ISTANBUL TECHNICAL UNIVERSITY★ GRADUATE SCHOOL OF SCIENCE ENGINEERING AND TECHNOLOGY

INVERSE METABOLIC ENGINEERING OF PROPOLIS-RESISTANT *Saccharomyces cerevisiae*

M.Sc. THESIS

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Department of Molecular Biology Genetics & Biotechnology
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Thesis Advisor: Prof. Dr. Zeynep Petek ÇAKAR

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İSTANBUL TEKNİK ÜNİVERSİTESİ ★ FEN BİLİMLERİ ENSTİTÜSÜ

TERSİNE METABOLİK MÜHENDİSLİK YAKLAŞIMIYLA PROPOLİSE DİRENÇLİ *Saccharomyces cerevisiae* ELDESİ

YÜKSEK LİSANS TEZİ

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HAZİRAN 2016

Filiz Demir, a **M.Sc.** student of ITU **Graduate School of Science Engineering and Technology** student ID 521131109, successfully defended the **thesis/dissertation** entitled "Inverse Metabolic Engineering of Propolis-Resistant *Saccharomyces cerevisiae*", which she prepared after fulfilling the requirements specified in the associated legislations, before the jury whose signatures are below.

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Jury Members	**:Prof. Dr. Ayten KARATAŞ** (İstanbul Technical University)	.....
Jury Members	**:Prof. Dr. Oğuz ÖZTÜRK** (İstanbul University)	.....

Date of Submission : 02 May 2016
Date of Defense : 06 June 2016

To my family,

FOREWORD

I would like to thank to my supervisor Prof. Dr. Zeynep Petek Çakar for giving me the chance to study in this thesis project and yeast research group. She always supported me in academic and business life and she enlightened me with important informations and experiences.

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I must express my special thanks to Eren Yılmaz for her ideas about propolis. Finally, I would like to thank to my family being with me all the time.

May 2016

Filiz Demir
(Biochemist)

TABLE OF CONTENTS

Page

ABBREVIATIONS

AVG **:** Average
CAPE **:** Caffeic Acid Phenyl Ester
EMS **:** Ethyl Methane Sulfonate
MPN **:** Most Probable Number
LP **:** Last Population
ROS **:** Reactive Oxygen Species
RS **:** Reference Strain
YMM **:** Yeast Minimal Medium
YPD **:** Yeast Extract Peptone Dextrose Medium
CDW **:** Cell Dry Weight
HPLC **:** High Pressure Liquid Chromotography
EEP **:** Ethanol Extract of Propolis

LIST OF TABLES

Page

LISTS OF FIGURES

Page

INVERSE METABOLIC ENGINEERING OF PROPOLIS-RESISTANT *Saccharomyces cerevisiae*

SUMMARY

Propolis is a resinous, sticky and dark colored substance that bees produce by mixing their own waxes with resins obtained from plants. Propolis is a resiny compound that bees collect and use as a building material and to protect their hives against fungi and bacteria. Propolis has been used at least to 300 BC and its use continues today in natural medicine and personal products. Chemical content of propolis is quite complex due to more than 300 ingredients such as polyphenols, phenolic aldehydes, sesquiterpene quinines, coumarins, amino acids, steroids and inorganic compounds, which have been identified in propolis samples.

Propolis has various biological activities such as antimicrobial activity, antitumor activity, antioxidant activity, antiinflammatory activity, immunomodulator, cytotoxic and therapeutic activity. The antimicrobial activity of propolis originates from flavonoids, aromatic acids and esters present in resin. Ferulic and caffeic acids also provide antibacterial effect to propolis. Antimicrobial effect of propolis is expressed with synergism between flavonoids, hydroxy acids and sesquiterpenes. Propolis mainly includes flavonoids and phenolic compounds and these compounds have antioxidant properties.

In propolis-exposed yeast cells, intracellular oxygen levels decrease. Changes also occur at mitochondrial proteome level, including antioxidant proteins. Therefore, increase in antioxidant protein levels ensures decreasing intracellular oxidation. Propolis is a significant antioxidant in the yeast *Saccharomyces cerevisiae* due to three important findings : (1) it promotes protection of membrane lipids from H_2O_2 stress, (2) O_2 stress provides menadione, and propolis resumes redox status by scavenging ROS. (3) it activates Cu/Zn-superoxide dismutase, one of the most important antioxidant enzymes.

S. cerevisiae is a eukaryotic organism, also named as baker's yeast or budding yeast. *S. cerevisiae* cells are mainly oval-shaped but cell size varies between 10 μm long and 5 μm wide, according to environmental conditions.

Culturing *S. cerevisiae* cells is easy and inexpensive. Basic nutritional sources are enough for cell growth. They can grow almost as rapid as bacteria in solid and liquid media, if the growth media have basic nutritional sources. *S. cerevisiae* is the first eukaryote the genome of which has been sequenced. It can be found in haploid or diploid form. Cells can proliferate when they are haploid and can then be easily isolated. Therefore, *S. cerevisiae* provides a highly suitable system to study basic biological processes that are relevant to many higher organisms, including human.

In the present study, propolis-resistant *S.cerevisiae* population was obtained under gradually increasing propolis stress levels, by using an inverse metabolic engineering strategy. Reference strain(905) and its mutagenised form (906) were screened under

increasing propolis stress levels to determine the initial stress level for selection. 150 µg/ml propolis was chosen as the initial propolis level and it was increased by 10 µg/ml at each step during selection. Totally, 57 mutant populations were obtained and their survival rates decreased, when propolis levels were increased. EMS mutagenised population (906) gained resistance and showed growth even at 710 µg/ml propolis concentration.

The final population was incubated on solid YMM plates and twelve individual mutant colonies were chosen randomly. These propolis-resistant colonies were tested for their propolis-resistance, using spot assay and MPN method. According to spot assay results, more resistant colonies were determined among twelve individual mutants. Colonies were named as FD7, FD8, FD10, FD11 and FD12. MPN method was used for quantification of propolis stress resistance of mutant colonies. MPN tests showed that FD10 and FD11 were the most resistant colonies to propolis.

Cross-resistance tests were applied to propolis-resistant mutants to determine their potential resistance against other stress types. *S.cerevisiae* mutants were grown on solid YMM containing ; 0.1-0.3-0.5-0.8 mM $NiCl_2$, 1-2-2.2 mM $CoCl_2$, 0.1-0.3-0.4-0.5-0.8 mM $CuSO_4$, 0.5-1-1.5 mM H_2O_2 , 2-2.5-3 mM $CrCl_3$, 10 mM $ZnCl_2$, 0.5-1-1.5 M $MgCl_2$, 15-25-30-35-40 mM NH_4FeSO_4, 15-20 mM $MnCl_2$, 8-12 % (v/v) ethanol , 12 mM $AlCl_3$, 0.5-1 M NaCl, 150 µg/ml geneticin, 10 mM caffeine, to determine their potential cross-resistances.

The genetic stability analyses were performed using FD10 and FD11 mutants, to test if their resistance is permanent or not. It was shown that the mutants tested were genetically stable. At last, growth curves and cell dry weight measurements of FD11 mutant and the reference strain were obtained and compared to each other. HPLC analysis was used to determine concentrations of important metabolites, such as residual glucose, glycerol, acetate and ethanol.Trehalose and glycogen levels were measured by an enzymatic assay. Finally, reactive oxygen species were detected by ROS assay for both reference strain and FD11, with and without propolis stress.

To conclude, a highly propolis-resistant and genetically stable *S. cerevisiae* mutant was obtained in this study. Physiological analyses revealed that the mutant was cross-resistant against caffeine and $NiCl_2$ stress and has lower levels of ROS generation. Future genomic, transcriptomic and proteomic analyses may help understand the molecular basis of propolis resistance and response in *S. cerevisiae*.

TERSİNE METABOLİK MÜHENDİSLİK YAKLAŞIMIYLA PROPOLİSE DİRENÇLİ *Saccharomyces cerevisiae* ELDESİ

ÖZET

Propolis, bal arılarının kovanlarını inşa etmek ve funguslar ile bakterilere karşı kovanlarını korumak için bitki ve ağaçlardan toplayarak oluşturdukları reçineli bir bileşiktir. Propolis eski zamanlardan beri yerel tıp alanında kullanılmaktadır.

Propolis, yapısında bulunan üçyüzden fazla bileşen ile karmaşık bir kimyasal içeriğe sahiptir. Bu bileşenler polifenoller, fenolik aldehitler, kumarinler, aminoasitler, steroid ve inorganik bileşenler olarak sıralanabilir. Propolis içeriği hangi bölgede üretildiğine göre değişir. Sıcaklık ve mevsimsel etki gibi doğal faktörler propolis bileşimini etkiler. Bitki türlerindeki çeşitlilik propolis içeriğini yüksek oranda değişken kılar. Örneğin, coğrafi bölgedeki farklılığa göre propolis içerisindeki antibakteriyel bileşikler değişebilir. Avrupa örneklerinde flavonoidler ve sinnamik asit bulunurken, Brezilya örneklerinde diterpenik asit ve kumarik asit bulunur.

Propolisin antimikrobiyal aktivite, antitümör aktivitesi, antioksidant aktivite, antiinflamatuar aktivite, sitotoksik aktivite ve terapötik aktivite gibi biyolojik aktivitelere sahip olması, onu ilgi çekici bir bileşik haline getirmiştir. Propolisin antimikrobiyal aktivitesi flavonodiler, aromatik asitler ve aromatik asit esterlerinden kaynaklanmaktadır. Flavonoidler antimikrobiyal etkilerini hidrolaz ve alkalin fosfataz gibi enzimleri inhibe ederek gerçekleştirirler. Propolisin antibakteriyel etkisi ferulik asit ve kafeik asitten kaynaklanmaktadır. Propolisin *Trichophyton* ve *Mycosporum* gibi türler üzerinde önemli bir antifungal etkisi bulunmaktadır ve antifungal ilaçlarla birlikte kullanılması ilaçların etkinliğini arttırmaktadır. Ayrıca propolis, çeşitli DNA ve RNA virüsleri üzerinde de etkilidir.

Propolis, bileşimindeki flavonoidler ve fenolik bileşikler sayesinde antioksidant özelliklere sahiptir. Propolis, hücreleri oksidatif stresin zararlarından korur. Oksidatif stress, serbest radikallerin oluşmasıyla gerçekleşir ve propolis yapısındaki dicaffeoylquinic asit türevleri, serbest radikalleri güçlü bir şekilde uzaklaştırır. Ayrıca propolis yapısında bulunan kafeik asit fenil ester bileşiği de serbest radikal oluşumunu durdurur. Propolis maya hücrelerine verildiğinde hücre içi oksijen seviyeleri düşer ve böylece serbest radikal oluşumu azalır. Ayrıca propolis, antioksidatif proteinlerin üretimini arttırarak hücre içi oksidasyonu da azaltır. Propolis, membran lipidlerini hidrojen peroksit stresinden korur ve bir antioksidant enzim olan Cu/Zn süperoksit dismutaz enzimini aktive eder.

Propolis lenfosit üretimini arttırarak memelilerde bağışıklık sisteminin korunmasına yardımcı olur. Brezilya propolisinden izole edilen artepilin C, kafeik asit ve quercetin bileşikleri, tümör hücreleri üzerinde sitotoksik etkiye sahiptir. Ayrıca propolis, akut ve kronik inflamasyona karşı antiinflamatuar etkiye sahiptir. Kafeik asit fenil ester bileşiği, inflamasyon oluşumunu engeller. Biyolojik aktivitelerine nazaran propolis,

toksik ve allerjen etkiye de sahiptir. Propolis bileşiği çeşitli ağır metaller içerebileceğinden hücreler üzerinde toksik etki yaratabilir.

Saccharomyces cerevisiae ökaryotik bir maya hücresidir ve fungus alemine aittir. Hücre yapısı yuvarlak ve hücre büyüklüğü 10 µm ile 5µm arasında değişmektedir. *S. cerevisiae* oksijen varlığında glukozu karbondioksit ve suya kadar parçalarken, oksijen olmadığında glukozu etanole çevirerek oksijensiz solunum yapar. Maya hücreleri tomurcuklanma ile aseksüel üreme gerçekleştirirler. *S. cerevisiae* genom dizisi belirlenen ilk ökaryotik organizmadır ve diploid veya haploid formda bulunabilir.

S.cerevisiae hücrelerinin kültivasyonu ucuz ve kolaydır. Temel besin kaynakları hücre üremesi için yeterlidir. Gelişme ortamı temel besin kaynaklarını içeriyorsa, bakteri kadar hızlı gelişebilirler. Hücre yapıları hayvan ve bitki hücresi gibi kompleks yapılara benzerdir. Tüm bu sebeplerden dolayı *S.cerevisiae* bilimsel çalışmalarda ökaryotik model organizma olarak kullanılmaktadır.

Bu tez çalışmasında tersine metabolik mühendislik yaklaşımıyla, propolise dirençli *S. cerevisiae* mayası elde edilerek fizyolojik açıdan incelenmiştir. Bu amaçla, öncelikle referans suş ve EMS ile rastgele kimyasal mutasyona uğratılmış *S.cerevisiae* suşu farklı konsantrasyonlarda propolis içeren ortamlarda büyümeye bırakılarak inhibe edici propolis konsantrasyonu ve seleksiyon deneylerinde kullanılacak propolis konsantrasyonu belirlenmiştir.

Başlangıçta uygulanan propolis konsantrasyonu 150 µg/mL iken, propolis konsantrasyonu yavaş yavaş arttırılarak 57 mutant popülasyon elde edilmiştir ve 57. popülasyonda 710 µg/mL propolis stresi uygulanmıştır. Böylelikle propolise yüksek dirençli bir popülasyon elde edilmiştir. Son popülasyon seyreltilip katı YMM besiyerine ekilerek bu besiyerinden 12 farklı koloni rastgele seçilmiştir. Seçilen bu kolonilerin propolis direnci çeşitli fizyolojik analizlerle belirlenmiştir.

Seçilen 12 mutant koloni, son popülasyon ve referans suşun propolis direncini belirlemek için öncelikle damlatma (spot) testleri gerçekleştirilmiştir. Hücreler farklı konsantrasyonlarda propolis içeren katı YMM ortamında üretilerek, üreme miktarları karşılaştırılmıştır. Damlatma test sonuçlarına göre, 12 mutant koloni arasından, en dirençli gözlenen 5 farklı koloni (FD7, FD8, FD10, FD11, FD12) seçilmiştir. Ayrıca 710 µg/mL propolis içeren katı besiyerinde mutant koloniler üreme güçlüğü çekmişlerdir. Bu durum, propolis stresinin katı ve sıvı ortamlardaki etkisinin farklı olabileceğini göstermektedir.

Seçilen beş mutant bireyin propolis stresine olan direncini gözlemlemek amacıyla Most Probable Number (MPN) testi uygulanmıştır. Mutant koloniler 200 µg/mL, 500 µg/mL ve 710 µg/mL propolis stresi içeren MPN platelerine ekilerek, oluşan bulanıklık miktarlarından yola çıkılıp canlı hücre sayısı MPN tablosu yardımıyla hesaplanmıştır. MPN sonuçlarına göre ; mutant koloniler en iyi üremeyi 200 µg/mL propolis konsantrasyonunda göstermiş olup, en iyi üreyen mutant birey de FD11 mutant bireyidir.

Çapraz direnç testinde ise propolise direnç geliştirmiş olan mutant bireylerin başka hangi stress türlerine de direnç kazandığı incelenerek karşılaştırma yapılmıştır. Bu amaç doğrultusunda 0.1-0.3-0.5-0.8 mM $NiCl_2$, 1-2-2.2 mM $CoCl_2$, 0.1-0.3-0.4-0.5-0.8

mM $CuSO_4$, 0.5-1-1.5 mM H_2O_2 , 2-2.5-3 mM $CrCl_3$, 10 mM $ZnCl_2$, 0.5-1-1.5 M $MgCl_2$, 15-25-30-35-40 mM NH_4FeSO_4, 15-20 mM $MnCl_2$, 8-12 % (v/v) etanol , 12 mM $AlCl_3$, 0.5-1 M NaCl, 150 µg/mL genetisin, 10 mM kafein içeren katı YMM besiyerinde damlatma testi uygulanmıştır. Test sonucuna göre mutant koloniler $NiCl_2$, NH_4FeSO_4, genetisin ve kafein bileşiklerine dirençlilik fakat etanol ve H_2O_2 bileşiklerine karşı ise duyarlılık göstermiştir. Damlatma sonuçlarını desteklemek amacıyla mutant bireylerin direnç ve duyarlılık gösterdiği stress koşullarında MPN testi de uygulanmıştır.

Genetik kararlılık testinde propolise karşı yüksek direnç gösteren FD10 ve FD11 mutant kolonilerinin propolise olan dirençlerinin kalıcı olup olmadığı araştırılmıştır. FD10 ve FD11 ardarda beş pasajlama boyunca propolis içermeyen taze besiyerinde üretilmiş ve bu suşlardan -80 ^oC stok kültürleri yapılmıştır. Daha sonra bu kültürler canlandırılarak YMM ve 250 µg/mL propolis içeren YMM ortamlarında MPN testi uygulanmıştır. MPN sonuçlarına göre FD10 ve FD11 kolonilerinin genetik olarak kararlı olduğu gözlenmiştir. FD11'in FD10'a göre daha yüksek bir üreme oranına sahip olduğu da görülmüştür.

FD11 suşunun üreme eğrilerinin eldesi için öncelikle doz tarama deneyi uygulanmış ve deney sonuçlarına göre 200 µg/mL propolis konsantrasyonu referans suş ve FD11 mutantı için uygun propolis konsantrasyonu olarak belirlenmiştir. Üreme eğrisi deneyleri 200 µg/mL propolis içeren ve içermeyen (kontrol) besiyeri ortamlarında gerçekleştirilmiştir.

Referans suş ve FD11'in 200 µg/mL propolis varlığında ve propolissiz ortamdaki optik yoğunluklarının 600 nanometre dalgaboyunda düzenli aralıklarla ölçümü ile üreme eğrileri elde edilip, birbiriyle kıyaslanmıştır. Üreme analizi sonunda, hücre kuru ağırlıkları da ölçülüp kıyaslanmıştır. Ayrıca; tüketilen glukoz, üretilen gliserol, asetat ve etanol gibi metabolitlerin miktarı yüksek basınçlı sıvı kromotografisi (HPLC) cihazı ile belirlenmiştir. Depo karbonhidratlarından trehaloz ve glikojen miktarları, enzimatik bir yöntem yardımıyla hesaplanmıştır. Son olarak, hücre içindeki oksidasyon düzeyleri reaktif oksijen deneyi ile saptanmıştır. Tüm bu çalışmalar referans suş ile propolise dirençli mutant suşun fizyolojik farklılıklarını belirlemek amacıyla yapılmıştır.

Sonuç olarak, bu çalışmada propolise yüksek düzeyde direnç gösteren ve genetik açıdan kararlı bir *S. cerevisiae* mutant suşu elde edilmiştir. Yapılan fizyolojik analizler, mutant suşun kafein ve $NiCl_2$ streslerine karşı çapraz direnç gösterdiğini ve hücre içi ROS düzeylerinin referans suşa kıyasla daha düşük olduğunu göstermiştir. Yapılacak genomik, transkriptomik ve proteomik analizler, *S. cerevisiae'*de propolis direnç ve tepkisinin moleküler altyapısının anlaşılmasına katkı sağlayabilecektir.

1. INTRODUCTION

1.1 The Yeast *Saccharomyces cerevisiae*

Saccharomyces cerevisiae is a eukaryotic organism also named as baker's yeast or budding yeast. As seen in table 1.1 it belongs to fungi kingdom, under ascomycota phylum, saccharomycetes class (Kurtzman et al., 1998).

Table 1.1: Taxonomic classification of *Saccharomyces cerevisiae.*

Kingdom	Phylum	Class	Order	Genus	Species
Fungi	Ascomycata	Saccharomycetes	Saccharomycetales	Saccharomyces	*S. cerevisiae*

S. cerevisiae cells are mainly oval shaped and their size varies according to environmental conditions. They have thick cell wall like other fungi (Alberts et al., 1991). Transmission electron microscopy images of a yeast show cell wall, nucleus, mitochondria, endoplasmic reticulum (ER), Golgi apparatus, vacuoles, microbodies and secretory vesicles. These organelles are not exactly free from each other and come into existence from an intramembranous structure (Walker, 2009).

S. cerevisiae requires macronutrients (sources of carbon, nitrogen, oxygen, sulfur, phosphorus, potassium and magnessium) and trace elements (e.g., Cu, Cu, Fe, Mn, and Zn) for growth. Malt extract or yeast extract with peptone and glucose are commonly used for cell growth.Yeast nitrogen base is a chemically defined medium component that includes ammonium sulphate and asparagine as a nitrogen sources, together with mineral salts, vitamins and trace elements. *S.cerevisiae* can thrive best from 20 ^{o}C to 50 ^{o}C and requires water at high concentration for its growth and metabolism. Additionally, it can grow optimally at pH values between 4.5 and 6.5 (Walker, 2009).

