ISTANBUL TECHNICAL UNIVERSITY ★ GRADUATE SCHOOL

# EVOLUTIONARY ENGINEERING OF RAPAMYCIN-RESISTANT YEAST

M.Sc. THESIS

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Department of Molecular Biology-Genetics and Biotechnology

Molecular Biology-Genetics and Biotechnology Programme

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**ISTANBUL TEKNİK ÜNİVERSİTESİ ★ LİSANSÜSTÜ EĞİTİM ENSTİTÜSÜ**

# EVRİMSEL MÜHENDİSLİKLE RAPAMİSİNE DİRENÇLİ MAYA ELDESİ

**YÜKSEK LİSANS TEZİ**

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**ARALIK 2022**

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***To my family,***

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## ABBREVIATIONS

| | |
|---|---|
| **°C** | : Degree Celsius |
| **µl** | : Microliter |
| **µM** | : Micromolar |
| **CLS** | : Chronological life span |
| **$dH_2O$** | : Distilled water |
| **h** | : Hour |
| **HOG** | : High-osmolarity glycerol |
| **MAP** | : Mitogen-activated protein |
| **ml** | : Milliliter |
| **mM** | : Millimolar |
| **ng** | : Nanogram |
| **OD** | : Optical density |
| **$OD_{600}$** | : Optical density at 600 nm |
| **ROS** | : Reactive oxygen species |
| **rpm** | : Revolutions per minute |
| **SNV** | : Single nucleotide variation |
| **SUMO** | : Small ubiquitin-like modifier |
| **TOR** | : Target of rapamycin |
| **TORC** | : Target of rapamycin complex |
| **U** | : Unit |
| **YMM** | : Yeast minimal medium |
| **YPD** | : Yeast extract-peptone-dextrose |

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**EVOLUTIONARY ENGINEERING OF RAPAMYCIN-RESISTANT YEAST**

## SUMMARY


The budding yeast *Saccharomyces cerevisiae*, a unicellular eukaryotic microorganism, is widely used in many industrial processes such as baking, alcohol fermentation, biofuel and recombinant protein production as well as in basic research to understand the complex biological processes of advanced eukaryotic organisms, including humans due to its well-characterized genome and proteome, ease of growth and manipulation, as well as the similarity of its genes and pathways to higher organisms.

Rapamycin is a macrolide compound that is produced by the bacterium *Streptomyces hygroscopicus*. In the medical field, rapamycin and its analogs are used to prevent organ transplant rejection, coat coronary stents and treat tumor cells. Many key metabolic pathways including cell growth and lifespan, protein synthesis and ribosome biogenesis, regulation of cell cycle and size, environmental stress response, nutrient uptake, starvation control, and autophagy in *S. cerevisiae* and also other higher eukaryotes are affected by rapamycin due to its inhibition of the target of rapamycin (TOR). Fkbp12 forms a complex with rapamycin that interacts with TOR resulting in its inhibition where the exact mechanism is still unknown. Fkbp12, immunophilin, is conserved in its structure and function among eukaryotes from yeasts to mammalians. Fpr1 found in *S. cerevisiae* is an orthologue of Fkbp12 found in humans.

In this study, an inverse metabolic engineering strategy, evolutionary engineering was used to obtain rapamycin stress-resistant *S. cerevisiae*. Thus, serial batch cultivation of the *S. cerevisiae* CEN.PK113-7D reference strain under gradually increasing rapamycin stress was carried out. Before selection, a screening experiment was performed and 3 ng/ml rapamycin stress was determined as the initial stress level. During the selection process, the concentration of rapamycin in the medium was gradually increased from 3 ng/ml to 200 ng/ml over 61 daily passages or populations. From the final population, fifteen individual colonies were randomly selected which were named R1 to R15. Every individual (R1 to R15) was highly resistant to rapamycin stress in comparison to the reference strain (905). Afterward, R1, R3, R7, R12 and R14 were selected to continue for genetic stability test in which they were found to be genetically stable and their resistance to rapamycin was shown to be permanent.

Genetically stable strains were tested by spot assay for their cross-resistance or sensitivity against various stress types, including 0.5 mM $NiCl_2$, 2.5 mM $CrCl_3$, 3 mM $CoCl_2$, 17.5 mM $MnCl_2$, 50 mM $NH_4Fe(SO_4)_2$, 10 mM $AlCl_3$, 20 mM $CuCl_2$, 15 mM LiCl, 50 mM $H_3BO_4$, 100 µM $AgNO_3$, 0.5 M NaCl, 15 mM caffeine, 4 mM vanillin, 200 ng/ml propolis, 1mM coniferyl aldehyde and 200 ng/ml cycloheximide.

R12 was found to be the mutant with the highest number of cross-resistance and sensitivities: R12 strain was cross-resistant to $CuCl_2$, $NH_4Fe(SO_4)_2$, NaCl, coniferyl aldehyde, vanillin, cycloheximide, propolis; and sensitive to $AlCl_3$, $CoCl_2$, $H_3BO_4$ and $AgNO_3$ in comparison to the reference strain (905).

In order to determine the cell wall integrity of the rapamycin-resistant mutant (R12), lyticase susceptibility assay was performed. The result of this experiment showed that R12 resisted lyticase more than 905, under nonstress condition. On the other hand, under rapamycin stress, 905 resisted lyticase more than R12. Furthermore, the presence of rapamycin stress did not change the lyticase resistance of the evolved strain R12, in comparison to the nonstress condition.

The chronological lifespan of rapamycin resistant strain (R12) was determined by using semi-quantitative and also quantitative chronological lifespan analysis. The result of the experiments correlated with each other in which R12 was found to have a shorter CLS, in comparison to 905.

Comparative whole genome sequencing analysis of the rapamycin-resistant mutant (R12) revealed four single nucleotide variations (SNVs). These SNVs were located in four different genes. The precise functions of these genes in rapamycin response and resistance in yeast should be examined in greater detail in future studies.

In conclusion, a rapamycin-stress-resistant and genetically stable *S. cerevisiae* strain (R12) was successfully obtained by using evolutionary engineering in this thesis study, and characterized at genomic and physiological levels. These results indicate that TOR pathway-related changes occurred in rapamycin-resistant mutant in order to overcome the high levels of rapamycin stress. However, in order to fully comprehend the molecular basis of rapamycin-resistance of R12, its comparative transcriptomic and metabolic analyses would be necessary as future studies.

**EVRİMSEL MÜHENDİSLİKLE RAPAMİSİNE DİRENÇLİ MAYA ELDESİ**

**ÖZET**

*Saccharomyces cerevisiae*, tomurcuklanan bir maya türü olup; gerek fırıncılık, alkol fermentasyonu, biyoyakıt ve rekombinant protein üretimi gibi pek çok endüstriyel süreçte, gerekse de ökaryotik bir mikroorganizma olması nedeniyle insanın da dahil olduğu ileri ökaryotik organizmaların karmaşık biyolojik süreçlerini anlayabilmek için yapılan temel araştırmalarda bir model organizma olarak yaygın şekilde kullanılmaktadır. Model organizma olarak kullanılmasının ana sebepleri, *S. cerevisiae*'nin iyi karakterize edilmiş genomu ve proteomu olması, büyüme ve manipülasyon kolaylığı ve ayrıca genlerinin ve yolaklarının daha yüksek organizmalara benzerliğidir.

*S. cerevisiae* mayası, endüstriyel üretim koşullarında çeşitli stres türlerine maruz kalır. Bunlar açlık (besin kısıtlılığı), oksidatif stres, donma-erime stresi, ozmotik stres veya tuz stresi gibi çeşitli streslerdir. Bu tip çoklu stres türlerine dirençli mayaların elde edilmesi ve stres tepkisi ile stres direncine neden olan moleküler faktörlerin belirlenmesi önem arz etmektedir. *S. cerevisiae*, çevresel değişikliklere ve streslere karşı çeşitli hücresel yanıtlar vererek, homeostazını yani hücresel dengesini yeniden kazanmak için hücresel yolaklarında değişiklikler yapar. *S. cerevisiae*'de stres yanıt elementleri (STRE) olarak adlandırılan yaklaşık 900 genin, çeşitli düzensizlikler üzerine çevresel stres yanıtına dahil olduğu gösterilmiştir. Bu bulgular, stres tepkisi ile stres direncinin moleküler altyapısının son derece karmaşık olduğunu göstermektedir. Mayada ve ileri ökaryotlarda stres tepkisi ve direncinin moleküler mekanizmaları, henüz tam anlamıyla aydınlatılamamış olduğu için halen üzerinde çalışılmakta olan, karmaşık ancak güncel bir konudur.

Rapamisin, *Streptomyces hygroscopicus* bakterisi tarafından üretilen bir makrolid bileşiktir. Tıp alanında, rapamisin ve analogları organ nakli reddini önlemek, koroner stentleri kaplamak ve tümör hücrelerini tedavi etmek için kullanılır. *S. cerevisiae*'de ve ayrıca diğer yüksek ökaryotlarda hücre büyümesi ve ömrü, protein sentezi ve ribozom biyogenezi, hücre döngüsü ve boyutunun düzenlenmesi, çevresel stres tepkisi, besin alımı, açlık kontrolü ve otofaji gibi birçok önemli metabolik yolak, rapamisinin TOR inhibisyonu nedeniyle etkilenir. Fkbp12, rapamisin ile kompleks oluşturarak mekanizması henüz bilinmeyen bir şekilde TOR'u inhibe eder. İmmünofilin olan Fkbp12, mayalardan memelilere kadar ökaryotlar arasında yapısı ve işlevi bakımından değişiklik göstermez. *S. cerevisiae*'de bulunan Fpr1, insanlarda bulunan Fkbp12'nin bir ortologudur. *S. cerevisiae* mayasında, iki ayrı gen (*TOR1* ve *TOR2*) iki ayrı TOR kompleksi kodlamaktadır, memeli TOR kompleksi (mTORC1 ve mTORC2) ise tek bir gen ile kodlanmaktadır. TOR, hem mayalarda hem de memelilerde Ser/Thr protein kinaz aktivitesine sahiptir. Bu organizmalar, ayrıca TOR fonksiyonu için önemli olan $Ser^{1972/1975}$, $Trp^{2042}$ and $Phe^{2049}$ rezidülerini de korumuştur.

Rapamisin, maya hücrelerinde retrograd sinyalleme yolağıyla amino asit biyosentezini etkiler ve bu da belirli amino asitlerin (çoğunlukla glutamin ve glutamat) üretiminin artmasına neden olur. TOR'un inhibisyonu, *S. cerevisiae*'de özellikle Msn2/4 ve Rim15'in etkinleştirilmesi yolağıyla Çevresel Stres Yanıtı (ESR) yolaklarını etkinleştirir. TOR ve Msn2/4 gibi faktörlerin *S. cerevisiae*'nin ısı, oksidatif stres ve tuz stresi tepkisine dair genleriyle ilişkili olduğu da belirlenmiştir. Benzer şekilde, rapamisinin ve TOR inhibisyonunun, hücre duvarının bütünlüğünün korunması ile ilgili metabolik yolağı da aktive ettiği gösterilmiştir.

Bu çalışmada, rapamisin stresine dayanıklı *S. cerevisiae* suşları elde etmek için, tersine metabolik mühendislik yaklaşımı olan evrimsel mühendislik kullanılmıştır. Bu doğrultuda, kademeli olarak artan rapamisin stresi altında *S. cerevisiae* CEN.PK113-7D referans suşuna kesikli kültürlerde seleksiyon uygulanmıştır. Seleksiyondan önce tarama deneyi yapılarak, başlangıç stres seviyesi 3 ng/ml rapamisin stresi olarak belirlendi. Seleksiyon sırasında, ortamdaki rapamisin konsantrasyonu, 61 günlük pasaj veya popülasyonda kademeli olarak 3 ng/ml'den 200 ng/ml'ye çıkarıldı. Nihai popülasyondan, R1 ila R15 olarak adlandırılan on beş bireysel koloni rastgele seçildi. Her birey (R1 ila R15), referans suşa (905) kıyasla rapamisin stresine karşı oldukça dirençli bulundu. Sonrasında, R1, R3, R7, R12 ve R14 genetik kararlılık testine devam etmek üzere seçildi ve genetik olarak kararlı oldukları bulundu. Böylelikle rapamisine karşı dirençlerinin kalıcı olduğu gösterildi.

Genetik olarak kararlı suşlar, 0.5 mM $NiCl_2$, 2.5 mM $CrCl_3$, 3 mM $CoCl_2$, 17.5 mM $MnCl_2$, 50 mM $NH_4Fe(SO_4)_2$, 10 mM $AlCl_3$, 20 mM $CuCl_2$, 15 mM LiCl, 50 mM $H_3BO_4$, 100 µM $AgNO_3$, 0.5 M NaCl, 15 mM kafein, 4 mM vanilin, 200 ng/ml propolis, 1mM koniferil aldehit ve 200 ng/ml sikloheksimit dahil olmak üzere çeşitli stres türlerine karşı çapraz dirençleri veya hassasiyetleri için damlatma testine (spot assay) tabi tutuldu. R12'nin en yüksek sayıda çapraz direnç ve duyarlılığa sahip mutant olduğu bulundu: R12 suşu referans suşa (905) kıyasla, $CuCl_2$, $NH_4Fe(SO_4)_2$, NaCl, koniferil aldehit, vanilin, sikloheksimit, propolis'e karşı çapraz dirençli; ve $AlCl_3$, $CoCl_2$, $H_3BO_4$ ve $AgNO_3$'e duyarlı bulundu.

Rapamisine dirençli mutantın (R12) hücre duvarı bütünlüğünü test etmek için litikaz duyarlılık testi yapıldı. Bu deneyin sonucu, R12'nin stressiz koşullar altında litikaza 905'ten daha fazla direnç gösterdiğini gösterdi. Öte yandan, rapamisin stresi altında, 905 litikaza R12'den daha fazla direnç gösterdi. Ayrıca, rapamisin stresinin varlığı, stressiz duruma kıyasla evrimleşmiş R12 suşunun litikaz direncini değiştirmedi.

Rapamisine dirençli suşun (R12) kronolojik ömrü, yarı kantitatif ve ayrıca kantitatif kronolojik ömür analizi kullanılarak belirlendi. Deneylerin sonuçları birbiriyle tutarlı olup, ayrıca R12'nin 905'e kıyasla daha kısa bir CLS'ye sahip olduğu görüldü.

Rapamisine dirençli mutantın (R12) karşılaştırmalı tüm genom dizileme analizi, dört tek nükleotit varyasyonunu (SNV'ler) ortaya çıkardı. Bu SNV'ler dört farklı gende tespit edildi. Bu genlerin mayadaki rapamisin yanıtı ve direncindeki kesin işlevlerini ortaya çıkarabilmek için, daha ayrıntılı çalışmaların yapılması gerekmektedir.

