosmotic stress response/resistance and internal cation homeostasis.

TERSİNE METABOLİK MÜHENDİSLİK YÖNTEMİ İLE KCl TUZUNA DİRENÇLİ *Saccharomyces cerevisiae* ELDESİ

ÖZET

Saccharomyces cerevisiae, tek hücreli, ökaryotik model bir organizmadır. Tomurcuklanan maya veya ekmek mayası olarak da bilinir. Yüksek ökaryotlar ile ortak birçok metabolik fonksiyona sahiptir. Bu nedenle yüksek ökaryotların karmaşık yapılarını anlamak için yararlı bir model organizmadır. Ayrıca *S. cerevisiae*, genomu dizilenen ilk ökaryotik organizmadır.

*S.cerevisiae'*nın kültivasyonu kolay ve ucuzdur. -80°C'de gliserol içinde saklanabilir ve agar plakalarında büyütülüp saflaştırılabilir. Bunun yanısıra, gıda, şarap ve biyoyakıt endüstrileri dahil olmak üzere birçok biyoteknolojik uygulamanın temel taşıdır. *S. cerevisiae*'nin ozmotik, oksidatif, donma-erime, yüksek sıcaklık ve etanol stresi gibi çevresel stres faktörlerine karşı direnci, *S. cerevisiae*'yi biyoteknoloji için avantajlı kılmaktadır.

KCl kokusu olmayan, beyaz bir metal tuzudur. Suda çözündüğünde K^+ ve Cl^- iyonlarına ayrışır. Potasyum iyonu, membran potansiyelinin dengelenmesi, enzim aktivasyonun sağlanması, ozmoregülasyon, pH regülasyonu ve protein translasyonu gibi çeşitli hücresel fonksiyonları sürdürmek için hayati bir öneme sahiptir. Sodyum (Na^+) ve lityum (Li^+) iyonlarının aksine, potasyum iyonu hücreler için toksik değildir. Normal hücresel fonksiyonlar için hücre içi K^+ konsantrasyonun Na^+ konsantrasyonundan yüksek olması gerekir. Hücresel K^+/Na^+ oranı yüksek olması membran potansiyelini dengeler ve diğer toksik katyonları hücre içerisine almak için gereken itici gücü azaltır.

Hücre içi potasyum homeostazının regulasyonu, HOG, Calcineurin ve Rim yolları dahil olmak üzere karmaşık sinyal ağları tarafından kontrol edilir. K^+'nın hücre içerisine alımına ve hücre dışarısına atılmasına membran üzerinde bulunan taşıyıcı (transporter) proteinler aracılık eder. K^+ iyonlarını hücre içerisine alımından Trk1,2 membran proteinleri sorumludur. Tok1, Nha1 ve Ena1-5, 6 membran proteinleri ise K^+ iyonun hücre dışına atımından sorumludur. Bunun yanı sıra, Nha1 ve Ena transporterları Na^+ ve Li^+ toksik iyonların hücre dışına atılmasından sorumludur. Membran üzerinde bulunan tüm transporter proteinleri membran potansiyelini korumak, turgor basıncını düzenlemek ve hücrelerin osmotik stres ile başa çıkabilmesi için koordineli olarak birlikte faaliyet gösterirler.

Normal büyüme koşulları altında, *S. cerevisiae*'nin hücre içi K^+ konsantrasyonu 200-300 mM civarındadır. Fakat hücre dışında, 400 mM'den fazla K^+ bulunması hiperozmotik strese yol açar. Hiperozmotik strese maruz kalma, ani su kaybına ve hücre büzülmesine neden olur. Hücreler osmotik stresin etkilerinden korunabilmek için HOG sinyal yolağı tarafından kontrol edilen ozmotik stres tepki mekanizmalarını tetiklerler.

Hiperozmotik strese karşı en etkili strateji, gliserol gibi ozmolitlerin hücre içindeki konsantrasyonunu arttırmaktır. Bu sayede hücreler, hücre içi çözünmüş madde miktarını arttırarak su kaybının önüne geçmiş olurlar. HOG yolunun aktivasyonu, stres yanıtı için 500'den fazla genin transkripsiyonunu düzenler.

Bu çalışmada, KCl tuzu kaynaklı hiperosmotik strese dayanıklı *S. cerevisiae* suşları elde etmek için, tersine metabolik mühendislik yaklaşımı olan evrimsel mühendislik kullanılmıştır. Evrimsel mühendislik ile elde edilen suşların fizyolojik ve metabolik analizleri yapılmıştır.

Evrimsel mühendislik için seçilim deneyi, kademeli olarak artan KCl stresi altında gerçekleştirilmiştir. Seçilim yapılmadan önce, *S. cerevisiae* referans (905) suşunun büyümesini inhibe eden minimum KCl stres konsantrasyonunu tayin edebilmek için tarama deneyi yapılmıştır. Tarama deneyi sonucunda 0.5 M KCl stresi, başlangıç stresi olarak belirlenmiştir. Stres seviyesi 22. popülasyona kadar her bir pasajda 0.05 M arttırılarak 1.5 M'a kadar çıkarılmıştır. Daha sonra artış miktarı 0.02 M'a düşürülerek stres seviyesi 2.5 M'a erişinceye kadar (72. populasyona kadar) seçilim devam ettirilmiştir.

Son popülasyon (72) elde edildikten sonra, katı besiyerinde KCl stresi varlığında rastgele seçilen 12 koloni izole edilip, K1-K12 olarak adlandırılmıştır. Spot testi yapılarak, izole edilen suşların KCl stresine karşı direnç seviyeleri analiz edilmiştir. İzole edilen suşlar arasından K1, K9, K10, K11 ve K12 suşlarının 1.75 M KCl stresine daha dirençli oldukları gözlemlenmiştir.

Yüzde hayatta kalma oranı hesaplamaları, 2 M KCl stresi varlığında K9 suşunun, referans (905) suşa kıyasla 1.9 kat daha fazla direnç gösterdiğini ortaya koymuştur. Akabinde, genetik stabilite testi yapılmış; K1, K9 ve K10 suşlarının genetik olarak kararlı oldukları tespit edilip, KCl'ye karşı dirençlerinin kalıcı olduğu gösterilmiştir.

Genetik olarak kararlı olan K1, K9 ve K10 suşlarının fizyolojik karakterizasyonları ve çapraz direnç testleri, çeşitli stres türleri (0.7 M NaCl, 5 mM LiCl, 0.8 M $CaCl_2$, 0.5 mM $NiCl_2$, 7.5 mM $AlCl_3$, 0.75 mM H_2O_2, %10 etanol, 15 mM kafein 1.5 M sorbitol, 1 M NH_4CI, 15 mM $MnCl_2$) ile yapılmıştır. K1 ve K10 suşları, birçok stres tipine karşı benzer davranış göstermiştir: Her ikisi de NaCl, H_2O_2 ve hafif düzeyde LiCl'ye karşı direnç kazanmıştır. Ancak K9 suşu NaCl, H_2O_2, LiCl, $AlCl_3$ ve kafeine karşı hassasiyet göstermiştir.

Bu kimyasalların dışında, katyonik ilaçlara (0.5, 0.8 ve 1 M TMA, 1.2, 2 ve 5 mM Spermine, 125 µg/mL Higromisin B) karşı direnç seviyeleri de test edilmiştir. Bahsi geçen toksik katyonlara karşı gösterilen tolerans, membran potansiyelinde bir azalmaya işaret eder. Ayrıca KCl-dirençli suşların farklı pH (3.5, 5.5 ve 7.5) değerlerinde çapraz dirençleri de test edilmiştir. Sonuçlar, düşük pH altında, KCl-dirençli suşların hiçbir direnç veya hassasiyet göstermediğini göstermiştir. Bununla birlikte, yüksek pH altında, KCl-dirençli suşlar hafif bir hassasiyet göstermiştir. Test edilen diğer kimyasalların aksine, katyonik ilaçların ve farklı pH değerlerinin direnç sonuçları; K9, K1 ve K10 suşları arasında korelasyon varlığına işaret etmektedir.

K1 ve K9 suşlarının büyüme profilleri çıkartılmış, metabolit (glukoz, asetat, etanol, gliserol) ve depo karbonhidratları (trehaloz ve glikojen) analizleri yapılmıştır. K9 suşu, çapraz direnç sonuçları K1 ve K10 suşlarından farklılık gösterdiği için seçilmiştir. K1 suşu ise hayatta kalma yüzdesi oranı, 1.75 M ve 2 M KCl streslerinde K10 suşundan daha iyi olduğu için seçilmiştir.

Sonuçlar, KCl stresine direnç kazandırılmış suşların yüksek miktarda gliserol, ve asetat ürettiğini ve trehaloz depoladığını göstermektedir. Öte yandan, K1 ve K9 suşlarının KCl stres yokluğunda da asetat üretimine devam ettiği gözlemlenmiştir.

K1 suşu ise, stres varlığında ve yokluğunda K9 ve referans suşundan daha fazla gliserol üretmiştir: Muhtemelen bu durum, yüksek KCl konsantrasyonunun hiperozmotik strese neden olduğuna ve evrimsel mühendislik sonucu HOG yolağında gerçekleşmiş olası mutasyonların gliserol ve asetat üretimine yol açtığına işaret etmektedir.

Sonuç olarak bu çalışmada, evrimsel mühendislikle KCl dirençli iki farklı suş (K1 ve K9) başarıyla elde edilmiştir. Bu dirençli iki suş, farklı çapraz direnç düzeyleri ve metabolit profilleri göstermiştir. Bu durum, farklı metabolik yolaklardaki çeşitli mutasyonlardan kaynaklanıyor olabilir. Hiperozmotik stres tepki veya direnci ile dahili katyon homeostazını düzenleyen karmaşık moleküler ağın aydınlatılabilmesi için, ileriki çalışmalarda genomik ve transkriptomik analizlerin de yapılması gerekmektedir.

1. INTRODUCTION

1.1 The Yeast "*Saccharomyces cerevisiae*"

Saccharomyces cerevisiae is a single-celled, model eukaryotic organism. Its unicellular nature makes *S. cerevisiae* a useful tool for understanding many complex biological functions of high eukaryotes (Parapouli et al., 2020). Because *S. cerevisiae* is easy to grow, non-pathogenic, has a well-defined genome, and most importantly, it has conserved metabolic mechanisms with eukaryotes including mammals (Schneiter, 2004). Apart from being a versatile model organism, *S. cerevisiae* is the cornerstone of many biotechnological applications including food, wine, and biofuel industries (Parapouli et al., 2020). *S. cerevisiae* belongs to the fungi kingdom of phylum ascomycetes (Table 1.1) and is also known as budding yeast or baker's yeast (Duina et al., 2014).

Table 1.1 : Taxonomy of *Saccharomyces cerevisiae.*

Classification	
Domain	Eukaryota
Kingdom	Fungi
Phylum	Ascomycota
Subphylum	Saccharomycotina
Class	Saccharomycetes
Order	Saccharomycetales
Family	Saccharomycetaceae
Genus	*Saccharomyces*
Species	*Saccharomyces cerevisiae*

The word Saccharomyces means sugar and cerevisiae means beer in old terminology (Mortimer, 2000). Yeasts have an immense impact on human life. Their utilization dates back to 10,000 B.C.E. Sumerians used yeast for beer making (Schneiter, 2004). And later, it was discovered that yeast could be added to sugars and plant materials for

a variety of alcoholic beverage production. In addition, yeast has been used in bread making for centuries. The carbon dioxide produced during fermentation is used to raise bread dough which results in light texture bread (Duina et al., 2014). However, the purification of unicellular yeast was achieved only in the late 1800s. Emil Christian Hansen who worked in Carlsberg Laboratory discovered how to isolate and purify yeast colonies. Hansen named the isolated strain *Saccharomyces carlsbergensis* (Greig & Leu, 2009). After that, strains of Saccharomyces were purified all over the world. Since mid-1930, *S. cerevisiae* has been studied at the genetic level (Mortimer, 2000).

S. cerevisiae can be stored at – 80 ℃ in glycerol and can grow and be purified on agar plates (Duina et al., 2014). Due to its easy handling properties and model organism characteristics, *S. cerevisiae* is the first eukaryotic organism whose genome was sequenced (Goffeau et al., 1996). The sequence results revealed that it has 12068 kilobases of (kb) genomic DNA organized in 16 chromosomes. It has 5570 protein-coding genes among approximately 6000 genes (Goffeau et al., 1996). Approximately 30 % of *S. cerevisiae* genes have already been characterized (Schneiter, 2004). As of 4/7/2022, the number of verified ORFs (Open reading frames) in reference strain S288C is 5234 (*Saccharomyces cerevisiae Genome Overview | SGD*, n.d.).

1.2 *S. cerevisiae* Life Cycle

S. cerevisiae cells can divide by budding at about 90 minutes under optimal growth conditions. The cell's unbudding form is approximately 5 μm in diameter. During budding, a daughter cell outgrows from the mother cell. Each of the mother cells can form 20-30 buds. (Schneiter, 2004) *S. cerevisiae* is polyploid and stable in both haploid and diploid states. The haploid state has two mating types, *MAT***a** (a cell) or *MAT*α (α cell). Both mating types can undergo mitotic cell division through budding. The cells can also switch between two mating types. *HO* endonuclease enzyme which is encoded by the *HO* gene is responsible for this switch. This enzyme makes double-stranded cuts at the mating-type locus (*MAT*) (Coughlan et al., 2020). However, laboratory haploid strains usually lack this endonuclease to have a stable mating type. These two cell types can also form a diploid state (*MAT***a**/*MAT*α (**a**/α cell) by mating. Before mating cell types secrete pheromones which leads to the formation of shmoo followed by mating (Figure 1.1).

Upon exposure to nutrient deficiency, diploid strains sporulate. The diploid cells undergo meiosis and form four haploid cells. These haploid strains later become encapsulated as spores. When optimum conditions are established the spores germinate and form haploid cells. Diploid cells can also divide mitotically and form daughter cells (Figure 1.1) (Duina et al., 2014). The diploid cell buds appear on the opposite side of the cell (axial budding). On the other hand, haploid cell buds appear adjacent to the previous one (radial budding) (Schneiter, 2004).

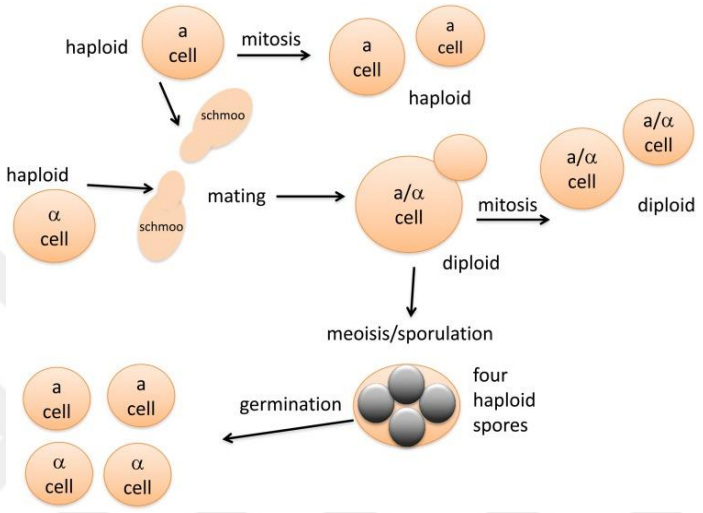


Figure 1.1 : A simplified life cycle diagram of *S. cerevisiae* (Duina et al., 2014).

1.3 *S. cerevisiae* in Biotechnology

S. cerevisiae is a useful organism for many biotechnological applications. It has been extensively used in the beverage and food industries for centuries, especially for wine and bread production (Parapouli et al., 2020). However, in recent years, its application in biofuel, fine chemicals, food ingredients, and protein drugs production gained substantive attention. Therefore, *S. cerevisiae* is considered an important cell factory for both high-value added and low-value added products (Figure 1.2) (Hong & Nielsen, 2012).

The reason why *S. cerevisiae* is so useful for biotechnology is that *S. cerevisiae* has a high fermentation capacity (Parapouli et al., 2020). *S. cerevisiae* can ferment approximately its own weight of glucose per hour (Schneiter, 2004). Additionally, it has the ability to produce alcohol and is resistant to environmental stresses like low pH and osmotic stress.

In addition, *S. cerevisiae* has a different carbon metabolism than the other cell factories such as *Escherichia coli*. This phenomenon is known as the Crabtree effect. Under aerobic conditions, *S. cerevisiae* initially metabolizes the available carbon source into ethanol instead of using its respiratory mechanism to produce biomass. The result of this high amount of alcohol production gives *S. cerevisiae* cells an advantage over the other microorganism. The accumulated ethanol is toxic to many microorganisms. In this way, *S. cerevisiae* can inhibit the growth of other microorganisms, eliminating competition for carbon sources. After that *S. cerevisiae* can utilize the early-produced ethanol for growth. This evolutionary advantage makes *S. cerevisiae* a better candidate for many biotechnological applications (Parapouli et al., 2020).

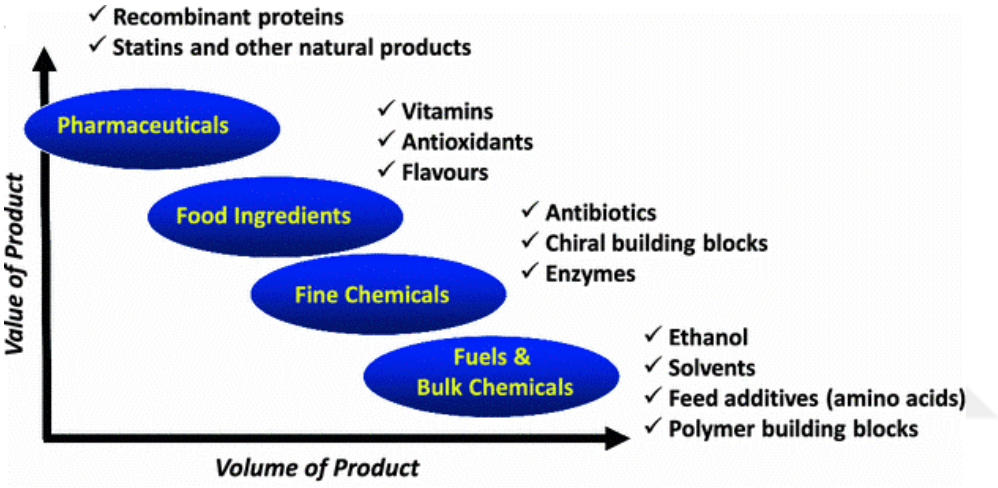


Figure 1.2 : Biotechnological product range of *S. cerevisiae* with the aspect of high value-added to low value-added products (Hong & Nielsen, 2012).

1.4 Potassium Homeostasis in *S. cerevisiae*

Potassium is an important alkali metal cation for many cellular functions, such as osmoregulation, regulation of pH, stable potential maintenance across the plasma membrane, enzyme activation, protein translation, pyruvate synthesis, and compensation of negative charges in macromolecules (Ariño et al., 2010; Cyert & Philpott, 2013). In contrast to sodium and lithium, potassium ion (K^+) is not toxic to the cell. Thus, it is highly accumulated in different types of cells including *S. cerevisiae*. Under normal growth conditions, *S. cerevisiae*'s intracellular potassium ion content is around 200-300 mM, and it requires ~ 30 mM K^+ for survival (Cyert & Philpott, 2013). *S. cerevisiae* is able to grow at external K^+ concentrations of 10 µ M to 2.5 M (Ariño et al., 2010). Despite Na^+ being highly abundant in the environment,

it is also highly toxic. Thus, cells selectively accumulate K^+ and extrude Na^+ to maintain a high intracellular ratio of K^+/Na^+ (Cyert & Philpott, 2013). Therefore, it is highly important to maintain a sufficient amount of intracellular K^+ concentration inside the cell. This balance is regulated by electro-chemical forces and proton fluxes across the plasma membrane (Kahm et al., 2012). K^+ is taken up and extruded by the cells via plasma membrane transporters (Figure 1.3). These transporters are coordinated and act together to maintain membrane potential, eliminate toxic cations (sodium, lithium), regulate turgor pressure, and help cells to cope with osmotic stress (Ariño et al., 2010).