S. cerevisiae converts a large fraction of glucose to ethanol and carbon dioxide under anaerobic conditions. However, in the presence of oxygen glucose is used to generate new biomass, carbon dioxide and water. Aerobic degradation of glucose is

energetically more favorable (Krull et al., 2015). However, when glucose concentration exceeds a critical threshold level, alcoholic fermentation may occur even under aerobic conditions. This circumstance is called as Crabtree effect (Erik et al., 1989).

Asexual reproduction, also named vegetative reproduction, exists in *S. cerevisiae* by budding. Vegetative cells are diploid or polyploid and vegetative reproduction overrides in the life cycle of the yeast (Joseph, 2014). Budding begins by the emergence of outpouching at some point on the surface of the cell. Parent cell remains constant in size, while the bud develops in size to emerge as a new cell. After a particular time, the new cell separates from the parent cell (Kurtzman et al., 1998). Figure 1.1 shows scanning electron micrographs of budding cells of *S.cerevisiae*.

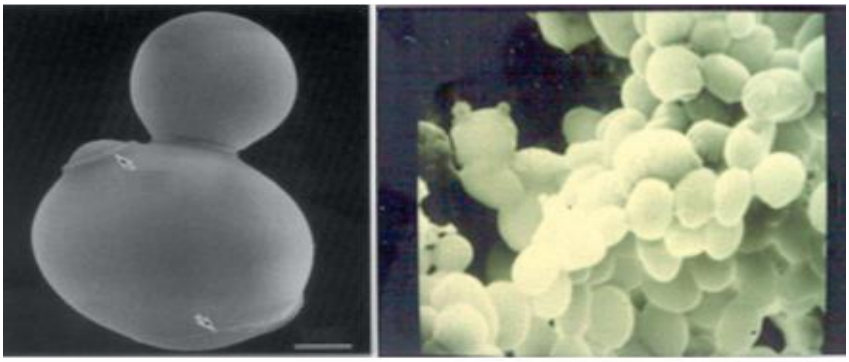

Figure 1.1: Scannig electron micrographs of budding yeast (a) Individual cell (b) Cluster of cells (Walker, 2009).

Parent and daughter cell walls are adjacent during bud development. Multilateral budding is prevalent in which daughter buds occur at different locations on the mother cell wall surface. In *S. cerevisiae,* cell size is asymmetrical at division and buds are smaller than mother cell when they leave. Figure 1.2 shows multilateral budding in *S.cerevisiae* (Walker, 2009).

Sexual reproduction occurs by the generation of the asci. Ascospores form directly following meiosis of the diploid nucleus. Acetate-containing media, such as acetate agar triggers sporulation of *S.cerevisiae* (Joseph, 2014). Figure 1.3 shows the sexual reproduction.

Mating of *S. cerevisiae* occurs by the conjugation of two haploid cells of opposite mating types. These mating types are called a and α factor. Pairing occurs by peptide mating pheromones known as a factor and α factor, depending on the allele (MATa and MATα) at the MAT locus (Esslinger, 2009).

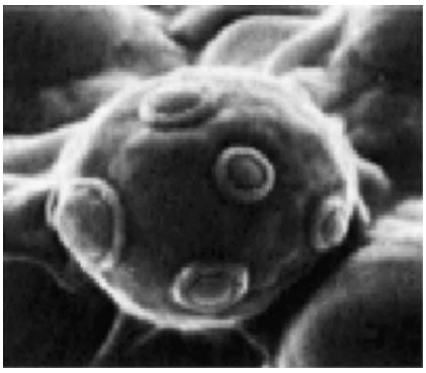

Figure 1.2 : Bud scars in a single cell of *S. cerevisiae.* The micrograph shows multilateral budding on the surface of an aged cell of *S.cerevisiae* (Walker, 2009).

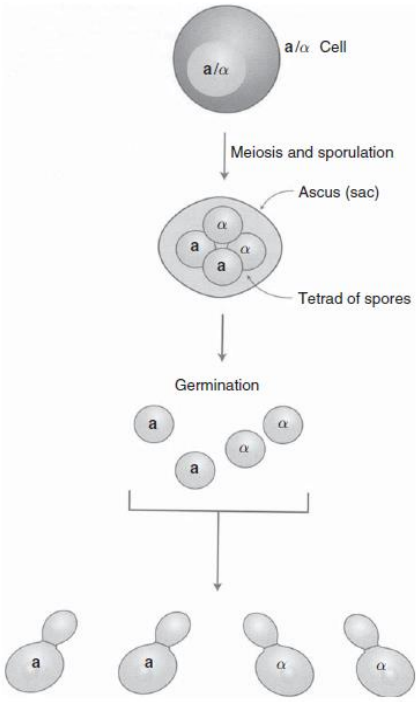

Figure 1.3: Sexual life cycle of *S. cerevisiae* (Madhani, 2007).

The conjugation of mating cells starts with touching of cell wall surfaces, and then plasma membrane fusion occurs to form a mutual cytoplasm. Diploid nucleus occurs as a result of nuclear fusion. Mitoic cell cycle proceeds by this diploid zygote in rich media, but if deprived of nitrogen, diploid cells sporulate to produce four haploid spores. Figure 1.4 shows mating and sporulation in *S.cerevisiae.*

Although laboratory strains of *S.cerevisiae* can exist in diploid or haploid form, industrial strains are usually diploid or aneuploid and can sporulate poorly (Johnson and Erasun, 2014).

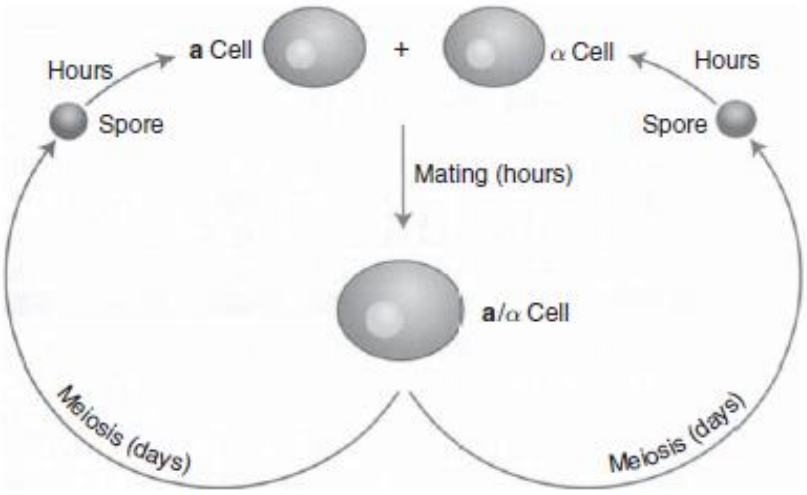

Figure 1.4: Meiosis and sporulation in *S. cerevisiae.* Diploid cells (a/α) can go through meiosis or sporulation to constitute spores. These spores can germinate a and α haploid cells (Madhani, 2007).

S. cerevisiae is the first eukaryote whose genome was sequenced. Haploid yeast genome includes 16 chromosomes. The total size of chromosomal DNA is 13,392 kb. *S.cerevisiae* genome is highly compact and its size is less than 1% that of a mammal and 3.5-fold the genome size of *E.coli* (Madigan et al., 2003).

1.2 Advantages of *S. cerevisiae* in Research and in Industry

S. cerevisiae has been chosen as a model organism in research due to its important properties. For example ;

- *S. cerevisiae* is a small single cell and it has a short doubling time of 1.25-2 h at 30 ^{o}C. Cultivation of *S.cerevisiae* is also very easy. Therefore, these properties ensure rapid production at low cost.
- *S. cerevisiae* can be manipulated genetically by addition or deletion of genes using modern recombination techniques. The genome sequence of *S. cerevisiae* was published in 1996 and has been updated routinely as *Saccharomyces* Genome Database. The genome includes 6275 genes. Cultivation of yeast species in haploid form allows easy isolation of mutants and haploid-diploid hybrids.

- Intracellular structure of *S.cerevisiae* is similar to those of animals and plants (Stewart, 2014).
- Using *S.cerevisiae* as a simple eukaryotic model organism is also important for medicine and human genetics, because of the ethical limits on experimenting with humans. Therefore, experiments with *S.cerevisiae* can provide valuable information on complex eukaryotic organisms like human (Karathina et al., 2011).

Moreover, its physiological properties and convenience for genetic manipulation make *S. cerevisiae* a desirable organism for many industrial applications. *S.cerevisiae* is classified as "Generally Recognized as Safe" (GRAS) in food industry, because of its long history of safe use and consumption and the absence of toxin production. *S.cerevisiae* is used as a production organism of innate and recombinant products (Stewart, 2014).

Yeasts have been used in traditional fermentation processes to produce beer, bread and wine. Owing to improvements in modern biotechnology, yeasts have also been used in important industrial areas like food, beverages, chemicals, industrial enzymes, pharmaceuticals, and environment. *S. cerevisiae* is very important for several fermentation and biomass conversion processes due to its ability to convert sugars and other carbon sources into ethanol in the absence of oxygen or into CO_2 and water in the presence of oxygen. Yeast is also a good food supplement and unusual source for vitamin B and low meat/vegeterian diets (Ratledge and Kristiansen, 2001). *S. cerevisiae* has also been used in agriculture. *S.cerevisiae* secures rumen of ruminant animals and enhances animal growth and milk yields by increasing nutrient availability (Walker, 2009).

Due to some advantages of *S. cerevisiae*, it has also been chosen as a model organism for medical research. So far, *S .cerevisiae* has continued its role as a model organism for studying disease mechanisms and mammalian cell biology. *S.cerevisiae* improves our knowledge about regulation of eukaryotic cell division. Also, yeast provides a cellular environment to investigate disease-related proteins that have no homologous copies in yeast (Mager, 2005). Table 1.2 shows that examples of human diseases where *S. cerevisiae* has been used as a model organism.

Table 1.2: Examples of human diseases where *S. cerevisiae* has been used as a model organism (Stewart, 2014).

Disease	Reference
Prion Related Disease	Nakayashiki et al. 2005
Alzheimer's Amyloid Disease	Von der Haar et al. 2007
Parkinson's Disease	Doostzadeh et al. 2007
Cancer	Botstein et al. 2003
Channelopathies	Wolfe and Pearce, 2006
Aging	Piper et al. 2006

1.3 Propolis

Propolis is a resinous, sticky and dark-colored substance that bees produce by mixing their own waxes with resins obtained from plants. The meaning of the word propolis is "defence of the city". The United States Department of Agriculture's 'United State Standards for Grades of Extracted Honey, effective May 23, 1985' (adapted from 7 CFR, 521394) defines propolis as follows (USDA, 1985) :

"Propolis means a gum that is gathered by bees from various plants it may vary in color from light yellow to dark brown. It may cause staining of the comb or frame and may be found in extracted honey" (Burdock, 1997).

Propolis is used as a building material and bees protect their hives against fungi and bacteria. Propolis has been used in folk medicine since ancient times because of its biological advantages (Cuesta et al., 2005). Propolis is shown at figure 1.5.

Figure 1.5: Samples of propolis (Krell, 1996).

1.3.1 Historical uses of propolis

Anti-digester property of propolis was known very well by Egyptians and they used it to embalm cadavers. Greek and Roman physicians discovered medicinal properties

of propolis; Aristoteles, Dioscrodies, Pliny and Galen. Incas used propolis as an anti-pyretic agent and London pharmacopoeias of the seventeenth century showed propolis as an approved drug. The drug was very popular in European countries between the seventeenth and twentieth century, especially due to its anti-bacterial activity (Castaldo and Capasso, 2002).

Propolis was used in Italy in the seventeeth century as an antiquarian non-personal product or medicinal agent. Stradivari used propolis to wax the stringed instruments. Today, propolis is applied to musical instruments to repair accordions (Burdock, 1997).

Propolis has been used at least since 300 BC and its use goes on today in natural medicine and personal products. Propolis has antiseptic, antimycotic, bacteriostatic, astringent, choleric, spasmolytic, anti-inflammatory, anaesthetic and antioxidant properties. Implementations of these properties require no prescription. Dermatological ointments are accepted useful in wound healing, tissue regeneration, cure of burns, neurodermatitis, leg ulcers, psoriasis, morphoea, herpes simplex, genitalis and pruritus (Burdock, 1997). Propolis also plays a role in drug industry in some European countries as a medication against prostate hyperplasia (Popova, 2005).

Propolis is commercialised for remedy of rheumatism and sprains and it has been used in dental medicine. Propolis is also used in toothpaste and mouthwash applications to heal gingivitis, cheilitis, and stomatitis. It is marketed as tablets, powders, and chewing gum. Propolis is also important in cosmetic industry, it is applied in face creams, oinments, lotions and solutions (Burdock, 1997).

1.3.2 Chemistry of propolis

Chemical content of propolis is quite complex due to more than 300 ingredients, such as polyphenols, phenolic aldehydes, sequiterpene quinines, coumarins, amino acids, steroids and inorganic compounds, which have been identified in propolis samples (de Castro, 2012).

The constituents of propolis are derived from three sources: plant exudate collected by bees; secreted substances from bee metabolism; and materials which are introduced during propolis elaboration. The plant origin of propolis has been searched by scientists. Bankova et al. discovered that propolis constitution is very

similar to bud exudates Marcucci et al., 1995). Surely, propolis is obtained from propolis sources like *Populus spp*. (*Populus alba, Populus pyrimidalis*, and *Populus tremulodies*) and *Salix spp*. (*Salix alba, Salix fragilis*) trees. In *Populus alba,* the basic components are chyrisin, ferulic acid and octadecanoic acid and if in *Salix alba* basic components, are glycosides, vanillin, ferulic acid and sesquiterpene (Silici and Kutluca, 2005).

Table 1.3: Main compounds from different sources which were found in propolis (Krell, 1996).

Components	Main substances	Abundance (%)
Resins	• Flavonoids • Terpenes • Cumarins • Phenolic acids and esters	45-55
Waxes and fatty acids	• Polyunsaturated fatty acids and waxes from bees and plants	25-35
Essential oils	• Volatiles	10
Pollens	• Proteins • Free amino acids	5
Other substances	• Vitamins (A, B, C, E, PP, etc.) • Trace elements (Cu, Mn, Fe, Zn, Al, Ag, Ca, Mg, Co, etc.) • Ketones • Lactones • Quinones • Steroids • Sugars	5

Composition of propolis varies depending on where it is produced by bees. Natural factors such as type of vegetation, zone of temperature, and seasonality affect its composition (Rafael, 2012). Because of the diversity of plant sources, the chemical composition of propolis is highly variable and due to differences between geographic regions, antibacterial compounds in propolis also vary. For example; flavonoids and cinnamic acid derivatives are found in European samples, and diterpenic acids and prenylated coumaric acids are found in Brazilian, etc. (Popova, 2005).

Regarding propolis species in Turkey, the major source is poplar bud exudate. It includes pentenly and aromatic caffates, pinocembrin, pinobanksin 3-O-acetate, and galangin, which are regarded as taxonomic markers for poplars of region Aigeiros (Popova, 2005).

Honeybees modify some flavones by an enzyme in the bee saliva. The propolis used to mend the honeycomb contains large amount of wax. Therefore, propolis ensures durability to honeycomb. However, if there is a thin layer of propolis on honeycomb, it comprises little or no wax. Propolis obtained from hives in Ohio includes lower concentration of methanol-insoluble wax compared to those in South Georgia. Simple fractionation of propolis is hard because of its complex composition. Therefore, alcohol and other solvents are used for fractionation of propolis. Fraction is soluble in alcohol and leaves the alcohol-insoluble and wax fraction. This alcohol-soluble form is called as 'propolis balsam' (Burdock, 1997).

1.3.3 Biological activities of propolis

Propolis has several biological activities such as antimicrobial and hepatoprotective effect, antitumor activity, antioxidative activity, antiinflamatory activity, immunomodulator, cytotoxic activity and therapeutical activity (Rafael, 2012).

1.3.3.1 Antimicrobial activity

Bees produce propolis to protect their hives and avoid accumulation of creatures killed by bees as a result of their hive invasion. Therefore, propolis is evaluated to have antimicrobial properties (Banskota et al., 2001). The antimicrobial activity of propolis reputedly stems from flavonoids, aromatic acids and esters present in resin. Galangin, pinocembrin and pinostrobin are most effective flavonoids against bacteria. Ferulic and caffeic acids also ensure antibacterial effect to propolis. Antimicrobial effect of propolis is expressed with synergism between flavonoids, hydroxy acids and sesquiterpenes (Marcucci, 1995).

Biochemical effects of flavonoids are divided into four sections : (1) binding affinity to biological polymers ; (2) binding of heavy metal ions; (3) catalysis of electron transport and (4) ability to scavenge free radicals. There are various instances about inhibition of a series of enzymes by flavonoids such as hyrolases and alkaline phosphatase (de Castro, 2011). Propolis possesses same effects by inhibiting

glycosyltransferases of *Streptococci*, myeloperoxidase activity of inflamation, ornithine decarboxtlase, lipooxygenase, tyrosine protein kinase and arachidonic acid metabolism (Burdock, 1997).

A minimum of 60-80 µg/ml propolis concentration was required for inhibition of *Bacillus subtilis* and *Staphylococcus aureus*, but a minimum of 600-800 µg/ml propolis concentration was required for inhibition of *Escherichia coli* (Serra and Escola, 1995).

Propolis samples have antimicrobial effect on some gram positive bacteria including *S.aureus, P.aeruginosa, B.subtilis, S.epidermidis and Streptococcus* sp. However, gram negative bacteria were not affected by propolis. The ethanol extract of propolis concentrate exactly inhibited the growth of *Pseudomonas aeruginosa* and *Escherichia coli*, but it posed no inhibition to *Klebsiella pneumoniae*. Extracts of propolis have exhibited similar effects to those of major antibiotics. The antibiotic effect was increased by the presence of propolis in medium (Fuantes and Hernandez, 1990).

Effect of crude propolis and fractions on *Helicobacter pylori*, considered to be related to gastric ulcer, was investigated. Propolis has anti-*H.pylori* activity and p-coumaric acid, 3-prenyl-4-dihydrocinnamoyloxycinnamic acid and artepilin compounds ensure the activity (Banskota et al., 2001). Scheller et al.(1999) studied synergism between the ethanol extract of propolis and antituberculosis drugs on the mycobacteria (Banskota, 2001).

Amaros et al. (1992a, 1992b) examined *in vitro* effect of propolis on several DNA and RNA viruses such as herpex simplex type 1, an acyclovir-resistant mutant, herpex simplex type 2, adenovirus type 2, vesicular stomatitis virus and poliovirus type 2. Flavonoids and aromatic acid derivatives ensure antiviral activity. The luteolin is more effective than quercetin, but less than caffeic acid. Caffeic acid poses weak antiviral activity against influenza, although vaccinia and adenovirus are more sensitive than polio and parainfluenza virus (Marcucci, 1995).

Antiviral activity of components of propolis, such as esters of substituted cinnamic acids, have been investigated *in vitro*. One of them, isopentyl ferulate exhibits antiviral activity against influenza virus. Similar results were obtained with 3-methyl-2enyl caffeate against herpex simplex virus (HSV-1) (Marcucci, 1995).

In Turkey, amount of phenolic compounds, flavones and flavanones in poplar propolis is important in terms of antimicrobial activity. Figure 1.6 shows that primary chemical components of Turkish propolis.

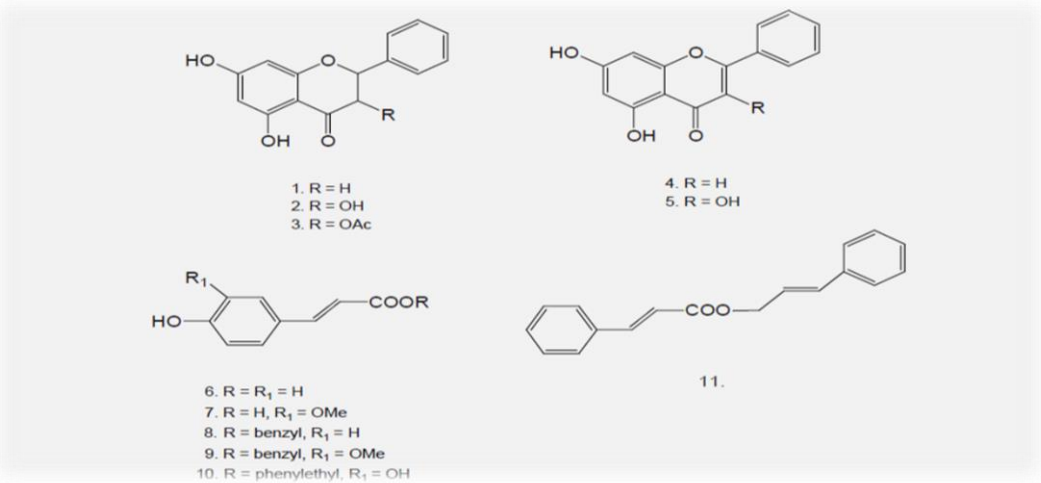

Figure 1.6: Primary chemical components of Turkish propolis from different areas (1) pinocembrin, (2) pinobanksin, (3) pinobanksin-3-O-acetate, (4) chrysin, (5) galangin, (6) coumaric acid, (7) ferulic acid, (8) benzyl-p-coumarete, (9) benzyl ferulate, (10) phenylethylcaffeate, (11) cinnamyl cinnamate (Popova, 2005).