Sonuç olarak, bu tez çalışmasında evrimsel mühendislik yöntemi kullanılarak rapamisin stresine karşı dirençli ve genetik olarak kararlı bir *S. cerevisiae* suşu (R12) başarıyla elde edilmiş olup, bu suşun genomik ve fizyolojik düzeyde karakterizasyonu gerçekleştirilmiştir. Bu sonuçlar, yüksek rapamisin stres seviyelerinin üstesinden gelebilmek için, rapamisine dirençli mutantta, TOR yolağı ile ilgili değişikliklerin meydana geldiğini göstermektedir. Bununla birlikte, R12'nin rapamisin direncinin moleküler temelini tam olarak anlayabilmek için, ileride karşılaştırmalı transkriptomik ve metabolik analizlerin de gerçekleştirilmesi uygun olacaktır.

# 1. INTRODUCTION

## 1.1 General information about *Saccharomyces cerevisiae*

Yeast is a fungus that mainly exists as a unicellular organism and yeasts are found in wide range of natural habitats which includes skin surfaces and intestinal tracts of animals where they can live symbiotically or as parasites (Schneiter, 2004). *Saccharomyces cerevisiae* is a species of budding yeast that is a member of the phylum Ascomycete and the kingdom Fungi. In addition, the most well-known and economically relevant yeasts are among the related species and strains of *Saccharomyces cerevisiae* (Kurtzman, 2005). Table 1.1 depicts the classification of *Saccharomyces cerevisiae*.

**Table 1.1 :** Scientific classification of *Saccharomyces cerevisiae*.

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

A single-celled eukaryote, *Saccharomyces cerevisiae* is often known as budding yeast, brewer's yeast, or baker's yeast that can proliferate both sexually and asexually. Furthermore, it has membrane-bound organelles including a nucleus, mitochondria and endomembrane system. The common name of *Saccharomyces cerevisiae* as budding yeast originates from its unique cell division process called budding which is a type of vegetative reproduction in which tinier daughter cells bud off the mother cell (Duina et al., 2014).

Sexual production of *S. cerevisiae* includes two mating types which are haploid a cell and haploid α cells. a-cells produce 'a-factor' which is a mating pheromone which attracts neighbouring α cells. Similarly, α cells produce 'α factor' which attracts a cell. The repression and MAT locus on chromosome III which contains MATa or MAT α alleles, regulates the activation of mating types. When under stress, diploid cells can go through meiosis to create four haploid spores, each of which contains two a and two α cells (Davey,1998). Figure 1.1 depicts the life cycle of a budding yeast.

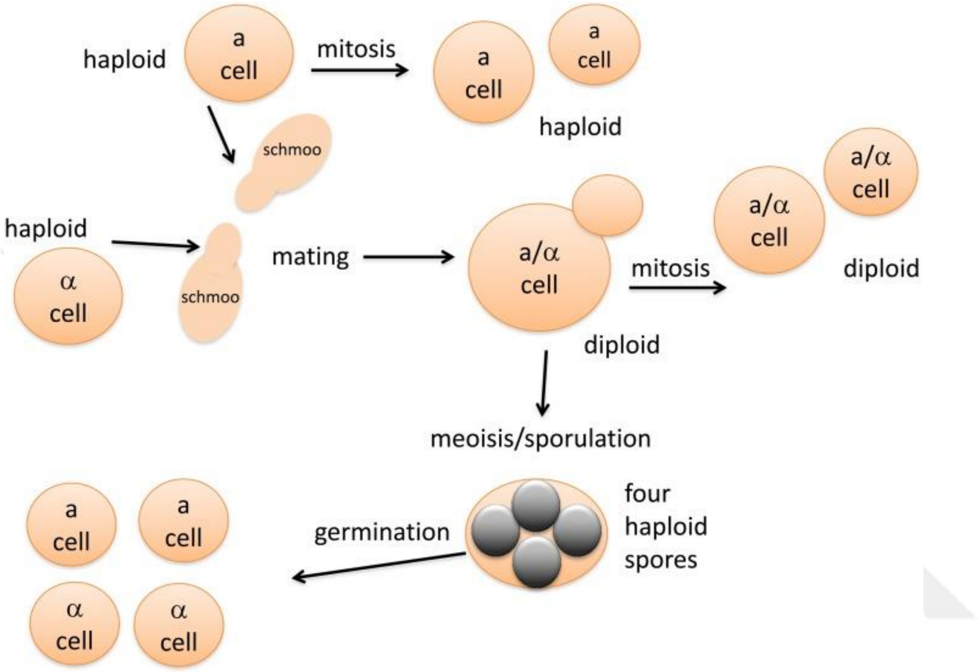


**Figure 1.1 :** A simplified representation of the life cycle of a budding yeast (Duina et al., 2014).

*S. cerevisiae* can generate ATP from sugars via two distinct pathways which are aerobic respiration and anaerobic fermentation. Both start with glycolysis where glucose is turned into pyruvate and ATP. In the absence of oxygen, pyruvate is utilized in the fermentation process in order to form ethanol, carbon dioxide and lesser ATP. In aerobic respiration; through TCA cycle and oxidative phosphorylation (OXPHOS), pyruvate is entirely oxidized to carbon dioxide which leads to more ATP generation. Additionally, with adequate concentrations of glucose and oxygen, *S. cerevisiae* can do both aerobic respiration and anaerobic fermentation simultaneously in a process called 'Respiro-Fermentative' metabolism which is also known as 'Crabtree effect'. In this process; ethanol that is accumulated in the surroundings through fermentation process that can be utilized for ATP generation in the absence of glucose (Pfeiffer and Morley, 2014). *S. cerevisiae* energy metabolism is shown in Figure 1.2.

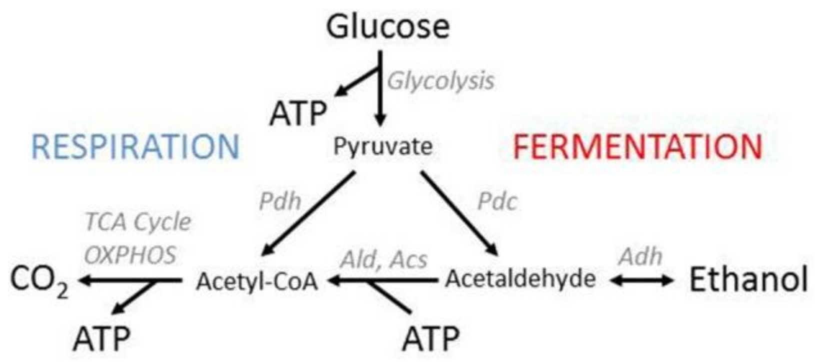


**Figure 1.2 :** *Saccharomyces cerevisiae* energy metabolism (Pfeiffer and Morley, 2014).

### 1.2 Clinical and industrial applications of *Saccharomyces cerevisiae*

Usage of yeast by humans have begun approximately 7000 years ago with the discovery process of fermentation of crushed grapes in water (Mortimer, 2000). At the present day; yeast takes part in various industrial applications such as modern baking, brewing and fermenting alcoholic beverages along with pharmaceutical applications like vaccine preparation, production of secreted proteins for instance, monoclonal antibodies and cytokines. Furthermore; yeast is already used in Brazil and China for production of biofuels through production of ethanol for fuel (Duina et al., 2014; Botstein & Fink, 2011). Biotechnological product spectrum of *S. cerevisiae* is shown in Figure 1.3.

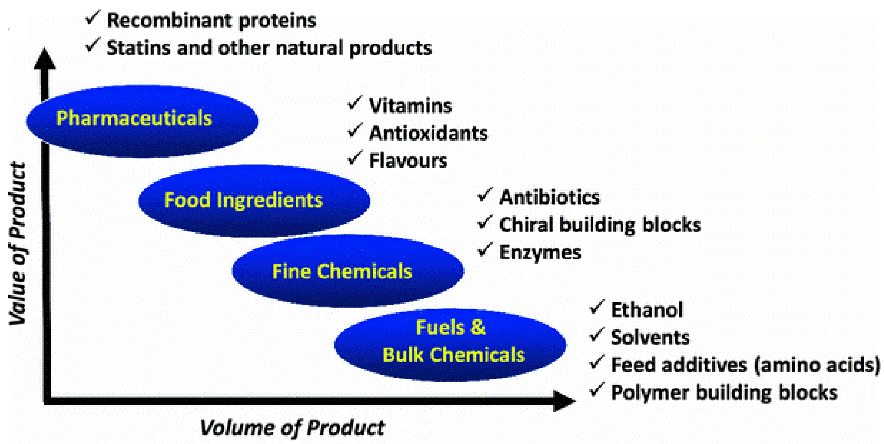


**Figure 1.3 :** Biotechnological product spectrum of *S. cerevisiae* (Hong & Nielsen, 2012).

*S. cerevisiae* provides an insight on genome organization and evolution by being the first fully sequenced eukaryotic genome in 1996. A haploid yeast cell contains approximately 12,000 kb of genomic DNA that is subdivided into 16 chromosomes which are thought to have evolved from an ancestral set of 8 distinct chromosomes via whole-genome duplication (Duina et al., 2014). Furthermore, most open reading frames of *S. cerevisiae*'s genome have been annotated; however, there is smattering information on the functions of noncoding RNA along with subcellular distribution of proteins and metabolites in the cell cycle (Botstein & Fink, 2011). Due to *S. cerevisiae*'s rapid generation time, low-cost cultivation and articulate genetics; its genome and proteome are extremely well-characterized so it has been widely used as a model organism in biochemistry, cell biology, molecular biology and systems biology studies due to its ease of growth and manipulation, as well as the similarity of its genes and pathways to higher organisms. In the light of these advantages, methods like metabolome, proteome and transcriptome analyses were first developed using yeast, before being widely used with other higher organisms. *S. cerevisiae* research is essential for establishing analogy between *S. cerevisiae* and humans in research areas including the unfolded protein response, lipid metabolism, mitochondrial metabolism, prion formation and aging (Smith et al., 2010; Cherry et al., 2012).

### 1.3 Environmental stress responses in *S. cerevisiae*

The maintenance of homeostasis is necessary for all organisms including *S. cerevisiae* in order to ensure optimal growth and resist against fluctuations such as available nutrients, osmolarity, temperature, and acidity of their environment and the variable presence of destructive agents such as toxic chemicals and radiation. Thus, the organism's genomic expression drastically changes in response to the environmental changes that can be either responsive to a variety of stresses which is called as 'environmental stress response' (ESR) or specific to particular environmental conditions (Gasch et al., 2000).

In order to minimize detrimental effects of stress; instantaneous molecular reactions to repair damage while preventing against further exposure to the stress is mainly achieved by stress proteins which improves cell's survival and tolerance to harsh environments. Yeast cells that are exposed to mild stress conditions would not only develop tolerance to higher concentrations of the same stress; but also develop tolerance against other stress agents, a phenomenon known as cross protection. In this context, general stress response element (STRE) is a common cis element that is found in the promoter region of stress genes which regulates the synchronized induction of several stresses (Estruch, 2000).

The yeast *S. cerevisiae* is exposed to various types of stresses under industrial production conditions such as starvation (nutrient restriction), oxidative stress, freeze-thaw stress, osmotic stress or salt stress. It is important to obtain yeasts resistant to multiple stress types and to determine the stress response and the molecular factors that cause stress resistance (Çakar et al., 2005; Çakar et al., 2012). *S. cerevisiae* modifies its cellular pathways to regain its homeostasis by giving various cellular responses to environmental changes and stresses. It has been shown that approximately 900 genes, called stress response elements (STRE) in *S. cerevisiae*, are involved in the environmental stress response on various disorders (Gasch et al., 2000). These findings show that the molecular infrastructure of stress response and stress resilience is extremely complex. Molecular mechanisms of stress response and resistance in yeast and advanced eukaryotes is a complex but current issue that is still being studied, as it has not been fully elucidated yet.

### 1.3.1 Rapamycin stress

Rapamycin is a secondary product of *Streptomyces hygroscopicus*, a bacterium from soil that comes from Rapa-Nui (Easter Island). Firstly, it was discovered as an antifungal agent in the 1970s (Vezina et al., 1975). Rapamycin was approved as a pharmaceutical drug by FDA for the anti-rejection drug in kidney transplants in 1999 (Li et al., 2014). After that, rapamycin and its analog drugs were used against tumors, cancer risks, Parkinson's disease, AIDS, and rheumatoid arthritis (Li et al., 2014).

Rapamycin affects many key metabolic pathways in *S. cerevisiae* and also other higher eukaryotes by inhibiting the target of rapamycin (TOR) pathway such as cell growth and lifespan, protein synthesis and ribosome biogenesis, regulation of cell cycle and size, environmental stress response, nutrient uptake, starvation control and autophagy (Loewith R., 2011; Conrad M., 2014). It was shown that rapamycin activity mainly depends on the binding of its target FKBP12 (FK506-binding protein 12 kDa). FKBP12-rapamycin complex interacts with TOR resulting in inhibition of TOR where the exact action mechanism is still unknown. TOR is a kind of kinase and FKBP12, immunophilin, are conserved in its structure and function among eukaryotes from yeasts to mammalians. Fpr1 (FK506-sensitive proline rotamase 1) found in *S. cerevisiae* is an orthologue of FKBP12 found in humans (Kasahara et al., 2020). *S. cerevisiae* encodes two TOR complexes through two separate genes *TOR1* and *TOR2*, mammalian TOR complex (mTORC1 and mTORC2) is encoded by one gene. TOR has Ser/Thr protein kinase activity in both yeasts and mammalians (Inoue & Nomura, 2018). Also, they have conserved $Ser^{1972/1975}$, $Trp^{2042}$ and $Phe^{2049}$ residues which are important for TOR function (Powell & Delgoffe, 2010). Major metabolic pathways that are regulated by the two mTOR complexes are shown in Figure 1.4.