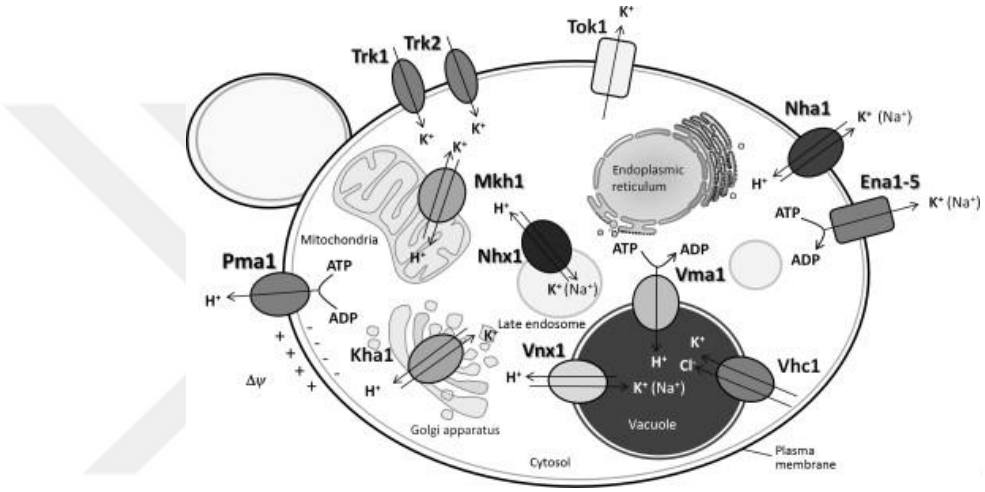


Figure 1.3 : The major transporters for uptake and efflux of alkali metal ions (Ariño et al., 2014).

The activity of most of these transporters is driven by proton gradient and membrane potential. The potential is generated by proton pumps. Plasma membrane (Pma1) and vacuolar (Vma1) H^+ ATPase are responsible for this activity (Figure 1.3) (Ariño et al., 2014). They regulate the concentration of protons in the cytosol (Cyert & Philpott, 2013). After many enzymatic reactions, cytosolic CO_2 is produced which is later converted into carbonic acid by the carbonic anhydrase enzyme. Carbonic acid is dissociated into HCO_3^- (bicarbonate) ions and protons (H^+). Pma1 can pump excess protons out of the cell. However, HCO_3^- remains inside and creates a negative charge.Therefore K^+ retention becomes vital to restoring the charge balance (Cyert & Philpott, 2013; Kahm et al., 2012).

The influx of K^+ is mediated by three classes of potassium transporters: the ACU (alkali cation uptake), the HAK (high-affinity K^+) transporters ATPases, and the TRK (transport of K^+) family. The ACU and HAK are less abundant and characterized

systems (Ariño et al., 2019). The members of the TRK family, Trk1 and Trk2, are two main uniporters responsible for necessary K^+ uptake. Trk1 is the major plasma membrane transporter, its activity plays a major role in pH homeostasis (Yenush et al., 2002). On the other hand, Trk2 is founded later and it was revealed that it has less effect on K^+ uptake (Petrezsélyová et al., 2011). Change in external potassium concentration also triggers uptake of K^+, a model proposed by Kahm et, al. (2012) suggests that an unidentified system would sense external potassium concentration change and triggers K^+ uptake coupled with Pma1 activity as a regulator (Kahm et al., 2012). Furthermore, Trk1 is a good target candidate for antifungal studies. Since the *TRK1* gene is responsible for this protein, it is present in all yeast, however, it has no homology with animal cells (Llopis-Torregrosa et al., 2016).

The export of K^+ is maintained by three different membrane transport systems. Tok1 is a voltage-gated channel that selectively effluxes K^+ over Na^+. It opens when the membrane potential is positive (Bertl et al., 1993). The other two transporters are Nha1 and Ena1. They export both toxic cations (Na^+, Li^+, Rb^+) and excess K^+ (Ariño et al., 2014). Nha1 is an antiporter that utilizes a gradient generated by Pma1 ATPase (Kinclová et al., 2001). The activity of Nha1 is pH-dependent, it operates under acidic conditions. The K^+ efflux activity of Nha1 is significant within a high concentration K^+ containing environment. Its activity is considered a rapid response of the cell against osmotic or salt stress (Ariño et al., 2019).

Therefore, Nha1 has a role in the regulation of osmotic pressure and also internal pH (Bañuelos et al., 2002). When the osmotic stress is caused by high concentrations of glucose or sorbitol, the activity of Nha1 is truncated to hinder potassium efflux to prevent water efflux (Ariño et al., 2019). The third transporter is Ena1 ATPase which hydrolyses ATP for transport. At high pH, Ena1 extrudes toxic cations (Na^+, Li^+, Rb^+) and K^+. Its function is characterized as detoxification (Ariño et al., 2010). *S. cerevisiae* cells usually contain various copies of *ENA* genes. The copy number is varied for different strains. For instance, S288C, A364A, and D273-10B strains contain five *ENA* repeats (*ENA*1-5) (Ariño et al., 2019). On the other hand, CEN.PK113-7D strains contain only one repeat (*ENA6)*. This atypical structure leads to hypersensitivity of CEN.PK113-7D against Na^+ and Li^+ toxicity (Daran-Lapujade et al., 2009). This hypersensitivity is specific to sodium and lithium ions. CEN.PK113-7D is not hypersensitive to K^+ or osmotic pressure (Daran-Lapujade et al., 2009).

These transport systems also exist at organellar membranes (Figure 1.3). Individual organelles also maintain their organellar volume and pH with potassium homeostasis (Ariño et al., 2010). Especially, vacuolar transport has significance for potassium homeostasis. Two antiporters (Nhx1 and Vnx1) arbitrate cation accumulation in vacuolar lumen. In addition, mitochondria also contain a potassium transfer system. This system enables the efficient exchange of protons for K^+ which is important for maintaining mitochondrial physiology (Ariño et al., 2014).

1.4.1 Regulation of potassium homeostasis in *S. cerevisiae*

The maintenance of potassium homeostasis is regulated by multiple signaling pathways at the post-transcriptional level (Figure 1.4) (Ariño et al., 2010). This regulation is largely mediated by phosphorylation/dephosphorylation of ion transporters. Mass spectroscopy analyses revealed that the aforementioned transporters have multiple phosphorylation sites. Several phosphates and protein kinases are involved in this regulatory mechanism (Ariño et al., 2014). For example, Ppz1 and Ppz2 are serine/threonine phosphatases that regulate Trk1,2 and Ena1,5 transporters. Their expression levels have also an effect on salt tolerance, cell wall integrity, and cell cycle regulation (Yenush et al., 2002). The activity of Ppz is negatively controlled by Hal3/Vhs3. They function as inhibitory subunits by binding the catalytic domain of the phosphatase (Nadal et al., 1998; Ruiz et al., 2004).

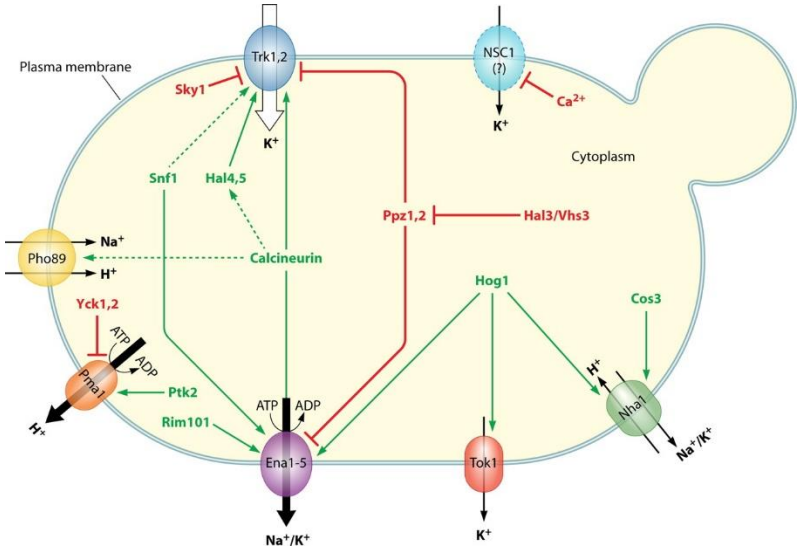


Figure 1.4 : Regulatory signaling network of potassium homeostasis (discontinuous lines represent not fully elucidated interactions) (Ariño et al., 2010).

Other regulatory elements of Trk1,2 potassium transporters are Hal4 and Hal5 protein kinases. They activate the Trk1,2 transporters and enhance uptake of K^+ (Mulet et al., 1999). The K^+ uptake results in a decrease in membrane potential and improve tolerance against toxic salts by reducing the driving force for their uptake (Mulet et al., 1999). Another regulatory mechanism involves osmotic stress and is controlled by High Osmolarity Glycerol (HOG) pathway. This pathway has a role in the regulation of Nha1, Tok1, and Ena family transporters (Figure 1.4) (Ariño et al., 2010). Hog1 MAP kinase is activated upon osmotic stress which phosphorylates Tok1 and Nha1 and stimulates their activity. This activation creates an initial response against hyperosmotic stress (Proft & Struhl, 2004).

1.5 Osmoregulation and Hyperosmotic Stress in *S. cerevisiae*

When solute concentration around the cells changes dramatically, cells are subjected to osmotic stress conditions. Exposure to high osmolarity causes low availability of water (Blomberg & Adler, 1992). Upon high osmolarity, cytoplasmic water flows out of the cell (Figure 1.5). This water loss results in a decrease in turgor pressure which is enforced by the plasma membrane upon the cell wall (Varela & Mager, 1996). The loss of or complete abolishment of turgor pressure results in cell shrinkage (Babazadeh, 2014).

Since water activity is vital for all biochemical reactions, cells tend to initiate a number of cellular responses to overcome the effect of hyperosmotic stress and repair the cellular damage (Hohmann, 2002). To re-establish water balance, *S. cerevisiae* cells accumulate compatible osmolytes such as glycerol. Other than glycerol, several molecules can also act as osmolytes like polyols, sugars, amino acids, urea, and methylamines (Yancey, 2005). Glycerol accumulation results in water regain and positive turgor pressure (Figure 1.5) (Blomberg, 2000; Hohmann, 2002). After cells reduce the effect of osmotic shock, they can continue to grow under hyperosmotic conditions (Babazadeh, 2014).

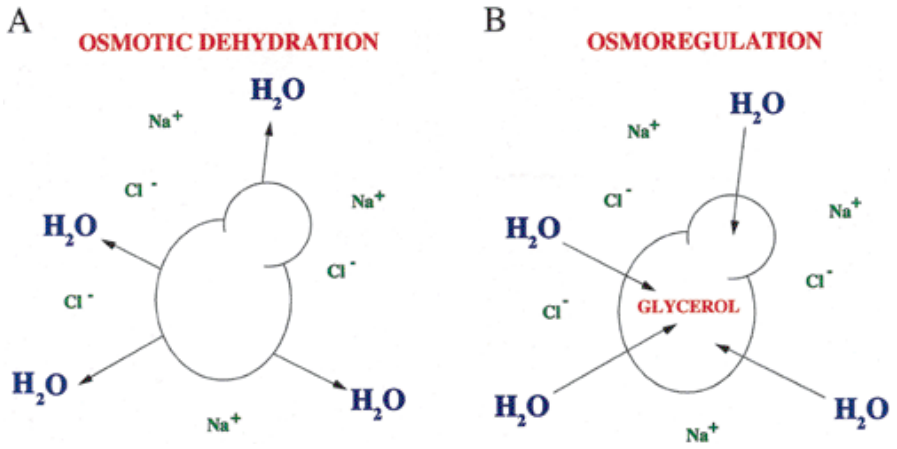


Figure 1.5 : Cellular response of yeast to osmotic shock. A: upon osmotic dehydration, cell loses water. B: within the osmotic regulatory mechanism, glycerol accumulation occurs which leads to water regain and cell volume increase (Blomberg, 2000).

However, when the effect of osmotic stress diminishes or cells are exposed to dilution stress, *S. cerevisiae* cells release the accumulated glycerol into the medium to prevent more water uptake and cell swelling (Figure 1.6). This phenomenon is also known as a hypo-osmotic shock or osmotic downshift (Hohmann, 2002; Varela & Mager, 1996).

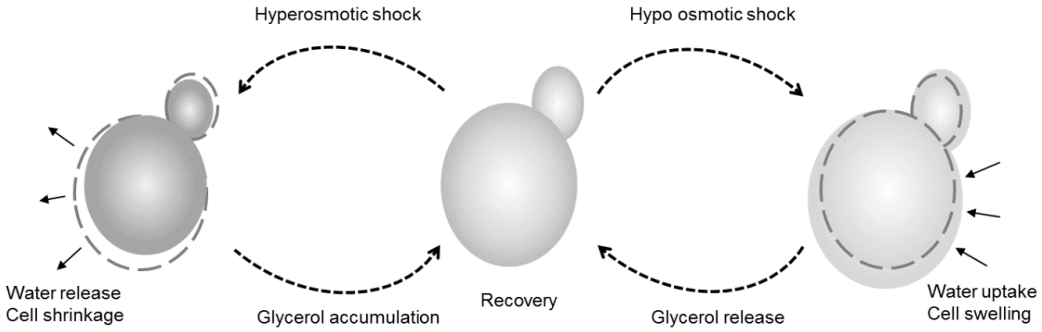


Figure 1.6 : Osmotic regulation in *S. cerevisiae* (Babazadeh, 2014).

1.5.1 Signal transduction involved in osmoadaptation

The osmoadaptation mechanism of *Saccharomyces cerevisiae* has been studied extensively, as resistance against osmotic stress has industrial importance (Hohmann, 2002), especially for wine and food industries (Tilloy et al., 2014). In addition, regulatory mechanisms that control osmoadaptation are conserved among the eukaryotes, which increases scientific interest in osmoregulation (Hohmann, 2002).

When osmolarity in the medium changes, osmosensor transmembrane proteins sense the osmotic stress and trigger cell response. Sln1p and Sho1p are two proteins that sense osmotic change and interact with the HOG pathway (Hohmann, 2002; Nevoigt

& Stahl, 1997). Two branches of upstream sensors: Sln1p and Sho1p activate Ssk2p or Ssk22p and Ste11p (MAPKKKs) mitogen-activated protein (MAP) kinase kinase kinases which in turn phosphorylate Pbs2p (MAPKK). Phosphorylated pbs2p later activates Hog1p (MAPK) (Figure 1.7) (Nadal et al., 2011; Hohmann, 2002; Varela & Mager, 1996).

Activation of Hog1p (MAP) kinase mediates the expression of many genes. Activated Hog1 can enter the nucleus and interact with DNA binding proteins like Hot1, Msn2/4, and Sko1 (Figure 1.8) (Babazadeh, 2014; Rep et al., 2001). These transcription factors control the transcription of hundreds of genes and play a major role in yeast's stress response (Capaldi et al., 2008; Nadal et al., 2011; Rep et al., 2001; Sadeh et al., 2011).

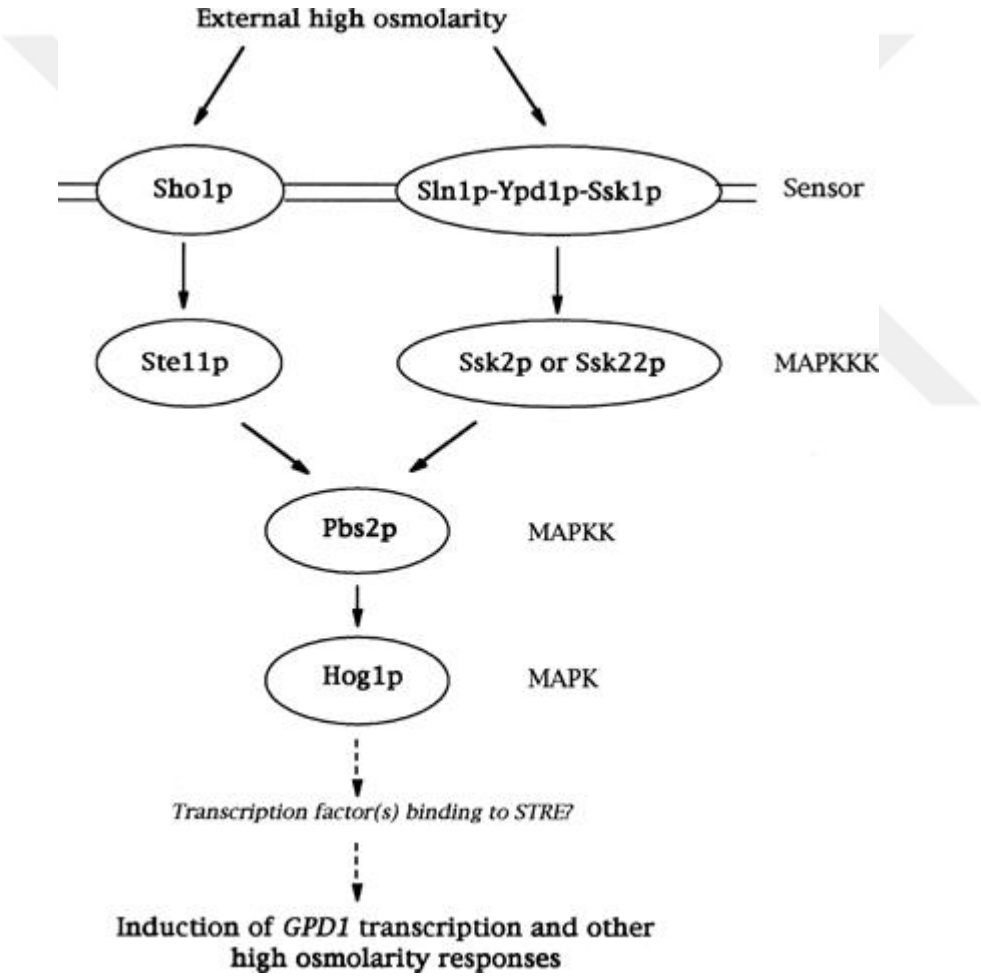


Figure 1.7 : High osmolarity signal pathway model in *S. cerevisiae* (Nevoigt & Stahl, 1997).

Hot1 is especially significant. It regulates the transcription of glycerol 3-phosphate dehydrogenase which is encoded by the *GPD1* gene (Navarro-Aviño et al., 1999). As explained earlier synthesis and accumulation of glycerol is crucial for osmotic stress

tolerance (Figure 1.8). Capaldi et al. (2008) also showed that in KCl hyperosmotic stress (0.4 M KCl) activates the HOG pathway and genes controlled by Msn2/Msn4 are also transcribed for general stress response (Capaldi et al., 2008).

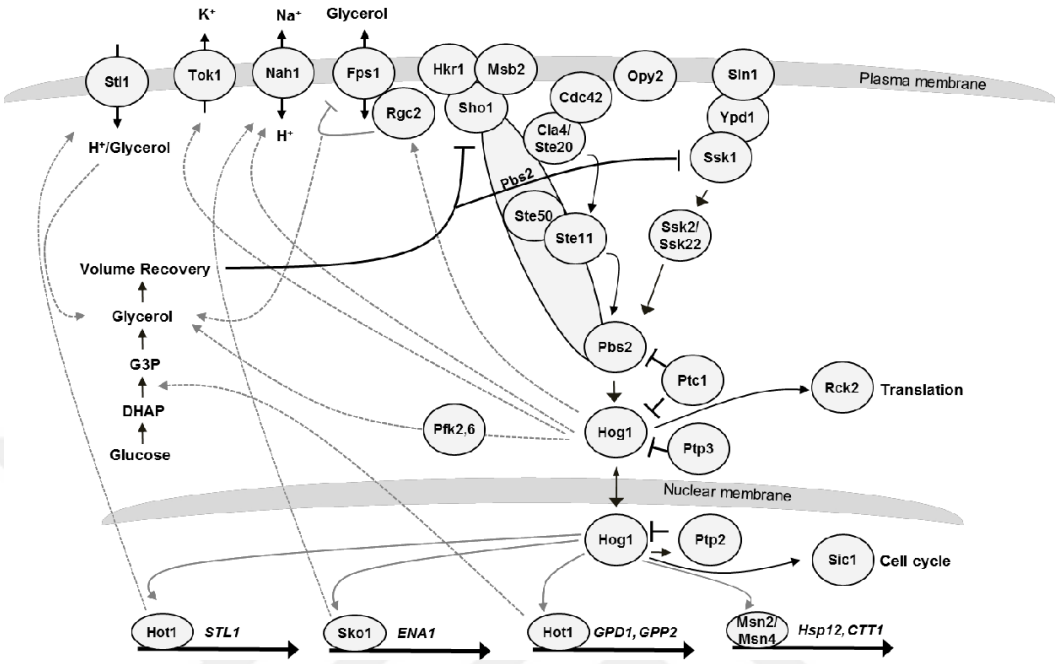


Figure 1.8 : HOG pathway overview (Babazadeh, 2014).