Propolis showed significant antifungal avtivity against *Trichophyton* and *Mycosporum* in the presence of propylene glycol. Use of propolis together with some antimycotic drugs enhanced drug activity against *Candida albicans* yeasts. The important synergistic effect was achieved when propolis was added to antifungal drugs. Antifungal activity of ethanol extract of propolis was considered against *C. albicans, C. paraplisosis, C. tropicalis* and *C. guilliermondii* ; 98% of fungi samples were sensitive. Antifungal activity of propolis was also studied on some plant fungi *in vitro* (Marcucci, 1995). Despite differences in chemical contents of propolis collected from different geographic locations, all propolis samples showed important antimicrobial activity. According to propolis studies, antimicrobial activity of propolis is not derived from one particular substance. Combination of different chemical compounds ensure this activity (Kujumgiev et al., 1990).

1.3.3.2 Antioxidant activity

Aerobic organisms cope with toxic effects of reactive oxygen species (ROS). ROS can be formed during stress conditions like heat shock, dehydration, toxic chemicals, UV and ionizing radiation. Aerobic respiration causes generation of ROS because

oxygen can be reduced during respiration. ROS represses the cellular antioxidant species and oxidative stress occurs. Oxidative stress enhances damages to cell structure such as proteins, lipids and nucleic acids. Changes in such molecules are associated with several diseases such as cancer, Alzheimer's disease, Amyotrophic Lateral Sclerosis (ALS) and process of aging (Rafael, 2012).

There are a variety of enzymatic and non-enzymatic factors that act as defence mechanisms against ROS-induced oxidative stress. Enzymatic factors include ezymes such as superoxide dismutases, glutathione transferases, catalase and other factors relevant to removal, repair or detoxification of damaged intracellular compounds. Moreover, non-enzymatic ones such as ascorbic acid (Vitamin C), α-tocopherol (Vitamin E), glutathione (GSH), carotenoids, flavonoids ensure removal of ROS and detoxification of constituents damaged by ROS. Therefore, components derived from the beehive such as honey, propolis and royal jelly become important (Rafael, 2012).

Propolis mainly includes flavonoids and phenolic compounds. These compounds have antioxidant properties. Therefore, propolis may protect humans against oxidative stress damages. Antioxidant properties of propolis and its active compounds have been studied by many research groups. Five different propolis samples from Brazil were studied regarding 1,1-diphenyl-2-picrylhyrazyl (DPPH) free radical and superoxide anion radical in the xanthine / xanthine oxidase (XOD) and α-nicotinamide adenin dinucleotide (NADH) / phenazyne (PMS) reactions. Four dicaffeoylquinic acid derivatives were isolated from water extract of propolis. These derivatives exhibited a stronger free radical scavenging activity than the most common antioxidants such as vitamin C, vitamin E, and caffeic acid. Moreover, dicaffeoylquinic acid derivatives have an inhibitory activity on nitrite formation in lipopolysaccharide-induced murine macrophages (Matsushige et al., 1996).

Propol is an antioxidant compound, obtained from water extract of Brazilian propolis and propol has stronger antioxidant activity than vitamin C and vitamin E. Propolis and propol inhibited Cu^{+2}-initiated low density lipoprotein (LDL) oxidation (Banskota et al., 2001). Another component obtained from propolis is caffeic acid phenyl ester (CAPE). CAPE has antitumor activity and inhibited 5-lipoxygenase and soybean 15-lipoxygenase at micromolar concentrations. Also, CAPE exactly stopped the production of ROS in human neutrophils and in the cell free xanthine/XOD system (Mirzoeva et al., 1997).

When propolis was applied to yeast cells, their intracellular oxygen levels decreased. Changes also occured at mitochondrial proteome level, including antioxidant proteins and proteins involved in ATP synthesis. Therefore, increase in antioxidant protein levels ensured decreasing levels of intracellular oxidation (Cigut et al., 2011).

According to propolis studies with the yeast *S. cerevisiae*, propolis is the up and coming antioxidant due to three important findings: (1) it promotes protection of membrane lipids from H_2O_2 stress, (2) O_2 stress provides menadione, and propolis resumes redox status by scavenging ROS. (3) it activates Cu / Zn-superoxide dismutase, one of the most substantial antioxidant enzymes (Rafael, 2012).

1.3.3.3 Antitumor activity

Propolis extracts have been investigated for *in-vitro* cytotoxic activity in different cell lines. Propolis cannot be used in untreated form and it should be extracted to remove ineffective part and protect the polyphenolic fraction. The etheral propolis fraction (DEEP) have most effective cytotoxic activity and secondary fractions of etheral propolis fraction also have good activity (Marcucci, 1995). Also ethanolic extract of propolis (EEP) excited attention of scientists due to its biological and pharmacological properties like immunomodulatory and anticancer effects. Cancer cell proliferation and tumor growth are prevented by EEP due to increase in cell-cycle halt and apaptosis (Szliszka, 2011).

13E-symhyoreticulic acid, 13Z-symhyoreticulic acid and 3-(2,2-dimethyl-8-prenylbenzopyran-6-yl) prepenoic acid, isolated from Brazilian propolis, possess cytotoxic effect. Also artepilin C has cytotoxic effect on tumor cells. It is isolated from Brazilian propolis. The cytotoxicity is ensured by the induction of apoptosis-like DNA fragmentation. The component have more cytotoxic activity than 5-FU against transplantable tumor cells. Artepilin C induces immune system and shows direct anti-tumor activity. Propolis provides decrease by 0.1 % and 0.01% on incidence and multiplicity of mammary carcinomas (Banskota, 2001).

Caffeic acid phenyl ester (CAPE), an active compound of Israeli propolis has important cytotoxic effect on various tumor cell lines. It was synthesized and used to prevent the growth of human leukaemia HL-60 cells. Tumor inhibition by CAPE was relevant to increased enterocyte apoptosis and proliferation (Huang et al., 1996).

The ethereal propolis fraction (DEEP) showed the strongest cytotoxic activity. The secondary fractions of ethylacetate and butanol DEEP exhibited a good activity. Flavonoids were tested to investigate the killing action of propolis. Hela cells were more sensitive to quercetin and rhamnetin, but less sensitive to galangin. KB and Hela cell line studies showed that the cytotoxic effect was derived from quercetin and caffeic acid phenyl ester components of propolis (Marucci, 1995).

1.3.3.4 Anti-inflammatory effect

Characteristics of inflammation divides it into groups such as acute, chronic, irritability- and immunity-related inflammation. There are three major factors that trigger inflammation; such as physical factors (bruises, burns, frostbite, radical damage), chemical factors (acid, alkali, allergens, mineral oil) and biochemical factors (microorganisms, parasites, endotoxins and animal toxins). Inflammatory media also contain histamine, bradykinin, prostaglandin, platelet activation factor, neutrophile hydrolase, inflammation prestimulation factors (TNF-α, IL-1, IL-6, cell chemotaxis factors), adherence cell, acute reaction protein (C reaction protein, LPS-combined protein, serum starched protein A) etc. (Hu et al., 2005).

Propolis is generally used to cure some skin inflammation diseases. According to studies, ethanol extract of propolis (EEP) and water soluble derivatives (WSD) possess inhibitory activity on leakage, oedema, conglomeration and increase of WBC. Therefore EEP and WSD have anti-inflammatory effect and reduce a broad spectrum of inflammatory reactions (Schmidt and Walter, 1994).

Exposing mice to water soluble derivative (WSD) of propolis avoided the cyclophasmide effects and increased survival rates of animals. Propolis induced cytokines production such as IL-1β and TNF-α by peritoneal macrophages. Six isolated compounds of propolis such as caffeoylquinic acid derivatives increased motility and spreading of macrophages. Applying propolis to rats enhanced antibody production. Propolis can regulate antibody synthesis as a part of adjuvant activity. Therefore, propolis has an important effect on different cells of congenital immune response. Propolis induced cytotoxic activity of natural killer cells against murine lymphoma. Natural killer cells are lymphocyte subpopulation and cytotoxic activity of natural killer cells ensures resistance against tumor development (Sforcin, 2007).

In conclusion, propolis is an anti-inflammatory agent against acute and chronic inflammation. Galangin and CAPE are the two phenolic compounds considered as

major constituents of propolis to prevent development of inflammation. Especially, CAPE is required for the anti-inflammatory effect of honeybee propolis (Borelli et al., 2002). Figure 1.7 indicates the chemical structure of CAPE.

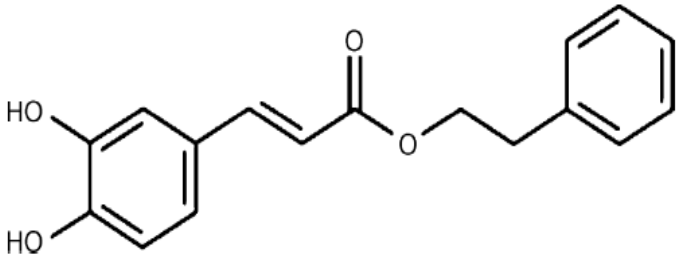

Figure 1.7: Chemical structure of caffeic acid phenyl ester (CAPE)

1.3.3.5 Toxic effect of propolis

Besides its various advantages, propolis also has toxic and allergenic effects. Propolis includes some constituents that cause toxicity. The bees may also collect hazardous materials when forming propolis: e.g. Cuban propolis contains metals such as iron (Fe), zinc (Zn), copper (Cu), and magnessium (Mg). Also, Brazilian propolis includes some heavy metals such as lead (Pb) (Banskota, 2001).

Propolis extracts have low toxicity, and flavonoids themselves are also of low toxicity. For instance, pinocembrin is the prevalent flavonoid in several extracts. It exhibited no toxicity when applied orally to mice at 1000 mg/ml (Banskota, 2001). A constituent of propolis, 1,1-dimethylallycaffeic acid, is responsible for allergy. The flavonoid tectochrysin was evaluated as a second allergen. Also, allergenic effects of prenylethyl and phenyl esters of caffeic acid were also investigated (Marucci, 1995).

1.4 Inverse Metabolic Engineering

Metabolic engineering is the improvement of cellular activities by modification of enzymatic, transport and regulatory functions of the cell by using recombinant DNA technology. Metabolic engineering is the multidisciplinary area between molecular biology, biochemical reaction engineering, applied microbiology and biomedical research (Bailey, 1991).

Classical or rational metabolic engineering has some limitations such as the need for extensive biochemical, enzymatic and genetic information on the metabolic system of interest, and the need for a high number of sitimulus-response experiments.

Because of these limitations of rational metabolic engineering, an alternative strategy, named as 'inverse metabolic engineering', is used. Bailey divided "inverse metabolic engineering" strategy into three steps ; the first step is identifying, building or calculating the requested phenotype ; the second step is identifying the genetic or environmental factors related to this phenotype ; and the third step is transferring this phenotype to another organism by genetic or environmental manipulation techniques (Bailey, 1991). Inverse metabolic engineering starts with a known and desired phenotype. Therefore, detailed information about metabolic pathways of desired organism is not required in contrast to rational metabolic engineering (Çakar, 2009).

As an inverse metabolic engineering strategy, evolutionary engineering is the application of continuous evolution procedures to obtain a desired phenotype (Butler et al., 1996). In nature, environmental effects such as mutagens cause changes in the gene pool of an organism. Nature performs selective pressure on this gene pool and some genes undergo changes with the changing conditions. Finally, environmently adapted organisms are obtained (Barton, 2007).

Under laboratory condintions, evolutionary engineering strategy begins with the application of mutagens for random mutagenesis of the gene pool of the organism of interest. UV light or chemicals are used for the random mutagenesis. Selective pressure is then applied to obtain a desired phenotype (Hahn Hagerdal et al., 2007). Thus, evolutionary engineering is a useful inverse metabolic engineering strategy to obtain desired phenotypes. Basic evolutionary engineering strategy were shown in Figure 1.8.

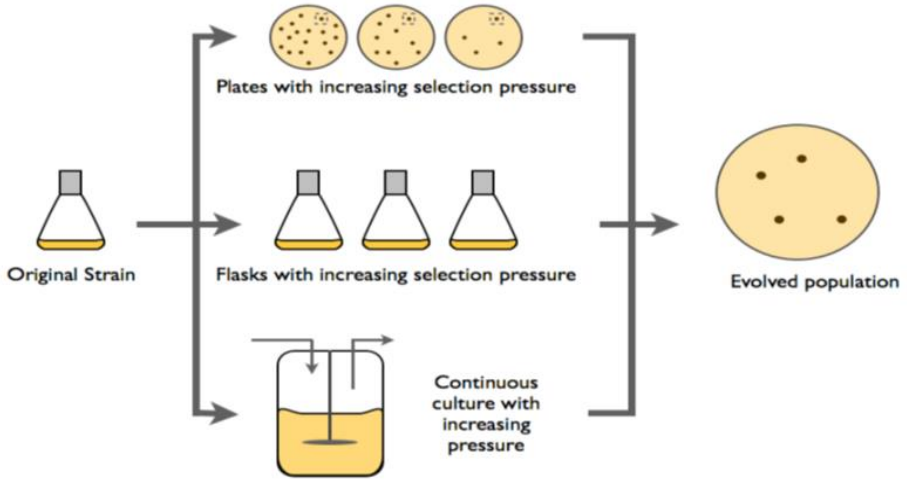

Figure 1.8: Basis of evolutionary engineering strategy (Hahn Hagerdal et al., 2007).

1.5 The Aim of the Study

The aim of the present study was to obtain propolis-resistant *Saccharomyces cerevisiae* strains by using an inverse metabolic engineering strategy, evolutionary engineering. Because propolis has a variety of biologically important effects, it was chosen as the selection factor. Turkish propolis was applied to a chemically mutagenized *S.cerevisiae* culture initially at low doses, and by increasing propolis concentration stepwise at each repetitive batch culture. The physiological analyses were then performed to compare the propolis-resistant yeast mutants to the reference strain.

The propolis-resistance of the mutants and the reference strain were determined semi-quantitatively by Most Probable Number (MPN) Method-based assay. The genetic stability of mutant strains were also determined. Additionally, cross-resistance of the propolis-resistant mutants to other stress types were also determined to identify the relationship between propolis-resistance and resistance to other stress factors.

2. MATERIALS AND METHODS

2.1. Materials

2.1.1 Strains and propolis

The reference strain CEN.PK 113-7D (*MATa, MAL2-8^c, SUC2*) *Saccharomyces cerevisiae* was kindly provided by Dr. Laurent Benbadis, University of Toulouse, France, and named as *905*. *905* was then randomly mutagenized with a chemical mutagen (ethyl methane sulfonate) as described previously (Lawrence, 1991), and the resulting population was named as *906*. Propolis was kindly provided by Prof.Dr. Oğuz Öztürk, Istanbul University. Ethanol extract of propolis was used in this study. Propolis was diluted with ethanol: water (60:40 v/v).

2.1.2 Culture media and preservation conditions

Yeast cultures were incubated at 30 ^oC and 150 rpm, using yeast minimal medium (YMM) or nutrient rich medium (YPD). After cultivation, 1000 µL of culture were placed in 1.5 mL microcentrifuge tubes and centrifuged at 10,000 rpm for 3 min. The culture was then washed with yeast minimal medium (YMM) and the supernatant was removed. 1000 µl of 30% glycerol (v/v) was added onto the cell pellet. This suspension was stored at -80 ^oC deep-freezer. For reviving and growing cultures after extended storage at -80 ^oC, 50 µL of cell suspension was placed to 50 mL culture tubes containing 10 mL YMM or YPD. The cultures were then incubated overnight at 30^oC and 150 rpm. The next day, cultures were inoculated into fresh medium to an initial OD_{600} value of 0.25.

2.1.3 Yeast culture media

2.1.3.1 Yeast minimal medium

Chemicals indicated in Table 2.1 were dissolved in deionized water to prepare yeast minimal medium (YMM) and autoclaved at 121 ^oC, for 15 min.

Table 2.1: Contents of yeast minimal medium (YMM).

Chemicals	Amount
Yeast nitrogen base without amino acids	6.7 g
Dextrose	20 g
Agar (for solid media)	20 g
Water	to 1 lt

2.1.3.2 Yeast extract peptone dextrose medium (YPD)

Chemicals indicated in Table 2.2 were dissolved in deionized water and autoclaved at 121 ^{o}C, for 15 min.

Table 2.2: Contents of yeast extract peptone dextrose medium (YPD)

Chemicals	Amount
Yeast nitrogen base without amino acids	10 g
Dextrose	20 g
Peptone	10 g
Agar (for solid media)	20 g
Water	to 1 lt

2.1.4 Laboratory Equipment

Laboratory equipment used during experiments are shown in Table 2.3.

Table 2.3: Laboratory instruments that are used in this study.

Equipment	Supplier
UV Visible Spectrophotometer	Shimadzu UV-1700 (Japan)
Vortex Mixer	Nüve NM 100 (Turkey)
Autoclaves	Tommy SX700E (China) Tuttnauer Systec Autoclave 2540 ml 2870ELCV (Switzerland)

Table 2.3 (continued): Laboratory instruments that are used in this study.

Equipment	Supplier
Light Microscope	Olympus CH30 (Japan)
Microfuge	Eppendorf Microcentrifuge-5424 (Germany)
Micropipettes	Eppendorf (Germany)
Balance	Precisa BJ 610 C (Switzerland)
Microbalance	Precisa 620C SCS
Laminar Flow Hood	Biolab Faster BH-EU 2003
Magnetic Stirrer	Labworld (Germany)
Benchtop Centrifuge	Eppendorf 5424 (Germany)
pH-Meter	Mettler Toledo MP220 (Switzerland)
Deep-freezer	-80 ^{o}C Sanyo Ultra Low MDT-U40865 (Japan)
Refrigerators and Deep-freezers	-20 ^{o}C Arçelik 3011 NY (Turkey) +4 ^{o}C Arçelik (Turkey)
Shaker	Thermo Scientific Orbital Shaker (USA)
Orbital Shaker Incubators	Certomat S-2 Sartorius (Germany)
Ultrapure Water System	TKA(Germany)
HPLC System	Shimadzu (Japan)
HPLC Column	Bio-Rad HPX-87H Aminex Ion-exclusion column, 300x7.8mm (USA)
UV-Visible Spectrophotometer	Bio-Rad Benchmark Plus^{TM}Microplate Reader Spectrometer (USA)

2.1.5 Chemicals

The chemicals used during this study are shown in Table 2.4.

Table 2.4: The chemicals used in this study.

Chemicals	Supplier
Propolis	Region of Kartal, Istanbul, Turkey
Nickel chloride hexahydrate ($NiCl_2.6H_2O$)	MERCK (Germany)
Cobalt chloride hexahydrate ($CoCl_2.6H_2O$)	Fluka (USA)
Copper (II) sulphate pentahydrate ($CuSO_4.5H_2O$)	Sigma ALDRICH (USA)
Hydrogen peroxide (H_2O_2)	MERCK(Germany)
Chrome chloride ($CrCl_3$)	Acros Organics (USA)
Zinc chloride ($ZnCl_2$)	Carlo Erba (Italy)
Magnesium chloride hexahydrate $MgCl_2.6H_2O$	MERCK (Germany)
Ammonium iron (II) sulphate hexahydrate $(NH_4)_2Fe(SO_4)_2.6H_2O$	MERCK (Germany)
Manganese (II) chloride tetrahydrate $MnCl_2.6H_2O$	MERCK (Germany)
Ethanol (C_2H_6O)	J.T Baker (The Netherlands)
Aluminium chloride hexahydrate ($AlCl_3 .6H_2O$)	MERCK (Germany)
Sodium chloride (NaCl)	MERCK (Germany)
Geneticin	Thermo Fisher (USA)
Caffeine	MERCK (Germany)
Acetic acid	MERCK (Germany)
Acetone (C_3H_6O)	MERCK KGaA (Germany)
Agar	BDDifco™ (USA)
Glycerol ($C_3H_8O_3$)	Duchefa Biochemie (The Netherlands)
Yeast Extract	MERCK (Germany)
Ethyl methane sulfonate (EMS)	Alpha-Aeasar (Germany)
Peptone	Riedel-de Haen (Germany)
Dextrose	Riedel-de Haen (Germany)
Sulphuric acid	Riedel-de Haen (Germany)

2.2. Methods

2.2.1 Screening at varying propolis concentrations

The *Saccharomyces cerevisiae* reference strain CEN.PK 113-7D (*MATa*, *MAL2-8^c*, *SUC2*), named as *905*, was randomly mutagenized using ethyl methane sulfonate (Lawrence, 1991), and named as *906*.

In order to obtain propolis-resistant *S.cerevisiae* mutants, an evolutionary engineering selection strategy was planned. For this purpose, it was necessary to determine the initial propolis concentration to be applied during selection experiments. Thus, 905 and *906* were screened under varying propolis levels: 60, 100, 150, 200, 250, 500, 550, 600, and 650 µg/ml. The propolis stress was applied continuously throughout the cultivation of 905 and 906, which lasted for 48h.