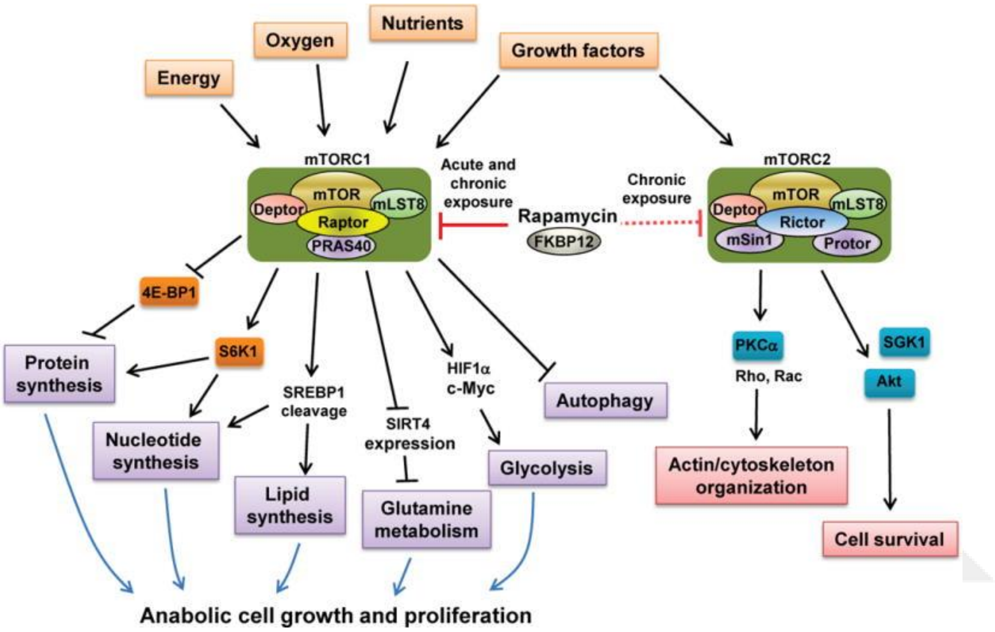


**Figure 1.4 :** Major metabolic pathways that are regulated by the two mTOR complexes (Li et al., 2014).

### 1.3.2 Caffeine stress

Caffeine is a methylxanthine alkaloid that is derived from the seeds, nuts, or leaves of numerous plants. It is chemically related to the bases adenine and guanine found in DNA and RNA molecules and present in coffee, tea, soft and energy drinks, chocolate, etc. (Ashihara, 2006). Furthermore; caffeine can enhance cognitive performance by stimulating central nervous system that reduces fatigue and drowsiness, it also enhances physical performance by improving athletic performance in aerobic and anaerobic conditions (Nehlig et al., 1992; Pesta et al., 2013). On the other hand; side effects of caffeine consumption include vasoconstriction, anxiety and dehydration (Dworzański, 2009).

Caffeine evokes pleiotropic effect that leads ultimately to cell’s death. Furthermore, targeting mitogen-activated protein kinase (MAPK), target of rapamycin 1/2 kinases

(TOR 1/2 kinase) and Ras/cAmp-dependent protein kinase signalling pathways, caffeine functions as a kinase inhibitor in *S. cerevisiae*. Phosphorylation of Mpk1p with caffeine induction negligibly activates main downstream of Rlm1p transcription factor. In addition; transcriptional programme that is evoked by caffeine resembles with response to rapamycin where caffeine inhibits TOR1/2 kinases which incidentally activates the Pkc1p-Mpk1p cascade (Kuranda et al., 2006). Major metabolic pathways that are affected by caffeine in yeast are shown in Figure 1.5.

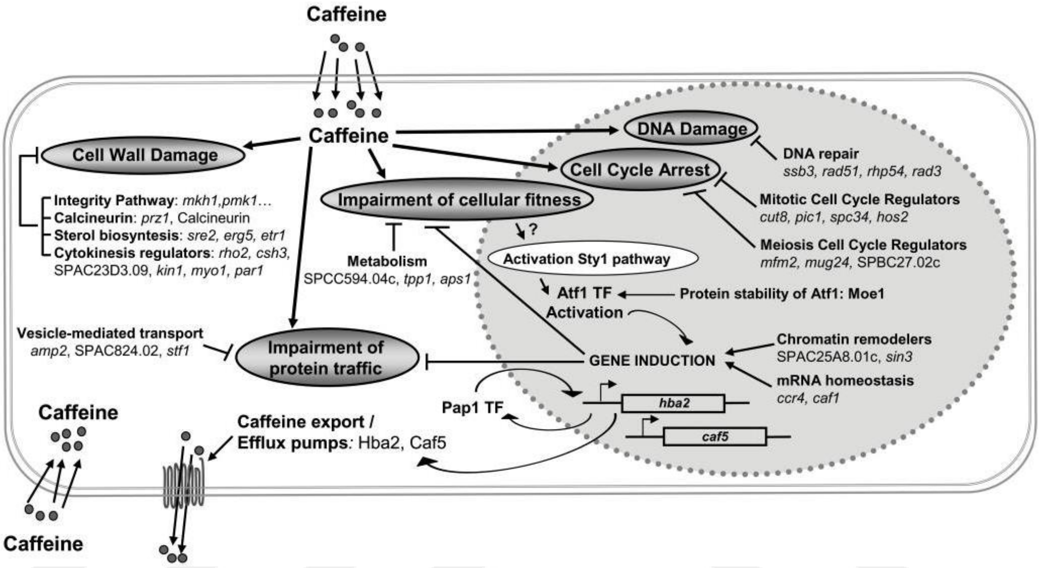


**Figure 1.5 :** Major metabolic pathways that are affected by caffeine in yeast (Calvo et al., 2009).

### 1.3.3 Coniferyl aldehyde stress

The industrial biofuel production through conversion of plant materials is a two-step process where the hydrolysis of plant lignocellulose is followed by the fermentation of resulting sugars. However, hydrolysis of lignocellulosic materials produces toxic by-products which include phenolic substances such as coniferyl aldehyde, ferulic acid and 4-hydroxybenzoic acid that severely inhibit yeast fermentation. Among these phenolic compounds, coniferyl aldehyde is the most hazardous one which induces reactive oxygen species (ROS) partly localized to mitochondria and fewer localized to endoplasmic reticulum (ER). Furthermore, it has been discovered that Zwf1p, which is a glucose-6-phosphate dehydrogenase enzyme, catalyzes the rate limiting step of pentose phosphate pathway, it reduces reactive oxygen species to promote coniferyl aldehyde tolerance, and when the cell is exposed to coniferyl aldehyde, Zwf1p localizes partly to the mitochondria and ER (Fletcher et al., 2019).

Typically, yeast cells respond to phenolic substances by activating stress response genes and multidrug resistance (MDR) pathways. The main reactions of *S. cerevisiae* to the drug include decreased drug absorption, activation of detoxification systems, and conversion of the drug to a less hazardous state. Therefore, coniferyl aldehyde is transformed into less harmful ferulic acid and alcoholic derivatives shown in Figure 1.6 (Wu et al., 2017; Adeboye et al., 2015).

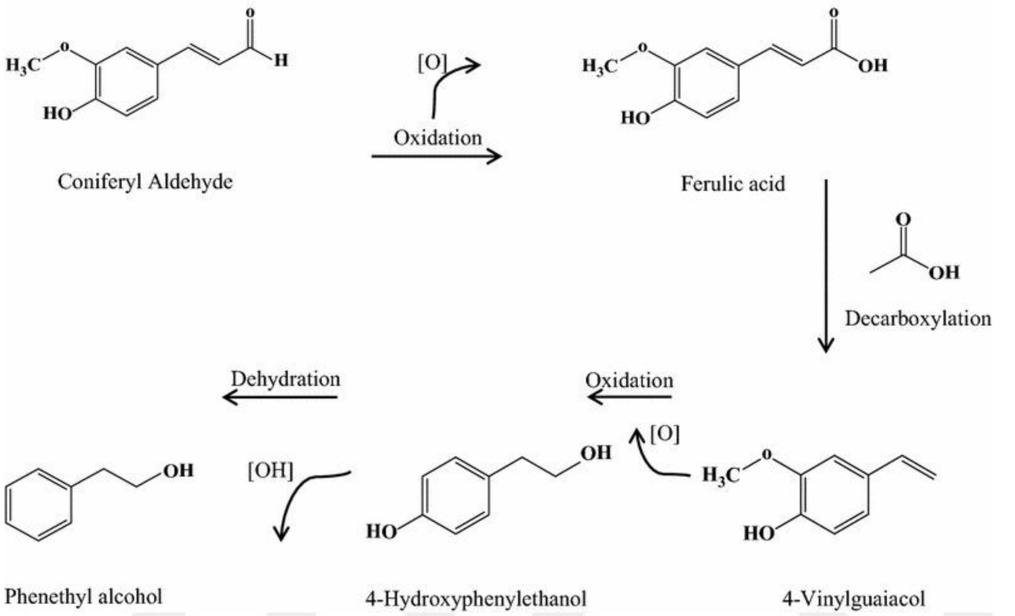


**Figure 1.6 :** Coniferyl aldehyde detoxification to phenyl ethyl alcohol (Adeboye et al., 2015).

### 1.3.4 Propolis stress

Propolis is a naturally occurring, non-toxic resinous mixture made by bees in order to close off gaps and prevent diseases and parasites from entering the hive as well as to stop bacterial and fungal growth. Furthermore; it has applications in food, pharmaceutical and cosmetic industry plus it is often employed in traditional medicine. Propolis contains over 300 different compounds which mainly consist of resin composed of polyphenols, wax, essential oils, pollen. Propolis functions as an antioxidant agent by reacting and scavenging ROS in yeast, while its exact protection mechanism is unknown (Cigut et al., 2011). After oxidative stress, propolis therapy has been shown to lower levels of ROS production and lipid peroxidation and it has been hypothesized that propolis may be working in concert with Cu/Zn-Sod (Copper-Zinc Superoxide Dismutase) as a result of an increase in Cu/Zn-Sod activity (de Sá et al., 2013).

## 1.4 Metabolic and evolutionary engineering

The field of microbial biotechnology known as metabolic engineering aims to improve cellular functions and characteristics by modifying the cellular enzymatic, transport, and regulatory functions using recombinant DNA technology. (Bailey, 1991). Rational or classical metabolic engineering involves making certain genetic changes to the organism's biochemical reactions network, which requires extensive knowledge of genetic and biochemistry of the metabolic pathways studied. In addition, due to the complexity of the cellular response mechanism, unexpected metabolic results may occur in rational metabolic engineering applications. In order to minimize such restrictive features of rational metabolic engineering, the inverse metabolic engineering approach has been introduced (Bailey et al., 1996). The steps of this approach are the opposite of the rational metabolic engineering steps: Foremost, the desired phenotype is identified, isolated or obtained under laboratory conditions, as the first step of this approach. In the second step, the molecular (genetic, etc.) and/or environmental factors causing the desired phenotype are determined using physiological analyses and the rapidly developing omics technologies (genomic, transcriptomic, proteomic, etc.). In the last step, the determined genetic factors are transferred to an industrial microorganism suitable for production (Bailey et al., 1996; Çakar et al., 2012).

Evolutionary engineering, which is an inverse metabolic engineering approach, is a powerful strategy to obtain industrially important and desired microbial phenotypes (Çakar et al., 2012). Evolutionary engineering consists of systematically repeated random mutation and selection cycles of the desired phenotype (Çakar et al. 2012; Mans et al. 2018). The evolutionary engineering approach was employed by the Çakar lab for obtaining stress-resistant *S. cerevisiae*. Examples include multi-stress resistance (Çakar et al. 2005), ethanol resistance (Turanlı-Yıldız et al., 2017), drug (caffeine) stress resistance (Sürmeli et al. al., 2019) and resistance to silver stress (Terzioğlu et al., 2020).

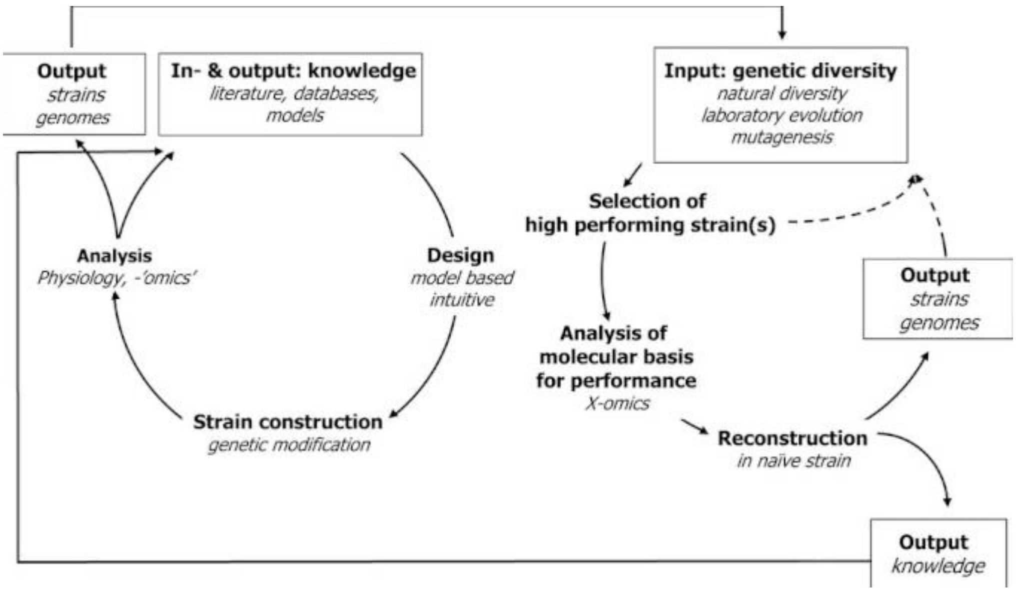


**Figure 1.7 :** The interactions between the "forward" and "reverse" metabolic engineering cycles (Oud et al., 2011).

### 1.5 Aim of the study

The aim of this study was to obtain rapamycin stress-resistant and genetically stable *S. cerevisiae* yeast strains by using evolutionary engineering which is an inverse metabolic engineering approach. Rapamycin stress may affect many key cellular activities in *S. cerevisiae* and higher eukaryotes including humans, such as cell development and lifespan, protein synthesis, ribosome biogenesis, regulation of cell cycle and size, environmental stress response, nutrient intake, starvation control, and autophagy. Within the scope of the thesis, genetically stable yeast strains with high rapamycin resistance were obtained by applying selection in batch cultures in the presence of increasing levels of rapamycin stress with the evolutionary engineering method. Yeast strains obtained by evolutionary engineering were tested for rapamycin resistance and genetic stability. In addition, physiological assays were performed to determine whether they acquired resistance to other types of stress. In order to determine the genetic factors responsible for rapamycin resistance, comparative whole genome sequencing was performed on rapamycin-resistant yeast and the reference yeast strain. In conclusion, the rapamycin-resistant yeasts obtained in this study were physiologically and genomically analyzed, and the findings can be used for clinical and industrial research and applications in the long term.

# 2. MATERIALS AND METHODS

## 2.1 Materials

### 2.1.1 Yeast strains

*S. cerevisiae* CEN.PK 113-7D (*MATa, MAL2-8c, SUC2*) strain was used as a reference strain that was generously provided by by Prof. Dr. Jean Marie François and Dr. Laurent Benbadis (University of Toulouse, France). In this study, S. *cerevisiae* CEN.PK 113-7D reference strain has been referred as ‘905’.