1.6 Metabolic and Evolutionary Engineering

The concept of metabolic engineering was first introduced by Jay Bailey in 1991 (Bailey, 1991). Metabolic engineering aims to improve the industrial performance of organisms by manipulating their cellular functions with the use of modern genetic tools (Bailey, 1999; Çakar, 2009; Nevoigt, 2008). These manipulations are targeted and knowledge-based manipulations to direct metabolic fluxes (Nevoigt, 2008). With the use of metabolic engineering; an organism's ability to produce naturally formed metabolites that have high industrial value can be improved, the ability to utilize atypical substrate can be introduced, or stress tolerance can be enhanced (Çakar et al., 2012; Nevoigt, 2008). To make these genetic manipulations, extensive information about metabolic pathways, enzymes, and their regulation is needed, and this approach is called rational metabolic engineering. Although there are some successful applications of rational metabolic engineering, due to the complex nature of metabolic networks, its hard to predict the outcome of the targeted modifications. Therefore, alternative approaches like inverse metabolic engineering and evolutionary engineering were developed (Çakar et al., 2012; Nevoigt, 2008).

Inverse metabolic engineering is a subdiscipline of metabolic engineering also introduced by Bailey (Bailey et al., 1996). It is described as “first, identifying, constructing, or calculating the desired phenotype; second, determining the genetic or the particular environmental factors conferring that phenotype; and third, endowing that phenotype on another strain or organism by directed genetic or environmental manipulation” (Bailey et al., 1996) (Figure 1.9). The inverse metabolic engineering follows a “bottom-up” strategy. It starts with the identification of the desired phenotype which is the last step of rational metabolic engineering (Çakar et al., 2012). The desired phenotype can be obtained by changing the environmental conditions of the host organism, finding naturally evolved strains that have a desired phenotype, or applying evolutionary engineering (Nevoigt, 2008). The second step involves the determination of the genetic basis of the phenotype. Genomics, proteomics, transcriptomics, and metabolomics analyses are branches of “omics technology" which utilize a systems-level approach to help identify cellular networks (Kim et al., 2012). Finally, in the last step, the potential genes determined by the second step are verified by genetic engineering (Nevoigt, 2008).

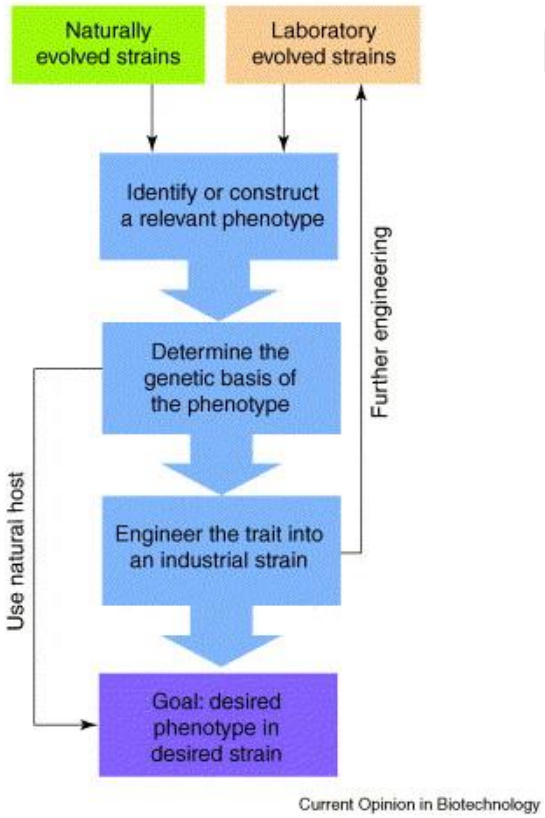


Figure 1.9 : Inverse metabolic engineering schematic diagram (Gill et al., 2003).

Evolutionary engineering is usually applied during the first step of inverse metabolic engineering strategies (Figure 1.9) (Çakar et al., 2012). The selection procedure is applied for generating the desired phenotype. With evolutionary engineering, genetic diversity is created by applying selective pressure in repeated batch cultivations. Throughout the selection procedure, fitter variants are formed and selected because fitter variants survive better than the starting population. For improving genetic variations, chemical mutagens or physical mutagens like UV light can be used (Çakar et al., 2012). *S. cerevisiae* is an industrially relevant microorganism for many biotechnological applications. Evolutionary engineering of stress resistance in *S. cerevisiae* creates great opportunities for the industry: multiple stress-resistant (Çakar et al., 2005), ethanol-resistant (Turanlı-Yıldız et al., 2017), phenolic inhibitor-resistant (Hacısalihoğlu et al., 2019), osmotic stress-resistant (Betlej et al., 2020; Tilloy et al., 2014) yeast strains have been successfully obtained using evolutionary engineering.

1.7 The Aim of the Study

In this study, evolutionary engineering was implemented to obtain KCl-induced hyperosmotic stress-resistant *S. cerevisiae* strains. Potassium ion is utilized in many cellular functions and is important in maintaining membrane potential. Although KCl salt is usually not toxic to *S. cerevisiae*, high concentrations of KCl create osmotic stress. The aim of this study was to investigate the KCl-induced hyperosmotic stress resistance in *S. cerevisiae*, using evolutionary engineering. For this purpose, a successive batch selection procedure was applied to the reference yeast strain without polar mutagenesis, in the presence of gradually increased KCl stress levels. After obtaining the KCl-resistant evolved strains, their physiological and metabolic analyses were performed, in comparison with the reference strain, to identify the key physiological and metabolic differences in the KCl-resistant, evolved strains.

2. MATERIALS AND METHODS

2.1 Materials

2.1.1 Strains

S. cerevisiae CEN.PK 113-7D (*MATa, MAL2-8c, SUC2*) strain which was kindly provided by Prof. Dr. Jean Marie François and Dr. Laurent Benbadis (University of Toulouse, France) is used as a reference strain. The reference strain has been renamed as "905".

2.1.2 Growth media

In this study, two different media were used for growth. Yeast complex medium (YPD) was mainly used for revival (Table 2.1), and Yeast minimal medium (YMM) was used for preculture and growth (Table 2.2).

Table 2.1 : Yeast extract peptone dextrose medium (YPD) composition.

Ingredient	Amount
Yeast Extract	10 g
Dextrose	20 g
Peptone	10 g
Agar (for solid media)	20 g
Water	to 1 L

Table 2.2 : Yeast minimal medium (YMM) composition.

Ingredient	Amount
Yeast Nitrogen Base without amino acids	6.7 g
Dextrose	20 g
Agar (for solid media)	20 g
Water	to 1 L

2.1.3 Chemicals

The chemicals used in this study are listed in Table 2.3

Table 2.3 : Chemicals used in this study.

Chemical	Supplier
Agar	BD Difco™
Yeast nitrogen base without amino acids	BD Difco™
Glycerol	Duchefa Biochemie
Dextrose	Riedel-de-Haen
Acetic acid	Merck
Ethanol	J.T Baker
Yeast extract granulated	Merck
Aluminum chloride hexahydrate	Merck
Potassium chloride	Merck
Sodium chloride	Merck
Lithium chloride	Sigma
Nickel(II) chloride hexahydrate	Merck
Manganese (II) chloride tetrahydrate	Merck
Ammonium chloride	Riedel-de Haen
Calcium chloride	Merck
D-Sorbitol	Sigma
Hydrogen peroxide	Merck
Caffeine	Merck
Tetramethylammonium chloride	Sigma
Spermine	Merck
Hygromycin B	Gibco™

2.1.4 Laboratory equipment

The laboratory equipment used in this study is listed in Table 2.4.

Table 2.4 : Laboratory equipment used in this study.

Equipment	Supplier
Autoclave	GR 110 GF-Zealway
Balance	Precisa XB220A
Deep freezer	-80℃ SANYO UltraLow
HPLC	Shimadzu Series 10A
HPLC column	Bio-Rad Aminex HPX-87H
Laminar Flow Cabinet	Faster BH-EN 2003
Light Microscope	Olympus CH30
Microcentrifuge	Eppendorf Microcentrifuge 5424
Orbital Shaker	Certomat S II Sartorius
UV-Visible Spectrophotometer	Shimadzu UV-1700
Vortex Mixer	Nüve NM 100

2.2 Methods

2.2.1 Screening for determination of initial KCl stress level

To determine the initial KCl stress level for evolutionary engineering, a screening experiment was performed with the reference strain (905) at various KCl concentrations.

Overnight cultures of the reference strain population were inoculated in 10 mL of YMM in 50 mL culture tubes containing KCl stress as well as the control without KCl stress. Incubation was performed at 30℃ and 150 rpm. Initial OD_{600} of cultures was set to 0.2 (approximately $2.8x10^6$ cells mL^{-1}), and cell growth was measured at 24^{th}, 48^{th}, and 72^{nd} hours. The KCl resistance level was expressed in terms of the percent survival rate. The percent survival rates were calculated by dividing OD_{600} of each stressed culture by that of their control cultures and then multiplying by 100.

2.2.2 Evolutionary engineering

Overnight cultures of the reference strain (905) were inoculated in 10 mL of YMM in 50 mL culture tubes containing 0.5 M KCl stress, as well as the control tube without

KCl stress. Incubation was performed at 30°C and 150 rpm. The initial OD_{600} of cultures was set as 0.2 and OD_{600} value was measured after 24^{th}-hour growth. After that, the stress-treated culture was named as the 1^{st} KCl-resistant population (first passage). This culture was inoculated into 0.55 M KCl stress containing YMM medium and cultivation was performed for the 2^{nd} KCl-resistant population. The same procedure was repeated for obtaining entire KCl-resistant populations and stock culture samples of each passage were prepared in 30% (v v^{-1}) glycerol and stored at -80°C. The KCl resistance level was expressed in terms of the percent survival rate. The percent survival rates were calculated by dividing OD_{600} of each stressed population by that of their control cultures and then multiplying by 100.

2.2.3 Selection of individual resistant strains

Overnight cultures of the final population (72^{nd} population) and the reference strain (905) were inoculated in 10 mL of YMM in 50 mL culture tubes. Growth was performed at 30°C and 150 rpm for 24 h. Cultures were diluted from 10^{-1} to 10^{-6} and spread onto YMM agar containing 1.75 M, 2 M, and 2.5 M KCl-containing plates and plates without KCl as a control. The growth of individual colonies was monitored at 30°C for 24, 48, 72, and 96 h.

Twelve individual colonies were randomly selected from a YMM plate containing 1.75 M KCl. After the selection of 12 individual colonies from the agar plates, each colony or evolved strain was inoculated into 10 mL of YMM in 50 mL culture tubes at 30°C and 150 rpm for 24 h. After the growth of each evolved strain, stock cultures were prepared in 30% (v v^{-1}) glycerol and stored at - 80°C. Each evolved strain was microscopically examined to verify that there was no contamination.

2.2.4 Estimation of KCl resistance of the evolved strains

2.2.4.1 Spot assay

OD_{600} values of overnight cultures of 12 individual evolved strains, the 72^{nd} population (final population) and the reference strain (905) were set to 0.2 in 10 ml YMM-containing 50 ml culture tubes. After 5-6-hours of growth, the required cell amounts for OD_{600} of 4.0 in 1 ml were prepared. For spot assay, cultures were serially diluted. Dilutions up to10^{-5} were prepared with dH_2O. YMM plates containing Control, 1.75 M, 2 M, and 2.5 M KCl were previously prepared. After that, 5 µL of each dilution

was spotted onto plates. Plates were incubated for 72 hours at 30°C. The results were then recorded.

2.2.4.2 Survival rate determination

Overnight cultures of the reference strain and five of the isolated strains were inoculated in 10 mL of YMM in 50 mL culture tubes containing 1.75 M and 2 M KCl stress, as well as the control without KCl stress. Incubation was performed at 30°C and 150 rpm. Initial OD_{600} of cultures were set to 0.2 and cell growth was measured at 24^{th}, 48^{th}, 72^{nd} hours. The KCl resistance level was expressed in terms of the percent survival rate.

Three biological replicates and three OD_{600} measurements were performed for statistical significance. The percent survival rates were calculated by dividing OD_{600} of each stressed culture by that of their control cultures then multiplying by 100.

2.2.5 Genetic stability analysis

For the determination of the genetic stability of selected KCl-resistant strains, genetic stability test was applied. Reference strain and selected evolved strains were revived in a 10 mL YPD medium-containing 50 mL culture tubes. After that, revived strains were precultured in a 10 mL YMM-medium containing 50 mL culture tubes. Overnight cultures of the reference strain and selected strains were inoculated in 10 mL of YMM in 50 mL culture tubes with no selective pressure. Incubation was performed at 30°C and 150 rpm. After 24 hours of cultivation, the cultures were inoculated into 10 ml fresh YMM medium in a 50 ml culture tube.. The same successive passaging procedure was applied for five times and stock cultures of each passage were prepared in 30% (v v^{-1}) glycerol and stored at - 80°C.

After that, spot assay was performed with the stored cultures of passage no 1, 3, and 5 in the presence of 1.75 M KCl stress, to determine the KCl resistance of each passage. For spot assay, reference strain (905) and stored strains were revived in YPD and precultured in YMM medium. OD_{600} values of overnight cultures were set to 0.2 in 10 mL YMM-containing 50 mL culture tubes. After 5-6-hours of growth, the required cell amounts for OD_{600} of 4.0 in 1 mL were prepared. For spot assay, cultures were serially diluted. Dilutions up to10^{-5} were prepared with dH_2O. YMM control plate and plates containing 1.75 M KCl were previously prepared. After that, 5 µL of each

dilution was spotted onto plates. Plates were incubated for 72 h at 30°C. The results were then recorded.

2.2.6 Determination of cross-resistance and sensitivity against other stress conditions

Genetically stable strains were tested with spot assay for their cross resistance or sensitivity against various stress types including cationic drugs like Tetramethylammonium (TMA), Spermine, Hygromycin B and different pH values. The stress conditions were follows: 0.7 M NaCl, 5 mM LiCl, 0.8 M $CaCl_2$, 0.5 mM $NiCl_2$, 7.5 mM $AlCl_3$, 0.75 mM H_2O_2, 10 % ethanol, 15 mM caffeine, 1.5 M sorbitol, 1 M NH_4Cl, 15 mM $MnCl_2$, 0.5-0.8-1 M TMA, 1.2-2-5 mM Spermine, 125 µg/mL Hygromycin B, and pH 3.5, 5.5, and 7.5.

Reference strain (905) and genetically stable evolved strains were taken from -80°C deep freezer stocks. After that, strains were inoculated into YPD medium. 100 µL of cell suspensions were transferred to 10 mL YPD medium-containing 50 ml culture tubes. The samples were incubated at 30°C for 24 h. After 24-hour cultivation, OD_{600} values were measured using a spectrophotometer, before preculture preparation. Precultures were made with YMM medium. Thus, centrifugation at 10000 g for 10 min was performed and supernatants (YPD medium) were discarded. For precultures, the OD_{600} values were set at 0.2 in 10 mL. Precultures were incubated overnight. For spot assay, cells had to be in their mid-exponential phase. For this purpose, after overnight cultivation, OD_{600} values were again set at 0.2 in 10 mL. After 5-6-hours of growth, the required cell amount for OD_{600} of 4.0 in 1 mL were prepared. For spot assay, cultures were serially diluted. Dilutions up to10^{-5} were prepared with dH_2O.

YMM plates and plates containing different stress factors were previously prepared. After that, 5 µL of each dilution were spotted onto plates. Plates were incubated for 72 h at 30°C. The, results were then recorded.

2.2.7 Growth profiles and cell dry weight (CDW) analysis

For the growth profile analysis, selected evolved strains and the reference strain (905) were revived in the YPD medium. After overnight pre-culturing in YMM medium, cells were inoculated into 1 L Erlenmeyer flasks containing 200 ml of YMM medium with and without 1.25 M KCl stress, to start the culture at an initial OD_{600} ~ 0.2.

Incubation was performed at 30℃ and 150 rpm for 72 h. At specific time intervals, culture samples were taken for OD_{600} measurements using a spectrophotometer and for cell dry weight (CDW) measurements.

For cell dry weight analysis, microcentrifuge tubes and 50 ml tubes were dried at 80 ℃ for 48 h and pre-weighed. At specific time intervals, 1.5 ml of the culture were transferred to pre-weighed microcentrifuge tubes and centrifuged at 10000 g for 5 min. The obtained pellets were washed with ddH_2O and centrifuged at 10000 g for 5 min. At 72^{nd} hour (the final measurement) 40 ml samples were transferred to 50 ml pre-weighed tubes instead of microcentrifuge tubes and centrifuged and washed using the same method. The pellets were then dried at 80 ℃ for 48 h and weighed for CDW calculation. Both growth profile and cell dry weight analyses were performed as three biological repeats, for statistical significance.

2.2.8 Metabolite analyses

The metabolites in culture samples such as ethanol, glycerol, acetate, and residual glucose were analyzed using high-performance liquid chromatography (HPLC) (Shimadzu Series 10A HPLC, Shimadzu Co., Kyoto, Japan). 5 mM H_2SO_4 was used as the mobile phase and HPX-87H Aminex ion-exclusion column (300 x 7.8 mm; Bio-Rad Laboratories, CA, USA) was used as the stationary phase. The flow rate of the mobile phase was set to 0.6 ml^{-1} and the column temperature was set to 65° C. Before analyzing the culture samples, standard curves of the metabolites of interest were obtained using standard solutions containing a mixture of metabolites at certain concentrations (Table 2.5). For the analysis, 1 ml of culture samples taken at specific time intervals were centrifuged at 10000 g for 5 min. The supernatant was filtered through a 0.22 μm pore-size filter, and 20 μl of the filtered sample was used by the device for the separation and detection of the metabolites, using the Shimadzu RID-10A refractive-index detector. Analyses were performed as three biological repeats, for statistical significance.

Table 2.5 : Standard solutions and their metabolite concentrations for HPLC analysis.

Standards	Glucose(g/L)	Glycerol(g/L)	Acetate(g/L)	Ethanol(g/L)
Std1	20	1	2	15
Std2	15	0.75	1.5	11.25
Std3	10	0.5	1	7.5
Std4	5	0.25	0.5	3.75
Std5	2.5	0.125	0.25	1.875
Std6	1.25	0.0625	0.125	0.9375

2.2.9 Storage carbohydrate (trehalose and glycogen) content analysis

Intracellular trehalose and glycogen content was measured using HPLC, as described previously (Divate et al., 2017; Parrou & François, 1997) with slight modifications. Samples from the growth experiment were used for storage carbohydrate analysis. After 72 h growth in 1 L Erlenmeyer flasks containing 200 ml of YMM medium with and without 1.25 M KCl stress, 1 ml samples were collected for each of the trehalose and glycogen analysis, by centrifugation at 10000 g for 5 min.

For the trehalose measurement, centrifuged samples were washed with dH_2O and centrifuged at 10000 g for 5 min. After that, the samples were dissolved in 1 ml dH_2O and incubated at 95 ℃ for 1 h. After incubation, samples were centrifuged at 10000 g for 5 min. Supernatants that contain trehalose were analyzed by HPLC (Shimadzu Series 10A HPLC, Shimadzu Co., Kyoto, Japan) with the same working conditions as described in Metabolite Analysis (section 2.2.8).

For glycogen analysis, harvested samples were suspended in 0.25 ml 0.25 M sodium carbonate and incubated at 95 ℃ for 1 h. After incubation, 0.6 mL 0.2 M sodium acetate (pH 5.2) and 0.15 mL 1 M acetic acid were added to adjust the pH at 5.2. After that, samples were treated with 1.2 U mL^{-1} amyloglucosidase (11202332001; Roche Diagnostics) and incubated overnight at 57°C with shaking. Upon incubation, samples were centrifuged at 10000 g for 5 min. Supernatants that contain glycogen were analyzed by HPLC (Shimadzu Series 10A HPLC, Shimadzu Co., Kyoto, Japan), as described previously.

3. RESULTS

3.1 Screening for Determination of the Initial KCl Stress Level for Evolutionary Engineering

To determine the initial KCl stress level for evolutionary engineering, a screening experiment was performed with the reference strain (905) at various KCl concentrations. Previously, spot assays were performed with reference strain (905) at 1 M to 2 M (data not shown). Based on the obtained results it was decided that the KCl concentrations should be between 0.25 M and 3 M (0.25 M, 0.375 M, 0.5 M, 0.75 M, 1 M, 1.25 M, 1.5 M, 1.75 M, 2 M, 2.25 M, 2.5 M, 3 M) for determining the initial stress concentration for selection experiments.

