ISTANBUL TECHNICAL UNIVERSITY ★ GRADUATE SCHOOL OF SCIENCE ENGINEERING AND TECHNOLOGY

INVERSE METABOLIC ENGINEERING AND MOLECULAR CHARACTERIZATION OF CAFFEINE-RESISTANT *Saccharomyces cerevisiae*

Ph.D. THESIS

Yusuf SÜRMELİ

Department of Molecular Biology – Genetics and Biotechnology

Molecular Biology – Genetics and Biotechnology Programme

MARCH 2020

ISTANBUL TECHNICAL UNIVERSITY ★ GRADUATE SCHOOL OF SCIENCE ENGINEERING AND TECHNOLOGY

INVERSE METABOLIC ENGINEERING AND MOLECULAR CHARACTERIZATION OF CAFFEINE-RESISTANT *Saccharomyces cerevisiae*

Ph.D. THESIS

Yusuf SÜRMELİ
(521132107)

Department of Molecular Biology – Genetics and Biotechnology

Molecular Biology – Genetics and Biotechnology Programme

Thesis Advisor: Prof. Dr. Zeynep Petek ÇAKAR

MARCH 2020

İSTANBUL TEKNİK ÜNİVERSİTESİ ★ FEN BİLİMLERİ ENSTİTÜSÜ

KAFEİNE DİRENÇLİ *Saccharomyces cerevisiae*'nin TERSİNE METABOLİK MÜHENDİSLİK İLE ELDESİ VE MOLEKÜLER KARAKTERİZASYONU

DOKTORA TEZİ

Yusuf SÜRMELİ
(521132107)

Moleküler Biyoloji – Genetik ve Biyoteknoloji Anabilim Dalı

Moleküler Biyoloji – Genetik ve Biyoteknoloji Programı

Tez Danışmanı: Prof. Dr. Zeynep Petek ÇAKAR

MART 2020

Yusuf SÜRMELİ, a Ph.D. student of ITU Graduate School of Science Engineering and Technology student ID 521132107, successfully defended the thesis/dissertation entitled "INVERSE METABOLIC ENGINEERING AND MOLECULAR CHARACTERIZATION OF CAFFEINE-RESISTANT *Saccharomyces cerevisiae*", which he prepared after fulfilling the requirements specified in the associated legislations, before the jury whose signatures are below.

Thesis Advisor :	**Prof. Dr. Zeynep Petek ÇAKAR** Istanbul Technical University	.....
Jury Members :	**Prof. Dr. Nevin Gül KARAGÜLER** Istanbul Technical University	.....
	Assoc. Prof. Dr. Mustafa TÜRKER Pakmaya Co. Inc.	.....
	Prof. Dr. Tuba GÜNEL Istanbul University	.....
	Dr. Öğr. Üyesi Bülent BALTA Istanbul Technical University	.....

Date of Submission : 12 February 2020
Date of Defense : 11 March 2020

To my precious son Mert SÜRMELİ,

FOREWORD

I would like to express my gratitude to my thesis advisor Prof.Dr. Zeynep Petek ÇAKAR for her significant guidance for my intellectual and practical improvement during my Ph.D. study

I would like to thank my steering committee members Prof.Dr. Nevin Gül KARAGÜLER and Assoc.Prof.Dr. Mustafa TÜRKER for their contributions to avoid experimental shortcomings and their valuable comments on the thesis results.

I would also like to thank the jury members Prof.Dr. Nevin Gül KARAGÜLER, Assoc.Prof.Dr. Mustafa TÜRKER, Prof.Dr. Tuba GÜNEL, Dr.Öğr.Üyesi Bülent BALTA, Prof.Dr. Fatma Neşe KÖK and Prof.Dr. Nazlı ARDA for allocating their time to read and comment on my Ph.D. thesis and to participate in my Ph.D. thesis defence.

I would like to thank my lab-mates Alican TOPALOĞLU, Dr.Burcu HACISALİHOĞLU, Can HOLYAVKİN, Dr.Mevlüt ARSLAN, Halil İbrahim KISAKESEN, Nazlı KOCAEFE, Ogün MORKOÇ and Ömer ESEN for their contribution in experimental support and data analysis. Additionally, I appreciate our former undergraduate students, İbrahim OĞUZ, Güney GÜVENÇ, Esra KATKAT, Nergis TABAŞ, Yusuf ŞEFLEKÇİ, Gizem KARABIYIK, Diler Kaan ATMACA, İsmail Can KARAOĞLU, Selvin YILDIZ and Mehmet Emre KENAR for their contribution to the experiments of evolutionary engineering and physiological characterization.