The initial optical density (OD_{600}) of the cultures was adjusted to 0.250 during inoculation with overnight fresh cultures, and the cultures were grown in 50mL culture tubes containing 10 mL of YMM at 30ºC and 150 rpm. After 24 and 48 h of incubation, OD_{600} values of the cultures were measured and survival rates were calculated by dividing "*OD_{600} of the strain under propolis stress*" by " *OD_{600} of the strain under control conditions*".

2.2.2 Obtaining propolis-resistant yeast populations

Previously, the chemically mutagenized yeast (906) culture frozen stock was cultivated in 10 mL yeast minimal medium (YMM) at 30˚C. After cultivation, a new pre-culture was prepared and incubated overnight for continous stress selection procedure. Two 50 mL-culture tubes that contained 10 mL YMM with and without propolis were inoculated with the same amount of precultures which were defined as the first population of propolis stress selection and its control. According to screening results, the initial propolis concentration for selection was determined as 150 µg/ml propolis and it was increased by 10 µg/ml at each successive cultivation, up to the final propolis concentration.

By this continuously applied selection strategy, several populations were obtained, which resisted against increasing levels of propolis stress. After 24 h of cultivation, the OD_{600} values were measured by using a UV-visible spectrophotometer and the survival rates were calculated by dividing OD_{600} population by OD_{600} control.

The propolis-resistant last population was diluted and plated on solid YMM media and twelve individual colonies were selected randomly to determine their propolis resistance.

2.2.3 Estimation of stress resistance

2.2.3.1 Spot assay

Individual twelve mutant colonies were selected from the final resistant population. Propolis-resistance levels of these colonies were determined by spot assay and MPN method, and compared to those of the reference strain.

Stock cultures of twelve individual mutants, the last population and the reference strain were inoculated into 10 mL of YMM medium and incubated overnight at 30^{o}C and 150 rpm. During exponential growth phase of the cultures, which were inoculated an initial OD_{600} of 2, OD_{600} units of 4 were measured, and the cultures were centrifuged at 13,000 rpm for 3 min. Pellets were diluted from 10^{-1} to 10^{-8} in YMM and inoculated into solid YMM plates including 200 µg/mL, 300 µg/mL, 500 µg/mL and 710 µg/mL propolis and control plates which did not contain propolis.

2.2.3.2 MPN method

MPN method is a statistical estimation of cell numbers using positive/negative turbidity data of microbial growth. Individual mutants with high propolis-resistance based on spot assay results were chosen for MPN assay to quantify their resistance to different propolis concentrations.

Viable cell numbers were estimated by serial dilutions in 96-well plates including 180 µL of YMM medium. Dilutions were made in the range of 10^{-1} to 10^{-8} for five parallel samples. Number of surviving cells was determined by statistical analysis of the presence/absence of growth in these dilutions. Quantification was made by using an MPN table which is based on Poisson regression (Russek and Colwell, 1983).

Twenty µL of individual mutants, the last population and reference strain cultures were inoculated into YMM with 200 µg/mL, 500 µg/mL, 710 µg/mL propolis concentration and YMM without propolis in 96-well plates with five replicates and were serially diluted in the range of 10^{-1} to 10^{-8}. After 96 h incubation, presence/absence of growth in the wells was monitored and viable cell numbers were estimated by using the MPN table.

2.2.4 Cross resistance tests

Strains that are resistant to a specific stress factor (e.g. propolis) may have cross-resistance against other stress factors. Thus, stress responses of mutant individuals, the last population and the reference strain were investigated against various stress factors, using spot assay and MPN method.

Cross resistance tests by spot assay were performed as follows: stock cultures of 12 individual mutants, the last population and the reference strain were inoculated into 10 mL of YMM medium and cultivated overnight at 30^{o}C and 150 rpm. During exponential growth phase of the cultures which were inoculated at an initial OD_{600} of 2, OD_{600} units of 4 were measured and the cultures were centrifuged at 13,000 rpm for 3 min. The cultures were then serially diluted up to 10^{-7} level by adding 20 µL of culture to 180 µL YMM. All dilutions were inoculated on YMM plates containing different stress factors : 0.1-0.3-0.5-0.8 mM NiCl , 1-2-2.2 mM $CoCl_2$, 0.1-0.3-0.4-0.5-0.8 mM $CuSO_4$, 0.5-1-1.5 mM H_2O_2 , 2-2.5-3 mM $CrCl_3$, 10 mM $ZnCl_2$, 0.5-1-1.5 M $MgCl_2$, 15-25-30-35-40 mM NH_4FeSO_4, 15-20 mM $MnCl_2$, 8-12% (v/v) ethanol, 12 mM $AlCl_3$, 0.5-1 M NaCl, 150 µg/ml geneticin, 10 mM caffeine.

Spot assay results revealed resistances and sensitivities of mutant colonies to other stress factors. Based on those results, MPN method was applied to the mutants for quantification of their cross resistance levels.

2.2.5 Genetic stability test

Genetic stability test was taken applied to verify the genetic stability of propolis resistance of the mutants obtained by evolutionary engineering strategy. Two mutant individuals with the highest propolis resistance according to spot assay and MPN method results were tested for their genetic stability. The frozen stock cultures of the mutants were inoculated into 10 mL YMM medium. After overnight incubation at 30^{o}C and 150 rpm, cultures were inoculated to fresh YMM again. This procedure was repeated five times, and at the end of each cultivation, culture samples were taken and stored at -80^{o}C.

At the end of the fifth cultivation, all frozen stocks from the previous and final cultures were inoculated into fresh YMM medium. After overnight incubation, the cultures were inoculated into MPN plates as five replicates and were serially diluted up to 10^{-8}. MPN test was performed in fresh YMM, with and without 250 µg/ml

propolis. 72nd hour MPN scores of cultures under 250 µg/ml propolis stress condition were read and the number of cells/ml were estimated from the MPN tables to quantify propolis stress resistance of each mutant culture, when grown in the absence of propolis stress for five successive passages.

2.2.6 Obtaining growth curves

Initially, the reference strain and the highest propolis-resistant mutant strain were grown in YMM medium with 50 µg/ml, 100 µg/ml, 150 µg/ml and 200 µg/ml propolis concentration and without propolis. OD_{600} values of cultures were measured for 10 h. Four different propolis concentrations were chosen to determine the optimum one for growth curve experiments with the mutant and the reference strain. 100 µL of stock culture of the mutant and reference strain were inoculated into 10 mL of YMM medium. After overnight incubation at 30°C and 150 rpm, cultures were inoculated into 500 mL flasks containing 100 ml YMM with 200 µg/ml propolis and without propolis, at an initial OD_{600} value of 0.25. OD_{600} values were measured regularly during 30 h and particular times and growth curves were obtained.

2.2.7 Cell dry weight (CDW) analysis

Cell dry weight measurements were taken during growth curve experiments. Firstly, empty microfuge tubes were weighed after drying at 80°C for 48 h. Two mL of samples were taken and centrifuged at 14'000 g for 5 min. Supernatants were removed and pellets were dried in an oven at 80°C for 48 h. Microfuge tubes containing the pellets were placed into a desiccator and kept there for 30 min. After 48 h, tubes were weighed again and compared with their first measurements when empty. Finally, cell dry weight was calculated as mg per ml of cell culture, based on the weight differences between empty tubes and tubes with dried pellets.

2.2.8 High performance liquid chromatography (HPLC) analysis of the reference strain and mutant individual *F11*

HPLC analysis was applied to determine the amount of various metabolites in culture samples obtained from the mutant and the reference strains. Samples were centrifuged at 14000 rpm for 5 min and the supernatants were filtered through 0.22 µm pore-size filter. HPX-87H Aminex ion-exclusion column (300 x 7.8 mm; Bio-

Rad, USA) was used for HPLC analysis at 60°C. 5 mM sulphuric acid was used as the mobile phase, at a flow rate of 0.6 ml/min. Sample volume was 20 µl. Shimadzu RID-10A refractive-index detector was used.

For quantification of metabolites, a standard curve was formed. Standard solutions were prepared for selected metabolites of interest, at particular concentrations. Stock solutions A and B were used to obtain the standard curve.

Table 2.5: The preparation of stock solutions A and B.

Stock Solution A		**Stock Solution B**	
Content	**Amount**	**Content**	**Amount**
Glucose	120 g	Acetate	4 g
		Glycerol	2 g
adjust final volume to 1 L with ddH_2O		Ethanol	30 g
		adjust final volume to 1 L with ddH_2O	

Table 2.6: The preparation of standard solutions.

Standard Solutions	**Mixing Volumes**	**Volume of Eluent (ml)**	**Final Volume (ml)**
Std 1	1 mL Solution A 3 mL Solution B	2.000	6
Std 2	0.750 mL Std 1	0.250	1
Std 3	0.500 mL Std 1	0.500	1
Std 4	0.250 mL Std 1	0.750	1
Std 5	0.125 mL Std 1	0.875	1
Std 6	0.063 mL Std 1	0.937	1

Sulfuric acid (5 mM) was used as the eluent for preparation of the standards.

Table 2.7: Various metabolite concentrations of HPLC standards.

Metabolite	**Std 1**	**Std 2**	**Std 3**	**Std 4**	**Std 5**	**Std 6**	**Retention Time(min)**
Glucose(g/L)	20	15	10	5	2.5	1.25	8.84
Glycerol(g/L)	1	0.750	0.500	0.250	0.1250	0.06250	13.53
Acetate(g/L)	2	1.500	1.000	0.500	0.2500	0.12500	15.13
Ethanol(g/L)	15	11.25	7.500	3.750	1.8750	0.93750	22.65

Standard solutions were prepared as shown in Table 2.5, Table 2.6 and Table 2.7 and 1 mL of each standard solution and samples were taken into the HPLC tubes. HPLC measurements were taken by using refractive index detector.

2.2.9 Estimation of trehalose and glycogen content through enzymatic reaction

Trehalose and glycogen contents were determined by using glucose oxidase/peroxidase asay (Parrou and François, 1997). At the end of the growth curve experiment (at 30^{th} hour) 25 OD_{600} unit cells were collected and centrifuged at 14000 rpm for 5 min. Supernatants were discarded and pellets were stored at -20ºC. Pellets were resuspended in 250 µl 0.25 M sodium carbonate and incubated at 95°C for 2-4 h. Then 150 µl 1 M acetic acid and 600 µl 0.2 M sodium acetate (pH 5.2) were added into samples and they were vortexed. Following that, 500µl of each sample were taken to new microfuge tubes. Consequently, each sample was divided into two microfuge tubes, one for trehalose and the other for glycogen analyses.

For trehalose analysis, 10µl trehalase enzyme was added onto half of the samples and they were incubated overnight at 37°C.

For glycogen determination, 20 µl alpha-glycosidase enzyme was pipetted into the second set samples and they were incubated at 57 °C, for overnight.

For both trehalose and glycogen measurements, glucose standards were prepared. Twenty µl of standards and samples were added to different wells of 96-well plates. Then, 200 µl of glucose oxidase/peroxidase reagent was pipetted onto the samples in wells. The glucose released was determined using the glucose oxidase/peroxidase method. After 30 min incubation at 37°C, absorbances of the samples and standards were measured at 490 nm, using a microplate reader.

2.2.10 Determination of reactive oxygen species (ROS) content

Five mM stock solution of dichlorofluorescein diacetate (DCF-DA) dissolved in ethanol was prepared and stored at -20°C. The solution was protected from light. Reference strain and the mutant were incubated overnight and then inoculated into fresh YMM and YMM containing propolis at 150 µg/ml concentration. Pre-cultures of reference strain and the mutant were cultivated until their mid-exponential phase of growth (OD_{600} 1-1.2). About $2x10^8$ cells were harvested by centrifugation. Harvested cells were pre-incubated for 10 min at 30ºC. DCF-DA was then added to

the medium at a final concentration of 10 μM or 15 μM, and it was incubated at 28°C for 30 min, in the dark, to allow probe uptake. After probe penetration, the cultures were centrifuged and the pellets were washed twice with sodium phosphate buffer or PBS. The pellet was re-suspended in 1000 μl buffer. Cells were vigorously vortexed for 1 min with glass beads and then kept on ice for 1 min. This cycle was repeated 10 times. After cell lysis, the solution was centrifuged at 13000 rpm for 5 min. The supernatant was then collected into fresh microfuge tubes. The supernatant was diluted six times for fluorescence measurements. Fluorescence was measured at an excitation wavelength of 488 or 504 nm and an emission wavelength of 520 or 524 nm.

3. RESULTS

3.1 Screening at Varying Propolis Concentrations

To determine the initial propolis stress level for selection, mutagenised yeast culture (906) and the reference strain (905) were cultured in 10 mL YMM including 60 µg/ml, 100 µg/ml, 150 µg/ml, 200 µg/ml, 250 µg/ml and 500 µg/ml propolis. Incubation was performed at 30^{o}C and 150 rpm. After 24 and 48 h of incubation, OD_{600} values were measured. The OD_{600} results are shown in Table 3.1 and survival rate values are shown in Table 3.2 and Figure 3.1.

Table 3.1: OD_{600} results of *905* and *906* grown in YMM at different propolis levels (0-500 µg/ml**)** after 48 h of incubation.

Propolis levels (µg/ml)	**OD_{600} of *905***	**OD_{600} of *906***
Control (0)	6.58	5.72
60	5.82	4.92
100	4.69	5.05
150	4.49	4.51
200	4.87	4.04
250	3.84	3.99
500	0.80	0.68

To determine the initial propolis level for selection experiments, *905* and *906* were screened under various propolis levels: 200 µg/ml, 300 µg/ml, 400 µg/ml, 450 µg/ml, 500 µg/ml, 550 µg/ml, 600 µg/ml and 650 µg/ml. The stress levels were applied continuously for 24 and 48h. OD_{600} values and survival rates of the reference strain (905) and 906 are given in Table 3.3, Table 3.4. and Figure 3.2.

Table 3.2: Survival rate values (normalized to those of the reference strain) and fold of reference strain values after 48 h of incubation, when grown in the presence of 60-500 µg/ml propolis.

Propolis levels (µg/ml)	Survival Rate of *905*	Survival Rate of *906*	Survival Rate of 906 (Normalized to reference strain value)
60	0.88	0.86	0.97
100	0.71	1.03	1.44
150	0.68	0.89	1.31
200	0.74	0.89	1.21
250	0.58	0.99	1.69
500	0.12	0.17	1.39

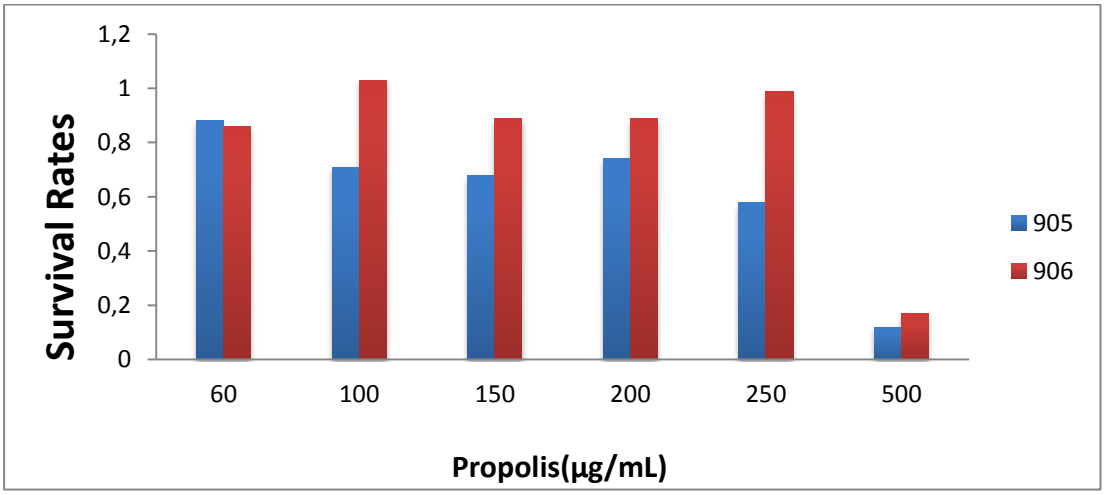

Figure 3.1: Survival Rate of *905* and *906* after 48 h of incubation, when grown in the presence of 60-500 µg/ml propolis.

Table 3.3: OD_{600} results of *905* and *906* grown in YMM at different propolis levels (200-650 µg/ml) after 48 h of incubation.

Propolis levels (µg/ml)	**OD_{600} of *905***	**OD_{600} of *906***
Control (0)	5.60	6.03
200	3.76	4.05
300	0.66	2.97
400	0.13	0.16
450	0.00	0.67
500	0.39	0.16
550	0.00	0.00
600	0.00	0.00
650	0.00	0.00

Table 3.4: Survival rate values after 48 h of incubation, when grown in the presence of 200-650 µg/ml propolis.

Propolis levels (µg/ml)	Survival Rate of *905*	Survival Rate of *906*
200	0.67	0.67
300	0.12	0.49
400	0.02	0.04
450	0.00	0.11
500	0.07	0.03
550	0.00	0.00
600	0.00	0.00
650	0.00	0.00

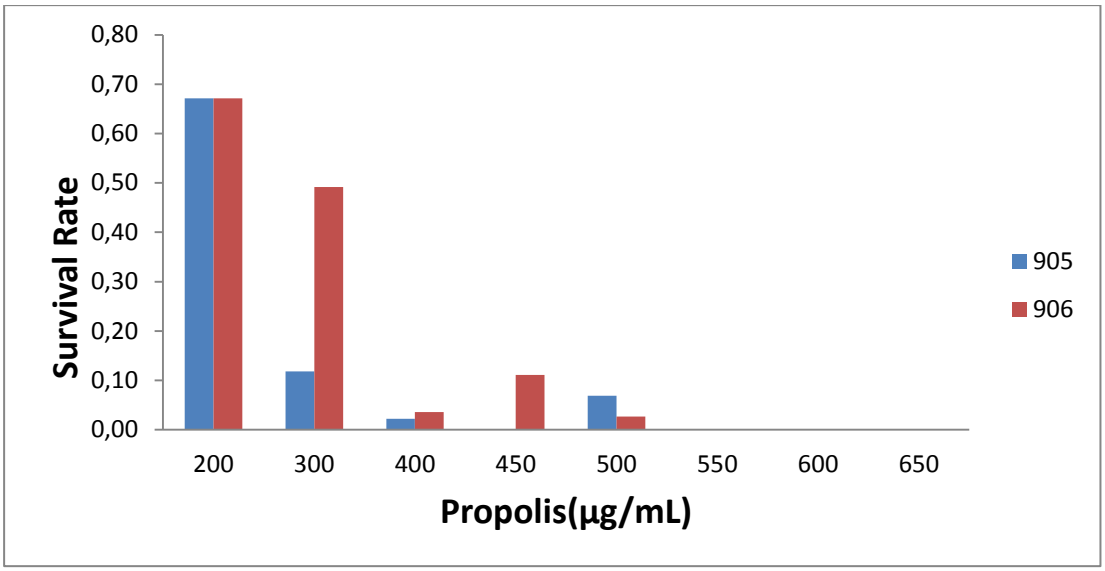

Figure 3.2: Survival Rate of *905* and *906* after 48 h of incubation, when grown in the presence of 200-650 µg/ml propolis.

3.2 Selection for Propolis Resistance

According to screening results, 150 µg/ml propolis concentration was chosen as the initial propolis stress level for selection. Propolis concentration was gradually increased from 150 µg/ml to 710 µg/ml, where the survival rates of successive populations decreased. By continuous selection strategy, 57 generations were obtained, which resisted against increasing levels of propolis stress up to 710 µg/ml. After 24 h of cultivation, the OD_{600} values were measured and the survival rates were calculated (OD_{600} population / OD_{600} control). These results are shown in Table 3.5 and survival rates upon increasing population numbers are given in Figure 3.3.

Table 3.5: Population data of propolis selection.

Population Number	Propolis (µg/ml)	OD_{600} control	OD_{600} stress	Survival rate	Incubation time (h)
1	150	4.02	1.53	0.38	24
2	160	4.77	3.15	0.72	24
3	170	4.89	3.55	0.73	24
4	180	4.75	1.84	0.39	24
5	190	4.39	3.55	0.81	24
6	200	5.12	4.18	0.82	24
7	210	5.12	3.51	0.69	24
8	220	5.23	4.09	0.78	24
9	230	5.21	2.15	0.41	24

Table 3.5 (continued): Population data of propolis selection.