The results of the screening experiment revealed that increasing KCl concentration decreased the survival rate (Table 3.1). 24^{th}-hour data revealed that after 0.25 M KCl concentration, survival rates started to decrease from 100 % (Table 3.2 and Figure 3.1). However, 24^{th}-hour data indicated that after 0.5 M KCl, the percent survival rate started to significantly decrease. Therefore, 0.5 M KCl stress was considered as a suitable initial KCl stress level for the selection experiment.

Table 3.1 : OD_{600} values of the reference strain (905) cultures after 24 h cultivation in YMM, in the presence of different KCl stress levels.

Concentration of KCl (M)	OD_{600} (905)
0 (Control)	5.28
0.25	5.34
0.375	4.95
0.5	4.48
0.75	2.87
1	2.32
1.25	2.01
1.5	1.49
1.75	0.77
2	0.20
2.25	0.18
2.5	0.17
3	0.14

Table 3.2 : Percent survival rates of the reference strain (905) cultures after 24 h cultivation in YMM, in the presence of different KCl stress levels.

Concentration of KCl (M)	Percent survival rate of (905)
0	100
0.25	101.12
0.375	93.79
0.5	84.90
0.75	54.45
1	43.97
1.25	38.12
1.5	28.39
1.75	14.45
2	3.91
2.25	3.49
2.5	3.20
3	2.68

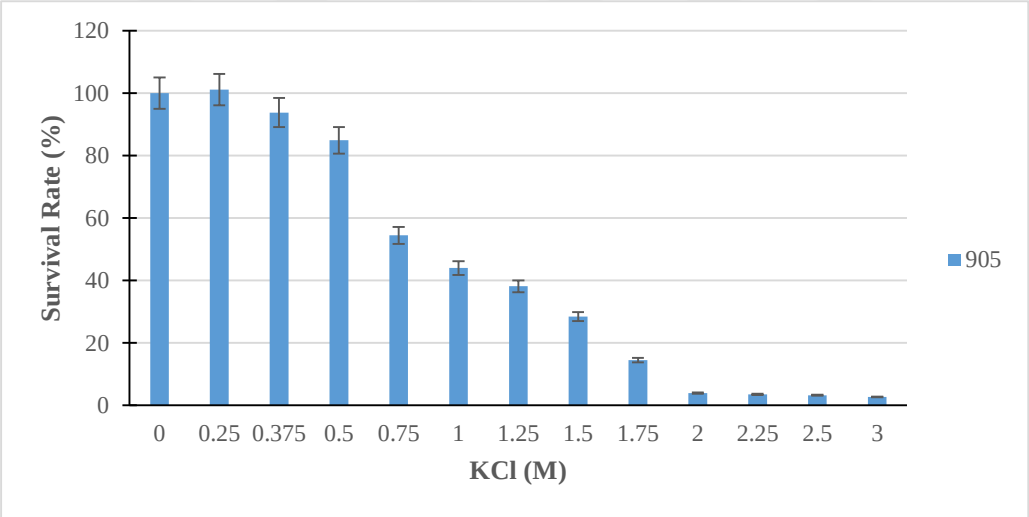


Figure 3.1 : The survival rates of the reference strain (905) cultures after 24 h cultivation in YMM with varying KCl stress concentrations.

3.2 Evolutionary Engineering of KCl Resistant Strains

Previously, screening experiment was performed to determine the inhibitory KCl stress concentration for the selection experiment. 24th-hour data of the screening experiment revealed that 0.5 M KCl concentration should be the initial stress level for the selection.

Selection of KCl-resistant strains by evolutionary engineering was performed by serial batch cultivation of the *S. cerevisiae* CEN.PK113-7D reference strain under increasing KCl stress conditions. Throughout 72 daily passages or populations, the concentration

of KCl in the medium was gradually increased from 0.5 M to 2.50 M (Figure 3.2). The stress increment level was firstly set as 0.05 M at each successive passage. It was then reduced to 0.02 M after the 22nd generation (at 1.5 M KCl stress concentration). The survival rates of each passage were calculated and are shown in Table 3.3.

Table 3.3 : KCl concentration of each passage, 24th hour OD_{600} values, and percent survival rates of stress and non-stress conditions during evolutionary engineering experiment.

Passage Number	KCl Concentration (M)	OD_{600} Control	OD_{600} Stress	Percent Survival Rate (%)
1	0.50	5.40	3.92	72.73
2	0.55	5.57	3.48	62.59
3	0.60	4.87	3.15	64.81
4	0.65	4.97	2.76	55.46
5	0.70	5.28	3.09	58.65
6	0.75	5.33	2.89	54.25
7	0.80	4.91	2.76	56.28
8	0.85	5.19	2.27	43.80
9	0.90	5.17	2.42	46.73
10	0.95	4.83	2.30	47.55
11	1.00	5.13	2.39	46.65
12	1.05	4.99	2.25	45.17
13	1.10	4.92	2.14	43.63
14	1.15	4.99	1.98	39.67
15	1.20	5.50	2.13	38.87
16	1.25	5.15	1.92	37.26
17	1.30	5.19	1.60	30.90
18	1.35	5.45	1.74	31.49
19	1.40	5.74	1.79	31.19
20	1.45	5.35	1.50	28.08
21	1.50	5.70	1.25	21.99
22	1.50	5.52	1.87	33.95
23	1.52	5.83	2.00	34.27
24	1.54	5.84	2.04	34.93
25	1.56	5.53	1.83	33.09
26	1.58	5.77	2.07	35.91
27	1.60	5.66	2.03	35.93
28	1.62	5.65	2.08	36.93
29	1.64	6.03	1.73	28.66
30	1.66	5.84	1.59	27.36
31	1.68	6.11	1.90	31.12
32	1.70	5.90	1.86	31.63
33	1.72	5.77	1.82	31.54
34	1.74	5.49	1.96	35.75
35	1.76	5.70	1.84	32.26
36	1.78	5.59	1.70	30.46

Table 3.3 : (Continued): KCl concentration of each passage, 24th hour OD_{600} values and percent survival rates of stress and non-stress conditions during evolutionary engineering experiment.

Passage Number	KCl Concentration (M)	OD_{600} Control	OD_{600} Stress	Percent Survival Rate (%)
37	1.80	5.75	1.88	32.74
38	1.82	5.45	1.65	30.25
39	1.84	5.58	1.60	28.76
40	1.86	5.53	1.70	30.86
41	1.88	5.39	1.51	28.10
42	1.90	5.10	1.57	30.75
43	1.92	5.49	1.43	26.14
44	1.94	5.42	1.57	29.00
45	1.96	5.42	1.70	31.42
46	1.98	5.43	1.79	32.91
47	2.00	5.38	1.57	29.20
48	2.02	5.55	1.58	28.56
49	2.04	5.21	1.58	30.27
50	2.06	5.58	1.50	26.96
51	2.08	4.88	1.28	26.26
52	2.10	5.57	1.52	27.38
53	2.12	5.15	1.33	25.85
54	2.14	5.27	1.19	22.64
55	2.16	5.17	1.36	26.38
56	2.18	5.24	1.26	24.11
57	2.20	5.22	1.25	24.08
58	2.22	5.32	0.92	17.41
59	2.24	5.26	1.06	20.27
60	2.26	5.43	1.16	21.41
61	2.28	5.15	1.20	23.29
62	2.30	5.03	0.97	19.34
63	2.32	5.41	1.02	18.86
64	2.34	5.25	1.07	20.53
65	2.36	5.27	0.78	14.93
66	2.38	5.03	0.81	16.18
67	2.40	5.38	0.94	17.55
68	2.42	5.11	0.70	13.71
69	2.44	5.41	0.89	16.50
70	2.46	5.07	0.63	12.55
71	2.48	4.79	0.64	13.40
72	2.50	5.15	0.85	16.48

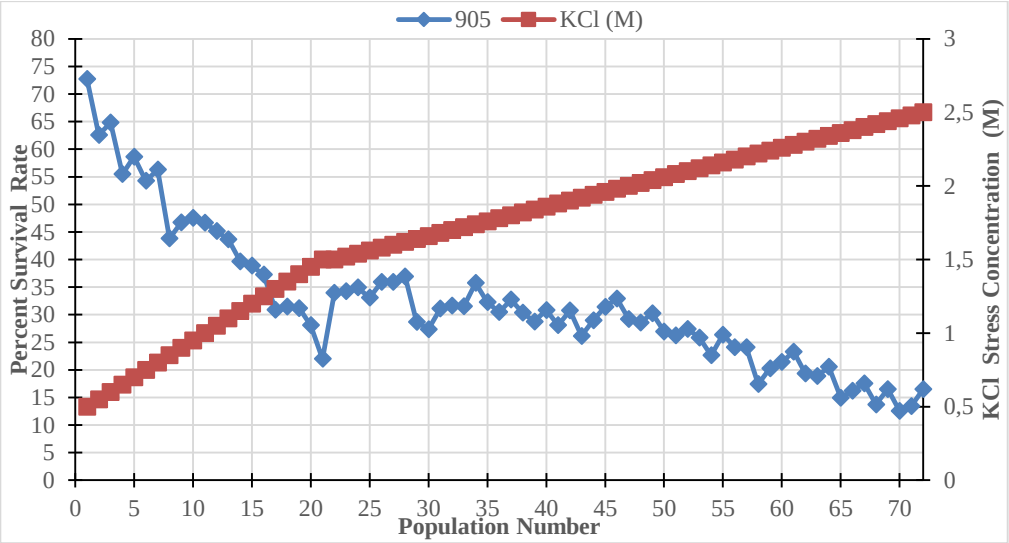


Figure 3.2 : Percent survival rate of the populations derived from the reference strain (905).

3.3 Selection and Estimation of KCl Resistance of Individual Evolved Strains Obtained from the Final Population

3.3.1 Selection of individual evolved strains

In this part of the study, 12 individual evolved strains were selected randomly from the final (72^{nd}) population. After selection, the KCl-resistance levels of the isolated strains were determined by using spot assay, and the percent survival rates of each strain were calculated upon batch cultivation.

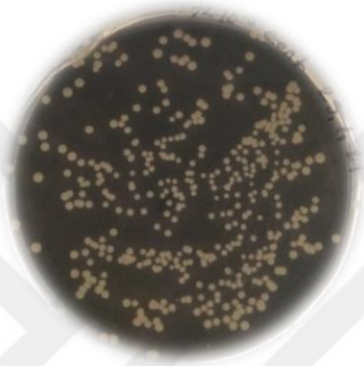
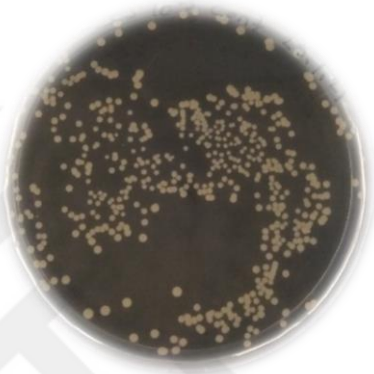
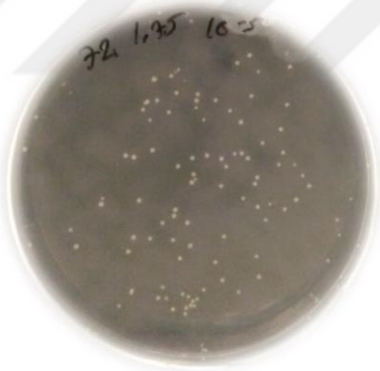
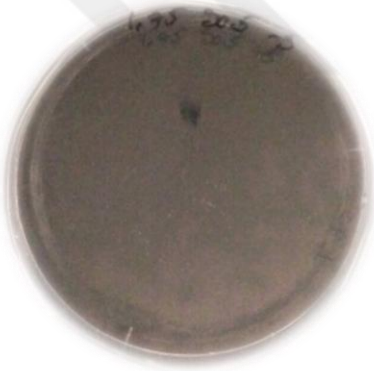
To isolate KCl-resistant individuals; the 72^{nd} population (final population) and 905 were spread onto YMM plates containing 1.75 M, 2 M, and 2.5 M KCl stress at various dilutions along with their control plates. After 96 h of growth at 1.75 M KCl at a dilution factor of 10^{-5}, it was observed that there was a difference between the final population and 905. As shown in Table 3.4 the final population at 1.75 M KCl at a dilution of 10^{-5} resulted in approximately 100 colonies. However, the 905 culture did not grow at all at the same concentration and dilution.

On the other hand, at 2 M and 2.5 M KCl no growth was observed for both the final population and 905. Therefore, the plate with 1.75 M KCl and 10^{-5} dilution was chosen for the selection of individual evolved strains.

Twelve colonies were randomly picked from the final population grown on 1.75 M KCl plate (Figure 3.3). These strains were named K1 to K12. After that, each

individual strain was stored at - 80°C as 30% (v v^{-1}) glycerol stocks. During stock preparation, samples from each strain were taken for light microscopic examination. It was morphologically confirmed that the selected strains were yeast cells, and there was no contamination. Examples of some microscopic images are shown in Figure 3.4.

Table 3.4 : Colony formation results of the final (72^{nd}) population and 905 at various KCl concentrations and dilutions.

KCl Stress Concentration and Dilution Factor	72^{nd} Population (final population)	Reference Strain (905)
Control (Dilution factor : 10^{-5})		
1.75 M (Dilution factor : 10^{-5})		

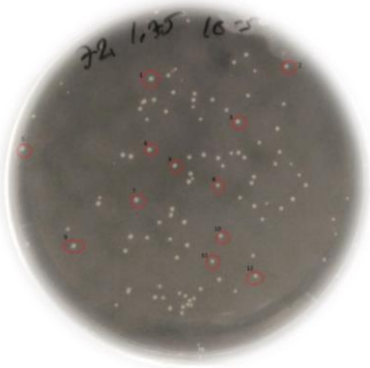


Figure 3.3 : Image of 12 randomly selected colonies on plates containing 1.75 M KCl at 10^{-5} dilution.

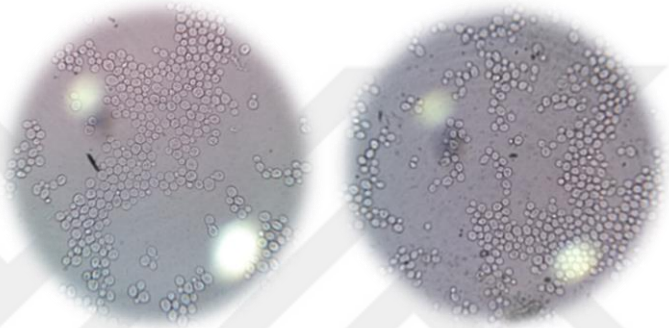


Figure 3.4 : Examples of light microscopic images of 12 randomly selected colonies (1000x magnification).

3.3.2 Estimation of KCl-resistance of individual colonies

3.3.2.1 Spot assay

After randomly selecting twelve colonies from the final population, the KCl resistance levels of isolated strains and final population were determined by spotting on KCl-containing agar plates. At 1.75 M KCl, the isolated strains could grow an average up to three spots (dilutions) within 72 h of incubation, while the reference strain could grow only by one spot (Figure 3.5). This indicates that the evolved strains gained resistance against KCl. No significant difference was observed between the isolated strains and the reference strain in terms of growth in KCl-free medium. The resistance level of the final population was also similar to those of the isolated strains. Compared to the reference strain (905), the final population (FP) and the individual evolved strains (K1-K12) all had significantly higher survival at 1.75 M KCl (Figure 3.5). On the other hand, there was no growth observed on 2 M KCl-containing culture plates (data not shown).

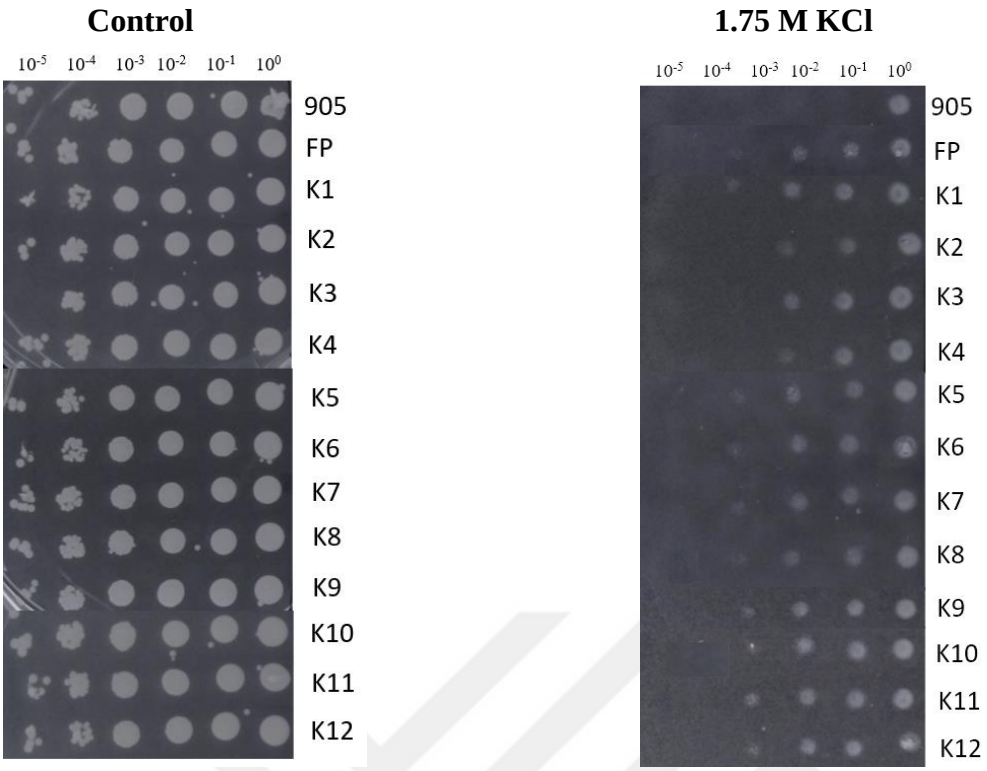


Figure 3.5 : 72nd hour spot assay results of selected individual strains (K1-K12), reference strain (905), and the final population (FP).

3.3.2.2 Determination of the percent survival rates

After obtaining the spot assay results, the percent survival rates of five selected strains were calculated at 1.75 M and 2 M KCl stress. The spot assay results indicated that K1, K9, K10, K11, and K12 showed relatively better performance. Therefore, they were chosen for the percent survival rate calculation.

At 1.75 M KCl stress, the 24-h growth results revealed that the evolutionary engineered strains had a relatively high survival rate, compared to the reference strain (Figure 3.6 and Table 3.5). There was approximately 1.5-fold improvement. The same result was also obtained at 48 and 72 hours. Among the selected strains, K9 showed slightly better performance than the other selected strains, regarding KCl resistance at 24, 48 and 72 h of growth (Figure 3.6 and Tables 3.6 and 3.7). At 2 M KCl stress, however, no growth was observed within 24 hours. After another 24 hour, reference strain and the selected strains started to grow.

The 48 h incubation results revealed that the selected strains had higher percent survival rates, compared to the reference strain (Figure 3.7 and Table 3.8). There was approximately 1.7 fold improvement. Among the selected strains, K9 also showed

better resistance than the others. The difference was clear at 2 M KCl stress. At 2 M KCl stress, K9 showed 1.9-fold higher resistance than the reference strain. The 72 h ıncubation results were also similar to the 48 h results (Figure 3.7 and Table 3.5)

Table 3.5 : OD_{600} values and percent survival rates of selected evolved strains, upon 24 h cultivation in non-stress and 1.75 M KCl stress conditions.

Concentration of KCl (M)	OD_{600} (Control)	OD_{600} (Stress)	Percent Survival Rate (%)
Ref	5.00	0.75	14.91
K1	4.89	0.98	20.10
K9	4.93	1.14	23.14
K10	5.25	0.95	18.18
K11	5.27	1.05	19.89
K12	4.72	1.03	21.89

Table 3.6 : OD_{600} values and percent survival rates of selected evolved strains, upon 48 h cultivation in non-stress and 1.75 M KCl stress conditions.

Concentration of KCl (M)	OD_{600} (Control)	OD_{600} (Stress)	Percent Survival Rate (%)
Ref	5.63	1.30	23.12
K1	5.12	1.61	31.43
K9	5.02	1.60	31.97
K10	5.30	1.50	28.30
K11	5.28	1.51	28.63
K12	5.12	1.56	30.48

Table 3.7 : OD_{600} values and percent survival rates of evolved strains, upon 72 h cultivation in non-stress and 1.75 M KCl stress conditions.