### 2.1.2 Growth medium

In this study, unless otherwise stated, Yeast complex medium (YPD) was mainly used for revivals (Table 2.1). In YPD medium, revivals were made by incubation at 30°C and 150 rpm, for 24 h. Yeast minimal medium (YMM) was mainly used for preculture and cultivations for stress applications (Table 2.2). In YMM medium, precultures were made by incubation at 30°C and 150 rpm, approximately for 16 h.

**Table 2.1 :** Composition of Yeast Peptone Dextrose Medium (YPD).

| Ingredient | Amount (w/v) |
|---|---|
| Yeast Extract | 1% |
| Dextrose | 2% |
| Peptone | 1% |
| Agar (for solid media) | 1% |

**Table 2.2 :** Composition of Yeast Minimal Medium (YMM).

| Ingredient | Amount (w/v) |
|---|---|
| Yeast Nitrogen Base without Amino Acids | 1% |
| Dextrose | 2% |
| Agar (for solid media) | 1% |

### 2.1.3 Chemicals

The chemicals that were used in this study are listed in Table 2.3.

**Table 2.3 :** Chemicals that were used in this study.

| Chemical | Producer |
|---|---|
| Agar | BD DifcoTM |
| Yeast nitrogen base without amino acids | BD DifcoTM |
| Yeast extract | Merck |
| Dextrose | Riedel-de-Haen |
| Peptone | Difco |
| Ethanol | J.T Baker |
| Rapamycin | Sigma |
| Caffeine | Merck |
| Vanilin | Sigma-Aldrich |
| Coniferyl aldehyde | Sigma-Aldrich |
| Nickel(II) chloride hexahydrate | Merck |
| Cobalt(II) chloride hexahydrate | Sigma |
| Manganase(II) chloride tetrahydrate | Merck |
| Aluminum chloride hexahydrate | Merck |
| Lithium chloride | Sigma |
| Boric acid | Merck |
| Sodium chloride | Merck |
| Glycerol | Duchefa Biochemie |
| Acetic acid | Merck |
| Hydrogen peroxide | Merck |

### 2.1.4 Laboratory equipment

The laboratory equipment that was used in this study are listed in Table 2.4.

**Table 2.4 :** Laboratory equipment that were used in this study.

| Equipment | Producer |
|---|---|
| Autoclave | GR 110 GF-Zealway |
| Balance | Precisa XB220A |
| Deep freezer | -80°C SANYO UltraLow |
| 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 |
| Refrigerator | +4°C Arçelik |
| Micropipettes | Eppendorf |
| Nanodrop 2000 | Thermo Fisher Scientific |

## 2.2 Methods

### 2.2.1 Screening for determination of the initial rapamycin concentration for evolutionary selection experiments

To determine the initial rapamycin stress level for evolutionary engineering, a screening experiment was performed with the reference strain (905) at various rapamycin concentrations. For this purpose, overnight cultures of reference strain were inoculated in 10 ml of YMM in 50 ml culture tubes containing rapamycin stress (1, 2, 3, 4, 5, 10, 15, 20, 25 and 50 ng/ml) as well as the control (YMM) tubes without rapamycin stress. Incubation was performed at 30°C and 150 rpm and initial $OD_{600}$ of cultures were set to 0.2 which is approximately $2.8x10^6$ cells $ml^{-1}$. Cell growth was measured at $24^{th}$ and $48^{th}$ hours. Rapamycin resistance levels were expressed in terms of percent survival rate that were calculated by dividing $OD_{600}$ of each stressed culture to their control cultures and then multiplying by 100. Rapamycin was dissolved in absolute ethanol.

### 2.2.2 Evolutionary engineering strategy for the selection of rapamycin-resistant mutants

As the evolutionary selection protocol, cells were grown in batch cultures in the presence of increasing rapamycin stress levels. Overnight cultures of the reference strain (905) were inoculated in 10 ml of YMM broth in 50 ml culture tubes containing 3.0 ng/ml rapamycin stress, along with the control tube without rapamycin stress. The initial $OD_{600}$ of cultures was set to 0.2 and $OD_{600}$ value was measured after $24^{th}$ hour growth. Later on, stress-treated culture was named as the $1^{st}$ rapamycin resistant population (first passage) which was then inoculated into 3.1 ng/ml rapamycin stress-containing YMM broth medium and cultivated for 24 hours in order to obtain the $2^{nd}$ rapamycin resistant population (second passage). Shortly, with each population (after 24 hours of incubation), rapamycin concentration of the medium (10 ml YMM) in which the cells (0.2 $OD_{600}$ for 10ml) were incubated, was increased by 0.1 ng/ml starting from 3.0 ng/ml (3.1, 3.2, 3.4… ng/ml), then by 0.2 ng/ml starting from 4.0 ng/ml, by 0.5 ng/ml starting from 9.0 ng/ml, and then by 1.0 ng/ml starting from 15.0 ng/ml up to 25.0 ng/ml followed by 50 ng/ml, 100 ng/ml and 200 ng/ml rapamycin stress, as the final stress level. Stock cultures of each passage were prepared in 30% (v/v) glycerol and preserved at - 80°C. Rapamycin resistance levels were expressed in terms of percent survival rate that were calculated by dividing $OD_{600}$ of each stressed culture to their control cultures and then multiplying by 100.

### 2.2.3 Selection of individual resistant mutants

After applying evolutionary selection strategy for 61 populations (rapamycin stress levels ranging between 3.0 ng/ml to 200.0 ng/ml), rapamycin resistant individuals were selected by using YMM agar plates. For this purpose, overnight cultures of the final population ($61^{st}$ population) and the reference strain (905) were inoculated in 10 ml of YMM broth in 50 ml culture tubes. Growth was performed at 30℃ and 150 rpm for 24 h. Cultures were diluted from $10^{-1}$ to $10^{-6}$ and spread onto YMM agar plates containing 150 ng/ml rapamycin and to control YMM plates without rapamycin. The growth of individual colonies was monitored at 30℃ for $24^{th}$, $48^{th}$, $72^{nd}$ hours.

Fifteen individual colonies were randomly selected from YMM agar plate containing 150 ng/ml rapamycin, grown at a dilution factor of $10^{-5}$. After the selection of 15 individual colonies from the agar plate, each evolved strain was inoculated into 10 ml of YMM broth in 50 ml culture tubes at 30℃ and 150 rpm for 24 h. Stock cultures of each individual colonies were prepared in 30% (v/v) glycerol and preserved at - 80℃. Evolved strains were examined under microscope in order to ensure there is no contamination.

### 2.2.4 Estimation of rapamycin stress resistance of evolved strains

Following the selection of the rapamycin resistant mutants, the most resistant individuals were identified by using the spot assay method. For this purpose, $OD_{600}$ values of overnight cultures of 15 individual evolved strains, $61^{st}$ population (final population of selection) and the reference strain (905) were set to 0.2 in 10 ml YMM broth in 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 up to $10^{-5}$ with $dH_2O$. YMM agar plates containing control and 200 ng/ml rapamycin were prepared. Afterwards, 5 µL of each dilution was spotted onto plates. Plates were incubated for 72 hours at 30°C and the results were then photographed.

### 2.2.5 Genetic stability analysis

In order to determine if the rapamycin-resistant mutants were genetically stable, genetic stability test was performed. For that purpose, overnight cultures of previously selected five evolved mutants and reference strain (905) inoculated in 10 ml of YMM broth in 50 ml culture tube in the absence of selective pressure. Incubation was performed at 30°C and 150 rpm. After 24 hours of cultivation, the cultures (0.2 of $OD_{600}$) were inoculated into 10 ml fresh YMM in a 50 ml culture tube. Successive passaging procedure was continued for 10 days and stock cultures of each passage were prepared in 30% (v/v) glycerol and preserved at - 80°C.

Afterwards, spot assay technique was carried out with $0^{th}$, $2^{nd}$, $4^{th}$, $6^{th}$, $8^{th}$ and $10^{th}$ days of passages and reference strain (905) which were revived in YPD broth and precultured in YMM broth. $OD_{600}$ values of overnight cultures of 5 individual evolved strains and the reference strain (905) were set to 0.2 in 10 ml YMM broth in 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 up to $10^{-5}$ with $dH_2O$. YMM agar plates containing control, 25 ng/ml and 50 ng/ml rapamycin were prepared. Afterwards, 5 µL of each dilution was spotted onto plates. Plates were incubated for 72 hours at 30°C and the results were then photographed.

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

Using spot assay, the genetically stable strains were tested for their cross resistance or sensitivity against various stress types including 0.5 mM $NiCl_2$, 2.5 mM $CrCl_3$, 3 mM $CoCl_2$, 17.5 mM $MnCl_2$, 50 mM $NH_4Fe(SO_4)_2$, 10 mM $AlCl_3$, 20 mM $CuCl_2$, 15 mM LiCl, 50 mM $H_3BO_4$, 100 µM $AgNO_3$, 0.5 M NaCl, 15 mM caffeine, 4 mM vanillin, 200 ng/ml propolis, 1mM coniferyl aldehyde and 200 ng/ml cycloheximide.

The spot assay technique was carried out with the genetically stable mutants and reference strain (905) which were revived in YPD broth and precultured in YMM broth. $OD_{600}$ values of overnight cultures of 5 individual evolved strains and the reference strain (905) were set to 0.2 in 10 ml YMM broth in 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 up to $10^{-5}$ with $dH_2O$. YMM agar plates containing control and various stress factors were prepared. Afterwards, 5 μL of each dilution was spotted onto plates. Plates were incubated for 72 hours at 30°C and the results were then photographed.

### 2.2.7 Lyticase susceptibility assay

In order to test the cell wall integrity of the selected rapamycin-resistant mutant, lyticase susceptibility assay was performed (Kuranda et al., 2006). For this reason, overnight cultures of the reference strain (905) and the rapamycin-resistant mutant were inoculated in 100 mL YMM broth in 500 mL flasks and grown at 30 °C and 150 rpm, beginning with an $OD_{600}$ of 0.2 under 2 ng/ml rapamycin stress and nonstress (control) conditions until the cells reached the stationary phase of growth. The cultures were harvested by centrifugation at 5500 g for 10 minutes. The pellets ($OD_{600}$ of 0.9 per ml) were dissolved in 10 ml of 10 mM Tris/HCl buffer (pH 7.4) including 40 mM β-mercaptoethanol, and incubated for 30 minutes at 25 °C. Later on, 2 U/ml of lyticase (β-1,3 glucanase from *Arthrobacter luteus*) was added to each sample along with the blank solution (10 mM Tris/HCl buffer (pH 7.4)) which were then incubated at 300 rpm and 30 °C, to follow the decrease in $OD_{600}$ of the samples over time. The experiment was performed in triplicate.

### 2.2.8 Chronological life span analysis

#### 2.2.8.1 Semi-quantitative chronological life span analysis

The quantitative CLS method can be labor-intensive and challenging. Therefore, the semi-quantitative CLS method was used first to determine the CLS of the evolved strain, as described previously (Arslan et al., 2018). For this purpose, overnight cultures of reference strain (905) and the evolved strain were inoculated in 20 ml of YMM broth in 100 ml flasks in the absence of rapamycin stress. Incubation was performed at 30°C and 150 rpm and initial $OD_{600}$ of cultures were set to 0.1. After 3 days of incubation, the cells have reached the end of the stationary phase of growth which was accepted as day 0 of the CLS experiment. Samples were withdrawn from cultures and $OD_{600}$ of cultures were set to 6 ($\approx 1.2 \times 10^8$ cells/ml). The serial dilutions were then performed up to $10^{-5}$ using $dH_2O$ and the spot assay was performed by spotting 5 μl from both cultures on YPD agar plates which were incubated at 30 °C for 48 hours and photographed. During semi-quantitative CLS experiment, the procedure including the adjustment of $OD_{600}$ to 6, serial dilutions and spotting onto

YPD agar plates, was repeated every second day. The experiment was performed in triplicate. At the end of day 10, all the plate photographs that were taken were joined together into a single image.

#### 2.2.8.2 Quantitative chronological life span analysis

In order to verify the semi-quantitative CLS method results and to quantitatively determine the CLS of the rapamycin stress-resistant mutant, the quantitative CLS method was performed with the reference strain (905) and the rapamycin stress-resistant evolved strain (Arslan et al., 2018). For this purpose, overnight cultures of reference (905) and evolved strains were inoculated into 100 ml of YMM broth in 500 ml flasks in the absence of rapamycin stress. Incubation was performed at 30°C and 150 rpm and initial $OD_{600}$ of cultures were set to 0.2. After 3 days of incubation, the cells have reached the end of the stationary phase of growth which was accepted as day 0 of the CLS experiment and the initial viability of the culture was assessed using the viable cell counting method. In order to perform this, 100 µl samples were withdrawn from cultures, diluted 10 times with $dH_2O$, placed onto YPD agar plates which were incubated at 30 °C for 72 hours. The viability of the cultures was evaluated every other day using the viable cell counting method, based on their initial viability set as 100% and until the viability decreases to 0.0001%. The experiment was performed in triplicate.

### 2.2.9 Whole genome sequencing of the evolved strain

Following overnight growth in 100 ml of YPD broth in 500 ml flasks at 150 rpm and 30°C, genomic DNA was isolated for whole genome sequencing of the reference strain and the rapamycin-resistant evolved strain using the MasterPure DNA Purification Kit (Epicentre), according to the manufacturer’s protocol. The Nanodrop spectrophotometer was used to measure DNA concentrations and quality. Comparative whole genome sequencing of rapamycin-resistant mutant and basic bioinformatic analysis were performed by Gen Era Diagnostik A.Ş. Illumina NextSeq 550 next-generation sequencing platform was used for whole genome resequencing. Quality control of the results obtained as a result of the resequencing process was performed using FastQC software (Babraham Bioinformatics). Alignment was performed on the *S. cerevisiae* CEN.PK113-7D reference genome using the Burrows-Wheeler alignment software MEM (Li & Durbin, 2009). Variant search was performed using the Genome Analysis Toolkit (DePristo et al., 2011) and nucleotide variations were examined in the Genome Browser (Golden Helix) software. High-quality point mutations were filtered using R software (Team, 2019).