Population Number	Propolis (µg/ml)	OD_{600} control	OD_{600} stress	Survival rate	Incubation time (h)
10	240	4.75	2.56	0.54	24
11	250	4.63	2.58	0.56	24
12	260	4.59	2.26	0.49	24
13	270	4.49	2.02	0.45	24
14	280	4.94	2.14	0.43	24
15	290	4.33	2.72	0.63	24
16	300	4.94	1.93	0.39	24
17	310	4.49	1.77	0.39	24
18	320	4.76	1.61	0.34	24
19	330	4.72	1.49	0.32	24
20	340	4.52	1.95	0.43	24
21	350	4.13	1.72	0.42	24
22	360	4.26	1.46	0.34	24
23	370	4.75	1.77	0.37	24
24	380	3.59	1.55	0.43	24
25	390	4.60	2.05	0.45	24
26	400	4.34	1.51	0.35	24
27	410	4.14	1.43	0.35	24
28	420	5.87	1.91	0.33	48
29	430	5.14	1.70	0.33	24
30	440	4.47	1.40	0.31	24
31	450	4.28	1.64	0.38	24
32	460	4.10	1.68	0.41	24
33	470	3.78	1.66	0.44	24
34	480	3.63	1.39	0.38	24
35	490	4.84	2.14	0.44	24
36	500	4.22	2.15	0.51	24
37	510	6.09	1.74	0.29	48
38	520	5.72	1.44	0.25	24
39	530	4.48	1.04	0.23	24
40	540	3.73	0.85	0.23	24
41	550	3.81	0.81	0.21	24
42	560	3.16	0.70	0.22	24
43	570	3.17	0.90	0.28	24
44	580	3.29	1.00	0.30	24
45	590	3.46	1.35	0.39	24
46	600	4.06	1.37	0.34	24
47	610	4.45	1.24	0.28	24
48	620	4.68	0.95	0.20	24

Table 3.5 (continued): Population data of propolis selection.

Population Number	Propolis (µg/ml)	OD_{600} control	OD_{600} stress	Survival rate	Incubation time (h)
49	630	3.75	0.94	0.25	24
50	640	4.10	1.18	0.29	24
51	650	3.74	1.31	0.35	24
52	660	4.37	1.25	0.29	24
53	670	4.19	1.43	0.34	24
54	680	4.08	1.18	0.29	24
55	690	4.70	1.24	0.26	24
56	700	4.73	1.15	0.24	24
57	710	4.66	1.48	0.32	24

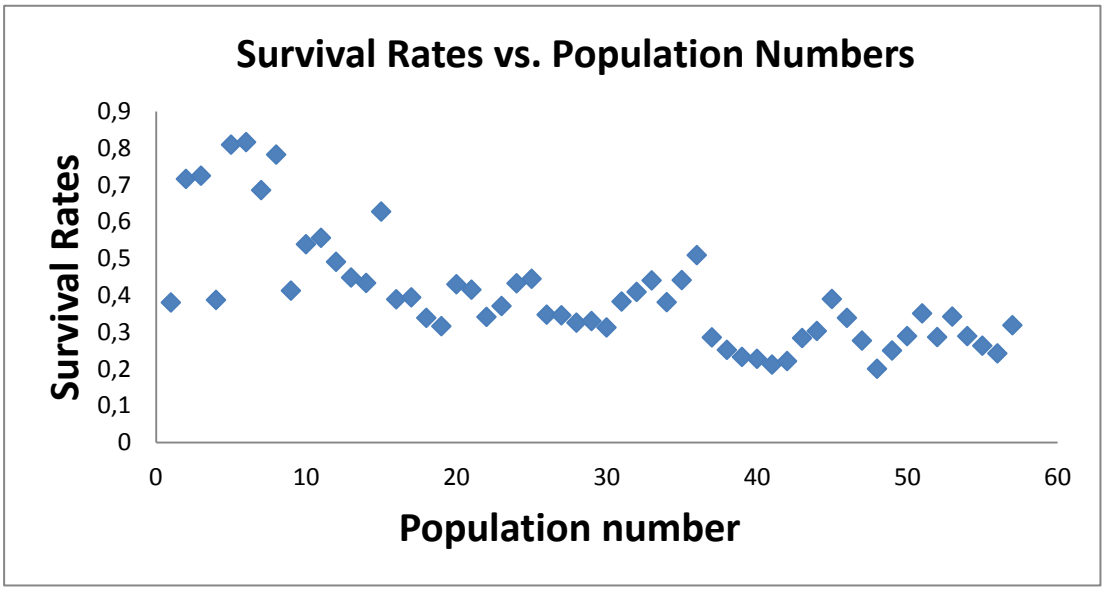

Figure 3.3: Survival rates versus population numbers during propolis selection.

3.3 Estimation of Stress Resistance

Individual colonies were randomly chosen from the final propolis-resistant population. These propolis-resistant colonies were compared with the reference strain, using spot assay and MPN method, for their propolis resistance.

3.3.1 Determination of propolis resistance by spot assay

Propolis-resistance of mutant colonies was determined first by spot assay. Mutant colonies, last population and the reference strain were inoculated onto solid YMM medium at different concentrations of propolis. Cultures were spotted in the range of 10^{-1} to 10^{-8} dilution and monitored after 72 h of incubation at 30°C . The images

were taken at 72 h and were are shown in Figure 3.4, Figure 3.5, Figure 3.6, Figure 3.7, and Figure 3.8.

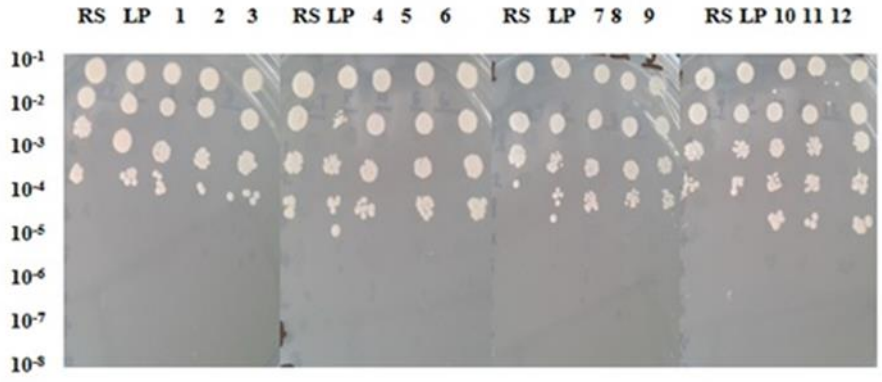

Figure 3.4: Spot assay results of individual mutants (FD1 to FD 12), 57th population and the reference strain (905) after 72 h incubation on solid YMM medium (control plates).

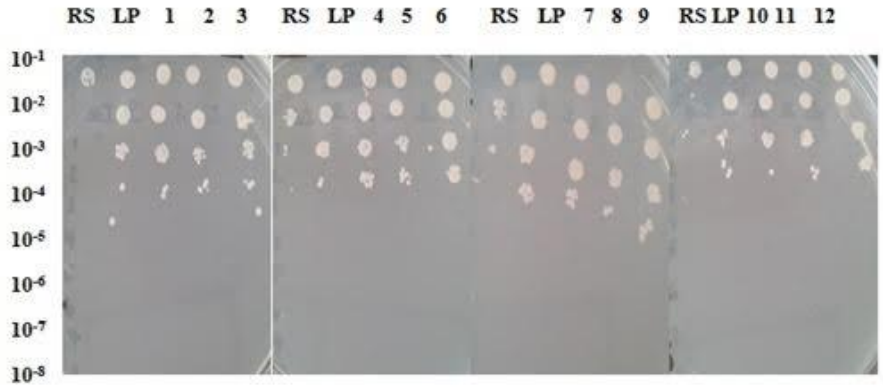

Figure 3.5: Spot assay results of individual mutants (FD1 to FD 12), 57th population and the reference strain (905) after 72 h incubation on solid YMM medium including 200 µg/mL propolis.

Individual mutants, the last population and the reference strain exhibited similar growth on control plates (0 µg/mL propolis). However, at 200 µg/mL propolis concentration, growth of the reference strain was inhibited and at higher propolis concentrations the reference strain showed no growth. According to spot assay results; FD7, FD8, FD10, FD11 and FD12 seemed to have better survival compared to the other mutants, at various propolis concentrations. FD7, FD8, FD10, FD11 and FD12 were thus chosen for quantification of their propolis-resistance by MPN method.

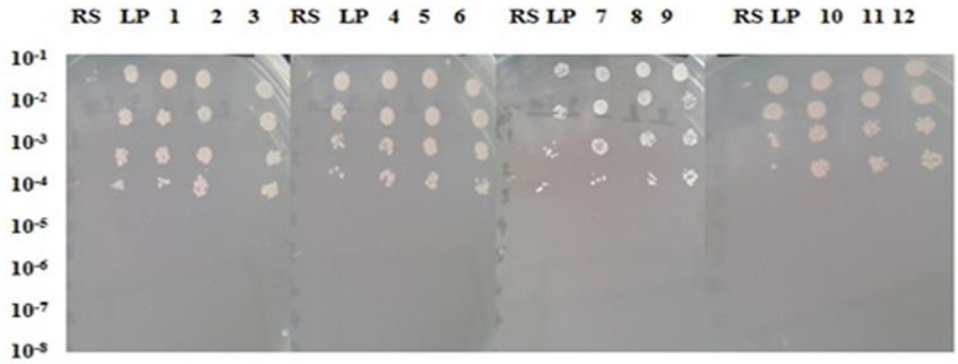

Figure 3.6: Spot assay results of individual mutants (FD1 to FD 12), 57th population and the reference strain (905) after 72 h incubation on solid YMM medium including 300 µg/mL propolis.

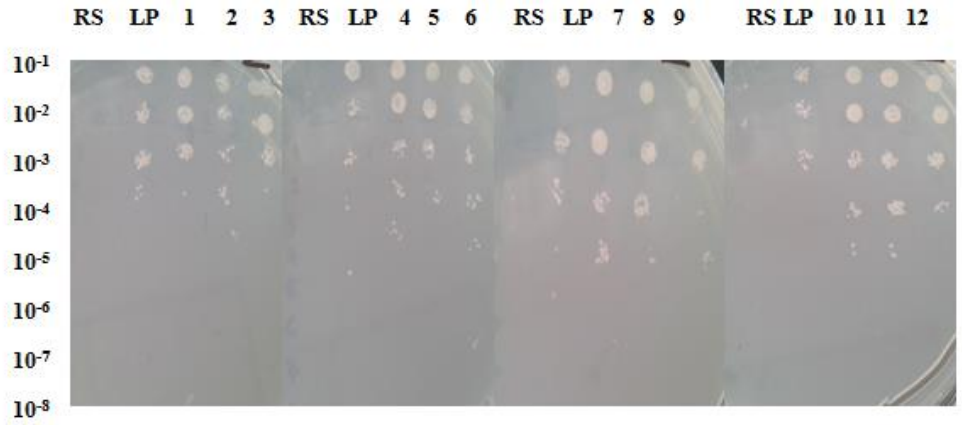

Figure 3.7: Spot assay results of individual mutants (FD1 to FD 12), 57th population and the reference strain (905) after 72 h incubation on solid YMM medium including 500 µg/mL propolis.

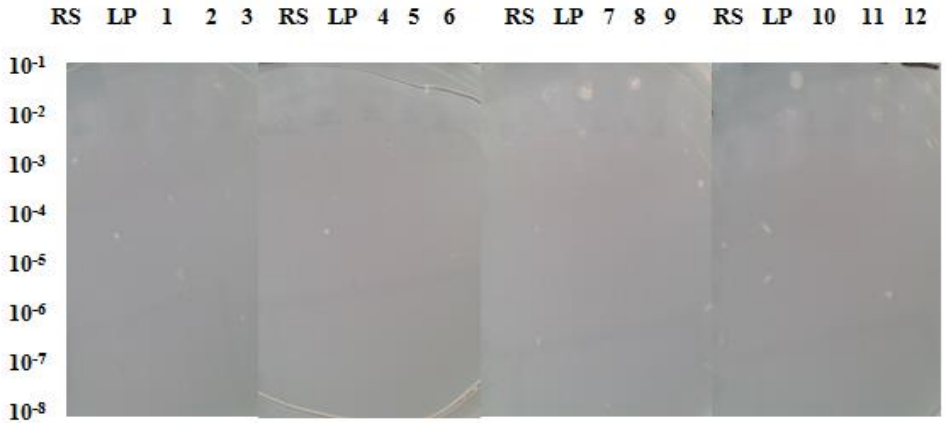

Figure 3.8: Spot assay results of individual mutants (FD1 to FD 12), 57th population and the reference strain (905) after 72 h incubation on solid YMM medium including 710 µg/mL propolis.

3.3.2 Determination of propolis resistance by MPN method

Individual mutants (FD7, FD8, FD10, FD11, FD12) were chosen according to spot assay results and they were compared according to their survival rates at various propolis concentrations, using MPN method.

MPN method was applied for quantitative estimation of propolis resistance. Thus, 200, 500, 710 µg/ml propolis concentrations were used for stress resistance estimation of individual mutants, the reference strain (905) and last population (Table 3.6). Survival rates of mutant individuals, reference strain and the last population are shown in Figure 3.9, and Table 3.7.

Table 3.6: Number of viable cells estimated by MPN Assay at 96 h of incubation, with and without propolis stress.

Number of cells/mL	Control	200µg/ml (Propolis)	500µg/ml (Propolis)	710µg/ml (Propolis)
FD7	16000000	3500000	920000	92000
FD8	16000000	2800000	1600000	24000
FD10	7000000	11000000	3500000	11000
FD11	5400000	22000000	2400000	540000
FD12	9200000	5400000	170000	350000
RS	16000000	1700	23	0
LP	16000000	5400000	1100000	240000

Table 3.7: Survival rates of mutant individuals, reference strain and the last population by MPN Assay at 96 h of incubation.

Survival Rate	200µg/ml	500µg/ml	710µg/ml
FD7	0.2188	0.0575	0.0058
FD8	0.1750	0.1000	0.0015
FD10	1.5714	0.5000	0.0016
FD11	4.0740	0.4444	0.1000
FD12	0.5870	0.0185	0.0380
RS	0.0001	0	0
LP	0.3375	0.0688	0.0150

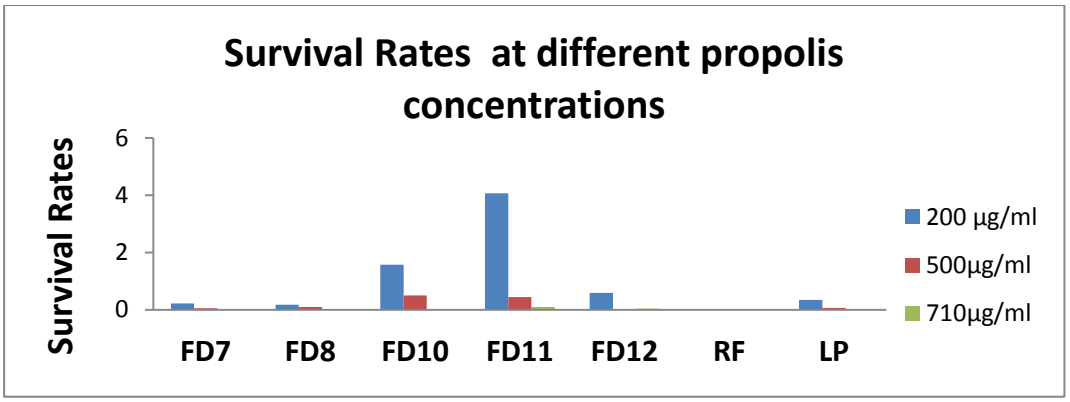

Figure 3.9: Survival rates of mutant individuals, reference strain and the last population at 96h of incubation.

According to MPN assay results, all mutants and the last population exhibited higher survival rates compared to the reference strain. Among all individual mutants tested, FD11 showed the highest survival rate at 200µg/ml propolis concentration.

3.4. Cross Resistance Tests With Spot Assay

Five individual *S.cerevisiae* mutants were grown in the presence of a wide range of stress conditions to determine their potential cross-resistance against other stress factors. Therefore, five individual *S.cerevisiae* mutants were grown on solid YMM

containing ; 0.1-0.3-0.5-0.8 mM $NiCl_2$, 1-2-2.2 mM $CoCl_2$, 0.1-0.3-0.4-0.5-0.8 mM $CuSO_4$, 0.5-1-1.5 mM H_2O_2 , 2-2.5-3 mM $CrCl_3$, 10 mM $ZnCl_2$, 0.5-1-1.5 M $MgCl_2$, 15-25-30-35-40 mM NH_4FeSO_4, 15-20 mM $MnCl_2$, 8-12 % (v/v) ethanol, 12 mM $AlCl_3$, 0.5-1 M NaCl, 150 µg/ml geneticin, and 10 mM caffeine. After 72 h of incubation, images of colonies were taken

3.4.1 Control plate

The images of control plates for cross-resistance assays are shown in Figure 3.10.

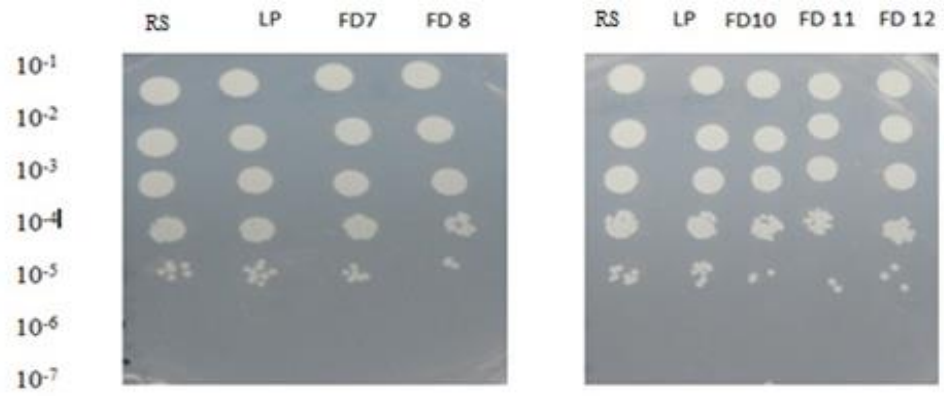

Figure 3.10 : Mutant colonies, the last population (LP) and the reference strain (RS) grown on control YMM plates, after 72 h of incubation.

3.4.2 0.8 mM $NiCl_2$ stress

Figure 3.11 showed that mutant individuals and the last population were cross-resistant against nickel stress.

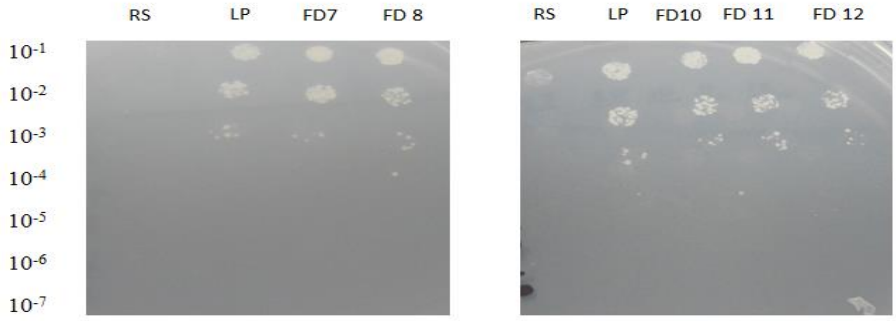

Figure 3.11: Mutant colonies, the last population (LP) and the reference strain (RS) on YMM plates including 0.8 mM $NiCl_2$, after 72 h of incubation.

3.4.3 2.2 mM $CoCl_2$ stress

Images of spot assay plates with 2.2 mM $CoCl_2$ are shown in Figure 3.12.

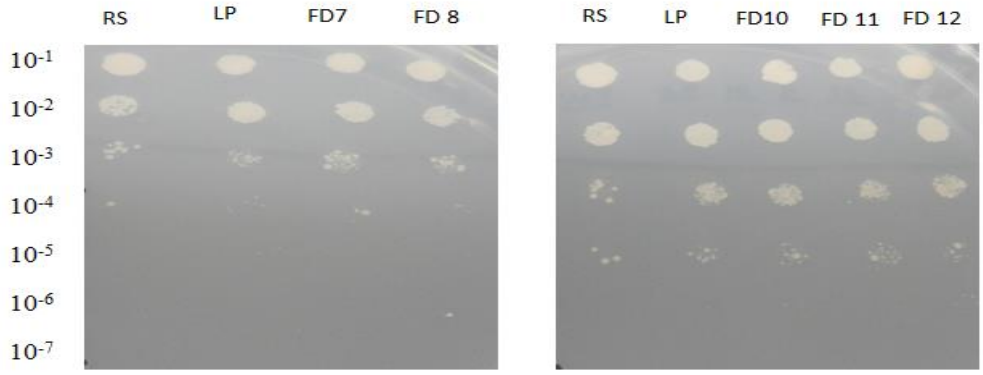

Figure 3.12: Mutant colonies, the last population (LP) and the reference strain (RS) grown on YMM plates including 2.2 mM $CoCl_2$, after 72 h of incubation.

According to spot assay results (1-2-2.2 mM $CoCl_2$), mutant individuals were not cross-resistant against cobalt stress.

3.4.4 0.4 mM $CuSO_4$ stress

Figure 3.13 shows that some of the mutant individuals seemed to be slightly cross-resistant against $CuSO_4$ stress.