Concentration of KCl (M)	OD_{600} (Control)	OD_{600} (Stress)	Percent Survival Rate (%)
Ref	5.86	1.34	22.98
K1	5.33	1.60	30.02
K9	4.90	1.65	33.79
K10	5.31	1.58	29.93
K11	5.25	1.63	31.15
K12	5.07	1.69	33.45

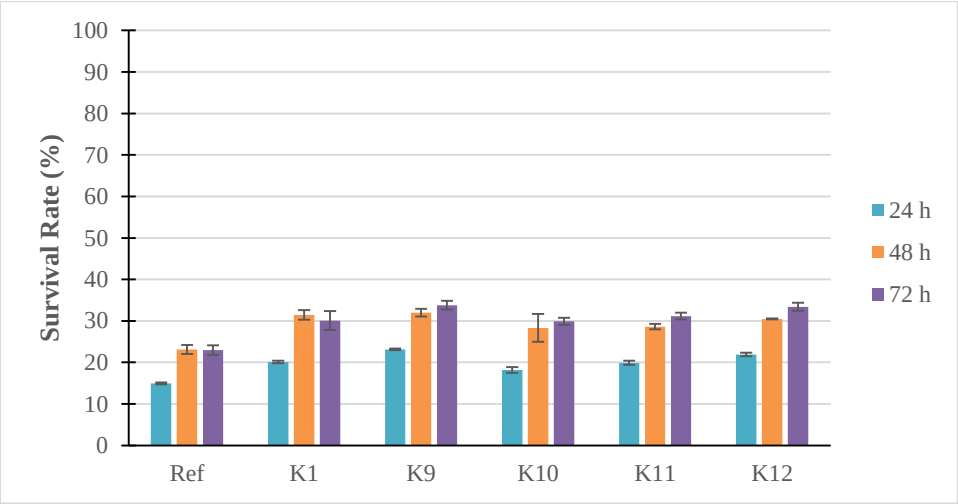


Figure 3.6 : Percent survival rates of selected evolved strains (K1-K12) and reference strain (Ref) upon 1.75 M KCl stress, at 24, 48, 72 h of cultivation.

Table 3.8 : OD_{600} values and percent survival rates of selected evolved strains, upon 48 h cultivation in non-stress and 2 M KCl stress conditions.

Concentration of KCl (M)	OD_{600} (Control)	OD_{600} (Stress)	Percent Survival Rate (%)
Ref	5.63	0.76	13.51
K1	4.91	1.11	22.75
K9	4.81	1.21	25.27
K10	5.26	1.27	24.15
K11	5.32	1.03	19.44
K12	5.16	0.97	18.93

Table 3.9 : OD_{600} values and percent survival rates of selected evolved strains, upon 72 h cultivation in non-stress and 2 M KCl stress conditions.

Concentration of KCl (M)	OD_{600} (Control)	OD_{600} (Stress)	Percent Survival Rate (%)
Ref	5.64	0.83	14.75
K1	5.12	1.30	25.41
K9	5.02	1.34	26.73
K10	5.29	1.29	24.50
K11	5.28	1.33	25.30
K12	5.07	1.29	25.51

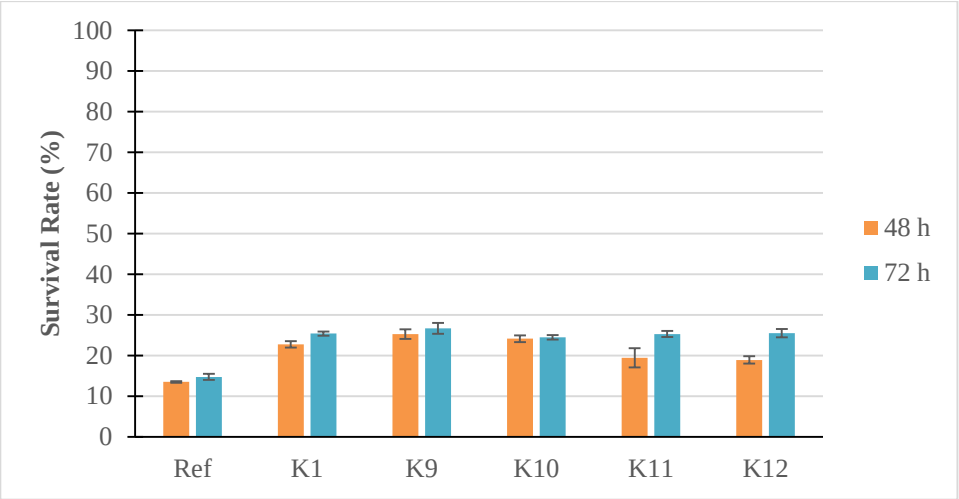


Figure 3.7 : Percent survival rates of selected evolved strains (K1-K12) and reference strain (Ref) upon 2 M KCl stress, at 48 and 72 h of cultivation.

3.4 Genetic Stability Analysis

KCl-resistant selected strains (K1, K9, K10, K11, and K12) were tested for their genetic stability. After 5 successive daily passages in YMM medium without selective pressure, it was observed that K1, K9, and K10 strains maintained their KCl resistance at 1.75 M KCl stress. However, K11 and K12 seemed to have lost their resistance against 1.75 M KCl stress after day 3. Therefore K1, K9, and K10 strains were chosen as genetically stable strains (Figure 3.8). Further analyses were continued with those strains.

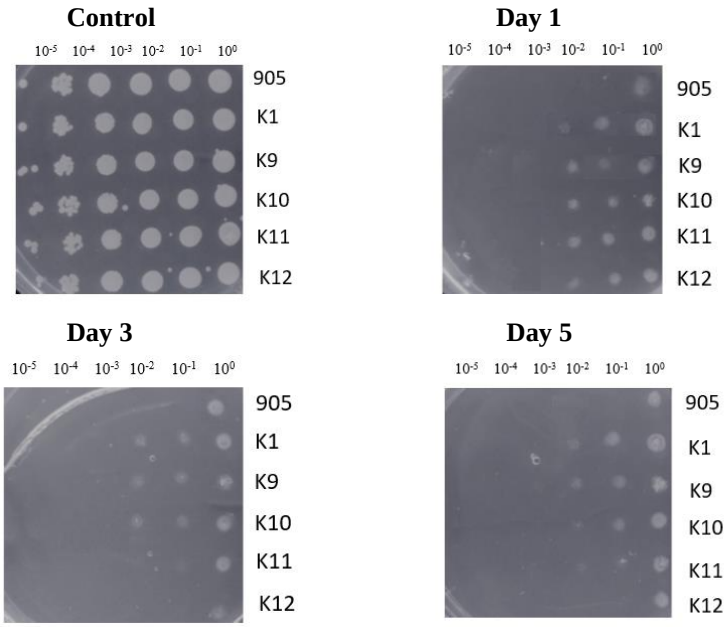


Figure 3.8 : Genetic stability analysis results of selected evolved strains, and the reference strain (905).

3.5 Cross-Resistance or Sensitivity Analyses

The genetically stable, evolved strains were tested for their cross-resistance or sensitivity against various stress types; including cationic drugs like Tetramethylammonium (TMA), Spermine, Hygromycin B and different pH values: The tested stress conditions were as follows: 0.7 M NaCl, 5 mM LiCl, 0.8 M $CaCl_2$, 0.5 mM $NiCl_2$, 7.5 mM $AlCl_3$, 0.75 mM H_2O_2, 10 % ethanol, 15 mM caffeine, 1.5 M sorbitol, 1 M NH_4Cl, 15 mM $MnCl_2$, 0.5-0.8-1 M TMA, 1.2- 2-5 mM Spermine, 125 µg/mL Hygromycin B, and pH 3.5, 5.5, 7.5.

Cross-resistance or sensitivities of K1, K9, and K10 strains were determined by spot assay. The results revealed that at 0.7 M NaCl stress, K1 and K10 showed resistance, however, interestingly K9 was extremely sensitive to NaCl stress (Figures 3.9 and 3.10). At 5 mM LiCl stress, K1 and K10 showed slight resistance. However, similar to NaCl results, K9 showed sensitivity against LiCl. On the other hand, at 0.8 M $CaCl_2$ stress, all the evolved strains (K1, K9, and K10) showed resistance (Figures 3.9 and 3.10). At 0.5 mM $NiCl_2$ stress, neither resistance nor sensitivity was observed. At 1 M NH_4Cl, all the evolved strains (K1, K9, and K10) showed slight resistance particularly at 48 h of growth (Figure 3.9). At 15 mM $MnCl_2$ stress, however, only the K9 strain showed slight resistance at 48 h of cultivation (Figure 3.9). However, at 7.5 mM $AlCl_3$ stress, only the K9 strain showed sensitivity. K1 and K10 showed neither sensitivity nor resistance according to the 72 h results (Figure 3.10).

Regarding oxidative stress (0.75 mM H_2O_2), it was observed that the K1 strain was resistant, K9 was sensitive, and K10 showed no sensitivity or resistance, according to both 48 h and 72 h results (Figures 3.9 and 3.10). Furthermore, at 10 % ethanol stress, no resistance or sensitivity was observed (Figures 3.9 and 3.10). At 15 mM caffeine stress, only the K9 strain showed slight sensitivity, K1 and K10 showed neither resistance nor sensitivity (Figure 3.10). Also at 1.5 M sorbitol, which causes osmotic stress, the evolved strains showed slight resistance based on 48^{th}-hour results (Figure 3.9).

To summarize, based on the 72 h incubation results, it was observed that the evolved strains were generally significantly cross-resistant to NaCl and $CaCl_2$ stress, with the exeption of K9, that was sensitive to NaCl stress. K9 strain showed sensitivities to other stress types as well, including LiCl $AlCl_3$ and caffeine stres

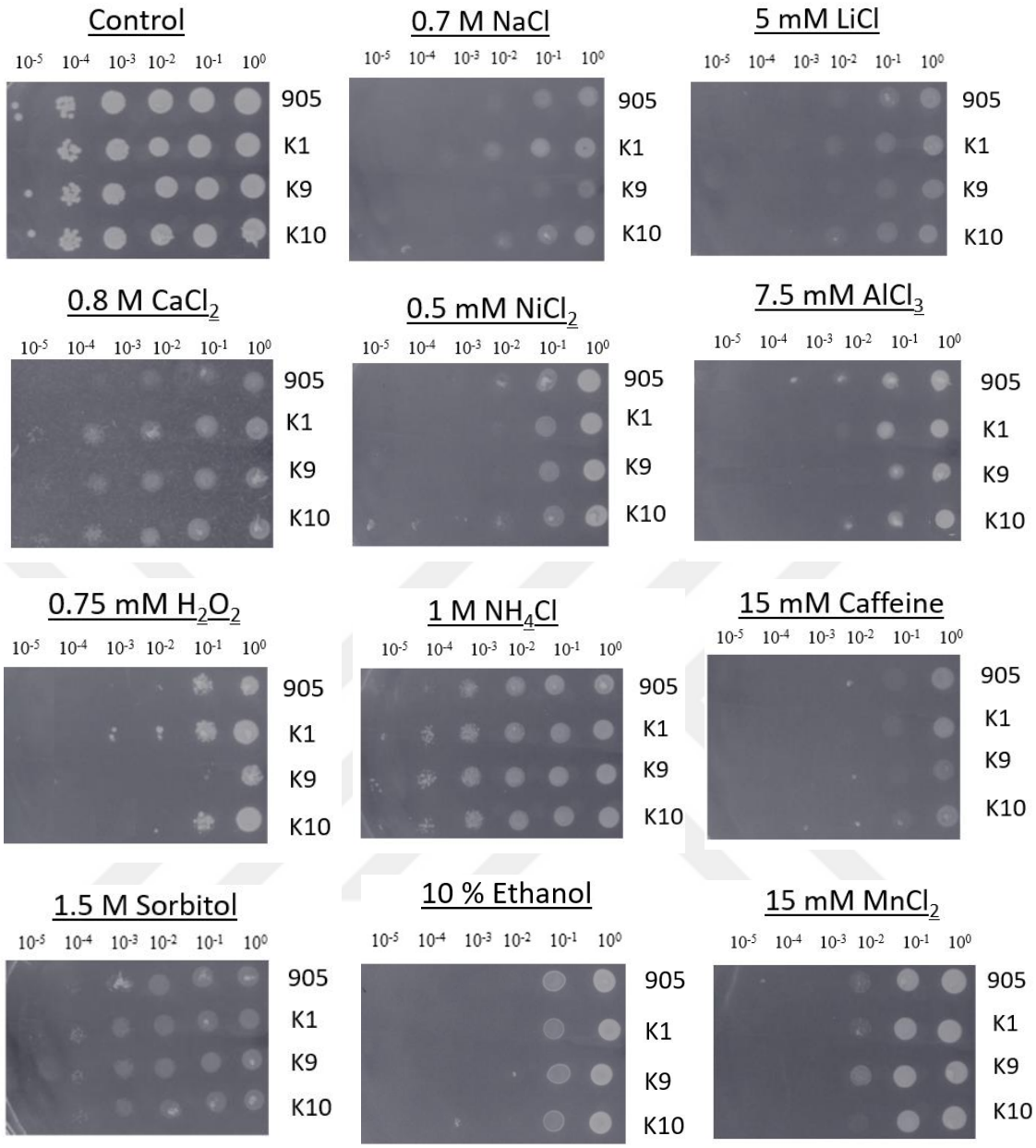


Figure 3.9 : 48th hour cross-resistance results of K1, K9, K10 strains and the reference strain (905), against different stress conditions, using spot assay.

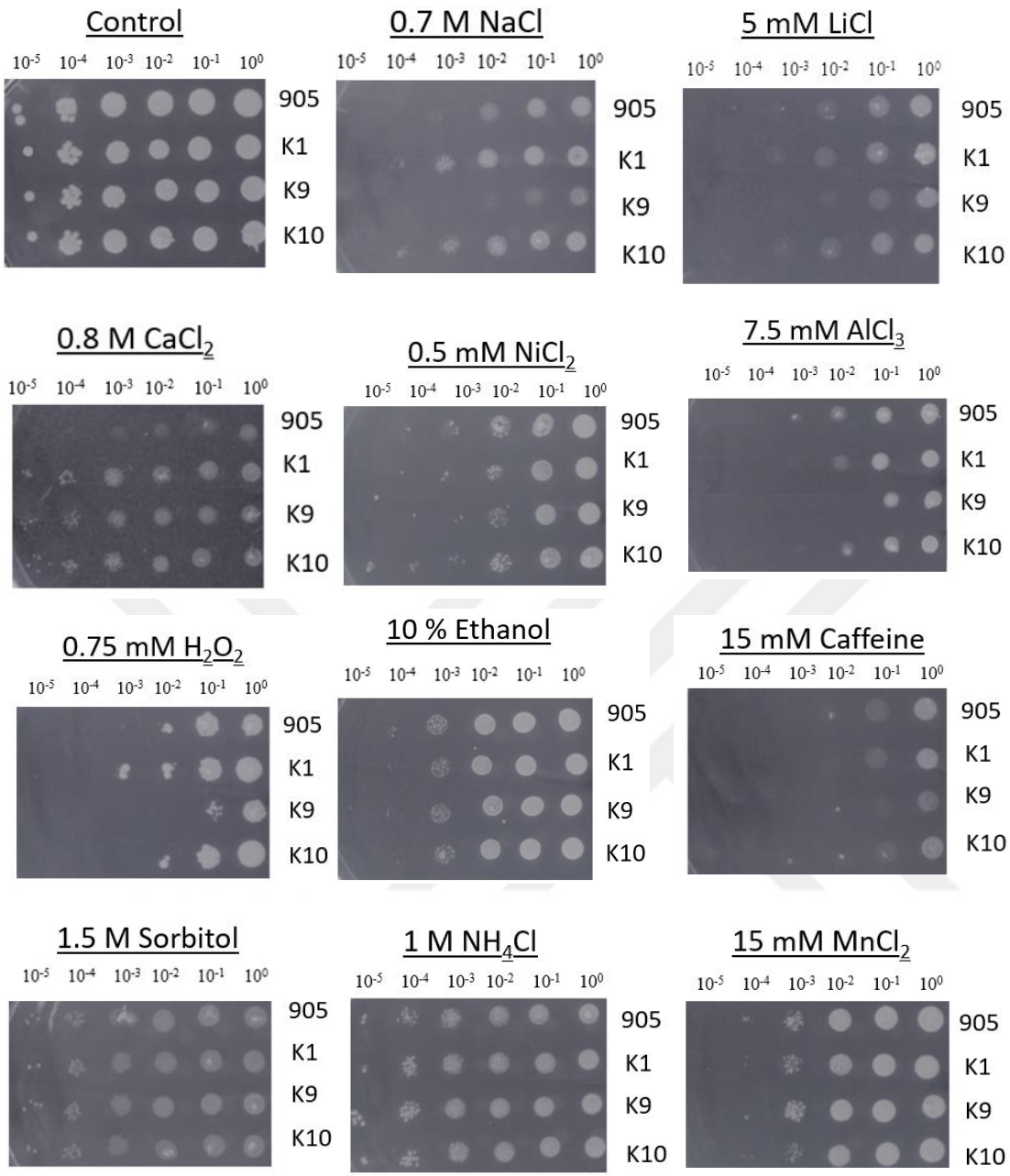


Figure 3.10 : 72nd hour cross-resistance results of K1, K9, K10 strains and the reference strain (905), against different stress conditions, using spot assay.

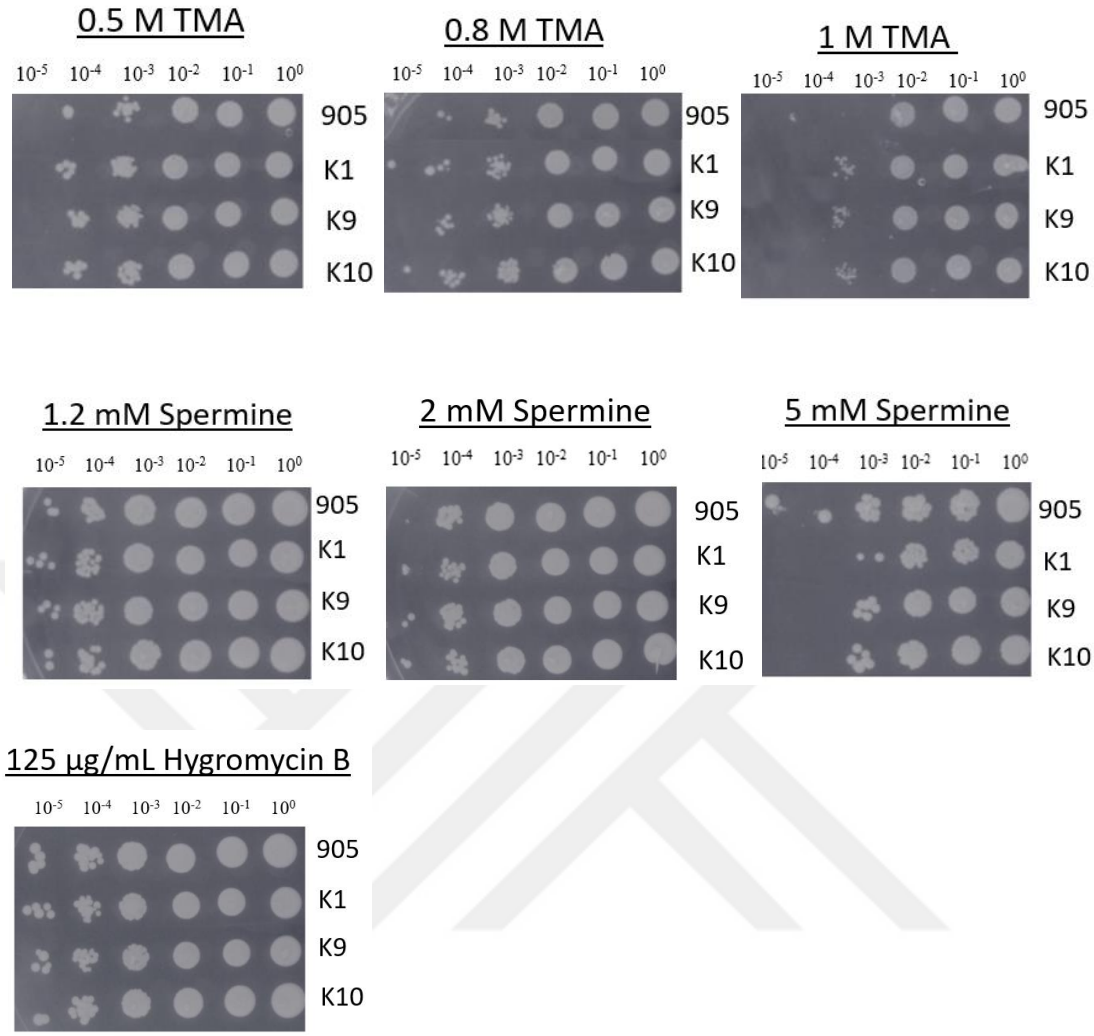


Figure 3.11 : 72nd hour cross-resistance test results of K1, K9, K10 strains and the reference strain (905), against cationic drugs, using spot assay.
TMA:Tetramethylammonium

The cross-resistance test results of the cationic drugs (Tetramethylammonium (TMA), Spermine, Hygromycin B) revealed that all evolved strains tested were resistant against 1 M TMA, and sensitive to 5 mM Spermine. No sensitivity or resistance was observed at 125 µg/mL Hygromycin B (Figure 3.11).