I would like to express my heartfelt thanks to Dr.Yetkin ÖZTÜRK, Dr.Havva Esra TÜTÜNCÜ, Naciye Durmuş İŞLEYEN, Öznur PEHLİVAN, Ceren SAĞDIÇ ÖZTAN, Dicle MALAYMAR PINAR, Doğuş ALTINÖZ, Ayşe Buse ÖZDABAK SERT, Hande MUMCU because we made many beneficial and lively conversions, which helped me survive the challenges in MOBGAM.

I heartily appreciate my wife Zeynep DURSUN SÜRMELİ for her patience throughout my Ph.D. study under challenging circumstances and for her endless contribution to keep me in a good mood.

I am so grateful to the universe since it enabled me to live a second life, along with my wife and son, starting from zero.

February 2020 Yusuf SÜRMELİ

TABLE OF CONTENTS

Page

ABBREVIATIONS

^{o}C	**:** degree Celsius
µg	**:** Microgram
µL	**:** Microliter
µM	**:** Micromolar
ABC	**:** ATP-binding cassette
$AlCl_3$	**:** Aluminium chloride
cDNA	**:** Complementary DNA
CDW	**:** Cell dry weight
CLS	**:** Chronological life span
CNS	**:** Central nervous system
$CoCl_2$	**:** Cobalt(II) chloride
cRNA	**:** Complementary RNA
cy3	**:** Cyanine 3
CYP1	**:** Cytochrome P450
DMSO	**:** Dimethyl sulfoxide
DNA	**:** Deoxyribonucleic acid
EDTA	**:** Ethylenediaminetetraacetic acid
EMS	**:** Ethyl methanesulfonate
EMP	**:** EMS-mutagenized population
GMO	**:** Genetically modified organism
H_3BO_4	**:** Boric acid
HCl	**:** Hydrochloric acid
HPLC	**:** High performance liquid chromatography
mL	**:** Milliliter
mM	**:** Millimolar
M	**:** Molar
$MnCl_2$	**:** Manganese(II) chloride
nM	**:** Nanomolar
NaCl	**:** Sodium chloride
NH_4Cl	**:** Ammonium chloride
$NH_4Fe(SO_4)_2$	**:** Ammonium iron(III) sulfate
$NiCl_2$	**:** Nickel(II) chloride
PDR	**:** Pleiotropic drug resistance
OCT	**:** Over-the-counter
OD_{600}	**:** Optical density at 600 nm
ORF	**:** Open reading frame
REF	**:** Reference strain
ROS	**:** Reactive oxygen species
RNA	**:** Ribonucleic acid
RPM	**:** Revolutions per minute
SAM	**:** S-adenosyl-L-methionine
SGD	**:** The *Saccharomyces* Genome Database

SRA : Sequence Read Archive
SNV : Single nucleotide variation
TE : Tris EDTA
TOR : Target of rapamycin
U : Unit
UV-Vis : Ultraviolet-Visible
YMM : Yeast minimal medium
YPD : Yeast extract-peptone-dextrose

LIST OF TABLES

Page

LIST OF FIGURES

Page

INVERSE METABOLIC ENGINEERING AND MOLECULAR CHARACTERIZATION OF CAFFEINE-RESISTANT *Saccharomyces cerevisiae*

SUMMARY

Caffeine, a natural purine alkaloid, is highly consumed worldwide as an ingredient of some beverages like coffee, tea and soft drinks. Although there are many reports on caffeine effects in many organisms like yeast, pleiotropic effects of caffeine, and molecular mechanisms of caffeine resistance and response are largely unknown.