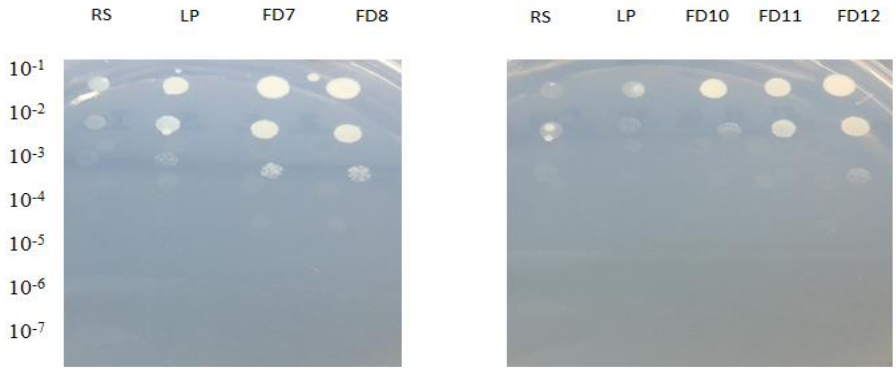

Figure 3.13: Mutant colonies, the last population (LP) and the reference strain (RS) grown on YMM plates including 0.4 mM $CuSO_4$, after 72 h of incubation.

According to cross-resistance tests with $CuSO_4$, some of the mutant individuals seemed to be slightly cross-resistant against $CuSO_4$ stress.

3.4.5 3 mM $CrCl_3$ stress

Figure 3.14 indicates that the mutant individuals were not cross-resistant against $CrCl_3$ stress.

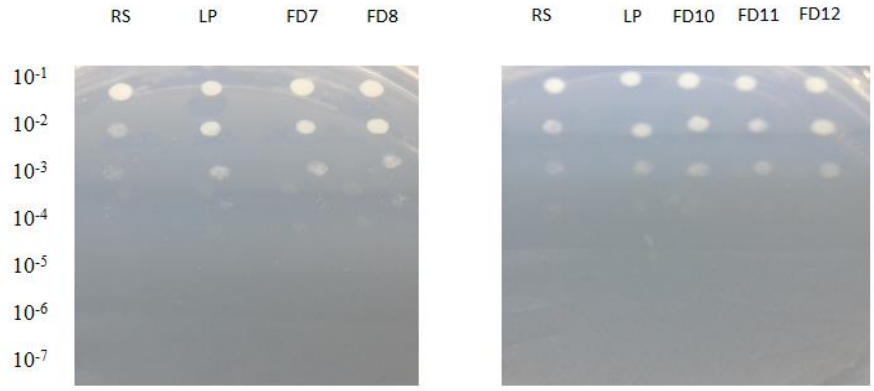

Figure 3.14: Mutant colonies, the last population (LP) and the reference strain (RS), grown on YMM plates including 3 mM $CrCl_3$, after 72 h of incubation.

3.4.6 10 mM $ZnCl_2$ stress

Mutant individuals and the reference strain showed almost the same growth in the presence of zinc stress, as it shown in Figure 3.15.

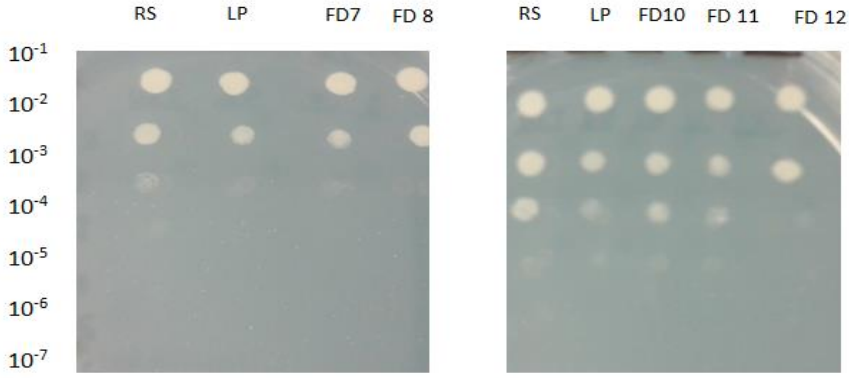

Figure 3.15: Mutant colonies, the last population (LP) and the reference strain (RS), grown on YMM plates containing 10 mM $ZnCl_2$, after 72 h of incubation.

3.4.7 1 M $MgCl_2$ stress

Images of spot assay plates with 1 M $MgCl_2$ are shown in Figure 3.16.

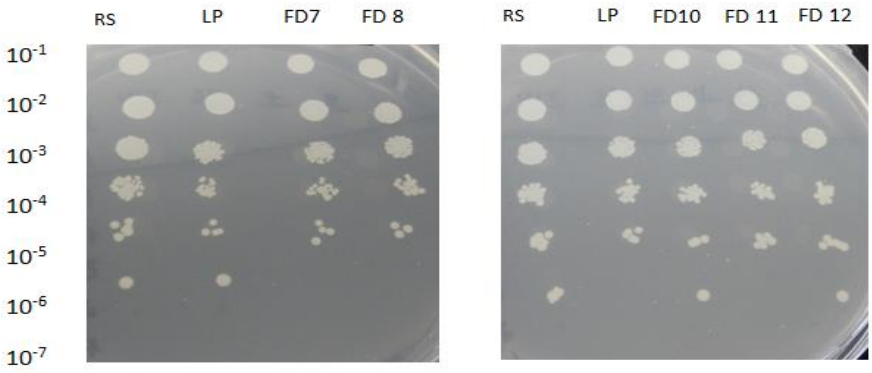

Figure 3.16: Mutant colonies, the last population (LP) and the reference strain (RS), grown on YMM plates containing 1M $MgCl_2$, after 72 h of incubation.

0.5 M $MgCl_2$, 1 M $MgCl_2$, and 1.5 $MgCl_2$ were applied to mutant colonies, the last population and the reference strain, but no cross-resistance aganist magnesium was observed.

3.4.8 15 mM $MnCl_2$ stress

0.5 mM, 15 mM and 20 mM $MnCl_2$ stress were applied mutant to colonies, the last population and the reference strain, but no cross-resistance against manganese stress was observed, as shown in Figure 3.17.

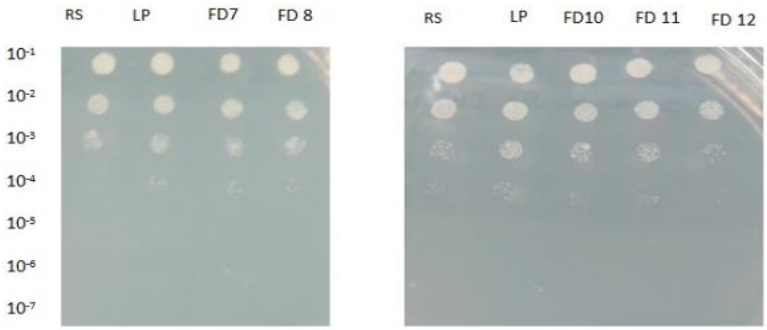

Figure 3.17: Mutant colonies, the last population (LP) and the reference strain (RS), grown on YMM plates containing 15 mM $MnCl_2$, after 72 h of incubation.

3.4.9 12 mM $AlCl_3$ stress

Figure 3.18 shows that propolis-resistant colonies did not exhibit cross-resistance to aluminium stress applied in YMM medium as12 mM $AlCl_3$.

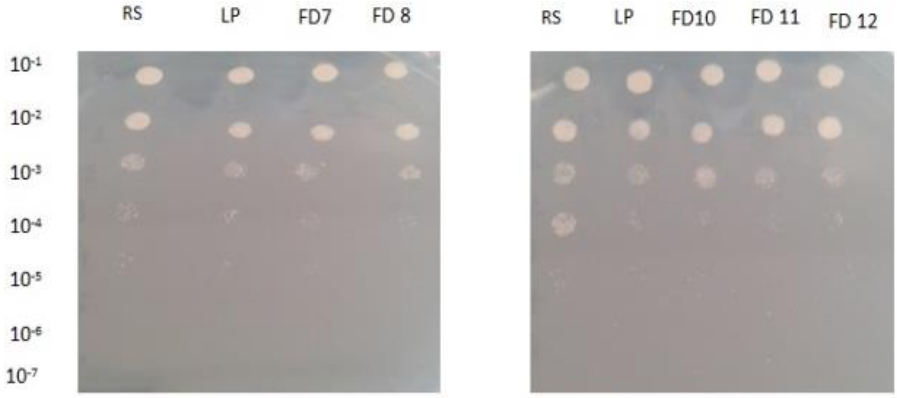

Figure 3.18: Mutant colonies, the last population (LP) and the reference strain (RS), grown on YMM plates containing 12 mM $AlCl_3$, after 72 h of incubation.

3.4.10 1 M NaCl stress

Figure 3.19 shows that mutant colonies did not gain cross-resistance against NaCl.

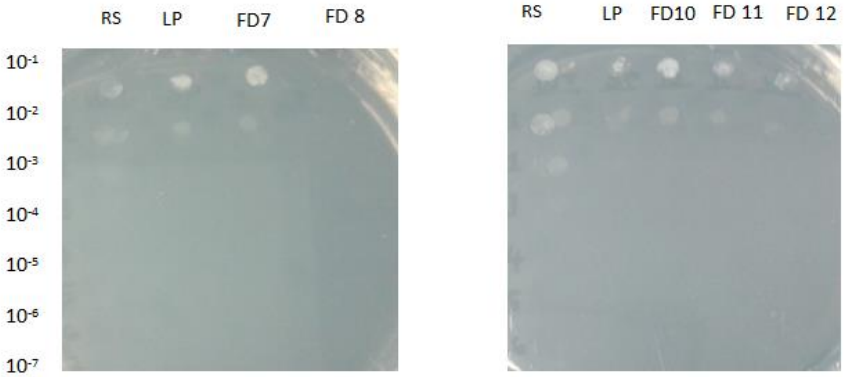

Figure 3.19: Mutant colonies, the last population (LP) and the reference strain (RS), grown on YMM plates containing 1 M NaCl, after 72 h of incubation.

0.5 M NaCl and 1 M NaCl stress were applied to mutant colonies, the last population and the reference strain.

3.4.11 40mM NH_4FeSO_4 stress

Figure 3.20 shows that mutant colonies and the last population were cross-resistant against iron stress.

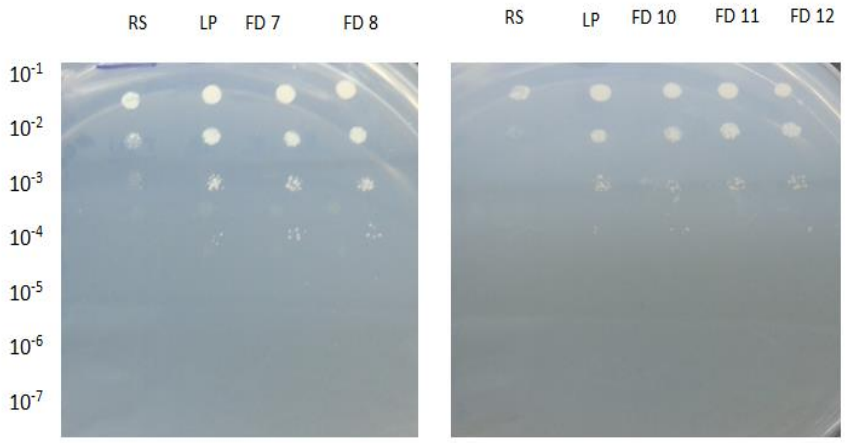

Figure 3.20 : Mutant colonies, the last population (LP) and the reference strain (RS), grown on YMM plates containing 40mM NH_4FeSO_4, after 72 h of incubation.

3.4.12 10mM Caffeine stress

Figure 3.21 shows that all mutant individuals tested were cross-resistant against caffeine stress.

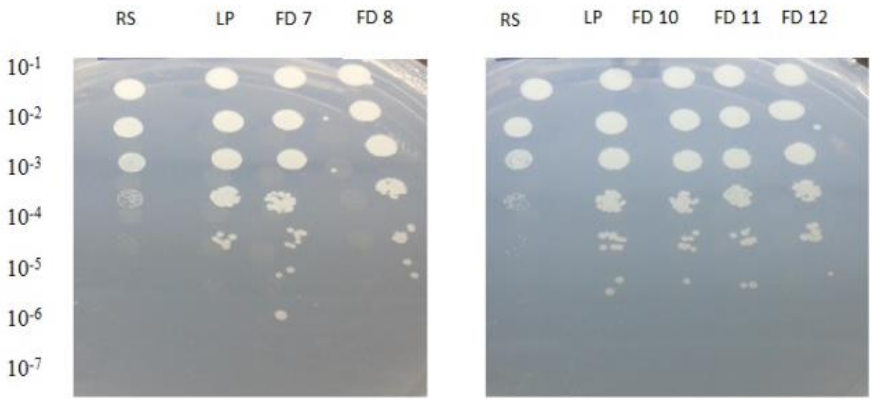

Figure 3.21: Mutant colonies, the last population (LP) and the reference strain (RS), grown on YMM plates containing 10 mM caffeine, after 72 h of incubation.

3.4.13 150 µg/ml Geneticin stress

Figure 3.22 shows that FD10, FD11 and FD7 seem to be slightly more resistant to geneticin, compared to the reference strain and other mutant individuals.

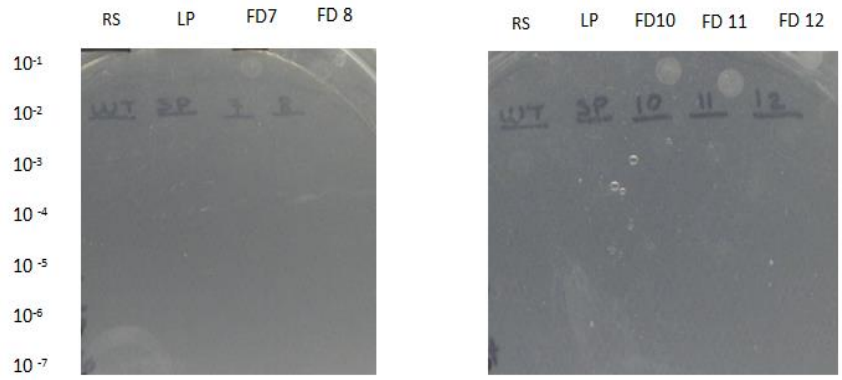

Figure 3.22: Mutant colonies, the last population (LP) and the reference strain (RS), grown on YMM plates containing 150 µg/ml Geneticin, after 72 h of incubation.

3.4.14 12 % (v/v) ethanol stress

8 % (v/v) and 12 % (v/v) ethanol stresses were applied and according to the results, the propolis-resistant mutant individuals became sensitive to ethanol stress (Figure 3.23).

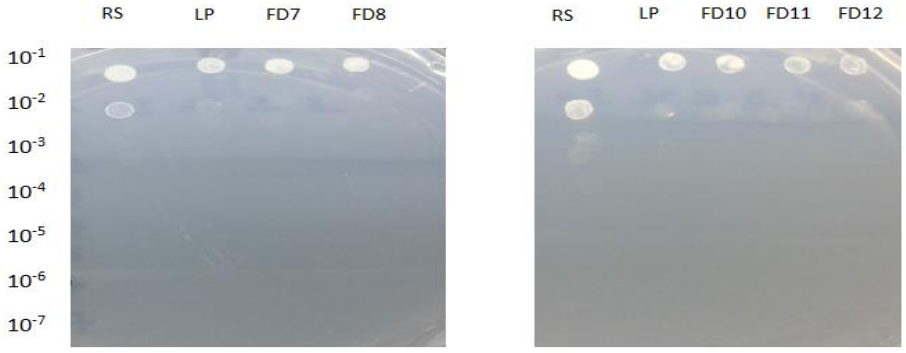

Figure 3.23: Mutant colonies, the last population (LP) and the reference strain (RS), grown on YMM plates containing 12% (v/v) ethanol, after 72 h of incubation.

3.4.15 0.5 mM H_2O_2 stress

0.5 mM H_2O_2 and 1mM H_2O_2 were applied to mutant colonies and the reference strain, as oxidative stress agents. Figure 3.24 showed that propolis-resistant mutant individuals were sensitive to H_2O_2 stress.

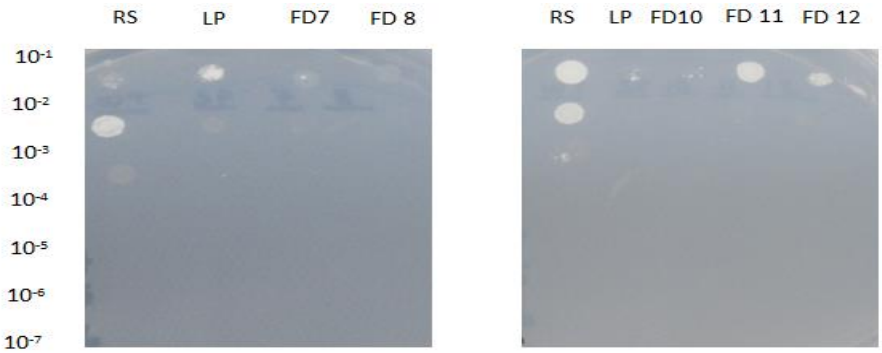

Figure 3.24 : Mutant colonies, the last population (LP) and the reference strain (RS), grown on YMM plates containing 0.5 mM H_2O_2 after 72 h of incubation.

3.5 Genetic Stability Test

Genetic stability test was performed using MPN method, after cultivating propolis-resistant mutants in propolis-free medium for five passages. The results of propolis mutants FD10 and FD11 after 72 h of incubation are shown in Tables 3.8 and 3.9, respectively. The results showed that both mutants were genetically stable. Genetic stability results are shown in Figure 3.25.

Table 3.8: Survival rates and percent survival rates of FD10 after 72 h of incubation in 250 µg/ml propolis-YMM.

Passage Number(72h)	**Number of cells/ml**		**Survival Rates**	**Percent Survival Rates**
FD10	**Control**	**250 µg/ml propolis**	**250 µg/ml propolis**	**250 µg/ml propolis**
1st passage	2200	2200000	1000	100000
2nd passage	4900	920000	187.8	18780
3rd passage	2400	1300000	541.7	54170
4th passage	4900	1300000	265.3	26530
5th passage	1700	1700000	1000	100000

Table 3.9: Survival rates and percent survival rates of FD11 after 72 h of incubation in 250 µg/ml propolis-YMM.

Passage Number (72h)	Number of cells/ml		Survival Rates	Percent Survival Rates
FD11	**Control**	**250 µg/ml propolis**	**250 µg/ml propolis**	**250 µg/ml propolis**
1st passage	1600	1700000	1062.5	106250
2nd passage	5400	2400000	444.4	44440
3rd passage	3500	2400000	685.7	68570
4th passage	2400	1100000	458.3	45830
5th passage	920	2400000	2608.7	260870

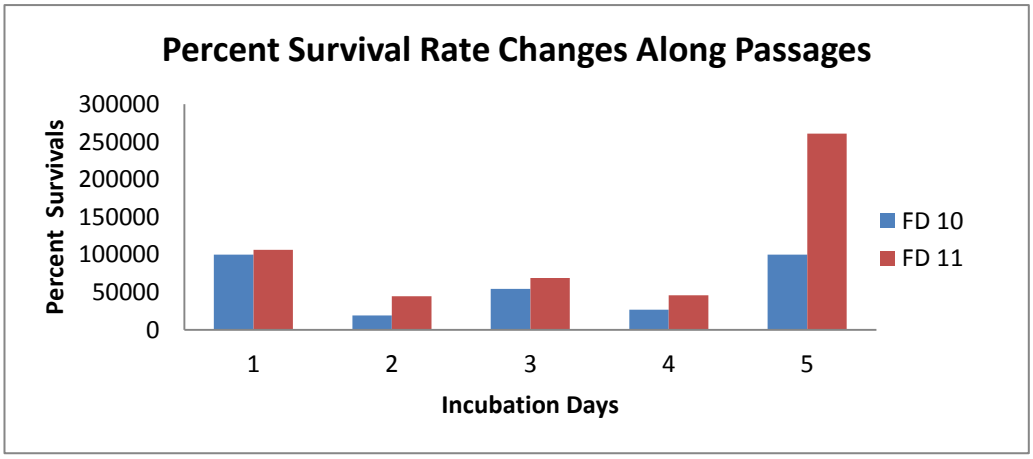

Figure 3.25 : The percent survival rate changes of FD10 and FD11 mutants along their five passages during genetic stability tests.

3.6 Quantitative Estimation of Cross Resistance Levels By MPN Method

According to spot assay, MPN and genetic stability test results, FD 11 was selected as the most resistant and genetically stable mutant against propolis stress. Thus, MPN assay was applied to FD 11 and the reference strain (RS), using 10 mM caffeine, 0.6 mM $NiCl_2$, 35mM NH_4FeSO_4, and 10 % (v/v) ethanol stress for determination of its cross-resistance levels in a quantitative way (Table 3.10).

Figure 3.26 shows that FD11 mutant individual was cross-resistant against caffeine and $NiCl_2$ stresses, but sensitive to ethanol and NH_4FeSO_4 stresses, although the cross-resistance spot test result for NH_4FeSO_4 stress indicated cross-resistance of FD11.