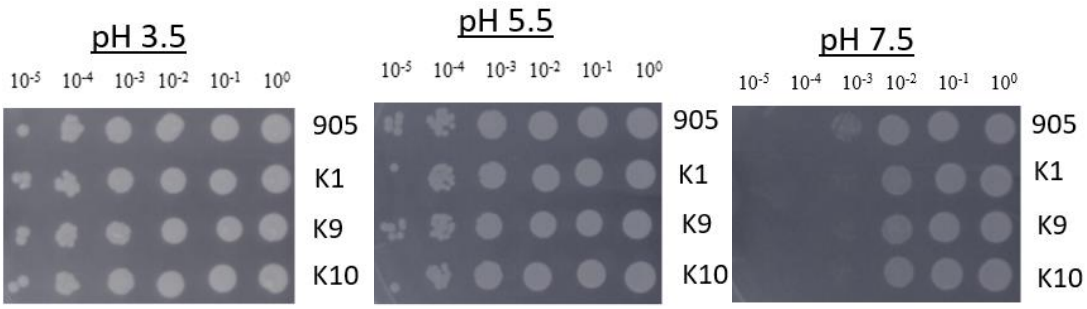


Figure 3.12 : 48th hour cross-resistance test results of K1, K9, K10 strains and the reference strain (905), against different pH, stress conditions, using spot assays.

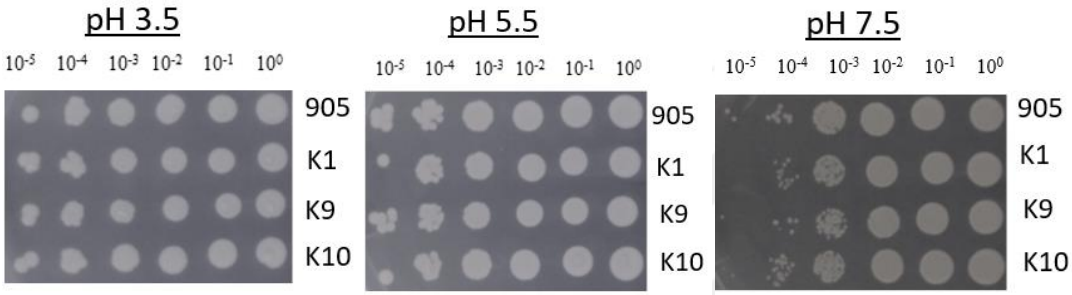


Figure 3.13 : 72nd hour cross-resistance test results of K1, K9, K10 strains and the reference strain (905), against different pH, stress conditions, using spot assay.

Cross-resistance tests of the evolved strains against different pH values were also performed. At pH 3.5 and 5.5, no differences were observed with the reference strain. All evolved strains and the reference strain could grow at those pH values without any inhibition. However, at pH 7.5 the evolved strains showed sensitivity (Figure 3.12).

3.6 Growth Profiles

Growth profile and cell dry weight analysis experiments of the reference strain and selected KCl-resistant evolved strains (K1, K9) were conducted by measuring OD_{600} absorbance at specific time intervals with 1.25 M KCl stress and without stress (YMM). Under nonstress conditions, the evolved strains and the reference strain showed similar growth profiles (Figure 3.14). At 12 h, they entered the stationary phase of the growth. After 72 h of growth, the final OD_{600} values of the reference, K1 and K9 strains were 6.37 ± 0.12, 5.97 ± 0.09, 6.31 ± 0.16 respectively. However, under 1.25 M KCl stress conditions, the reference strain and the evolved strains showed an extended lag phase and they entered the stationary phase at about 18 h (Figure 3.14). Under stress conditions, the final OD_{600} values of the reference, K1 and K9 strains after 72 h growth were 2.13± 0.03, 2.70± 0.13, and 2.94± 0.06 respectively. Additionally, the final cell dry weights (CDW), and maximum specific growth rates (μ_{max}) of the reference strain and evolved strains were lower than those without stress (Table 3.10). The evolved strains K1 and K9 showed higher growth rates than the reference strain, under stress condition. Their cell dry weights were also significantly higher than that of the reference strain during later hours of growth, under stress condition (Figure 3.15).

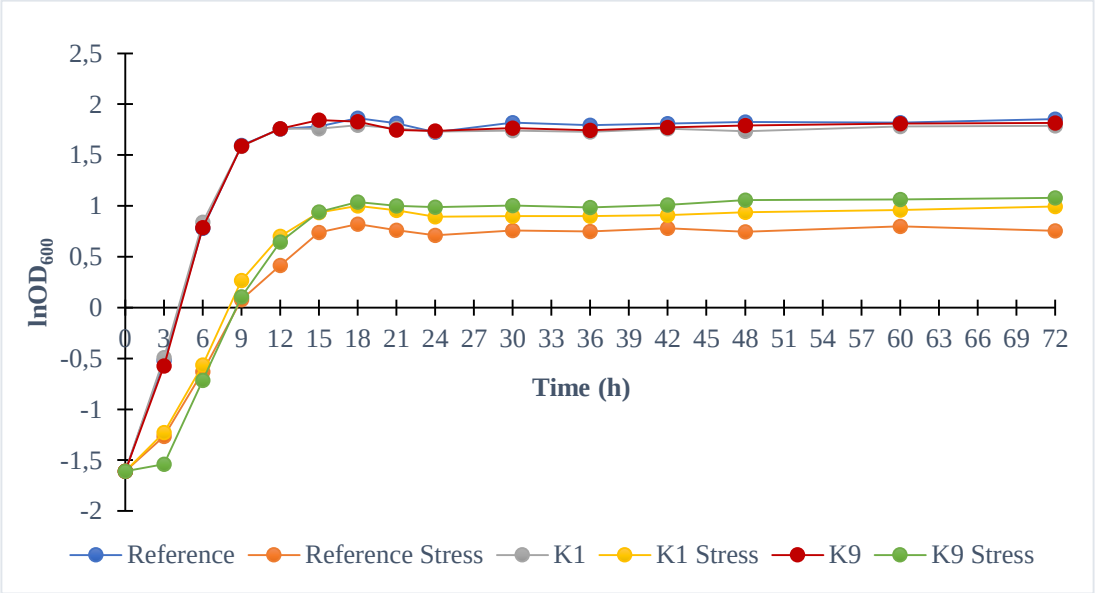


Figure 3.14 : Growth profiles of the reference strain and the evolved strains (K1, K9) with stress (1.25 M KCl) and without stress.

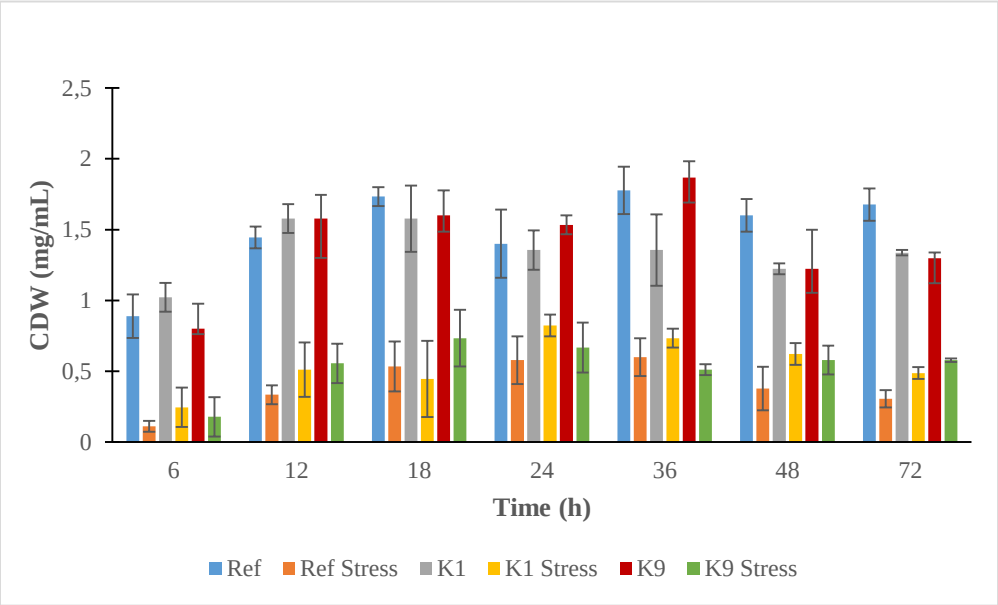


Figure 3.15 : Cell dry weight (CDW) analysis result of the reference strain (REF) and the evolved strains (K1, K9) with and without 1.25 M KCl stress.

Table 3.10 : Maximum specific growth rates (μ_{max}), final cell dry weight, ethanol, glycerol, and acetate yields of the reference strain and evolved strains (K1, K9) with stress (1.25 M KCl) and without stress.

Strain	μ_{max} (h^{-1})	Final cell dry weight (mg mL^{-1} 72 h)	Ethanol yield (g g^{-1}glucose consumed)	Glycerol yield (g g^{-1} glucose consumed)	Acetate yield (g g^{-1}glucose consumed)
0 M KCl					
Reference	0.3665±0.0040	1.6767±0.1140	0.3529±0.0040	0.0208±0.0006	0.0172±0.0002
K1	0.3460±0.0040	1.3366±0.0190	0.3547±0.0080	0.0253±0.0008	0.0177±0.0002
K9	0.3620±0.0050	1.2983±0.0392	0.3741±0.0050	0.0171±0.0001	0.0146±0.0002
1.25 M KCl					
Reference	0.1917±0.0040	0.3050±0.0600	0.2237±0.0130	0.0774±0.0020	0.0129±0.0004
K1	0.2207±0.0040	0.4866±0.0420	0.2729±0.0120	0.1006±0.0009	0.0220±0.0008
K9	0.2466±0.0140	0.5775±0.0130	0.2740±0.0080	0.0890±0.0002	0.0189±0.0004

3.7 Metabolite Analysis Results

High-performance liquid chromatography (HPLC) was used for residual glucose, ethanol, glycerol, and acetate measurements. Standard curves were obtained before the measurement (Figure 3.16). Measurements were done at specific time intervals throughout 72 h, of cultivation.

Under nonstress conditions, the reference strain and evolved strains had similar glucose consumption profiles. Glucose (~20 g/L) was depleted in the culture medium at about 15 hours. On the other hand, under 1.25 M KCl stress condition, the evolved strains K1 and K9 strains totally consumed glucose by about 36th hour of cultivation (Figure 3.17). However, the reference strain under stress could not consume all the glucose in the medium, even by the end of the cultivation (72 h) (Figure 3. 17).

The acetate production behavior of the reference and evolved strains showed different patterns under both control and stress conditions. The produced acetate levels of the reference strain began to decline after 30 and 36 hours with and without stress conditions, respectively, and then remained relatively constant (Figure 3.18). The decrease in acetate levels after 30 h was more pronounced under control conditions for the reference strain. However, the evolved strains continued to produce acetate under control conditions (Figure 3.18). In the presence of 1.25 M KCl stress, the K1 strain had the highest acetate production yield (Table 3.10). Even after the depletion of glucose, K1 and K9 strains continued with acetate production (Figure 3.17 and 3.18)..

For all strains, the glycerol production significantly increased in the presence of KCl stress (Figure 3.19). Under stress conditions, K1 and K9 strains produced glycerol at the highest yield (Table 3.10). K1 produced more glycerol than K9 and the reference strain, under both stress and non-stress conditions. Interestingly, K9 produced less glycerol than K1, although it is more resistant to KCl stress (Figure 3.19).

The ethanol production profiles of the reference and evolved strains were similar under non-stress conditions (Figure 3.20). However, under stress condition, the reference strain had lower ethanol production levels and yield than the evolved strains (Table 3.10, and Figure 3.20). Overall 1.25 M KCl stress condition reduced the ethanol yields of all strains (Table 3.10).

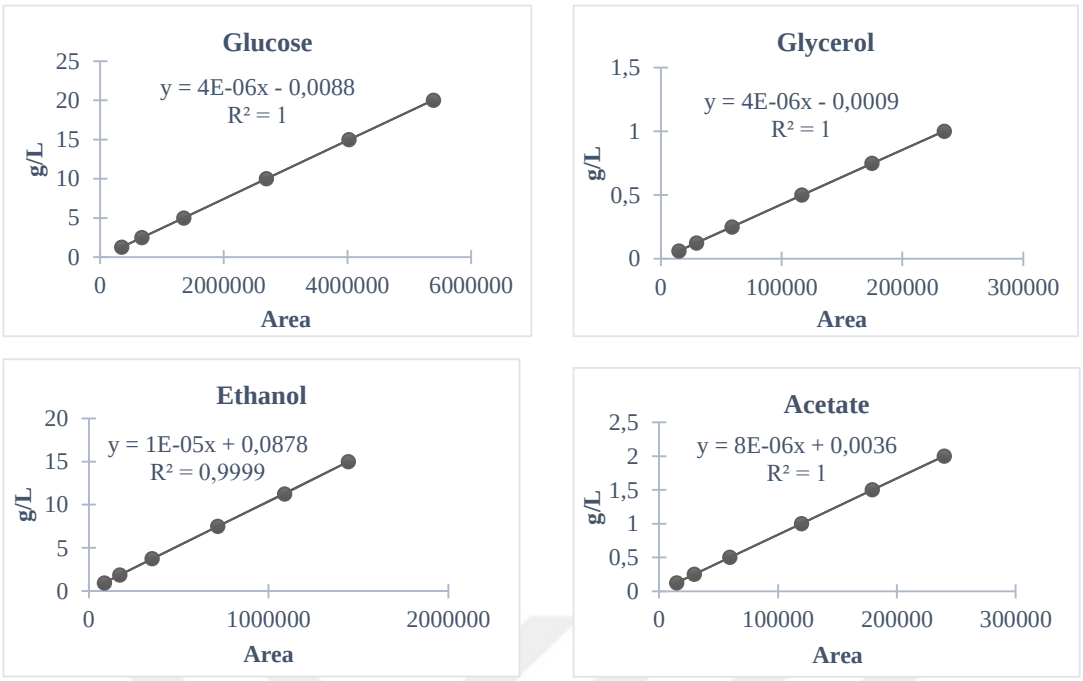


Figure 3.16 : HPLC standard curves for glucose, glycerol, ethanol and acetate measurements. The equations for the lines and and R^2 values are shown in the figure.

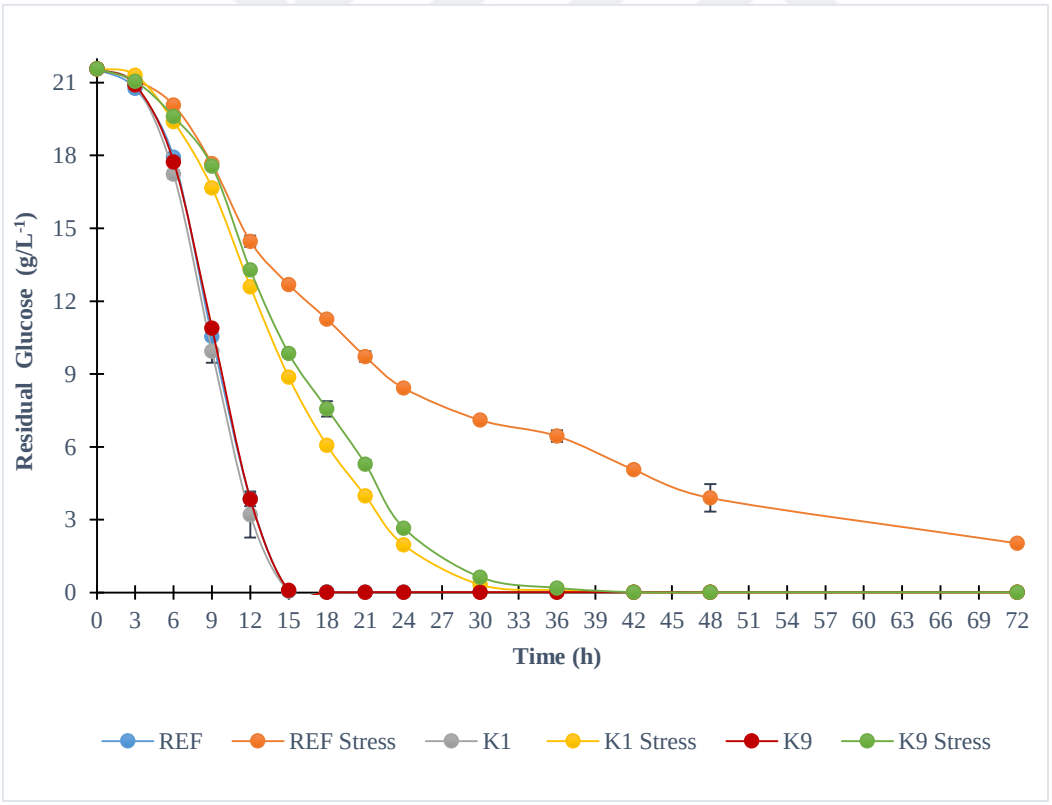


Figure 3.17 : Glucose consumption profiles of the reference strain (REF) and the evolved strains (K1, K9), under stress (1.25 M KCl) and control conditions.

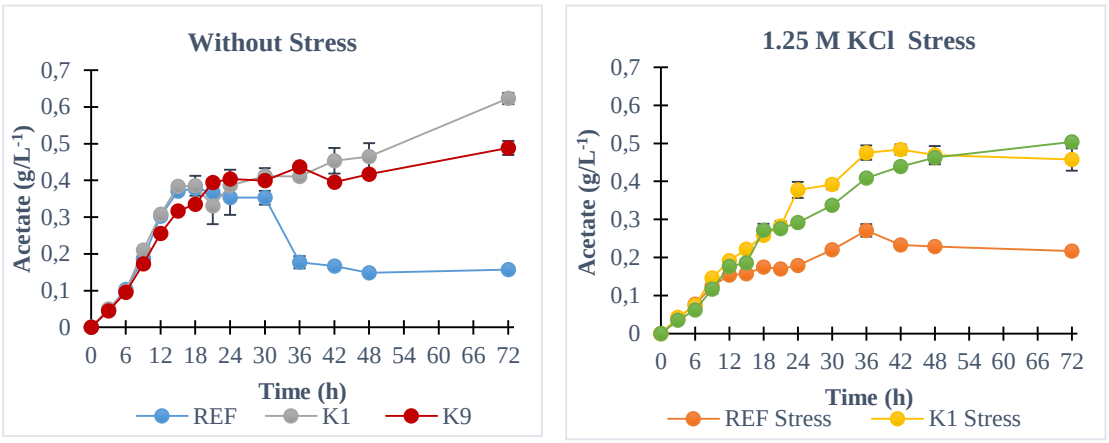


Figure 3.18 : Acetate production profiles of the reference (REF) strain and the evolved strains (K1, K9) under control (left) and 1.25 M KCl stress (right) conditions.