# 3. RESULTS

## 3.1 Screening for determination of the initial rapamycin concentration for evolutionary selection experiments

A screening experiment was carried out with the reference strain (905) at various rapamycin concentrations in order to determine the initial rapamycin stress level for evolutionary engineering experiments. Based on the literature, it was decided that rapamycin concentrations should be between 1.0 ng/ml and 50 ng/ml (1, 2, 3, 4, 5, 10, 15, 20, 25 and 50 ng/ml) (Dikicioğlu et al., 2018; Wride et al., 2014). The results generally indicated that, with increasing rapamycin concentration, survival rates decreased. 24$^{th}$ hour screening data indicated that, after 3.0 ng/ml rapamycin stress, survival rates started to decline drastically (Figure 3.1). Thus, 3.0 ng/ml was decided as the initial rapamycin concentration for selection experiment.

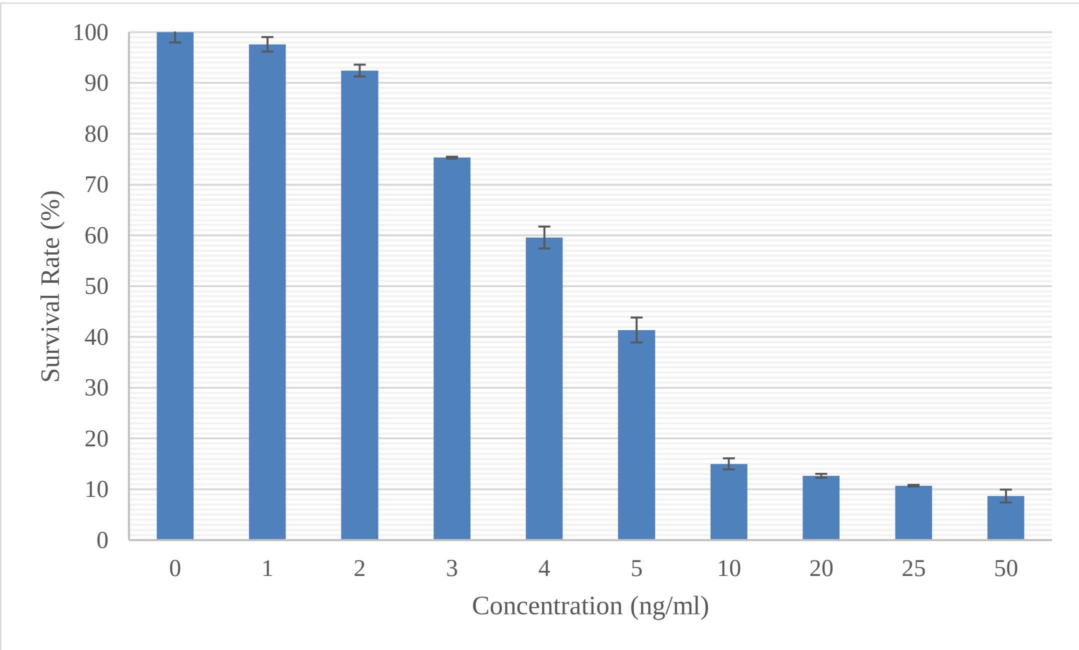


**Figure 3.1 :** The survival rates of the reference strain (905) cultures after 24 h incubation in YMM with various rapamycin stress concentrations.

### 3.2 Evolutionary engineering strategy for the selection of rapamycin-resistant mutants

According to previous rapamycin screening results, initial stress level for the selection was determined as 3.0 ng/ml. Serial batch cultivation of the *S. cerevisiae* CEN.PK113-7D reference strain under increasing rapamycin stress conditions was used to select rapamycin-resistant strains via evolutionary engineering method. The concentration of rapamycin in the medium was gradually increased from 3.00 ng/ml to 200 ng/ml over 61 daily passages or populations (≈ 275 generations) (Table 3.1). The survival rates of each population are shown in Figure 3.2.

**Table 3.1 :** Rapamycin concentration of each passage, 24$^{th}$ hour $OD_{600}$ values of stress and non-stress conditions and percent survival rates during evolutionary engineering selection.

| Passage Number | Rapamycin Concentration (ng/ml) | $OD_{600}$ Control | $OD_{600}$ Stress | Percent Survival Rate (%) |
|---|---|---|---|---|
| 1 | 3.0 | 5.05 | 4.25 | 84.14 |
| 2 | 3.1 | 5.45 | 4.64 | 85.19 |
| 3 | 3.2 | 5.61 | 3.86 | 68.82 |
| 4 | 3.3 | 5.26 | 2.87 | 54.55 |
| 5 | 3.4 | 5.66 | 3.03 | 53.47 |
| 6 | 3.5 | 4.99 | 0.88 | 17.70 |
| 7 | 3.6 | 5.09 | 4.71 | 92.48 |
| 8 | 3.7 | 4.92 | 4.75 | 96.56 |
| 9 | 3.8 | 5.16 | 4.82 | 93.57 |
| 10 | 3.9 | 5.15 | 4.66 | 90.51 |
| 11 | 4.0 | 5.41 | 5.23 | 96.75 |
| 12 | 4.2 | 5.22 | 4.01 | 76.81 |
| 13 | 4.4 | 5.03 | 3.25 | 64.64 |
| 14 | 4.6 | 3.84 | 3.81 | 99.13 |
| 15 | 4.8 | 5.22 | 4.51 | 86.40 |
| 16 | 5.0 | 5.10 | 4.29 | 84.12 |
| 17 | 5.2 | 5.66 | 5.45 | 96.20 |
| 18 | 5.4 | 4.61 | 4.39 | 95.19 |
| 19 | 5.6 | 4.25 | 4.09 | 96.17 |
| 20 | 5.8 | 5.38 | 3.68 | 68.37 |
| 21 | 6.0 | 4.61 | 3.56 | 77.31 |
| 22 | 6.2 | 4.63 | 3.66 | 79.17 |
| 23 | 6.4 | 5.33 | 4.89 | 91.62 |
| 24 | 6.6 | 4.71 | 4.21 | 89.49 |
| 25 | 6.8 | 5.07 | 4.55 | 89.63 |
| 26 | 7.0 | 5.31 | 4.63 | 87.25 |
| 27 | 7.2 | 4.87 | 4.47 | 91.80 |
| 28 | 7.4 | 5.11 | 4.79 | 93.60 |

**Table 3.1 (continued) :** Rapamycin concentration of each passage, 24th hour $OD_{600}$ values of stress and non-stress conditions and percent survival rates during evolutionary engineering selection.

| Passage Number | Rapamycin Concentration (ng/ml) | $OD_{600}$ Control | $OD_{600}$ Stress | Percent Survival Rate (%) |
|---|---|---|---|---|
| 29 | 7.6 | 5.07 | 4.92 | 97.20 |
| 30 | 7.8 | 4.91 | 4.29 | 87.41 |
| 31 | 8.0 | 4.50 | 4.30 | 95.49 |
| 32 | 8.2 | 4.44 | 3.66 | 82.38 |
| 33 | 8.4 | 4.79 | 4.30 | 89.80 |
| 34 | 8.6 | 4.79 | 4.40 | 91.87 |
| 35 | 8.8 | 5.27 | 4.91 | 93.18 |
| 36 | 9.0 | 4.92 | 4.84 | 98.44 |
| 37 | 9.5 | 5.09 | 4.71 | 92.53 |
| 38 | 10.0 | 4.96 | 4.69 | 94.57 |
| 39 | 10.5 | 5.21 | 5.00 | 96.01 |
| 40 | 11.0 | 5.28 | 5.17 | 97.87 |
| 41 | 11.5 | 5.18 | 5.05 | 97.61 |
| 42 | 12.0 | 5.11 | 4.84 | 94.70 |
| 43 | 12.5 | 5.20 | 5.03 | 96.76 |
| 44 | 13.0 | 5.07 | 4.27 | 84.18 |
| 45 | 13.5 | 4.92 | 4.73 | 96.12 |
| 46 | 14.0 | 5.20 | 4.91 | 94.53 |
| 47 | 14.5 | 4.52 | 4.26 | 94.17 |
| 48 | 15.0 | 5.52 | 5.32 | 96.35 |
| 49 | 16.0 | 5.09 | 4.91 | 96.43 |
| 50 | 17.0 | 5.54 | 5.32 | 96.00 |
| 51 | 18.0 | 5.18 | 5.14 | 99.20 |
| 52 | 19.0 | 5.36 | 5.16 | 96.28 |
| 53 | 20.0 | 5.24 | 5.16 | 98.45 |
| 54 | 21.0 | 5.04 | 4.96 | 98.37 |
| 55 | 22.0 | 5.10 | 4.90 | 96.17 |
| 56 | 23.0 | 5.67 | 5.60 | 98.78 |
| 57 | 24.0 | 4.97 | 4.87 | 98.01 |
| 58 | 25.0 | 5.41 | 5.29 | 97.64 |
| 59 | 50.0 | 5.56 | 5.26 | 94.50 |
| 60 | 100.0 | 5.24 | 3.68 | 70.29 |
| 61 | 200.0 | 5.51 | 5.20 | 94.41 |

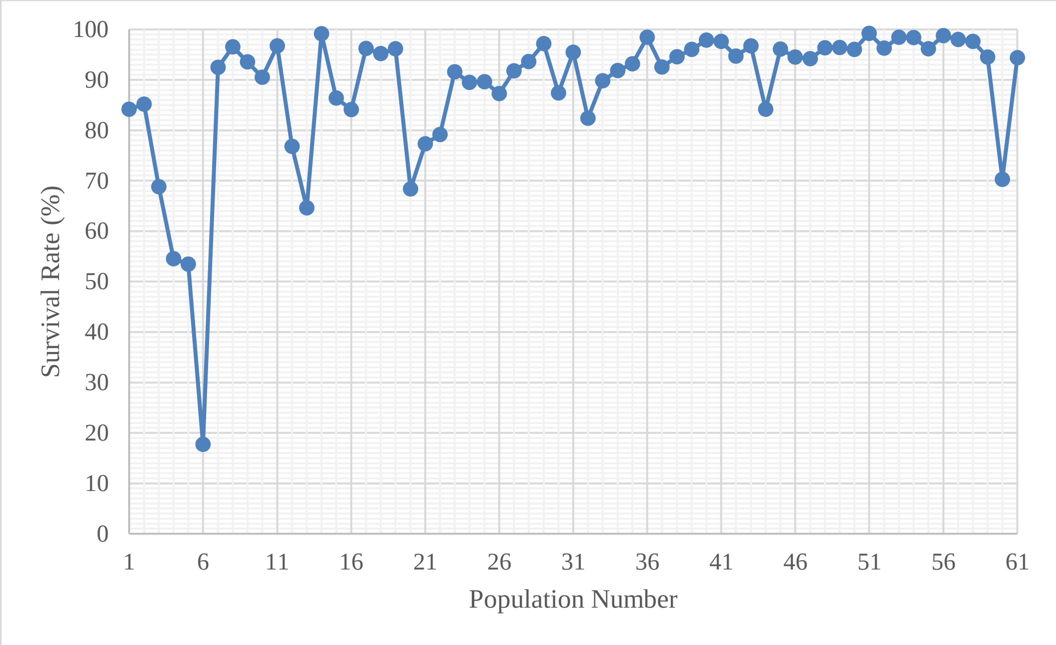


**Figure 3.2 :** Survival rates of populations descended from the reference strain (905) during evolutionary selection procedure.

### 3.3 Selection of individual resistant mutants

In order to isolate rapamycin resistant single colonies from the final population of evolutionary selection procedure ($61^{st}$ population), the $61^{st}$ population (final population) and the reference strain (905) were spread onto YMM agar plates containing stress (150 ng/ml rapamycin) and to control plates at various dilutions ($10^0$ to $10^{-6}$). After 72 hours of incubation on YMM agar plates, the plate with the dilution factor $10^{-5}$ and with 150 ng/ml rapamycin stress contained 30-300 colonies for $61^{st}$ population (final population) and no colonies were observed on the plate for the reference strain (905). Thus, the plate with $10^{-5}$ dilution and 150 ng/ml rapamycin was chosen for the selection of individual evolved strains. Fifteen individual colonies were randomly selected from the YMM agar plate containing 150 ng/ml rapamycin with dilution factor of $10^{-5}$ which were named as R1 to R15 (Figure 3.3). Stock cultures of each individual colonies were prepared in 30% (v/v) glycerol and preserved at - 80°C. Furthermore, samples from each mutant were examined for contamination by checking their morphology under the light microscope, and there was no contamination (data not shown).

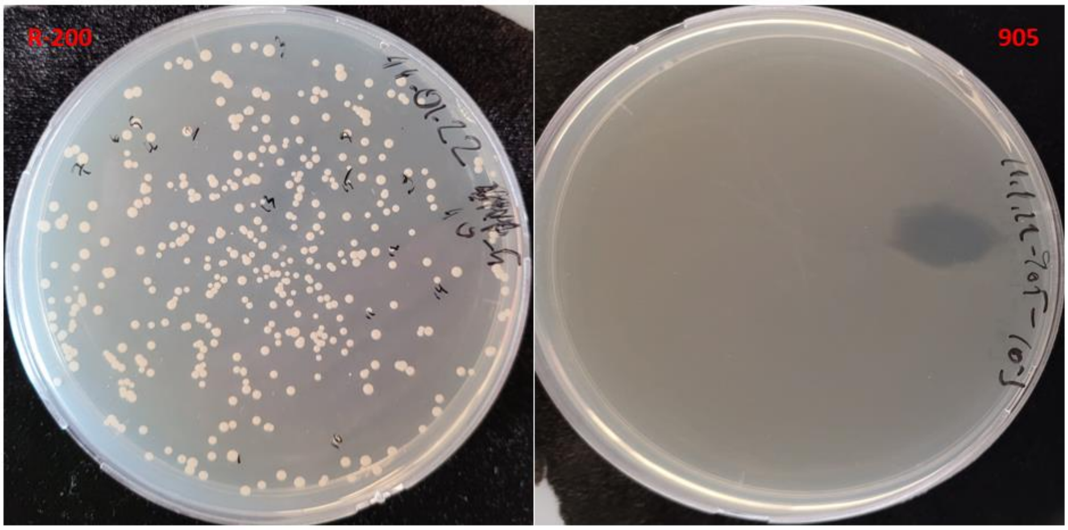


**Figure 3.3 :** Left: Selected individual colonies from final (61st) population of selection were marked (1 to 15) on 150 ng/ml rapamycin containing YMM agar plate ($10^{-5}$ dilution). Right: No colony was observed on 150 ng/ml rapamycin-containing YMM agar plate ($10^{-3}$ dilution) of the reference strain (905).

### 3.4 Estimation of rapamycin stress resistance of evolved strains

Following the selection of the 15 rapamycin-resistant mutant colonies, the most resistant individuals were identified by using the spot assay method. YMM agar plates with stress (200 ng/ml rapamycin) and nonstress (control) conditions were incubated for 72 hours. Every individual (R1 to R15) could grow up to 5 spots of serial dilution except R2 which could grow up to 4 spots while the reference strain (905) could only grow up to two spots. Furthermore, resistance level of final population (R200) was found to be similar to those of the individual mutants (Figure 3.4). Thus, it can be stated that every individual (R1 to R15) colony was resistant to 200 ng/ml rapamycin stress in comparison to the reference strain (905). R1, R3, R7, R12 and R14 mutants were selected for further analysis.