Table 3.10: Percent survival rates of FD11 as fold of the reference strain at various stress conditions, at 72 h of incubation.

Percent Survival Rates (Normalized to reference strain)	10mM Caffeine	0.6 mM $NiCl_2$	35 mM NH_4FeSO_4	10% (v/v) Ethanol
FD 11	50.165	7.327	0.015	0.007

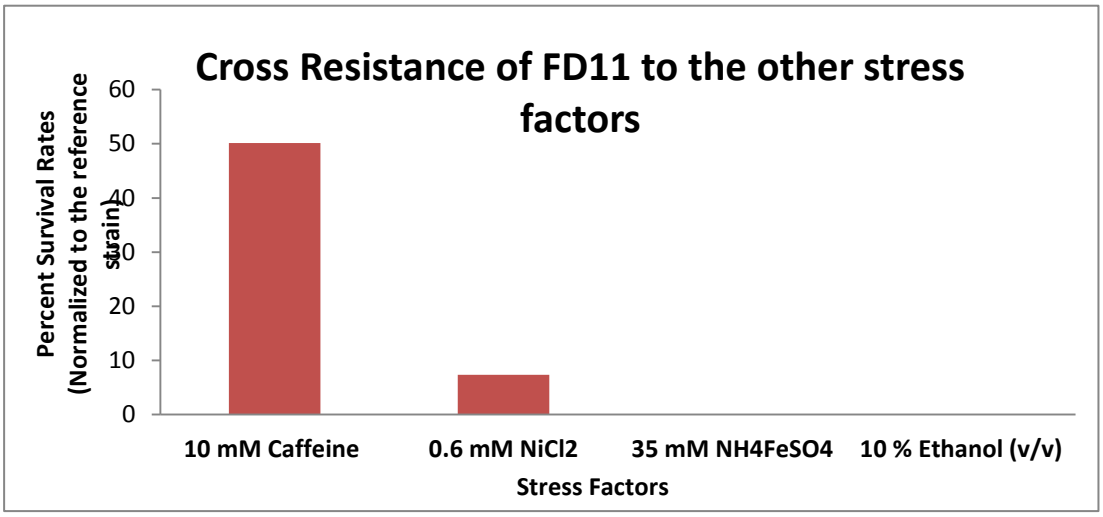

Figure 3.26 : Cross resistance of the individual mutant (FD11) to 10 mM caffeine, 0.6 mM $NiCl_2$, 35mM NH_4FeSO_4, and 10 % (v/v) ethanol stress, as determined by MPN method, upon incubation at 30^{o}C for 72 h**.**

3.7 Growth Behavior of FD11 and the Reference Strain

The reference strain (RS) and FD11 were grown in YMM (control) and YMM containing 50 µg/ml, 100 µg/ml, 150 µg/ml and 200 µg/ml propolis and their growth curves were obtained. Figure 3.27, 3.28, 3.29, 3.30, and 3.31 show these growth curves. According to these preliminary growth curves, 200 µg/ml propolis concentration was chosen for the major growth experiment, as it is a stress level that makes a significant difference between the reference strain's and the mutant strain's growth behavior (Figure 3.31).

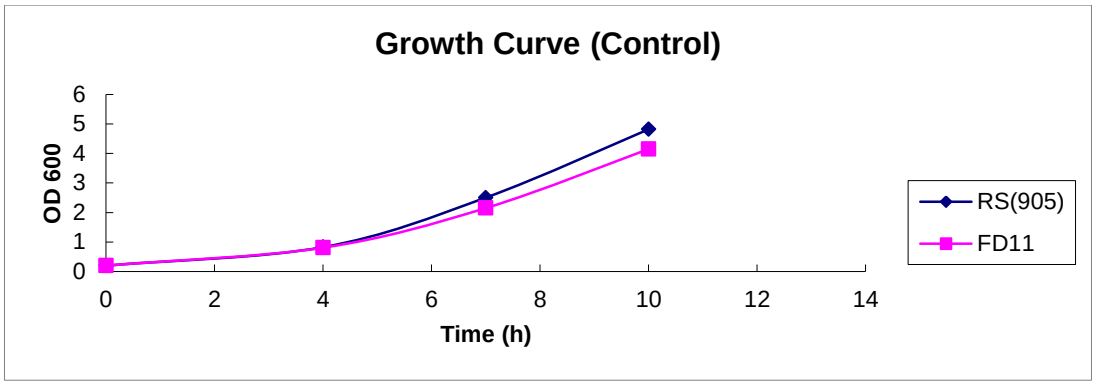

Figure 3.27: The growth curves of FD11 and the reference strain grown in YMM.

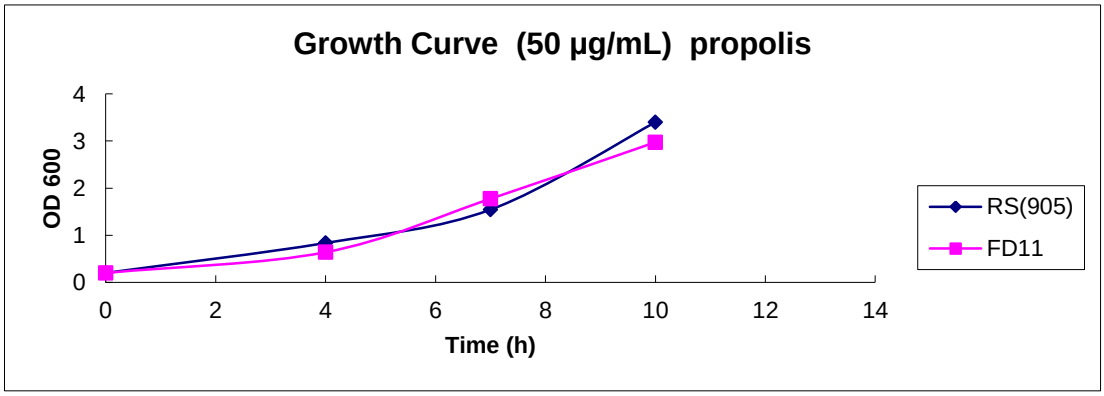

Figure 3.28: The growth curves of FD11 and the reference strain grown in YMM containing 50 µg/mL propolis.

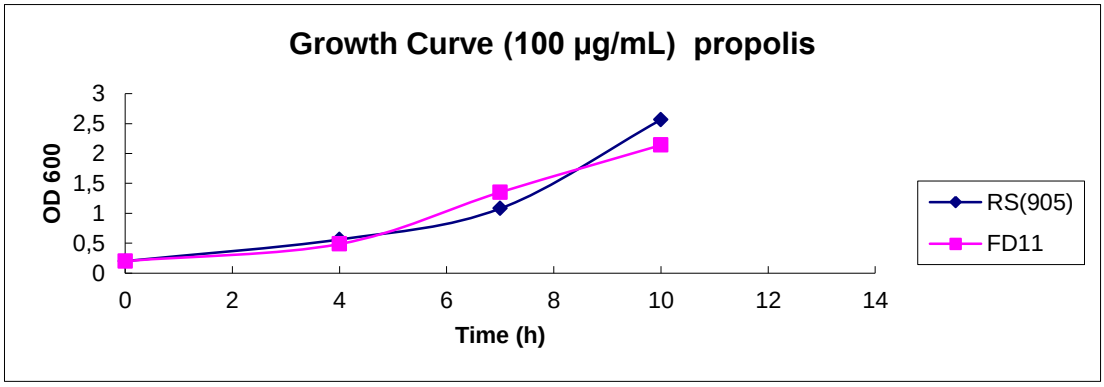

Figure 3.29 : The growth curves of FD11 and the reference strain grown in YMM containing 100 µg/mL propolis.

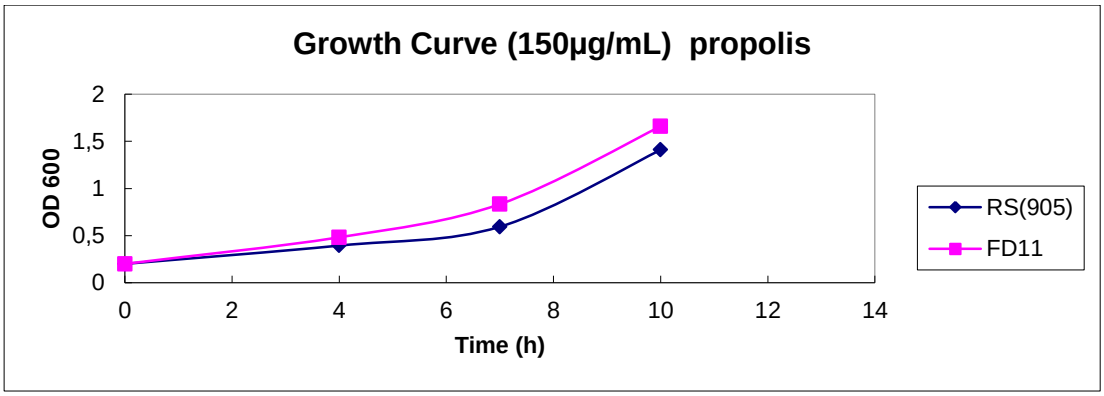

Figure 3.30: The growth curves of FD11 and the reference strain grown in YMM containing 150 µg/mL propolis.

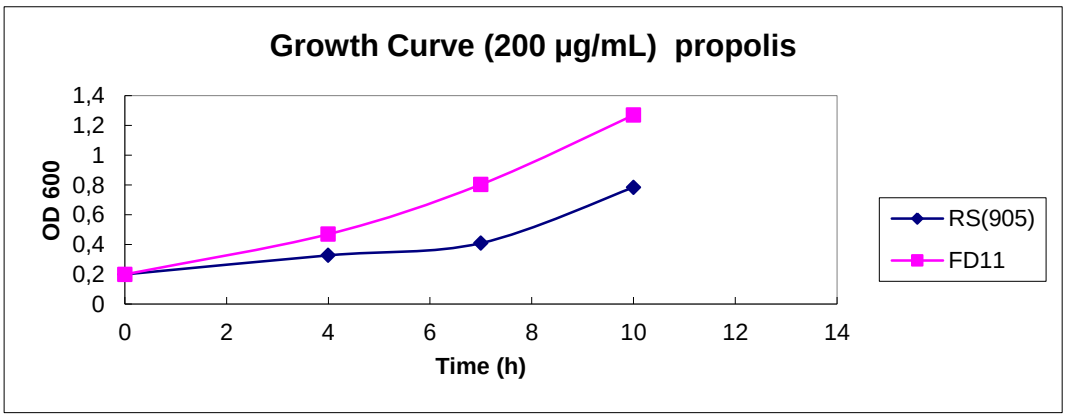

Figure 3.31: The growth curves of FD11 and the reference strain grown in YMM medium containing 200 µg/mL propolis.

Growth curves of FD11 and the reference strain (RS) in YMM and YMM including 200µg/mL propolis were obtained by measuring OD_{600} values regularly (Table 3.11). Cultures were incubated at 30°C for 30h. Figure 3.32 indicates growth curves of the reference strain and FD11.

FD 11 and the reference strain that was incubated in YMM medium, as well as FD11 that was incubated in YMM medium containing 200 µg/mL propolis have short lag phase, the cells entered exponential phase rapidly at about 4.5th h of cultivation. The reference strain that was exposed to 200 µg/mL propolis stress entered both exponential and stationary phases of growth later than FD 11 exposed to propolis stress, FD11 and the reference strain without propolis stress, indicating a strong growth inhibition of the reference strain by 200 µg/mL propolis levels.

Table 3.11: OD_{600} values of FD11 and the reference strain measured during growth experiments.

Time (hour)	FD11 OD_{600} AVG	RS OD_{600} AVG	200 µg/mL Propolis, FD11 OD_{600} AVG	200 µg/mL Propolis, RS OD_{600} AVG
0	0.23	0.22	0.31	0.32
1.5	0.34	0.32	0.26	0.30
3	0.58	0.64	0.36	0.29
4.5	1.05	1.02	0.53	0.30
7.5	2.98	3.06	0.90	0.33
9	4.09	4.07	1.62	0.40
11	4.93	4.81	2.54	0.65
16	5.86	5.86	4.72	1.16
24	5.82	6.06	4.70	4.06
30	6.45	6.22	4.89	4.37

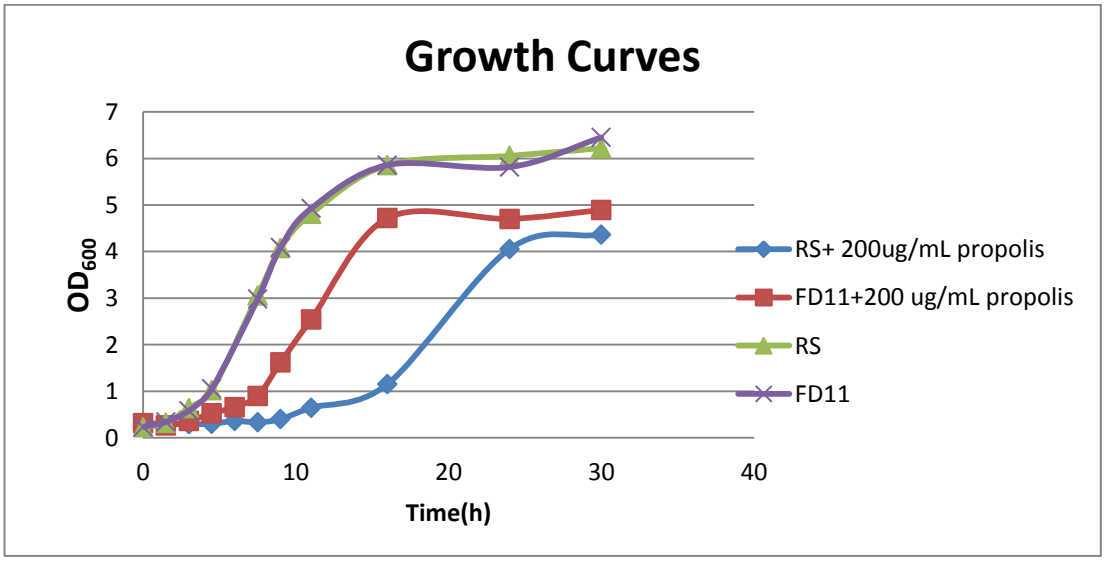

Figure 3.32: Growth curves of FD11 and the reference strain (RS) grown in the absence and presence of 200 µg/mL propolis stress.

3.8 Cell Dry Weight (CDW)

Cell dry weights (CDW) of reference strain and FD11 were determined in triplicate, both in the presence and absence of propolis. Cell dry weight values of the reference strain and FD11 are shown in Figure 3.33.

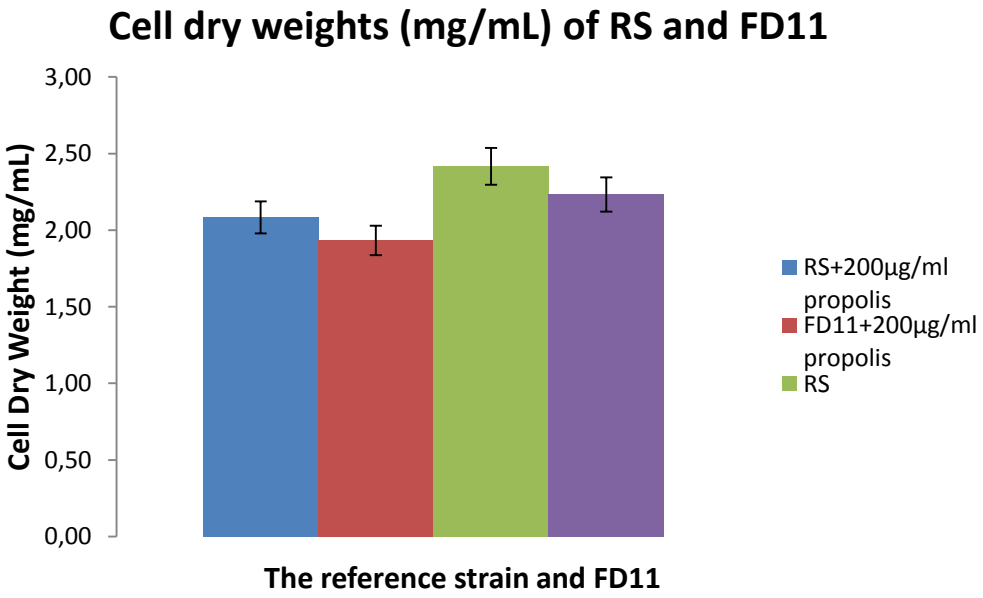

Figure 3.33: CDW values of the reference strain and FD11 with propolis stress and without propolis stress, at 30 h of cultivation.

As shown in Figure 3.33, RS and FD 11 had higher CDW values, compared to FD11 and RS exposed to propolis. Reference strain had slightly higher CDW values than FD11, both in the absence and presence of propolis stress.

3.9 Metabolite Production by FD11 and RS

Metabolite concentrations (residual glucose, glycerol, ethanol, and acetate) were measured using HPLC analysis. During 30 h of cultivation, samples were collected for metabolite analysis by HPLC. Standard curves for HPLC measurements of metabolites are shown in Figure 3.34.

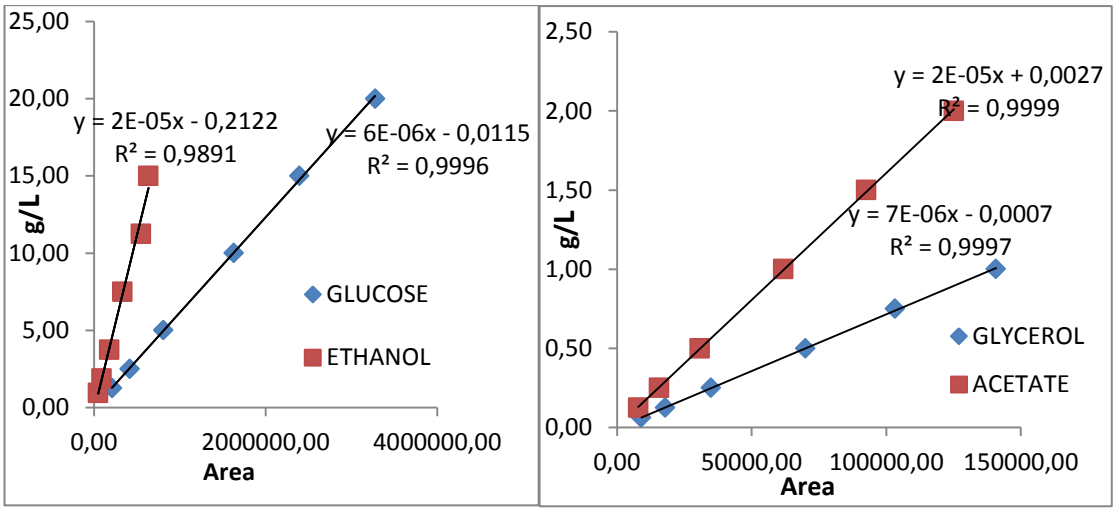

Figure 3.34: HPLC standard curves for glucose, glycerol, ethanol and acetate. Equations and R^2 values are shown.

Glucose consumption profiles of FD11 and the reference strain (RS) in YMM medium with and without are shown in Figure 3.35.

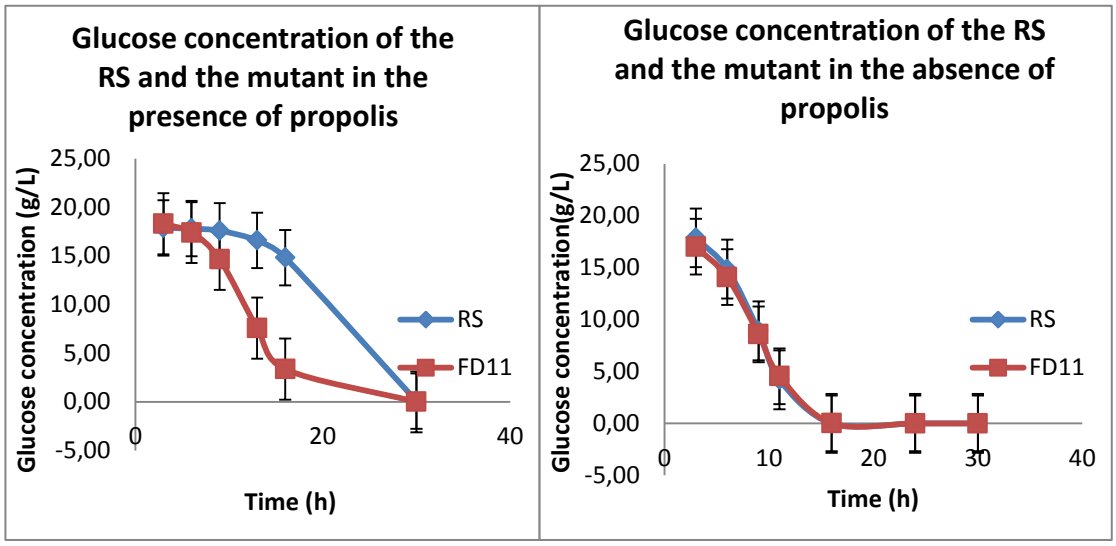

Figure 3.35: Change of glucose concentration (g/L) versus time (h) during cultivation of RS and FD11 with and without propolis.