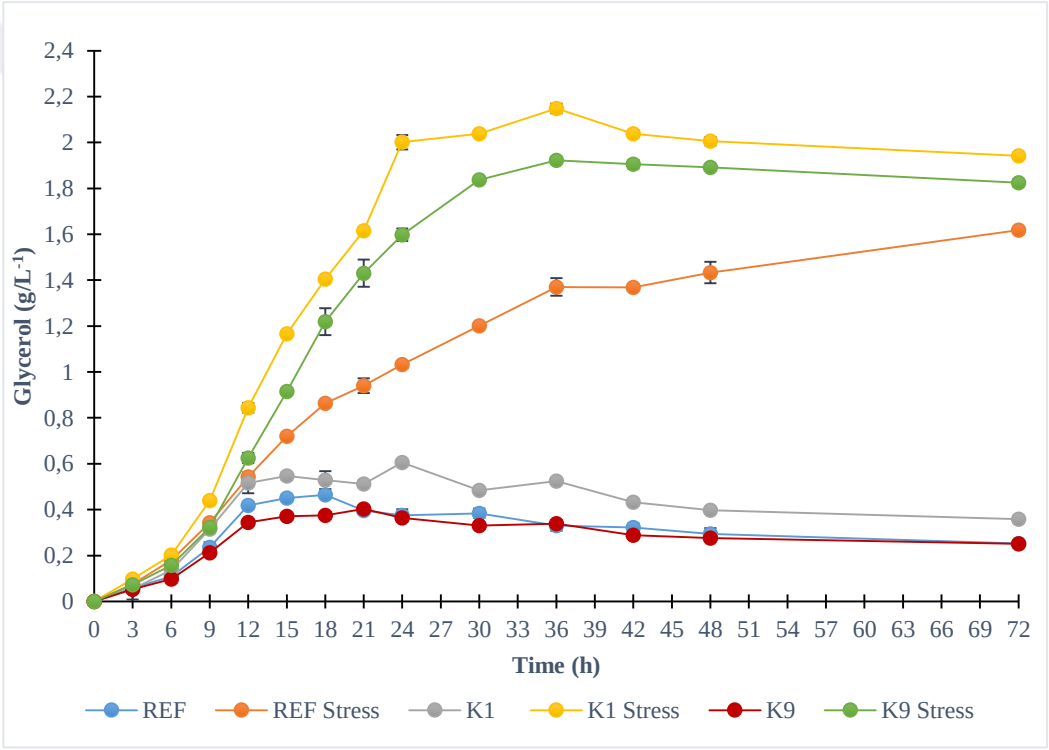


Figure 3.19 : Glycerol production profiles of the reference strain (REF) and the evolved strains (K1, K9) with and without 1.25 M KCl stress.

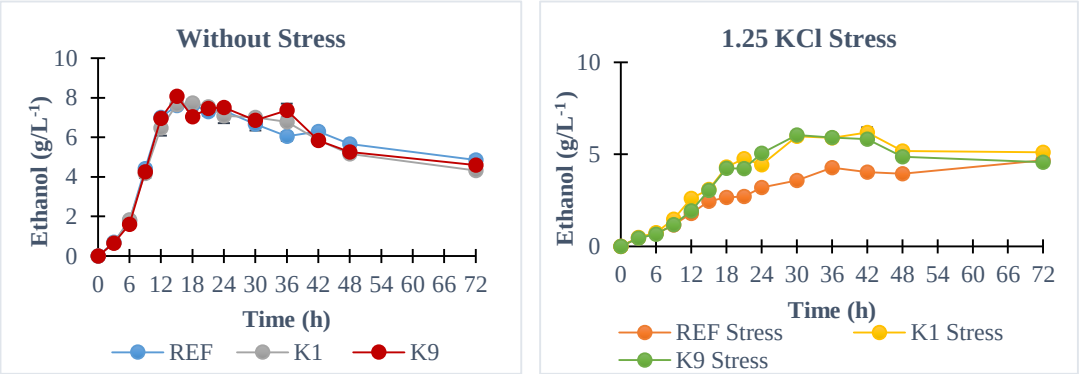


Figure 3.20 : Ethanol production profiles of the reference strain (REF) and the evolved strains (K1, K9) under control (left) and 1.25 M KCl stress (right) conditions.

3.8 Storage Carbohydrates

After 72 hours of cultivation, culture samples of the reference strain and the evolved strains were collected and treated as described in the method section for HPLC analysis. CDW data for the growth experiment were utilized for intracellular storage carbohydrate content calculations.

In general, in the presence of KCl stress, higher trehalose contents were obtained (Figure 3.21). K1 strain stored more trehalose than K9 and the reference strain under KCl stress condition. However, in the absence of KCl stress, the evolved strains did not produce significant amounts of trehalose (Figure 3.21).

The glycogen content of all strains also increased in the presence of KCl stress. The glycogen content of the reference strain was about two-fold of those of the evolved strains, under KCl stress conditions (Figure 3.22).

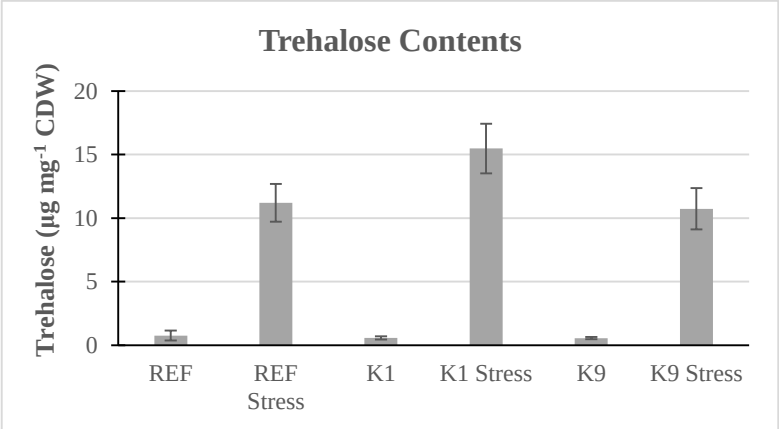


Figure 3.21 : Intracellular trehalose contents of the reference strain (REF) and the evolved strains (K1, K9), in the presence and absence of 1.25 M KCl stress.

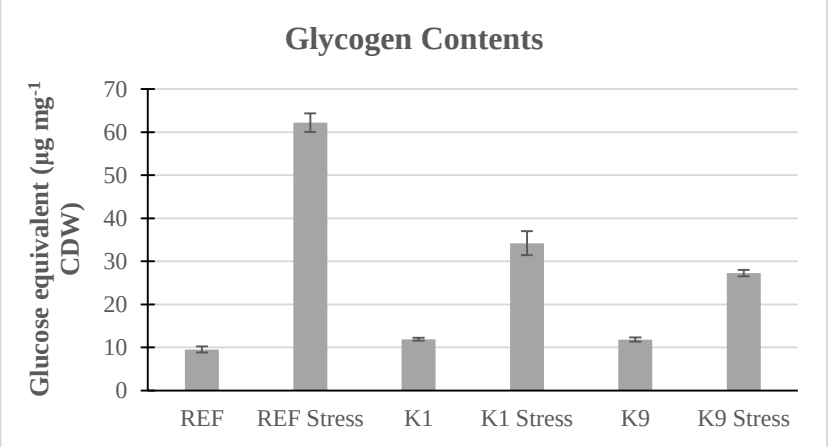


Figure 3.22 : Intracellular glycogen contents of the reference strain (REF) and the evolved strains (K1, K9), in the presence and absence of 1.25 M KCl stress. Glycogen content is given as glucose equivalents.

4. DISCUSSION

In this study, evolutionary engineering which is an inverse metabolic engineering strategy was used to obtain KCl stress-resistant *S. cerevisiae* strains. High concentrations of KCl create a hyperosmotic stress and activate the HOG pathway which initiates the transcription of many genes for stress response (Capaldi et al., 2008). To determine the initial KCl-stress level for evolutionary engineering, a screening experiment was performed with the reference strain (905) at various KCl concentrations. The results of the screening experiment revealed that increasing KCl concentration decreased the survival rate and 0.5 M KCl was a suitable concentrations as the initial stress level for evolutionary engineering selection experiments.

Selection of KCl-resistant strains was carried out by serial batch cultivation under increasing KCl-stress levels. Throughout the daily 72 passages, the concentration of KCl in the medium was gradually increased from 0.5 M to 2.5 M. The stress levels were initially increased by 0.05 M at each successive passage. It was then increased by 0.02 M after the 22^{nd} population (at 1.5 M KCl stress concentration). After the 72^{nd} population (2.5 M KCl stress), the percent survival rates and OD_{600} values decreased significantly. Thus, the 72^{nd} population was set as the final population of the selection. From the final population, individual colonies were chosen, using agar plates containing 2.5 M, 2 M and 1.75 M KCl. Twelve individual mutant colonies were selected from the 72^{nd} population. The randomly selected strains were named as K1-K12. After selection, the KCl-resistance levels of the isolated strains were determined by using spot assay, and the percent survival rates of each strain were calculated following batch cultivation. The results clearly revealed that the selected strains gained KCl resistance. However, the level of this gained resistance is low compared to the previous evolutionary engineering studies (Arslan et al., 2018; Hacısalihoğlu et al., 2019). Tilloy et al. (2014) conducted a selection study with KCl as an osmotic stressor, with successive 200 generations up to 2.4 M KCl stress levels.

This study revealed that the evolved strains were not more resistant than ancestral strain, but showed a gain of fitness which resulted in redirection of the carbon flux towards glycerol (Betlej et al., 2020; Tilloy et al., 2014). Further anayses are required to clarify and understand the diffrences in the results of that study and this thesis study.

The KCl-stress resistance results indicated that K1, K9, K10, K11 and K12 were relatively more resistant than the other evolved strains. Therefore, they were chosen for genetic stability analysis. The genetic stability analysis showed that three strains (K1, K9, and K10) were genetically stable. After determining the genetic stability of K1, K9, and K10 strains, cross-resistance or sensitivity of those evolved strains against various stress types (0.7 M NaCl, 5 mM LiCl, 0.8 M $CaCl_2$, 0.5 mM $NiCl_2$, 7.5 mM $AlCl_3$, and 0.75 mM H_2O_2, 10 % ethanol, 15 mM caffeine, 1.5 M sorbitol, 1 M NH_4Cl, and 15 mM $MnCl_2$) was investigated.

The cross-resistance test results revealed that K1 and K9 strains, which were both significantly resistant to KCl stress, behaved differently against NaCl and LiCl streses. It is most likely that this difference is caused by mutations in different genes or alteration of the expression levels of different genes that may lead to the activation of different metabolic pathways. Both sodium and lithium are toxic cations. However, potassium is not toxic to the cells. Therefore, cells tend to efflux Na^+ and accumulate a large amount of intracellular K^+. This influx and efflux system across the plasma membrane is crucial for the maintenance of potential across the plasma membrane (Ariño et al., 2010). K9 strain showed high sensitivity to both NaCl and LiCl. On the other hand, K1 and K10 showed resistance. This difference might be related to differences in K9 and other strains' membrane potential, toxic cation efflux capacity, or regulation of K^+ homeostasis (Petrezselyova et al., 2010).

A study conducted by Daran-Lapujade et al. (2009) showed that CEN.PK113-7D (905) strain is hypersensitive to sodium and lithium ions due to the atypical organization of the PMR2 locus (Daran-Lapujade et al., 2009), which usually harbors one to five tandemly arranged *ENA* genes (*ENA1-5*). However, PMR2 locus in CEN.PK113-7D is too small to harbor more than one *ENA* gene (Daran-Lapujade et al., 2003). It only harbors an allele called *ENA6*. Therefore, all important alleles including the *ENA1* gene are missing in CEN.PK113-7D genome. *ENA1* encodes Na^+ ATPase that functions as Na^+- and Li^+- exporting system in *S. Cerevisiae* (Daran-Lapujade et al., 2009). *ENA6* has a similar function to *ENA1* and is highly homologous to *ENA1*,

ENA2, and *ENA5*. However, some differences in the specific regions of the *ENA6* protein sequence, and a low copy number of *ENA* genes lead to hypersensitivity to Na^+ and Li^+. Daran-Lapujade et al. (2005) also showed that overexpression of the *ENA6* gene could restore the hypersensitivity of CEN.PK113-7D strain against Na^+ and Li^+. When they deleted the *ENA6* in CEN.PK, the growth in the medium containing Na^+ and Li^+ was completely terminated, which has no effect on tolerance to potassium and sorbitol (Daran-Lapujade et al., 2009). These results are also consistent with the results found in this thesis study. Overexpression of *ENA6* gene might have resulted in resistance against Na^+ and Li^+, which needs to be confirmed by further omic-level analyses. K1, K9, and K10 strains are also resistant to osmotic stress (the final population was obtained from 2.5 M KCl stress). Osmotic stress leads to activation of the HOG (high-osmolarity glycerol) pathway which results in phosphorylation and activation of the HOG1 mitogen-activated protein (MAP) kinase (de Nadal et al., 2002). Upon Hog1 activation *ENA1* expression increases (Ariño et al., 2010). In CEN.PK113-7D, this might lead to the overexpression of *ENA6.* This may also be the case with K1 and K10 strains. However, the extreme sensitivity of the K9 strain against Na^+ and Li^+ requires further omic-level investigation.

All evolved strains, showed resistance against 0.8 M $CaCl_2$ stress. The 0.8 M $CaCl_2$ is also considered as a source of hyperosmotic stress. Therefore, resistance against high concentrations of $CaCl_2$ was expected. On the other hand, calcium ion is a second messenger and acts as an enzyme cofactor. Normally, free cytosolic Ca^+ concentration is very low, excess amount of Ca^+ is usually removed from the cytosol by vacuoles, then extruded via exocytosis (Busti et al., 2016; Cui et al., 2009). In response to high extracellular Ca^+, sensor protein calmodulin can bind and activate phosphatase protein calcineurin (Cui et al., 2009), which is sensitive to osmotic stress and has a role in high-affinity potassium uptake (Mulet et al., 1999). Calcineurin also induces transcription of *ENA1*. Thus, a possible mutation that might have occurred during evolutionary engineering might have led to overexpression of calcineurin that enables resistance against $CaCl_2$ stress. This is yet to be confirmed by further genomic studies.

Furthermore, against oxidative stress, K1 strain showed resistance, but K9 strain showed sensitivity. Response to oxidative stress is also related to HOG (high-osmolarity glycerol) pathway. Sko1p is a repressor protein that is involved in regulation of HOG pathway which is also responsible for transcription of

oxidoreductases (Rep et al., 2001). Those oxidoreductases can repair oxidative damage. This might explain the resistance of K1 against oxidative stress. However, the sensitivity of K9 requires further investigation.

Sensitivity or resistance to cationic drugs may provide information about alteration in the membrane potential, independently of their toxicity mechanism. However, alteration of the electrochemical gradient is a very complex issue and involves a mutations or changes in many genes (i.e., *TRK1*,*2*, *SKY1*, *HAL4*,*5 PTK2*, and *PPZ1*), which mainly control alkali metal homeostasis in cells (Barreto et al., 2011).

Yenush et al. (2002) stated that "tolerance to cationic drugs correlates with a reduction in the membrane potential, which decreases the voltage-dependent uptake of cations" (Yenush et al., 2002). This statement seems to be in line with this thesis experimental results at 1 M TMA, and might also help us to explain the resistance of K1 and K10 to NaCl and LiCl. However, at 5 mM spermine all the evolved strains showed sensitivity. This might be due to a high concentration of the drug that might have cause another toxicity or another mechanism of regulation.

Changes in external pH also affect membrane potential. At high pH, ATPase activity was increased if potassium is not present in the medium (Pera et al., 1972). At pH 7.5 the evolved strains showed sensitivity, which might be due to increased activity of ATPase that may lead to growth defects, as the evolved strains might have overexpressed some of the K^+-uptake transporters like Trk1, Trk2. This, however, is yet to be further investigated by genomic and transcriptomic analyses.

Growth physiological analysis results demonstrated that KCl-resistant evolved strains (K1, K9) have growth rates similar to that of the reference strain in KCl stress-free medium. This indicates that gaining resistance to KCl did not affect the growth rate. However, the presence of 1.25 M KCl stress in the medium slowed down the growth and extended the lag phase for both reference strain and the evolved strains. This was also consistent with CDW results: under KCl stress, both reference and evolved strains had significantly less CDW than in the absence of stress (Figure 3.15). On the other hand, under KCl stress, the K9 strain had a significantly higher maximum specific growth rate than K1 and the reference strain (Table 3.10).

The metabolite analysis results showed that glucose consumption rates of the reference and the evolved strains were very similar to each other under non-stress conditions.

After depletion of all glucose at the 15th hour, it was observed that glycerol and ethanol production of these strains had stopped. After that, ethanol content started to decline, which indicates that both reference and the evolved strains utilized ethanol as a carbon source after the 15th hour of growth.

Unlike the previously conducted KCl-induced osmotic stress evolutionary engineering studies (Tilloy et al., 2014; Betlej et al., 2020) acetate production profiles demonstrated that the evolved K1 and K9 strains produced a higher amounts of acetate (approximately two-fold) than the reference strain under osmotic stress. Even in the absence of stress, the evolved strains kept producing acetate. This may imply that there migh be a relation between acetate production and KCl-induced osmotic stress. Acetate production in yeast is a complicated metabolism and is related to redox balance in cells (Aranda & del Olmo, 2003). Aldehyde dehydrogenase (ALDH) enzymes oxidize acetaldehyde to acetate. Aldehyde dehydrogenase (ALDH) enzyme complex is an enzyme family which is encoded by five different genes. They are located in different compartments of the cell: the cytosol and mitochondria (Remize et al., 2000). ALD6 is a major enzyme for acetate production which is located in the cytosol and is activated by Mg^{2+}. On the other hand, enzymes such as ALD4p and ALD5p are located in mitochondria and are activated by K^{+}. They use NAD and NADP as cofactor (Navarro-Aviño et al., 1999). Furthermore, it was found that one of the ALD family genes such as *ALD2* is induced by osmotic stress (Miralles & Serrano, 1995). The produced acetate can be converted to acetyl-CoA or secreted to culture media (Boubekeur et al., 1999). The dependence of K^{+} and the presence of genes induced by osmotic stress might explain the high-yield acetate production of evolved strains.

Regarding the glycerol production profiles, it can be concluded that the presence of 1.25 M KCl significantly increase glycerol production for all strains. It is well known that glycerol is accumulated when *S. cerevisiae* cells are subjected to osmotic stress (Nevoigt & Stahl, 1997). Under KCl stress, glycerol production of both reference and evolved strains K1 and K9 increased. However, K1 and K9 were able to produce more glycerol than the reference strain. These results are consistent with the previously conducted KCl-induced osmotic stress evolutionary engineering studies (Tilloy et al., 2014; Betlej et al., 2020). Both studies revealed that evolutionary engineering for KCl resistance leads to enhanced glycerol production and reduced ethanol yield. However,

under KCl-stress conditions, the ethanol yields of K1 and K9 were relatively higher than that of the reference strain. This difference might be the result of the utilization of different strains with different genetic backgrounds and different implementation of evolutionary engineering strategies as well as mutations or changes in different genes, which remains to be investigated further.

Furthermore, semi-quantitative RT-PCR analysis result of a previous KCl-evolutionary engineering study (Betlej et al., 2020) showed that the expression levels of *GPD1*, *GPP1*, and *GPP2* genes (which are responsible for glycerol synthesis) slightly increased after evolutionary engineering. GPD genes encode glycerol 3-phosphate dehydrogenase which reduces dihydroxyacetone phosphate to glycerol 3-phosphate. The synthesis of glycerol continues with dephosphorylation of glycerol 3-phosphate by glycerol 3-phosphatase which is encoded by *GPP1*, and *GPP2* (Nevoigt & Stahl, 1997). The expression of those genes is also controlled by HOG (High Osmolarity Glycerol) pathway (Albertyn et al., 1994). It is revealed that activation of Hog1p leads to the expression of genes responsible for glycerol synthesis (Babazadeh et al., 2014).

It is also known that glycerol formation contributes to the cellular redox balance. Overproduction of glycerol leads to the synthesis of acetate, 2,3-butanediol, and succinate (Remize et al., 1999). Acetate production has a certain impact on cytosolic redox balance. Because 1 mole of acetate production yields 2 moles of NAD(P)H during oxidation of acetaldehyde to acetate by aldehyde dehydrogenase enzyme (Remize et al., 1999). The produced NAD(P)H are consumed during glycerol synthesis by NAD(P)H dependent dihydroxyacetone phosphate reduction reaction catalyzed by glycerol 3-phosphate dehydrogenase (Navarro-Aviño et al., 1999).

The synergistic relation between acetate and glycerol production is seems to be consistent with the experimental results of this thesis study. Under KCl stress, glycerol and acetate production of K1 and K9 strains increased, compared to the reference strain. On the other hand, without KCl-stress, only the production of acetate increased.

Among the evolved strains, only the K1 strain had a slightly higher glycerol yield. Genes responsible for glycerol synthesis in the K1 strain might have been upregulated. Further genomic and transcriptomic analyses of K1 and K9 strains will help to elucidate the molecular mechanisms behind this phenotype.