In this study, highly caffeine-resistant *Saccharomyces cerevisiae* mutants were obtained for the first time by evolutionary engineering, an inverse metabolic engineering strategy, based on batch selection under gradually increasing caffeine stress, with and without applying any mutagen to the initial population before selection. The selection process was initiated at 7.5 mM caffeine concentration, and continued until 50 mM, for 48 populations. Genetically stable, caffeine-resistant mutant strains were isolated from the final populations of the selection, where they resisted as high as 50 mM caffeine, a concentration that has not been documented for *S. cerevisiae* so far. Among those mutants, three strains (Caf905-2, Caf906-11, and Caf906-12) had the highest caffeine resistance, and they were also cross-resistant to a variety of stress conditions like coniferyl aldehyde, antimycin A, and rapamycin. One of the caffeine-hyperresistant strains (Caf905-2) was characterized at physiological, transcriptomic and genomic levels. Caf905-2 did not have a reduction of growth under 10 mM caffeine stress, where its maximum specific growth rate (μ_{max}) was nearly three-fold of that of the reference strain. Expectedly, Caf905-2 entered the stationary phase at 12 h, but the reference strain at 30 h. Biomass levels of the two strains showed an increase under 10 mM caffeine stress, compared to the absence of caffeine. Also, ethanol yield decreased in the reference strain under 10 mM caffeine stress, but not in Caf905-2. Similarly, glycerol yield decreased in Caf905-2 under caffeine stress, but not in the reference strain. The reference strain also accumulated more intracellular trehalose than Caf905-2, particularly under caffeine stress condition. The resistance of Caf905-2 against lyticase, a β-1,3-glucan-degrading enzyme, was higher than that of the reference strain, under both caffeine stress and nonstress conditions.

DNA microarray analysis identified a total of 745 overexpressed and 741 repressed genes with statistically changes of gene expression levels (corrected $p < 0.05$) encompassing at least 2-fold alteration in Caf905-2. Functional categorization of transcriptomic analysis results of Caf905-2 suggested that the genes implicated in energy and protein fate pathways, oxygen and radical detoxification, and drug/toxin transport associated with pleiotropic drug resistance were stimulated, whereas the genes implicated in transcription, protein synthesis, cellular transport pathways, and nucleotide metabolism were repressed in the nonstress condition. Whole genome re-sequencing analysis results unearthed only three single nucleotide variations,

distributed in three different genes; *PDR1*, *PDR5*, and *RIM8* in Caf905-2, relative to the reference strain. The exact roles of these genes in caffeine response and resistance in yeast should be investigated in detail in future studies.

KAFEİNE DİRENÇLİ *Saccharomyces cerevisiae*'NİN TERSİNE METABOLİK MÜHENDİSLİK İLE ELDESİ VE MOLEKÜLER KARAKTERİZASYONU

ÖZET

Kafein, doğal bir pürin alkaloid olup, dünya genelinde çok sayıda insan tarafından çoğunlukla kahve, çay ve enerji içeceklerinin bir bileşeni olarak tüketilmektedir. Kafeinin, mayanın da içinde olduğu birçok organizma üzerindeki etkisi ile ilgili çok sayıda bilimsel veri olmasına karşın, kafein yanıtının ve dirençliliğinin karmaşık moleküler mekanizması ve pleiotropik etkisi hala büyük ölçüde bilinmemektedir.

Bu çalışmada, yüksek düzeyde kafeine dirençli ve genetik olarak kararlı olan üç adet *Saccharomyces cerevisiae* mutantı, ilk kez, kesikli kültürde seleksiyon işlemine dayalı bir strateji olan evrimsel mühendislik yoluyla, seçilim öncesinde bir başlangıç popülasyonu olarak herhangi bir mutajen kullanarak ve kullanmayarak, gittikçe artan kafein stres konsantrasyonu varlığında geliştirildi. Seçilim süreci, 7.5 mM kafein konsantrasyonu ile başlatıldı ve kafein düzeyi 50 mM'a ulaştığında sonlandırıldı. Seçilim süreci sonunda, 48 popülasyon elde edildi. EMS (etilmetan sülfonat) mutajeni ile elde edilen populasyon ve mutajene maruz bırakılmayan referans suş kullanılarak başlatılan seçilim süreci, 50 mM kafeine dirençli iki ayrı popülasyon olarak noktalandırıldı. Bu gelinen son konsantrasyonda (50 mM), başlangıçta seçilim süreci için kullanılan EMS'ye maruz bırakılmış populasyon ile referans suş gelişme göstermezken, bu başlangıç populasyonlarından elde edilen son popülasyonlar ise yaklaşık olarak %60 oranında sağkalım oranına sahip olmuşlardır. Genetik olarak kararlı olan ve kafeine dirençli mutant suşlar, *S. cerevisiae* için literatürde yer almayacak düzeyde yüksek kafein (50 mM) varlığında, seçilim süreci sonunda elde edilen son populasyonlarından izole edilmiştir. Kantitatif bir yöntem olan 5-tüplü En Muhtemel Sayı Yöntemi ile 15 mM kafein varlığında, 24, 48 ve 72 saatler sonunda en iyi gelişebilen kafeine dirençli üç suş (Caf905-2, Caf906-11, and Caf906-12), sonraki deneysel analizler için seçilerek analiz edilmiştir. Bu üç suş, kafeinin yanı sıra, koniferil aldehit, antimisin A, propolis ve rapamisin gibi çeşitli bileşiklere karşı çapraz direnç göstermiştir. Sonrasında, kafeine yüksek direnç gösteren Caf905-2 suşu, daha ileri fizyolojik, genomik ve transkriptomik karakterizasyonlar için seçilerek incelenmiştir. Kafeine yüksek direnç gösteren Caf905-2, konferil aldehit, rapamisin ve antimisin A'nın yanı sıra, dimetilsülfoksite (DMSO) ve özellikle siklohekzimide karşı yüksek düzeyde çapraz direnç sergilemiştir. Kafeine dirençli Caf905-2 ayrıca, hemen hemen test edilen bütün ağır metallerin yanı sıra, etanol, 2-feniletanol, tuz, glifosat, hidrojen peroksit, ısı, ve alkali pH streslerine karşı farklı düzeylerde duyarlılık sergilemiştir. Referans suştan farklı olarak, kafeine yüksek düzeyde direnç gösteren Caf905-2, 10 mM kafein stresi varlığında, gelişme hızında bir düşüş sergilemezken, referans suşun gelişme hızı önemli ölçüde düşmüştür: Caf905-2'nin maksimum spesifik gelişme hızı (μ_{max}), referans suşun maksimum spesifik gelişme hızına kıyasla yaklaşık olarak üç kat fazladır. Kafeine yüksek düzeyde direnç sergileyen Caf905-2, gelişme eğrisinin logaritmik gelişme fazının başlangıcından yaklaşık 12 saat sonra durağan fazına girmişken, referans suş ise