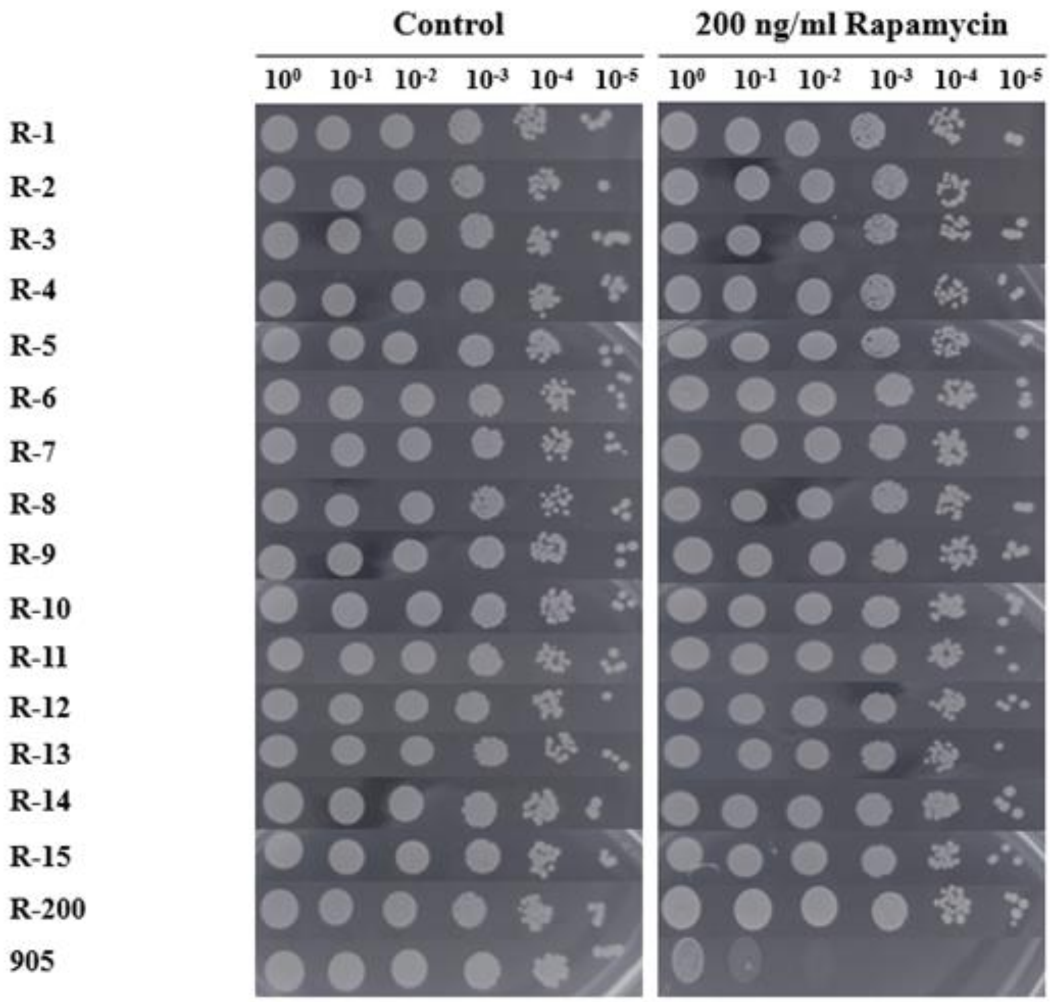


**Figure 3.4 :** Rapamycin resistance of selected mutants (R-1 to R-15), reference strain (905) and final population (R-200) under 200 ng/ml rapamycin stress and control conditions upon 72 h incubation.

### 3.5 Genetic stability analysis

Rapamycin-resistant selected mutants (R1, R3, R7, R12, and R14) were tested for their genetic stability. During 10 successive daily passages in YMM medium without selective pressure (rapamycin stress), stocks were prepared. Genetic stability analysis was continued by applying spot assay to $0^{th}$, $2^{nd}$, $4^{th}$, $6^{th}$, $8^{th}$ and $10^{th}$ day of stock cultures against 25 ng/ml, 50 ng/ml rapamycin stress and nonstress (control) conditions. It was observed that all evolved strains tested maintained their rapamycin resistance, according to the genetic stability analysis results (Figure 3.5).

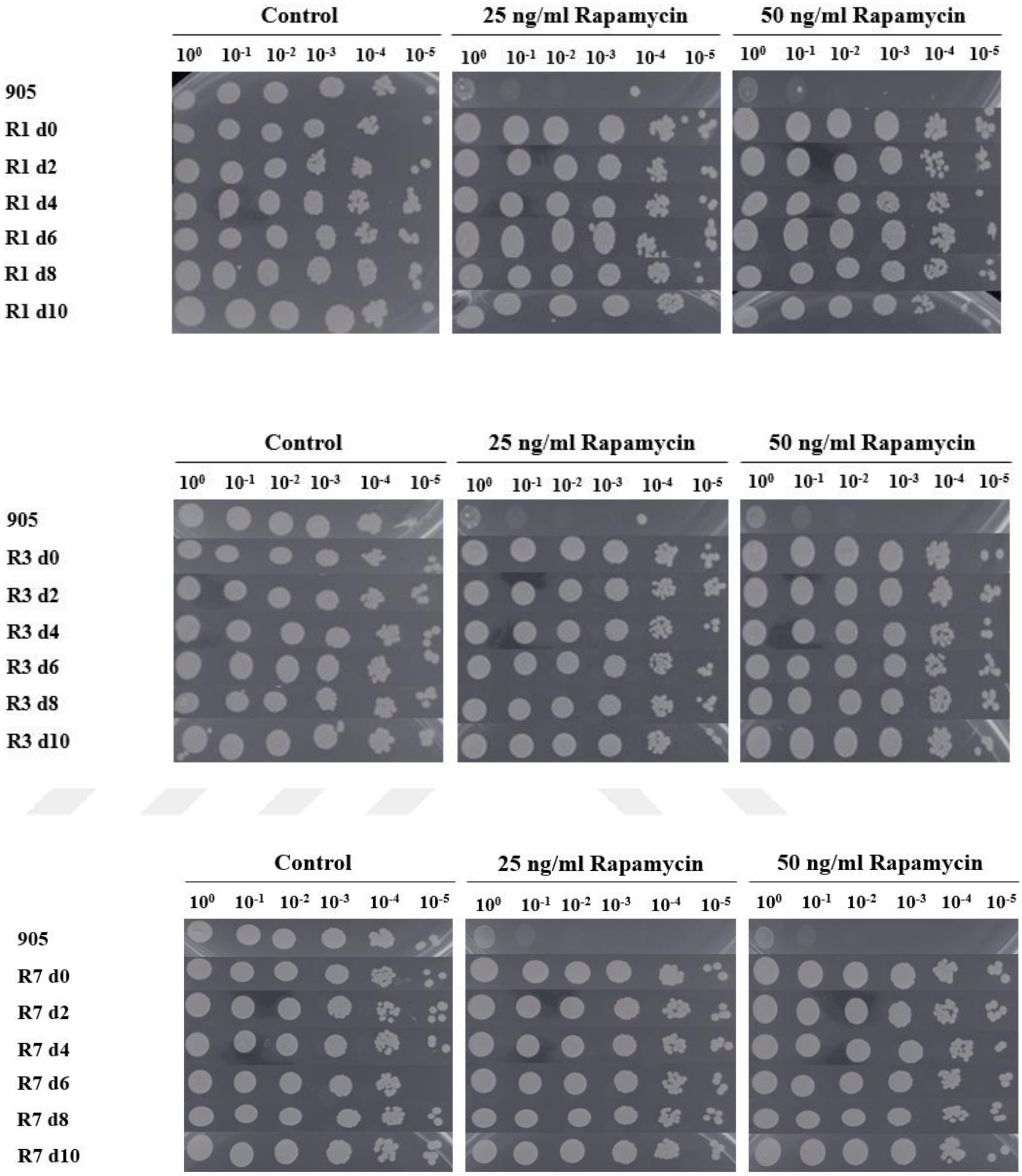


**Figure 3.5 :** Genetic stability test results of R1, R3, R7, R12 and R14 mutants. Spot assay photos were taken at 72 h of incubation and d0 to d10 indicate the day of passage in YMM medium without selective pressure.

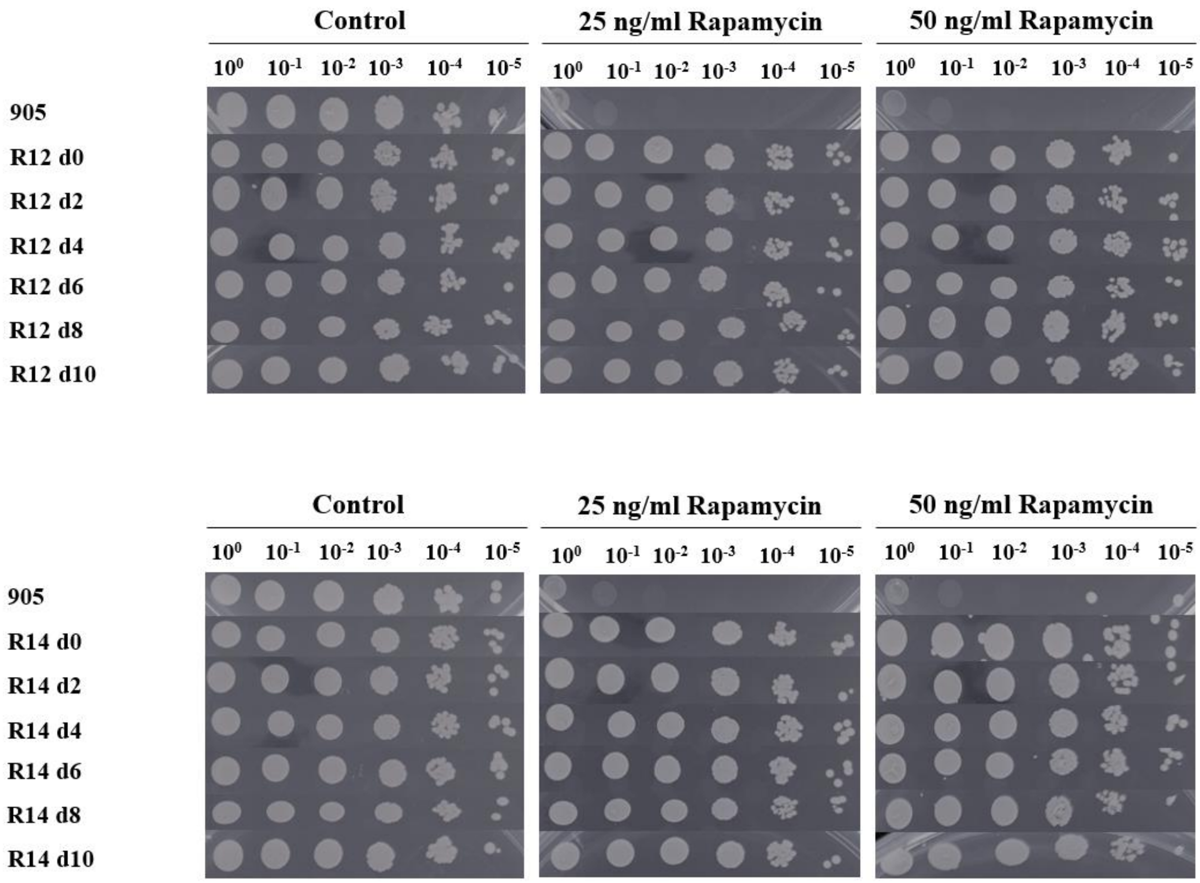


**Figure 3.5 (continued) :** Genetic stability test results of R1, R3, R7, R12 and R14 mutants. Spot assay photos were taken at 72 h of incubation and d0 to d10 indicate the day of passage in YMM medium without selective pressure.

## 3.6 Determination of cross-resistance and sensitivity against other stress conditions

Genetically stable strains were tested by spot assay for their cross-resistance or sensitivity against various stress types, including 0.5 mM $NiCl_2$, 2.5 mM $CrCl_3$, 3 mM $CoCl_2$, 17.5 mM $MnCl_2$, 50 mM $NH_4Fe(SO_4)_2$, 10 mM $AlCl_3$, 20 mM $CuCl_2$, 15 mM LiCl, 50 mM $H_3BO_4$, 100 µM $AgNO_3$, 0.5 M NaCl, 15 mM caffeine, 4 mM vanillin, 200 ng/ml propolis, 1mM coniferyl aldehyde and 200 ng/ml cycloheximide (Figure 3.6). According to cross-resistance and sensitivity analysis results, R12 was found to be the mutant with the highest number of cross-resistance and sensitivities. R12 strain was cross-resistant to $CuCl_2$, $NH_4Fe(SO_4)_2$, NaCl, coniferyl aldehyde, vanillin, cycloheximide, propolis; and sensitive to $AlCl_3$, $CoCl_2$, $H_3BO_4$ and $AgNO_3$. It was neither resistant nor sensitive to $NiCl_2$, $CrCl_3$, LiCl, $MnCl_2$ and caffeine, in comparison to the reference strain (905) (Table 3.2). By taking cross analysis results into consideration, R12 mutant was selected for further analysis.