Trehalose and glycogen are intracellular carbohydrates that are synthesized under stress conditions. Their production is the first metabolic response against exposure to stresses like heat, carbon limitation, oxidative stress, etc.(François & Parrou, 2001). Especially trehalose is mainly considered as a protecting agent rather than a reserve carbohydrate (Wiemken, 1990). On the other hand, it was suggested that reserving glycogen does not contribute to the internal osmotic pressure (Wilson et al., 2010; Silljé et al., 1999). The results of this thesis study showed that under KCl stress conditions, both evolved and reference strains stored a relatively high amount of trehalose and glycogen. However, without stress, K1 and K9 did not able to store these carbohydrates at high amounts.

5. CONCLUSION

By using evolutionary engineering, KCl-stress-resistant and genetically stable *S. cerevisiae* strains were successfully obtained in this thesis study and characterized at physiological level. The two evolved strains showed different cross-resistance behaviour and metabolite profiles. The evolved strains obtained under KCl-induced osmotic stress selection, especially K1, had higher yields of glycerol, acetate, and trehalose than the reference strain. At the same time, they consumed glucose at a slower rate. It appears that to survive at KCl-induced osmotic stress, the evolved strains might have directed their carbon flux toward glycerol, acetate, and trehalose, which may have reduced their maximum specific growth rates. Interestingly, the reference strain also behaved similarly; however, its maximum specific growth rate and product yields were relatively lower than those of K1 and K9 under KCl stress condition. In addition, the K1 strain had higher glycerol, acetate, and trehalose yields. However, K9 had higher KCl resistance and better growth performance under KCl stress. Interestingly, K9 had also sensitivity against some other stress factors. Therefore, to understand the differences between K1 and K9 strains and the molecular basis of KCl resistance, comparative genomic and transcriptomic analyses of the evolved strains would be necessary as future studies.

REFERENCES

Albertyn, J., Hohmann, S., & Prior, B. A. (1994). Characterization of the osmotic-stress response in *Saccharomyces cerevisiae*: osmotic stress and glucose repression regulate glycerol-3-phosphate dehydrogenase independently. *Current Genetics*, *25*(1), 12–18.

Aranda, A., & del Olmo, M. lí. (2003). Response to acetaldehyde stress in the yeast *Saccharomyces cerevisiae* involves a strain-dependent regulation of several ALD genes and is mediated by the general stress response pathway. *Yeast*, *20*(8), 747–759.

Ariño, J., Aydar, E., Drulhe, S., Ganser, D., Jorrín, J., Kahm, M., Krause, F., Petrezsélyová, S., Yenush, L., Zimmermannová, O., Van Heusden, G. P. H., Kschischo, M., Ludwig, J., Palmer, C., Ramos, J., & Sychrová, H. (2014). Systems Biology of Monovalent Cation Homeostasis in Yeast. The Translucent Contribution. In *Advances in Microbial Physiology* (Vol. 64), 1-63.

Ariño, J., Ramos, J., & Sychrova, H. (2019). Monovalent cation transporters at the plasma membrane in yeasts. *Yeast*, *36*(4), 177–193.

Ariño, J., Ramos, J., & Sychrová, H. (2010). Alkali Metal Cation Transport and Homeostasis in Yeasts. *Microbiology and Molecular Biology Reviews*, *74*(1), 95–120.

Arslan, M., Holyavkin, C., Kısakesen, H. İ., Topaloğlu, A., Sürmeli, Y., & Çakar, Z. P. (2018). Physiological and Transcriptomic Analysis of a Chronologically Long-Lived Saccharomyces cerevisiae Strain Obtained by Evolutionary Engineering. *Molecular Biotechnology*, *60*(7), 468–484.

Babazadeh, R. (2014). *Integrative analysis of osmoregulation in yeast Saccharomyces cerevisiae*. (Doctoral dissertation). University of Gothenburg, Department of Chemistry and Molecular Biology, Microbiology, Göteborg, Sweden.

Babazadeh, R., Furukawa, T., Hohmann, S., & Furukawa, K. (2014). Rewiring yeast osmostress signalling through the MAPK network reveals essential and non-essential roles of Hog1 in osmoadaptation. *Scientific Reports*, *4*, 1–7.

Bailey, J. E. (1999). Lessons from metabolic engineering for functional genomics and drug discovery. *Nature Biotechnology*, *17*(7), 616–618.

Bailey, James E., Sburlati, A., Hatzimanikatis, V., Lee, K., Renner, W. A., & Tsai, P. S. (1996). Inverse metabolic engineering: a strategy for directed genetic engineering of useful phenotypes. *Biotechnology and Bioengineering*, *52*(1), 109-121.

Bailey, James E. (1991). Toward a Science of Metabolic Engineering. *Science*, *252*(5013), 1668–1675.

Bañuelos, M. A., Ruiz, M. C., Jiménez, A., Souciet, J. L., Potier, S., & Ramos, J. (2002). Role of the Nha1 antiporter in regulating K+ influx in *Saccharomyces cerevisiae*. *Yeast*, *19*(1), 9–15.

Barreto, L., Canadell, D., Petrezsélyová, S., Navarrete, C., Marešová, L., Peréz-Valle, J., Herrera, R., Olier, I., Giraldo, J., Sychrová, H., Yenush, L., Ramos, J., & Ariño, J. (2011). A genomewide screen for tolerance to cationic drugs reveals genes important for potassium homeostasis in *Saccharomyces cerevisiae*. *Eukaryotic Cell*, *10*(9), 1241–1250.

Bertl, A., Slayman, C. L., & Gradmann, D. (1993). Gating and conductance in an outward-rectifying K+ channel from the plasma membrane of *Saccharomyces cerevisiae*. *The Journal of Membrane Biology*, *132*(3), 183–199.

Betlej, G., Bator, E., Oklejewicz, B., Potocki, L., Górka, A., Slowik-Borowiec, M., Czarny, W., Domka, W., & Kwiatkowska, A. (2020). Long-term adaption to high osmotic stress as a tool for improving enological characteristics in industrial wine yeast. *Genes*, *11*(5).

Blomberg, A. (2000). Metabolic surprises in *Saccharomyces cerevisiae* during adaptation to saline conditions: Questions, some answers and a model. *FEMS Microbiology Letters*, *182*(1), 1–8.

Blomberg, A., & Adler, L. (1992). Physiology of osmotolerance in fungi 1. *Advances in Microbial Physiology*, *33*, 145–212.

Boubekeur, S., Bunoust, O., Camougrand, N., Castroviejo, M., Rigoulet, M., & Guérin, B. (1999). A mitochondrial pyruvate dehydrogenase bypass in the yeast *Saccharomyces cerevisiae*. *Journal of Biological Chemistry*, *274*(30), 21044–21048.

Busti, S., Mapelli, V., Tripodi, F., Sanvito, R., Magni, F., Coccetti, P., Rocchetti, M., Nielsen, J., Alberghina, L., & Vanoni, M. (2016). Respiratory metabolism and calorie restriction relieve persistent endoplasmic reticulum stress induced by calcium shortage in yeast. *Scientific Reports*, *6*(May), 1–17.

Çakar, Z. P., Seker, U. O. S., Tamerler, C., Sonderegger, M., & Sauer, U. (2005). Evolutionary engineering of multiple-stress resistant *Saccharomyces cerevisiae*. *FEMS Yeast Research*, *5*(6–7), 569–578.

Çakar, Z. P., Turanli-Yildiz, B., Alkim, C., & Yilmaz, Ü. (2012). Evolutionary engineering of *Saccharomyces cerevisiae* for improved industrially important properties. *FEMS Yeast Research*, *12*(2), 171–182.

Çakar, Z., P., (2009). Metabolic and evolutionary engineering research in Turkey and beyond. *Biotechnology Journal*, *4*(7), 992–1002.

Capaldi, A. P., Kaplan, T., Liu, Y., Habib, N., Regev, A., Friedman, N., & O'shea, E. K. (2008). Structure and function of a transcriptional network activated by the MAPK Hog1. *Nature Genetics*, *40*(11), 1300–1306.

Coughlan, A. Y., Lombardi, L., Braun-Galleani, S., Martos, A. A. R., Galeote, V., Bigey, F., Dequin, S., Byrne, K. P., & Wolfe, K. H. (2020). The yeast mating-type switching endonuclease HO is a domesticated member of an unorthodox homing genetic element family. *ELife*, *9*.

Cui, J., Kaandorp, J. A., Sloot, P. M. A., Lloyd, C. M., & Filatov, M. V. (2009). Calcium homeostasis and signaling in yeast cells and cardiac myocytes. *FEMS Yeast Research*, *9*(8), 1137–1147.

Cyert, M. S., & Philpott, C. C. (2013). Regulation of cation balance in *Saccharomyces cerevisiae. Genetics*, *193*(3), 677–713.

Daran-Lapujade, P., Daran, J. M., Kötter, P., Petit, T., Piper, M. D. W., & Pronk, J. T. (2003). Comparative genotyping of the *Saccharomyces cerevisiae* laboratory strains S288C and CEN.PK113-7D using oligonucleotide microarrays. *FEMS Yeast Research*, *4*(3), 259–269.

Daran-Lapujade, P., Daran, J. M., Luttik, M. A. H., Almering, M. J. H., Pronk, J. T., & Kötter, P. (2009). An atypical PMR2 locus is responsible for hypersensitivity to sodium and lithium cations in the laboratory strain *Saccharomyces cerevisiae* CEN.PK113-7D. *FEMS Yeast Research*, *9*(5), 789–792.

De Nadal, Eulàlia, Alepuz, P. M., & Posas, F. (2002). Dealing with osmostress through MAP kinase activation. *EMBO Reports*, *3*(8), 735–740.

De Nadal, Eulélia, Ammerer, G., & Posas, F. (2011). Controlling gene expression in response to stress. *Nature Reviews Genetics*, *12*(12), 833–845. h

Divate, N. R., Chen, G. H., Divate, R. D., Ou, B. R., & Chung, Y. C. (2017). Metabolic engineering of *Saccharomyces cerevisiae* for improvement in stresses tolerance. *Bioengineered*, *8*(5), 524–535.

Duina, A. A., Miller, M. E., & Keeney, J. B. (2014). Budding yeast for budding geneticists: A primer on the *Saccharomyces cerevisiae* model system. *Genetics*, *197*(1), 33–48.

François, J., & Parrou, J. L. (2001). Reserve carbohydrates metabolism in the yeast *Saccharomyces cerevisiae*. *FEMS Microbiology Reviews*, *25*(1), 125–145.

Gill, R. T., Edwards, J. S., & Kauffman, K. J. (2003). Enabling inverse metabolic engineering through genomics. *Current Opinion in Biotechnology*, *14*(5), 484–490.

Goffeau, A., Barrell, G., Bussey, H., Davis, R. W., Dujon, B., Feldmann, H., Galibert, F., Hoheisel, J. D., Jacq, C., Johnston, M., Louis, E. J., Mewes, H. W., Murakami, Y., Philippsen, P., Tettelin, H., & Oliver, S. G. (1996). Life with 6000 Genes. *Science*, *274*(5287), 546–567.

Greig, D., & Leu, J. Y. (2009). Natural history of budding yeast. *Current Biology*, *19*(19), 886–890.

Hacısalihoğlu B., Holyavkin C., Topaloğlu A., Kısakesen H.I. & Çakar Z.P. (2019). Genomic and transcriptomic analysis of a coniferyl aldehyde-resistant *Saccharomyces cerevisiae* strain obtained by evolutionary engineering, *FEMS Yeast Res. 19*(3):foz021.

Hohmann, S. (2002). Osmotic stress signaling and osmoadaptation in yeasts. *Microbiology and Molecular Biology Reviews : MMBR*, *66*(2), 300–372.

Hong, K. K., & Nielsen, J. (2012). Metabolic engineering of Saccharomyces cerevisiae: A key cell factory platform for future biorefineries. *Cellular and Molecular Life Sciences*, *69*(16), 2671–2690.

Kahm, M., Navarrete, C., Llopis-Torregrosa, V., Herrera, R., Barreto, L., Yenush, L., Ariño, J., Ramos, J., & Kschischo, M. (2012). Potassium starvation in yeast: Mechanisms of homeostasis revealed by mathematical modeling. *PLoS Computational Biology*, *8*(6), 1–11.

Kim, I. K., Roldão, A., Siewers, V., & Nielsen, J. (2012). A systems-level approach for metabolic engineering of yeast cell factories. *FEMS Yeast Research*, *12*(2), 228–248.

Kinclová, O., Ramos, J., Potier, S., & Sychrová, H. (2001). Functional study of the *Saccharomyces cerevisiae* Nha1p C-terminus. *Molecular Microbiology*, *40*(3), 656–668.

Llopis-Torregrosa, V., Hušeková, B., & Sychrová, H. (2016). Potassium uptake mediated by Trk1 is crucial for *Candida glabrata* growth and fitness. *PLoS ONE*, *11*(4), 1–18.

Miralles, V. J., & Serrano, R. (1995). A genomic locus in *Saccharomyces cerevisiae* with four genes up-regulated by osmotic stress. *Molecular Microbiology*, *17*(4), 653–662.

Mortimer, R. K. (2000). Evolution and variation of the yeast (Saccharomyces) genome. *Genome Research*, *10*(4), 403–409.

Mulet, J. M., Leube, M. P., Kron, S. J., Rios, G., Fink, G. R., & Serrano, R. (1999). A Novel Mechanism of Ion Homeostasis and Salt Tolerance in Yeast: the Hal4 and Hal5 Protein Kinases Modulate the Trk1-Trk2 Potassium Transporter. *Molecular and Cellular Biology*, *19*(5), 3328–3337.

de Nadal, E., Clotet, J., Posas, F., Serrano, R., Gomez, N., & Ariño, J. (1998). The yeast halotolerance determinant Hal3p is an inhibitory subunit of the Ppz1p Ser/Thr protein phosphatase. *Proceedings Of The National Academy Of Sciences*, *95*(13), 7357-7362.

Navarro-Aviño, J. P., Prasad, R., Miralles, V. J., Benito, R. M., & Serrano, R. (1999). A proposal for nomenclature of aldehyde dehydrogenases in *Saccharomyces cerevisiae* and characterization of the stress-inducible *ALD2* and *ALD3* genes. *Yeast*, *15*(10 A), 829–842.

Nevoigt, E. (2008). Progress in Metabolic Engineering of *Saccharomyces cerevisiae. Microbiology and Molecular Biology Reviews*, *72*(3), 379–412.

Nevoigt, E., & Stahl, U. (1997). Osmoregulation and glycerol metabolism in the yeast *Saccharomyces cerevisiae*. *FEMS Microbiology Reviews*, *21*(3), 231–241.

Parapouli, M., Vasileiadis, A., Afendra, A. S., & Hatziloukas, E. (2020). *Saccharomyces cerevisiae* and its industrial applications. In *AIMS Microbiology* (Vol. 6, Issue 1).

Parrou, J. L., & François, J. (1997). A Simplified Procedure for a Rapid and Reliable Assay of both Glycogen and Trehalose in Whole Yeast Cells. *Analytical Biochemistry*, *248*(1), 186–188.

Pera, A., Puyou, E., Cinco, G., & Tuena, R. I. (1972). Effect of the pH of the Incubation Medium on *cerevisiae*. *Archives of Biochemistry and Biophysics*, *153*(4), 413–425.

Petrezsélyová, S., Ramos, J., & Sychrová, H. (2011). Trk2 transporter is a relevant player in K+ supply and plasma-membrane potential control in *Saccharomyces cerevisiae*. *Folia Microbiologica*, *56*(1), 23–28.

Petrezselyova, S., Zahradka, J., & Sychrova, H. (2010). *Saccharomyces cerevisiae* BY4741 and W303-1A laboratory strains differ in salt tolerance. *Fungal Biology*, *114*(2–3), 144–150.

Proft, M., & Struhl, K. (2004). MAP kinase-mediated stress relief that precedes and regulates the timing of transcriptional induction. *Cell*, *118*(3), 351–361.

Remize, F., Andrieu, E., & Dequin, S. (2000). Engineering of the pyruvate dehydrogenase bypass in *Saccharomyces cerevisiae*: Role of the cytosolic Mg2+ and mitochondrial K+ acetaldehyde dehydrogenases Ald6p and Ald4p in acetate formation during alcoholic fermentation. *Applied and Environmental Microbiology*, *66*(8), 3151–3159.

Remize, F., Roustan, J. L., Sablayrolles, J. M., Barre, P., & Dequin, S. (1999). Glycerol overproduction by engineered *Saccharomyces cerevisiae* wine yeast strains leads to substantial changes in by-product formation and to a stimulation of fermentation rate in stationary phase. *Applied and Environmental Microbiology*, *65*(1), 143–149.

Rep, M., Proft, M., Remize, F., Tamás, M., Serrano, R., Thevelein, J. M., & Hohmann, S. (2001). The *Saccharomyces cerevisiae* Sko1p transcription factor mediates HOG pathway-dependent osmotic regulation of a set of genes encoding enzymes implicated in protection from oxidative damage. *Molecular Microbiology*, *40*(5), 1067–1083.

Ruiz, A., Muñoz, I., Serrano, R., González, A., Simón, E., & Ariño, J. (2004). Functional characterization of the *Saccharomyces cerevisiae VHS3* gene: A regulatory subunit of the Ppz1 protein phosphatase with novel, phosphatase-unrelated functions. *Journal of Biological Chemistry*, *279*(33), 34421–34430.

***Saccharomyces cerevisiae Genome Overview | SGD*. (n.d.)**. Retrieved April 7, 2022, from https://www.yeastgenome.org/genomesnapshot

Sadeh, A., Movshovich, N., Volokh, M., Gheber, L., & Aharoni, A. (2011). Fine-tuning of the Msn2/4–mediated yeast stress responses as revealed by systematic deletion of Msn2/4 partners. *Molecular Biology of the Cell*, *22*(17), 3127.

Schneiter, R. (2004). *Genetics , Molecular and Cell Biology of Yeast.* Universitat Freiburg Schwarz, 4-10

Silljé, H. H. W., Paalman, J. W. G., Ter Schure, E. G., Olsthoorn, S. Q. B., Verkleij, A. J., Boonstra, J., & Verrips, C. T. (1999). Function of trehalose and glycogen in cell cycle progression and cell viability in *Saccharomyces cerevisiae*. *Journal of Bacteriology*, *181*(2), 396–400.

Tilloy, V., Ortiz-Julien, A., & Dequin, S. (2014). Reduction of ethanol yield and improvement of glycerol formation by adaptive evolution of the wine yeast *Saccharomyces cerevisiae* under hyperosmotic conditions. *Applied and Environmental Microbiology*, *80*(8), 2623–2632.

Turanlı-Yıldız, B., Benbadis, L., Alkım, C., Sezgin, T., Akşit, A., Gökçe, A., Öztürk, Y., Baykal, A. T., Çakar, Z. P., & François, J. M. (2017). In vivo evolutionary engineering for ethanol-tolerance of *Saccharomyces cerevisiae* haploid cells triggers diploidization. *Journal of Bioscience and Bioengineering*, *124*(3), 309–318.

Varela, J. C. S., & Mager, W. H. (1996). Response of *Saccharomyces cerevisiae* to changes in external osmolarity. *Microbiology*, *142*(4), 721–731.

Wiemken, A. (1990). Trehalose in yeast, stress protectant rather than reserve carbohydrate. *Antonie van Leeuwenhoek*, *58*(3), 209–217.

Wilson, W. A., Roach, P. J., Montero, M., Baroja-Fernández, E., Muñoz, F. J., Eydallin, G., Viale, A. M., & Pozueta-Romero, J. (2010). Regulation of glycogen metabolism in yeast and bacteria. *FEMS Microbiology Reviews*, *34*(6), 952–985.

Yancey, P. H. (2005). Organic osmolytes as compatible, metabolic and counteracting cytoprotectants in high osmolarity and other stresses. *The Journal of Experimental Biology*, *208*(Pt 15), 2819–2830.

Yenush, L., Mulet, J. M., Ariño, J., & Serrano, R. (2002). The Ppz protein phosphatases are key regulators of K+ and pH homeostasis: Implications for salt tolerance, cell wall integrity and cell cycle progression. *EMBO Journal*, *21*(5), 920–929.

CURRICULUM VITAE

Name Surname **:** Ogün Morkoç

EDUCATION

- **B.Sc.** **:** 2018, Yıldız Technical University, Chemical and Metallurgical Faculty, Department of Bioengineering
- **M.Sc** **:** 2022, Istanbul Technical University, Molecular Biology Genetics & Biotechnology

PROFESSIONAL EXPERIENCE AND REWARDS:

(2019 – present) : Research and teaching assistant, Turkish-German University, Department of Molecular Biotechnology

(2018) : Graduated as the highest ranked student, Yıldız Technical University, Department of Bioengineering

PUBLICATIONS, PRESENTATIONS AND PATENTS ON THE THESIS:

-