RS and FD11 consumed glucose similarly when there was no propolis stress, but FD11 used glucose faster than RS in the presence of 200 µg/ml propolis stress.

Glycerol production of FD11 and the reference strain (RS) in YMM medium with and without propolis during the growth experiment is shown in Figure 3.36.

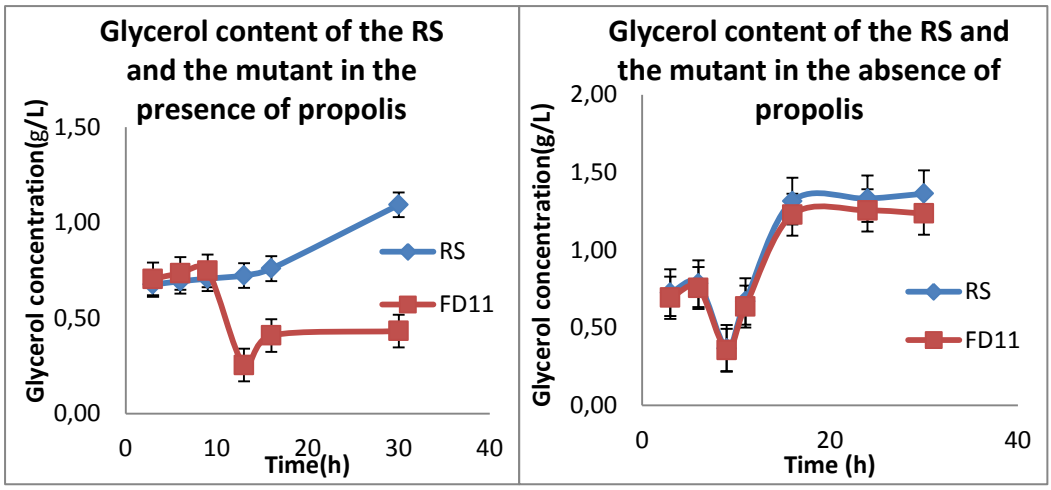

Figure 3.36: Glycerol production (g/L) versus time (h) during cultivation of RS and FD11 with and without propolis.

While FD11 and RS produced glycerol similarly in the absence of propolis, RS produced more glycerol compared to FD 11 in the presence of propolis stress. FD11 also seems to have consumed glycerol during later phases of growth.

Ethanol production of FD11 and the reference strain (RS) in the absence and presence of propolis during the growth experiment is shown in Figure 3.37.

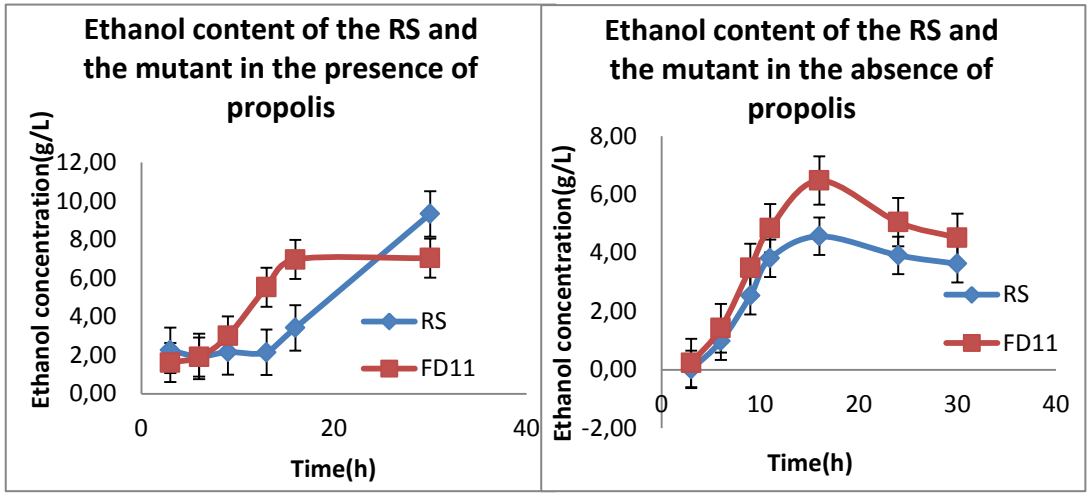

Figure 3.37 : Ethanol production (g/L) versus time (h) during cultivation of RS and FD11 with and without propolis.

Figure 3.37 shows that FD11 produced more ethanol than RS in the absence of propolis. RS and FD11 produced more ethanol in the presence of propolis, compared to control conditions. Acetate production of FD11 and the reference strain (RS) in YMM medium with and without propolis during the growth experiment is shown in Figure 3.38

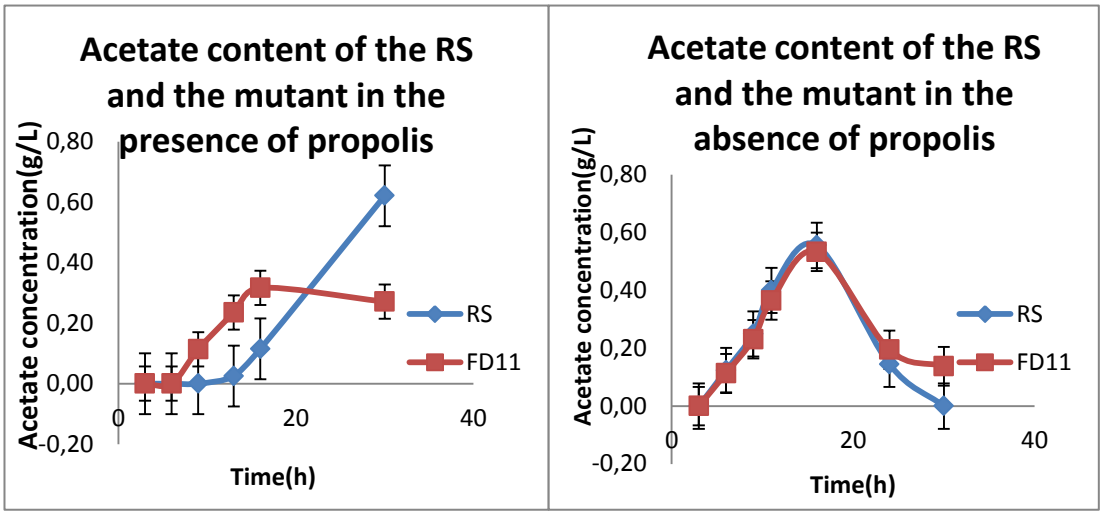

Figure 3.38: Acetate production (g/L) versus time (h) during cultivation of RS and FD11 with and without propolis.

According to HPLC results, RS and FD11 produced acetate similarly, in the absence of propolis. In the presence of propolis, FD11 produced more acetate compared to RS in exponential phase, but according to the 30th hour measurement, RS had higher acetate levels than FD11. FD11 seems to have partially consumed acetate during later hours of the cultivation (Figure 3.38).

3.10 Determination of Trehalose and Glycogen Content by Enzymatic Reaction

Reference strain and FD11 culture samples were collected at the 30th hour of cultivation. OD_{600} measurements and enzymatic assay were used to determine trehalose and glycogen content of the cultures. Also, cell dry weight measurements, were performed for calculating glycogen and trehalose concentrations.

Table 3.12: Intracellular trehalose and glycogen contents (mg glucose equivalents mg^{-1} CDW) of RS and FD11 cultures.

Storage carbohydrate	RS	RS+200µg/mL Propolis Stress	FD11	FD11+200µg/mL Propolis Stress
Trehalose content	0.016±0	0.022±0	0.019±0.002	0.030±0.05
Glycogen content	0.017±0.006	0.027±0.004	0.021±0.007	0.037±0.017

FD11 that was exposed to propolis stress had the highest amounts of trehalose and glycogen, and FD11 strain produced more glycogen and trehalose compared to the reference strain, both in the presence and absence of propolis. Also, reference strain

had higher trehalose and glycogen production, when propolis stress was applied (Figures 3.39 and 3.40).

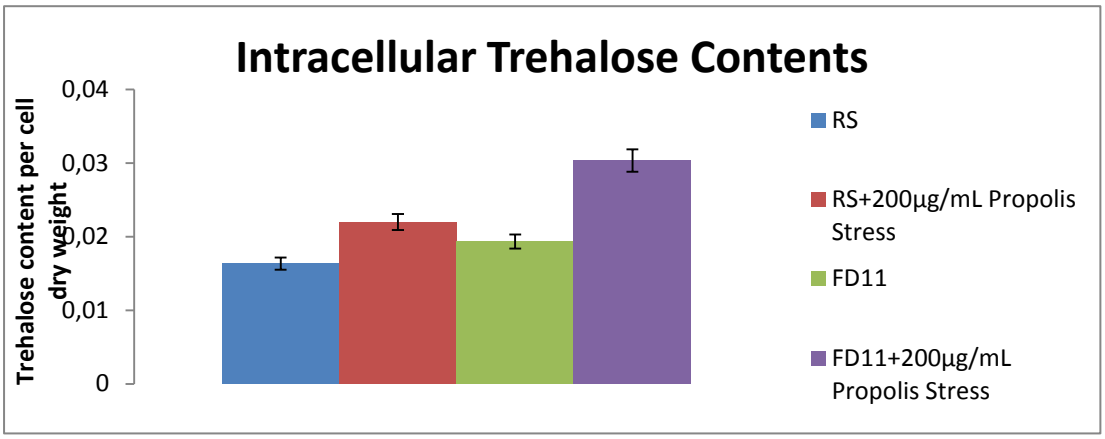

Figure 3.39: Trehalose contents (per cell dry weight) of RS and FD11 in the presence and absence of propolis.

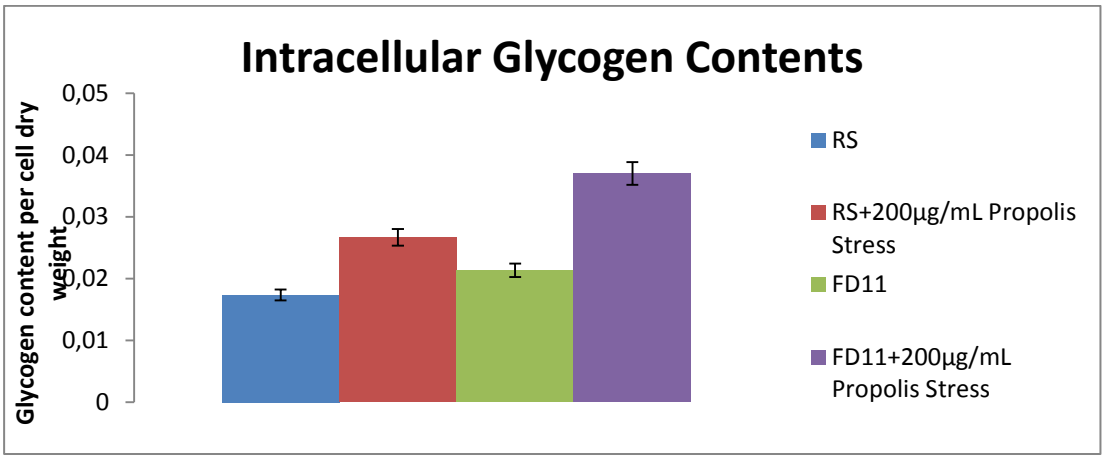

Figure 3.40: Glycogen contents (per cell dry weight) of RS and FD11 in the presence and absence of propolis.

3.11 Estimation of ROS Levels

ROS assay allows determination of the amounts of reactive oxygen species. Intracellular oxidation levels of RS and FD11 were determined in triplicate, with and without 150 µg/ml propolis. Reference strain had higher ROS production levels, both in the presence and absence of propolis. When strains were treated with propolis, ROS production decreased both in RS and FD11. ROS levels of FD11 were generally lower than those of RS, both in the presence and absence of propolis (Figure 3.41).

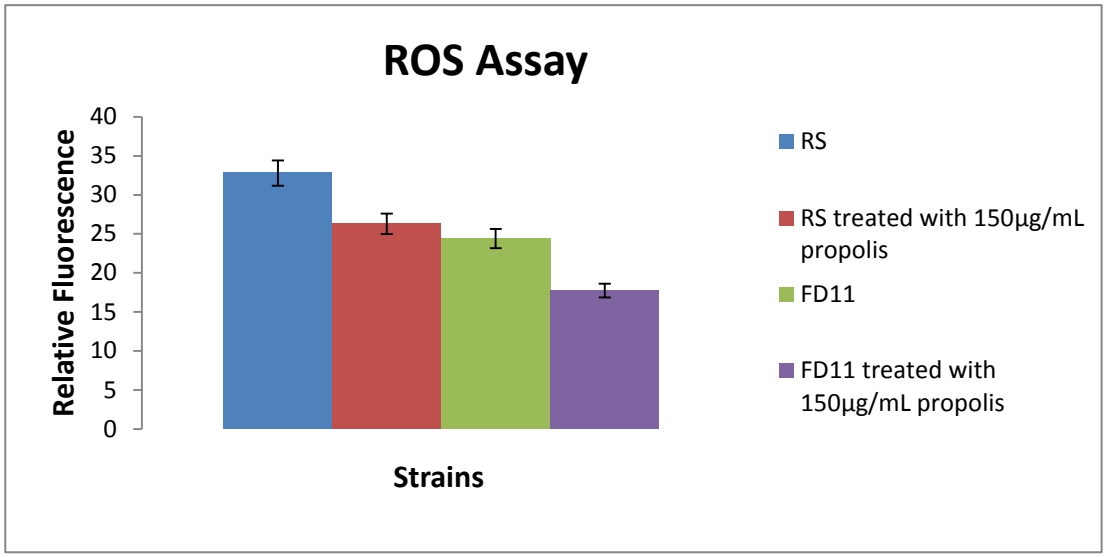

Figure 3.41: ROS production of RS and FD11 in the presence and absence of 150 µg/ml propolis.

4. DISCUSSION AND CONCLUSIONS

Propolis-resistant *S.cerevisiae* mutant populations and individuals were obtained in this study, by using an inverse metabolic engineering approach. After propolis-resistant mutant populations were obtained, physiological analyses were made: Twelve individual mutants were randomly picked from the final mutant population and their resistance levels were investigated firstly by spot assay. MPN method was then applied for quantification of propolis stress resistance levels of mutant individuals, and cross-resistance tests were performed to determine any potential cross-resistance against other stress factors. Also, genetic stability tests were applied to selected mutants with the highest propolis resistance, to verify the persistance of propolis resistance. Finally, growth profiles of the propolis-resistant mutant (FD11) and the reference strain were obtained in YMM with and without propolis.

Reference strain (905) and initial mutant population (906) were screened under varying propolis stress levels to determine the initial stress level for selection experiments. Screening results showed that both 905 and 906 exhibited no growth after 500 µg/ml propolis concentration at 48 h of incubation. At 150 µg/ml propolis concentration, there was a slight difference between the OD_{600} values of 905 and 906. 150 µg/ml propolis level was chosen as the initial propolis level and it was increased by 10 µg/ml gradually while obtaining mutant populations. Totally, 57 mutant populations were obtained and their survival rates decreased when propolis levels were increased. While 650 µg/ml propolis was inhibitory to 905 and 906 at the beginning, the selected final population derived from 906 gained propolis resistance and showed growth even at 710 µg/ml propolis concentration.

Twelve individual mutant colonies were randomly chosen from the final population, upon plating on solid YMM. Colonies were named as FD1 to FD12. Spot assay was applied to all mutants, the reference strain and the last population to determine their propolis resistance levels. According to spot assay results, all mutant colonies had higher resistance against propolis stress, compared to the reference strain. Among them, FD7, FD8, FD10, FD11, and FD12 were determined as the most resistant

mutants. Also, for quantitative estimation of propolis-resistance levels, MPN method was applied to mutants, reference strain and the last population. According to MPN results, the reference strain could not grow at 710 µg/ml propolis concentration while mutant individuals could grow at that concentration. Mutant colonies could grow very well at 200 µg/ml propolis concentration, and FD10 and FD11 had the highest propolis resistance for all concentrations tested.

Cross-resistance analyses were applied to identify possible relationships between propolis stress and other stress types. Cross-resistance results obtained with spot assay showed that mutant colonies were cross-resistant against 0.8 mM $NiCl_2$, 40 mM NH_4FeSO_4, 10 mM caffeine and 150 µg/ml geneticin, but sensitive to 10% (v/v) ethanol and 0.5 mM H_2O_2. Additionaly, colonies were not cross-resistant against $CuSO_4$, $CoCl_2$, $ZnCl_2$, $MgCl_2$, $MnCl_2$, $AlCl_3$, NaCl and acetic acid stress. Cross-resistance or sensitivity levels of FD11 and the reference strain to other stress factors were quantified by MPN assay. According to MPN results, resistance of FD11 to caffeine and $NiCl_2$ was confirmed, but unlike spot assay results, FD11 was not found to be resistant against NH_4FeSO_4 stress. The sensitivity of FD11 to ethanol stress was also confirmed by MPN assay.

Bee products may include very low concentrations of some metals like Cd, Ni, Pb, Fe, Mg and Zn. Honeybees collect samples away from their hives or their hives may be located in regions of high industrial or agricultural activity. Nickel content in propolis was not correlated with any of the other metals tested (Formicki et al., 2006). The cross-resistance of individual mutants against nickel stress may result from low levels of nickel that may be present in propolis samples.

Caffeine is a natural analogue of purine bases that causes pleiotrophic effects inducing cell death (Kuranda et al., 2006). It is also known that cell apoptosis and necrosis are induced by propolis (De Castro et al., 2011). The similar effects of propolis and caffeine and the caffeine cross-resistance of propolis-resistant mutants may help better understand the molecular mechanisms of propolis resistance.

According to cross-resistance results, individual mutants were sensitive to hydrogen peroxide. Propolis shows its effects by three different ways: (1) it promotes protection of membrane lipids from H_2O_2 stress, (2) O_2 stress provides menadione, and propolis resumes redox status by scavenging ROS. (3) it activates Cu/Zn-

superoxide dismutase, one of the most important antioxidant enzymes (De Castro et al., 2011). Propolis may have exhibited its antioxidant property without protecting membrane lipids from H_2O_2 stress. Thus, propolis-resistant mutants became sensitive to H_2O_2 stress.

Propolis may increase ROS generation, and it is more lethal when *S. cerevisiae* grows in the presence of glycerol and ethanol as a carbon source (De Castro et al., 2011). Spot assay and MPN assay results showed that propolis-resistant mutant (FD11) was sensitive to ethanol.

Genetic stability test results showed that the propolis-resistance of FD11 and FD10 did not decrease upon successive cultivation in nonselective media. Thus, FD10 and FD11 are genetically stable. As FD11 is more resistant to propolis than FD10, it was chosen for detailed physiological analysis and growth experiments, as the propolis hyper-resistant mutant individual.

Growth curves were obtained both in the presence and absence of 200 μg/mL propolis, and according to results, FD11 and the reference strain grown in YMM and FD11 grown in YMM containing 200 μg/mL propolis had short lag phases, compared to the reference strain incubated at 200 μg/mL propolis. Additionally, under propolis stress conditions FD11 had a higher growth rate than the reference strain. Also, the reference strain entered the exponential phase and stationary phase later, when grown in the presence of 200 μg/mL propolis, compared to FD11. Cell dry weight measurements revealed that strains grown under control conditions had higher CDW than strains grown under propolis stress conditions.

HPLC analysis was performed to reveal potential metabolic differences between the reference strain and propolis-resistant mutant. In control medium, FD11 and RS consumed glucose at the same level at different hours. However, in medium containing 200 μg/mL propolis, FD11 consumed glucose more rapidly, compared to the reference strain. Under stress conditions, the reference strain grew slowly and also consumed glucose slowly. FD11 and the reference strain produced other metabolites at similar levels in control medium. When propolis stress was present, FD11 produced high levels of acetate, ethanol and glycerol during exponential phase of growth. Also, according to HPLC results, FD11 produced higher concentrations of

ethanol compared to the reference strain under both control and propolis stress conditions.

Trehalose and glycogen are storage carbohydrates and stress protectants. (François & Parrou, 2001). FD11 accumulated higher trehalose and glycogen than the reference strain. This increase may result from propolis stress.

ROS assay results showed that FD11 had lower amounts of reactive oxygen species compared to the reference strain, under both control and propolis stress conditions. When yeast cells are exposed to propolis, intracellular oxygen levels decrease. Changes also occur at mitochondrial proteome level, including antioxidant proteins and propolis resumes redox status by scavenging ROS (De Castro, 2012).

In brief, highly propolis-resistant and genetically stable mutant individuals were successfully obtained by inverse metabolic engineering strategy. Two mutants (FD10 and FD11) were found to be more resistant to propolis than the others tested. Physiological analyses showed that mutant colonies had gained cross-resistance or became sensitive to other stress types. Genomic, transcriptomic and proteomic analyses to be performed could provide significant information to understand the molecular basis of propolis-resistance and response in the model eukaryote *S. cerevisiae* and more complex eukaryotes.

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