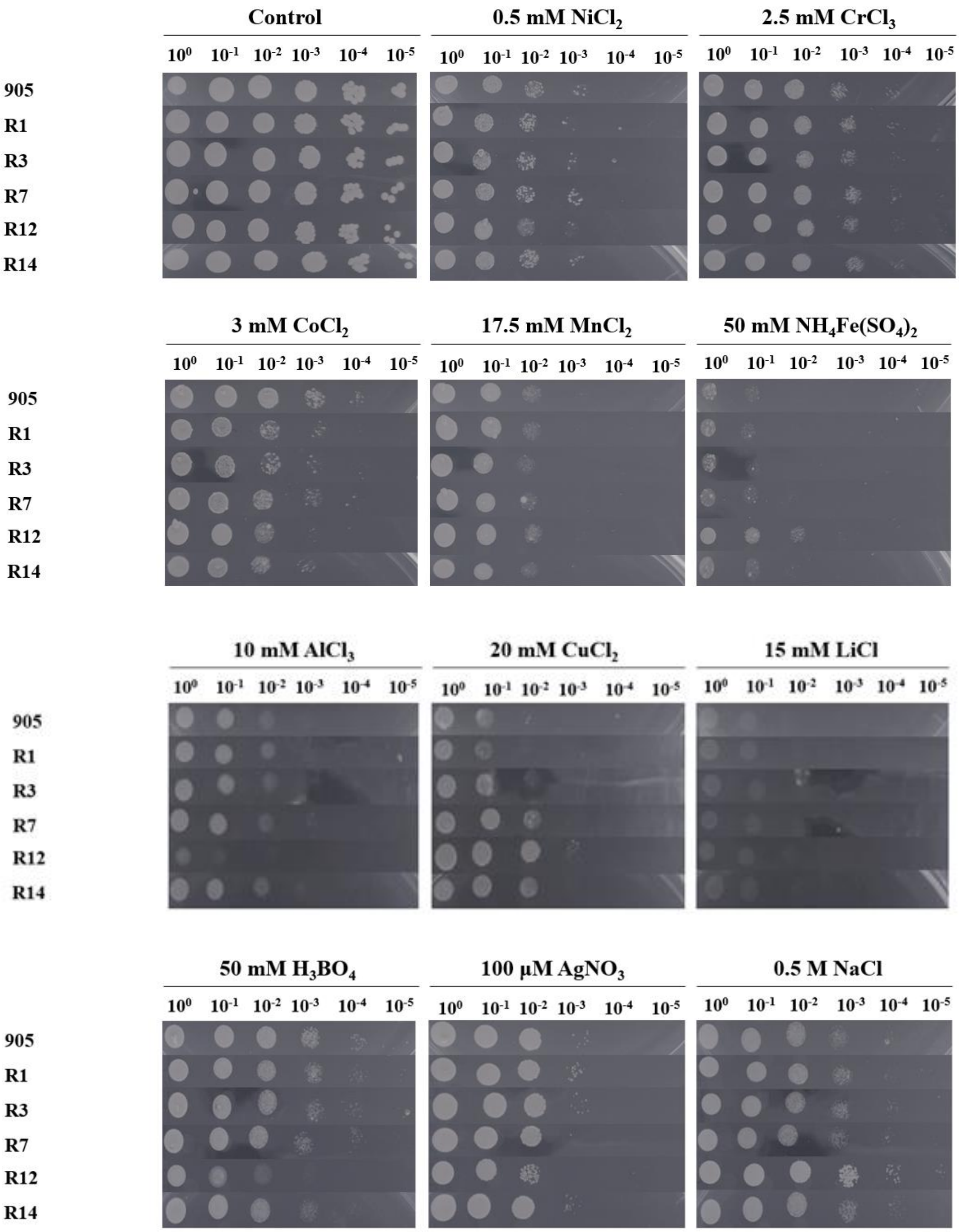


**Figure 3.6 :** Cross-resistance results of R1, R3, R7, R12, R14 strains and the reference strain (905) upon different stress conditions after 72 hours of incubation by using spot assay technique.

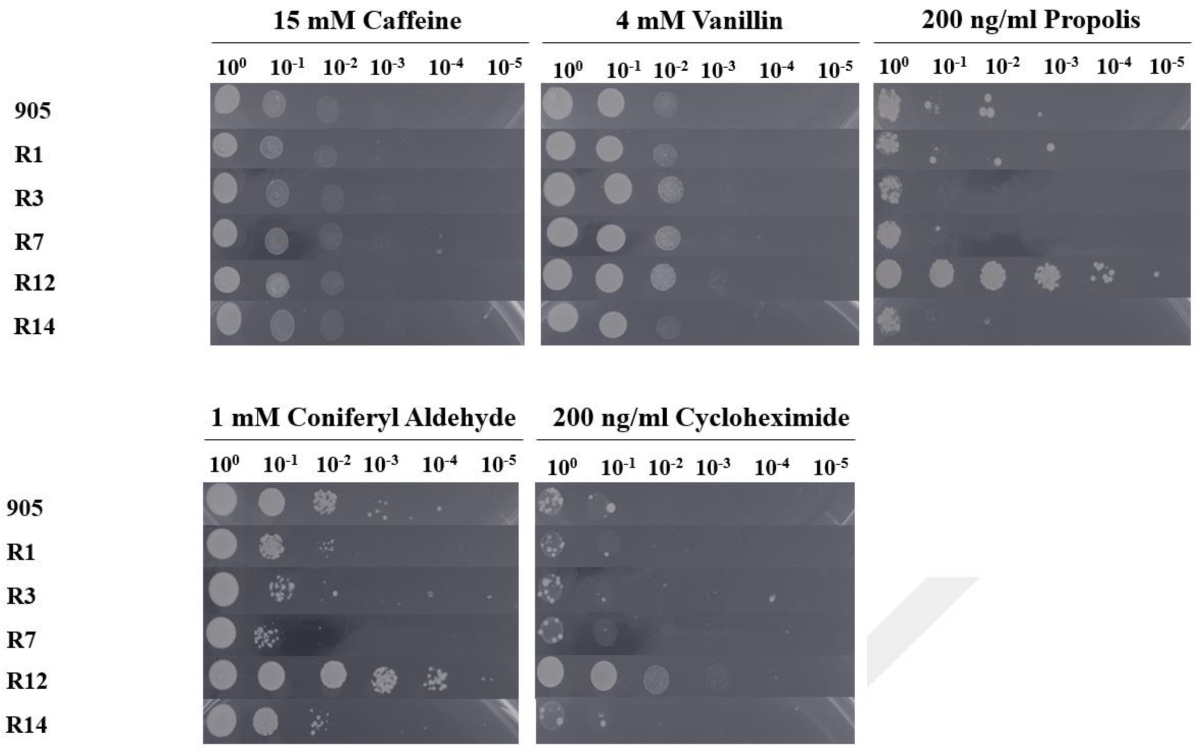


**Figure 3.6 (continued) :** Cross-resistance results of R1, R3, R7, R12, R14 strains and the reference strain (905) upon different stress conditions after 72 hours of incubation by using spot assay technique.

**Table 3.2 :** Cross resistance and sensitivities of R12 strain in comparison to reference strain according to spot assay results.

| Cross-Resistances | Sensitivities |
|---|---|
| $CuCl_2$ | $AlCl_3$ |
| $NH_4Fe(SO_4)_2$ | $CoCl_2$ |
| NaCl | $H_3BO_4$ |
| Coniferyl aldehyde | $AgNO_3$ |
| Vanillin | - |
| Cycloheximide | - |
| Propolis | - |

### 3.7 Lyticase susceptibility assay

In order to determine the cell wall integrity of the rapamycin-resistant mutant (R12), lyticase susceptibility assay was performed. By using cell wall β-1,3-glucan-degrading enzymes such as zymolyase or lyticase, the decrease in $OD_{600}$ with time can be used to track cell lysis spectrophotometrically (Kuranda et al., 2006). The result of this experiment showed that rapamycin-resistant R12 resisted lyticase more than the reference strain (905), under nonstress condition. On the other hand, under 2.0 ng/ml rapamycin stress, the reference strain (905) resisted lyticase more than the rapamycin-resistant R12. Furthermore, the presence of 2.0 ng/ml rapamycin stress did not change the lyticase resistance of the evolved strain R12, in comparison to the nonstress condition (Figure 3.7).

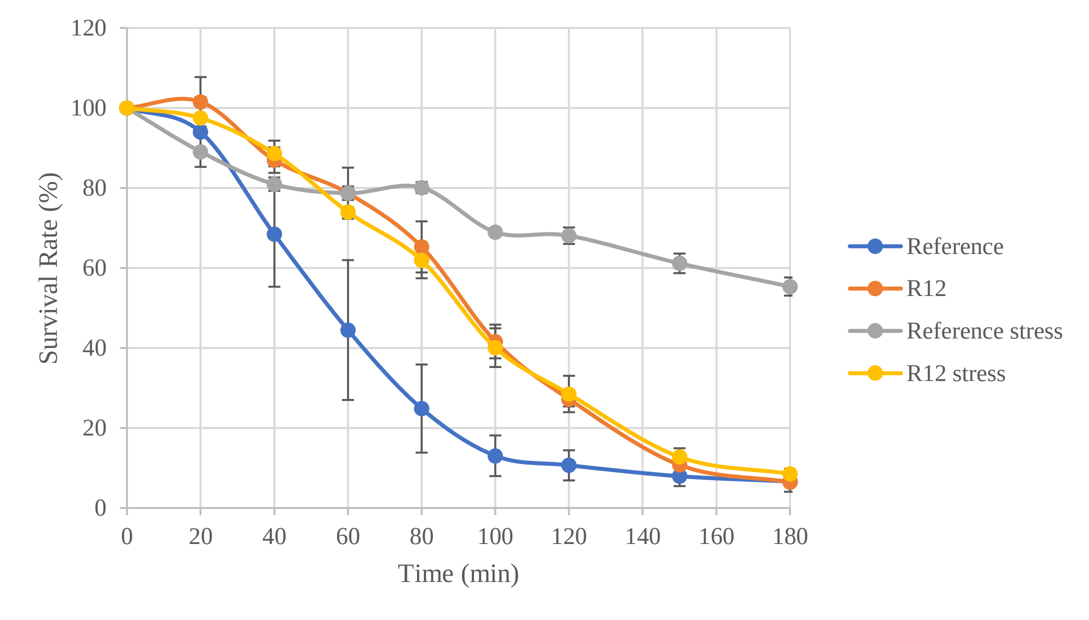


**Figure 3.7:** Lyticase susceptibility test results of rapamycin-resistant mutant (R12) and the reference strain (905), in the presence and absence of of 2.0 ng/ml rapamycin stress.

## 3.8 Chronological life span analysis

### 3.8.1 Semi-quantitative chronological life span analysis

The amount of time that cells stay alive in stationary phase is known as the chronological life span (CLS) (Cao et al., 2016). The chronological lifespan of rapamycin resistant strain (R12) was determined by using semi-quantitative chronological lifespan analysis. After rapamycin-resistant strain (R12) and the reference strain (905) have reached the stationary phase of growth, they were incubated in the absence of rapamycin stress for 10 days, where samples were withdrawn from the cultures every other day and $OD_{600}$ of the cultures were set to 6. The samples were then serially diluted up to $10^{-5}$ and spotted onto YPD agar plates. The results of the experiment showed that after day 4, growth of the rapamycin-resistant mutant (R12) ceased, whereas the reference strain could grow until day 8

(Figure 3.8). Thus, it can be stated that R12 has a shorter CLS in comparison to 905, according to semi-quantitative CLS experiment.

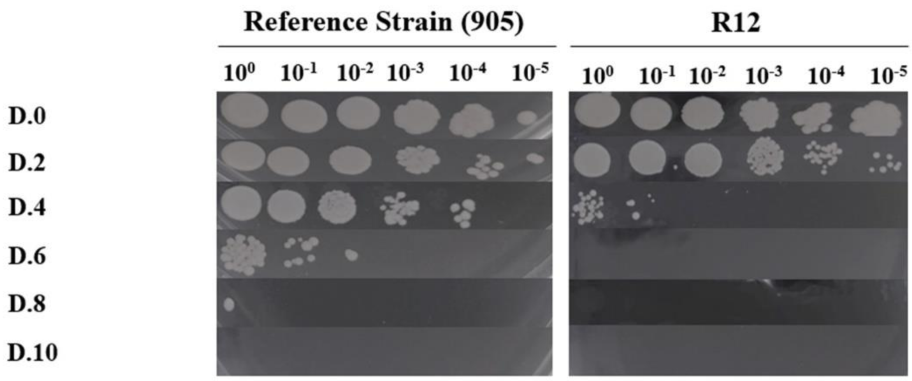


**Figure 3.8 :** Semi-quantitative CLS results of the reference strain (905) and rapamycin-resistant mutant (R12) upon 72 hours of incubation, using spot assay.D.0 to D.10 indicates the day of passage in YMM medium without selective pressure.

### 3.8.2 Quantitative chronological life span analysis

In order to verify the semi-quantitative CLS method and quantitatively determine the CLS of the rapamycin stress-resistant mutant (R12), the quantitative CLS method was performed, using the reference strain (905) and the rapamycin stress-resistant evolved strain (R12). After rapamycin-resistant strain (R12) and the reference strain (905) have reached the stationary phase of growth, they were incubated in the absence of rapamycin stress for 10 days, samples were withdrawn from the cultures every other day and their viability was assessed by viable cell counting method. The result of the experiment are in line with the semi-quantitative CLS analysis results, where R12 was found to have a shorter CLS, in comparison to 905 (Figure 3.9).

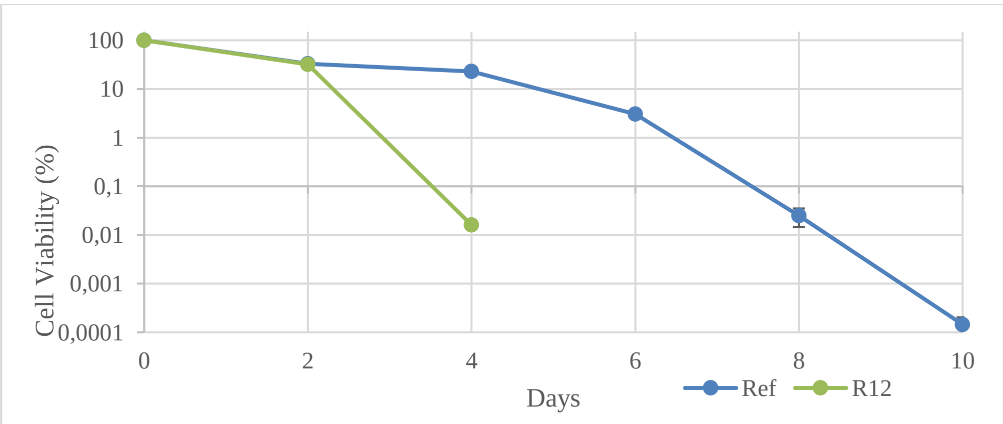


**Figure 3.9:** Quantitative CLS results of the reference strain (905) and rapamycin resistant mutant (R12).

### 3.9 Whole genome sequencing of the evolved strain

Comparative whole genome sequencing analysis of the rapamycin-resistant mutant (R12) revealed four single nucleotide variations (SNVs). These SNVs were located in four different genes (*FPR1*, *DAL1*, *LDB19* and *TUP1*) where the SNVs in *DAL1*, *LDB19* and *TUP1* were intragenic, missense SNVs; and the one in *FPR1* was a nonsense SNV. The peptidyl-prolyl cis-trans isomerase (PPIase) coding gene *FPR1* had a transition SNV (G198A), where tryptophan was replaced by a stop codon (W66*). The allantoinase-coding gene *DAL1* had a transversion SNV (C683G), where alanine was replaced by glycine (A228G). The alpha-arrestin-coding gene *LDB19* had a transversion SNV (A1727T), where lysine was replaced by isoleucine (K576I). Lastly, the general transcriptional corepressor-coding gene *TUP1* had a transition SNV (A1963G) where lysine was replaced by glutamate (K655E).

# 4. DISCUSSION

By inhibiting the TOR pathway, rapamycin is known to affect many key metabolic pathways in *S. cerevisiae* and higher eukaryotes, such as cell growth and lifespan, protein synthesis and ribosome biogenesis, regulation of cell cycle and size, environmental stress response, nutrient uptake and starvation control, and autophagy (Loewith R., 2011; Conrad M., 2014). In this study, a rapamycin stress-resistant *S. cerevisiae* strain (R12) was obtained using evolutionary engineering, which is an inverse metabolic engineering approach, by applying selection in batch cultures in the presence of increasing rapamycin stress levels. To determine the initial rapamycin-stress level for evolutionary engineering, a screening experiment was performed with the reference strain (905) at various rapamycin concentrations. The results of the screening experiment revealed that increasing rapamycin concentration decreased the survival rate and 3 ng/ml rapamycin was a suitable concentration as the initial stress level for evolutionary engineering selection experiments. Throughout 61 daily passages or populations, the concentration of rapamycin in the medium was gradually increased from 3.00 ng/ml to 200 ng/ml. From the final population (61$^{st}$ population), 15 individual colonies were randomly selected, using agar plates containing 150 ng/ml rapamycin. The randomly selected rapamycin-resistant strains were named as R1 to R15.

Despite some studies in the literature on the positive effects of rapamycin on yeast cells and their amino acid production, stress response and resistance (Dikicioglu et al., 2018; Crespo and Hall, 2002; Rallis et al., 2013; Shamji et al., 2000); yeast cells that are highly resistant to rapamycin or that can grow in the presence of high levels of rapamycin have not been obtained by a systematic approach such as evolutionary engineering. In a study found in the literature, rapamycin-resistant *S. cerevisiae* strains were obtained as a result of random chemical mutagenesis of a yeast mutant with a deletion in the *PDR1* gene associated with multidrug resistance, and short-term incubation in solid medium in the presence of rapamycin. Furthermore, mutation analysis of the rapamycin-resistant yeast strains was performed by whole genome sequencing. However, physiological (resistance to other stresses, etc.) or metabolic (amino acid production, etc.) characteristics of the mutants were not reported (Wride et al., 2014).

In the present study, after selection procedure, rapamycin resistance levels of the 15 isolated mutants were determined by using spot assay which revealed that all of the selected strains gained rapamycin resistance. However, R1, R3, R7, R12 and R14 mutants were chosen for genetic stability analysis which revealed that all five strains (R1, R3, R7, R12 and R14) were genetically stable. Afterwards, cross-resistance or sensitivity of those genetically stable R1, R3, R7, R12 and R14 mutants against various stress types (0.5 mM $NiCl_2$, 2.5 mM $CrCl_3$, 3 mM $CoCl_2$, 17.5 mM $MnCl_2$, 50 mM $NH_4Fe(SO_4)_2$, 10 mM $AlCl_3$, 20 mM $CuCl_2$, 15 mM $LiCl_2$, 50 mM $H_3BO_4$, 100 μM $AgNO_3$, 0.5 M NaCl, 15 mM caffeine, 4 mM vanillin, 200 ng/ml propolis, 1mM coniferyl aldehyde and 200 ng/ml cycloheximide) was investigated. According to cross-resistance and sensitivity analysis results, R12 was found to be the mutant with the highest number of resistance and sensitivities. R12 strain is resistant to $CuCl_2$, $NH_4Fe(SO_4)_2$, NaCl, coniferyl aldehyde, vanillin, cycloheximide, propolis; sensitive to $AlCl_3$, $CoCl_2$, $H_3BO_4$, $AgNO_3$; neither resistant nor sensitive to $NiCl_2$, $CrCl_3$, $LiCl_2$, $MnCl_2$, caffeine in comparison to the reference strain (905). According to these cross-resistance results, R12 mutant was chosen for further analysis.

An essential component of cellular homeostasis maintenance is the capacity to detect and respond to a variety of environmental signals. Cells must quickly activate certain stress responses in response to challenging environments in order to adapt, endure, and multiply. Many proteins go through biophysical and metabolic changes to ensure control over stress conditions (Hotamisligil and Davis, 2016). The Cy8-Tup1 repressor complex of *S. cerevisiae* is essential for the osmotic induction of HOG-dependent genes and also under conditions of hyperosmotic stress, the yeast transcription corepressor complex Cyc8-Tup1 is SUMOylated and biomolecularly condensed under the control of the Hog1 MAPK (Marquez et al., 2018; Nadel et. al, 2019). Hog1 is known to be a key protein in osmoregulation which helps to accumulate intracellular osmolytes, mainly glycerol, in yeast cells that results in the re-entry of water back into the cell (Blomberg, 2022). Any changes in this pathway may have caused NaCl resistance in rapamycin stress-resistant R12 strain. It was also found that the rapamycin-resistant R12 strain has a transition SNV (A1963G) in *TUP1* gene where lysine was replaced by glutamate (K655E). Tup1 is a general transcription repressor that interacts with histones and controls the placement of nucleosomes; it interacts with Cyc8p to form a repressor complex (Wong and Struhl, 2011). This SNV may be related to the NaCl resistance of R12.

Cycloheximide is a cell-permeable anti-fungal agent that particularly inhibits eukaryotic cytosolic translation by hindering ribosomal translocation (Buchanan et al., 2016). It is known that cycloheximide causes the endocytosis of multispan plasma membrane proteins (Galan and Haguenauer-Tsapis, 1997). In *S. cerevisiae*, cycloheximide-induced endocytosis of Can1 relies on activation of the arrestin-like adaptor protein Art1/Ldb19 that targets the Rsp5 ubiquitin ligase to particular cargoes at the plasma membrane that can be inhibited by Npr1p-mediated phosphorylation (Lin et al., 2008). Furthermore, it has been found that TORC1 control of ubiquitin-mediated endocytosis occurs through regulation of Art1/Ldb19 function (MacGurn et al., 2011). Any changes in this pathway may have caused cycloheximide resistance in rapamycin stress-resistant R12 strain. It was also found that the rapamycin-resistant R12 has a transversion SNV (A1727T) in *LDB19* gene where lysine was replaced by isoleucine (K576I). Ldb19/Art1 is an alpha-arrestin which localizes to the Golgi, cytoplasm and plasma membrane, binds to ubiquitin protein ligase enzymes and functions in ubiquitin-dependent endocytosis and control of receptor internalization (Marquez and Duncan; 2018). Furthermore, TORC1 control of ubiquitin-mediated endocytosis occurs through the regulation of Art1/Ldb19 function (MacGurn et al., 2011). This SNV may be associated with the cycloheximide resistance of R12.

The industrial biofuel production through conversion of plant materials is a two-step process where the hydrolysis of plant lignocellulose is followed by the fermentation of resulting sugars. However, hydrolysis of lignocellulosic materials produces toxic chemicals, including phenolic substances, among which coniferyl aldehyde is one of the most toxic inhibitors (Hacısalihoğlu et al., 2019). Coniferyl aldehyde can be converted by *S. cerevisiae* into less hazardous intermediates, vanillin and ferulic acid. The ferulic acid route either converges to the vanillin route to produce guaiacol or uses an alternative pathway to produce 2-phenyl ethanol (Adeboye et al., 2015; Wu et al., 2017). Vanilin and coniferyl aldehyde are also the primary components of propolis (Osés et al., 2020; Górecka et al., 2014). Any changes in these pathways may have caused resistance to coniferyl aldehyde, vanillin and propolis resistance in the rapamycin stress-resistant R12 strain. However, further detailed metabolic analyses of R12 and reference strains would be necessary.

After cross-resistance analysis of the rapamycin-resistant mutants (R1, R3, R7, R12 and R14), cell wall integrity of the rapamycin-resistant strain R12 was tested by lyticase susceptibility assay following incubation of 905 and R12 in rapamycin stress and control conditions. By using cell wall β-1,3-glucan-degrading enzymes such as zymolyase or lyticase, the decrease in $OD_{600}$ with time can be used to track cell lysis spectrophotometrically (Kuranda et al., 2006). The yeast cell wall is a solid yet flexible structure that is crucial for both advancing through the cell cycle and maintaining cell shape and integrity. Cell wall integrity (CWI) signaling pathway is primarily in charge of the highly controlled and polarized cell wall remodeling that occurs during development and morphogenesis as well as in response to environmental stresses (Levin, 2011). For the purpose of overcoming the stress circumstances, different cellular processes, such as cell wall remodeling, are triggered in conjunction with the inactivation of the TOR pathway by rapamycin or nutritional deprivation. TOR regulates the PKC-mediated mitogen-activated protein kinase (MAPK) pathway in *S. cerevisiae*. Rapamycin specifically inhibits Tor1 and Tor2, and Mpk1 is quickly activated by a mechanism mediated by Sit4 and Tap42. Along with that, Mpk1 is known to regulate maintenance of cell wall integrity (Torres et al., 2002, Kim and Levin, 2011). Furthermore, it has been suggested that TOR inhibition is detected by the PKC pathway at the cell wall which is also essential for cell wall remodeling (Torres et al., 2002; Heinisch and Rodicio, 2017). Any changes in these pathways may have caused lyticase resistance of the rapamycin-resistant R12 strain in comparison to the reference strain (905), under nonstress condition. Furthermore, under 2.0 ng/ml rapamycin stress, reference strain (905) resisted lyticase more than the rapamycin-resistant R12. Furthermore, the presence of 2.0 ng/ml rapamycin stress did not change the lyticase resistance of evolved strain R12, in comparison to the nonstress condition. It was also found that the rapamycin-resistant R12 strain has a nonsense SNV (G198A) in *FPR1* gene where tryptophan was replaced by a stop codon (W66*) that results in truncated Fpr1. *FPR1* gene product is known to inhibit TOR1 when bound to rapamycin

(Kasahara et al., 2020). *FPR1* found in *S. cerevisiae*, encoding a peptidyl-prolyl cis-trans isomerase (PPIase) is an orthologue of FKBP12 found in humans (Kasahara et al., 2020). Rapamycin forms a complex with FKBP12 which inhibits the TORC1 protein kinase complex, resulting in the inhibition of cell division and growth, ribosome synthesis, along with the induction of autophagy (Schmelzle and Hall, 2000). Furthermore, FK506–FKBP12 binary complex inhibits calcineurin which results in cation hypersensitivity in yeast. Additionally, FKBP12 of *S. cerevisiae* physically binds to the promoter of the RPGs and encourages their transcription (Kasahara et al. 2020). Furthermore, FKBP12 of human functions in the downregulation of MDM2 which directly improves the sensitivity of cancer cells to chemotherapeutic agents and nutlin-3 therapy (Liu et al., 2016). The truncated Fpr1 in R12 cannot function properly under rapamycin stress, therefore it cannot inhibit TOR pathway in rapamycin-resistant R12. Therefore, rapamycin stress does not affect R12 strain as much as the reference strain (905), and the R12 strain does not require cell wall remodeling in the presence of rapamycin stress, as much as the reference strain. This SNV in *FPR1* seems to be a critical mutation which may explain the molecular mechanism of the observed rapamycin resistance in R12.

The amount of time that cells stay alive in stationary phase is known as the chronological life span (CLS) (Cao et al., 2016). The chronological lifespan of rapamycin-resistant strain (R12) was determined first by using semi-quantitative chronological lifespan analysis. Later on, results of semi-quantitative chronological lifespan analysis were confirmed by quantitative chronological lifespan analysis which indicates that rapamycin resistant R12 have shorter CLS in comparison to the reference strain (905). It is known that by decreasing TOR pathway signaling, chronological life span of *S. cerevisiae* can be extended (Powers et al., 2006). Rapamycin resistant mutant R12 may have increased TOR pathway signaling in order to overcome rapamycin stress which is known to be an inhibitor of TOR (Lamming, 2016). Therefore, R12 may have shorter CLS in comparison to the reference strain (905), as observed in this study.

Allantoin (5-ureidohydantoin) is converted to allantoic acid by Dal1 which is involved in the utilization of purines as secondary nitrogen sources when primary sources are scarce (Buckholz and Cooper, 1991). By promoting the binding of Tap42 to Sit4 in the presence of a high-quality nitrogen sources like ammonium or glutamine, TOR maintains Sit4 inactive. In the conditions that inactivate TOR such as nitrogen starvation or rapamycin treatment, activation of Sit4 occurs by release from Tap42. Activated Sit4 dephosphorylates target proteins such as Npr1 and Gln3 which take part in nitrogen utilization (Stracka et al., 2014; Cutler et. al, 2001). The *DAL1* mutation in the rapamycin-resistant R12 strain may also change its utilization pattern of secondary nitrogen sources. This needs to be verified, however, by further metabolic analyses of R12 and the reference strain.

# 5. CONCLUSION

By using evolutionary engineering, a rapamycin-stress-resistant and genetically stable *S. cerevisiae* strain (R12) was successfully obtained in this thesis study, and characterized at genomic and physiological levels. According to cross-resistance test results, rapamycin-stress-resistant R12 strain has been found out to be resistant to $CuCl_2$, $NH_4Fe(SO_4)_2$, NaCl, coniferyl aldehyde, vanillin, cycloheximide, propolis; and sensitive to $AlCl_3$, $CoCl_2$, $H_3BO_4$, $AgNO_3$ stress conditions, in comparison to reference strain (905). Results from the lyticase susceptibility assay showed that the rapamycin-resistant R12 strain was more resistant to lyticase under nonstress condition, but less resistant to lyticase under rapamycin stress condition, compared to the reference strain (905). Furthermore, the presence of rapamycin stress did not change the lyticase resistance of the evolved strain R12 in comparison to the nonstress condition. Chronological life span analysis demonstrated that R12 was found to have shorter CLS in comparison to 905. According to comparative whole genome sequencing results, rapamycin-resistant mutant (R12) had four single nucleotide variations (SNVs). These SNVs were located in four different genes (*FPR1*, *DAL1*, *LDB19* and *TUP1*) where the SNVs in *DAL1*, *LDB19* and *TUP1* were intragenic, missense SNVs and the one in *FPR1* was a nonsense SNV. To sum up, these results indicate that TOR pathway-related changes occurred in rapamycin-resistant mutant in order to overcome the high levels of rapamycin stress. However, in order to fully comprehend the molecular basis of rapamycin-resistance of R12, its comparative transcriptomic and metabolic analyses would be necessary as future studies.

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