durağan faza 30 saat sonra girebilmiştir. Ayrıca, hem referans suşun hem de kafeine yüksek düzeyde direnç gösteren Caf905-2'nin biyokütle veriminde, 10 mM kafein stresi altında, kafeinin olmadığı ortama nazaran önemli bir artış gözlemlenmiştir. Buna ilaveten, referans suşun üretmiş olduğu etanolün veriminde, 10 mM kafein stresi varlığında, kafeinsiz ortama nazaran, bir düşüş olmasına karşın, kafeine yüksek düzeyde direnç gösteren Caf905-2'nin etanol veriminde bir değişiklik gözlemlenmemiştir. Gliserol veriminde ise, etanol veriminin aksine, kafein stresi altında, kafeine yüksek düzeyde direnç gösteren Caf905-2'de bir düşüş görülürken, referans suşta bir değişiklik gözlemlenmemiştir. Bunların yanı sıra, referans suş, gerek kafein stresinin olmadığı ortamda, gerekse de özellikle 10 mM kafein stresi varlığında, kafeine yüksek düzeyde direnç gösteren Caf905-2 suşuna nazaran, hücre içi trehalozu daha yüksek düzeyde birikmiştir. β-1,3-glucan'ın parçalanmasından sorumlu bir enzim olan litikaza karşı referans suşun ve kafeine yüksek direnç gösteren Caf905-2'nin duyarlılık düzeyi birbirinden farklılık göstermektedir. Buna göre, kafeine dirençli Caf905-2'nin, referans suşa nazaran, kafeinin olmadığı stressiz koşulda litikaza karşı daha dirençli olduğu gözlemlenmiştir. Ancak kafein, referans suşun litikaza karşı duyarlılığını ortadan kaldırarak referans suşu, Caf905-2 suşunun litikaza karşı dirençliliğine benzer bir seviyeye taşımıştır.

DNA mikrodizin analizine dayanan gen anlatım analiz sonuçları, kafeine yüksek düzeyde direnç sergileyen Caf905-2 suşunda, istatistiksel olarak anlamlı olan (doğrulanmış $p < 0.05$) en az iki kat değişiklikle 745 genin anlatım düzeyinde artış gözlemlenirken, 741 genin anlatımında ise düşüş olduğunu göstermiştir. Bu şekilde toplamda 1489 gende, farklı düzeylerde değişiklikler meydana gelmiştir. Kafeine yüksek düzeyde dirençli suş olan Caf905-2'nin transkriptomik analizinin işlevsel gruplandırılmasına göre, karbonhidrat ve ikincil metabolizma gruplarını içeren metabolizma, enerji, protein akibeti (fate), protein bağlanma işlevi ve hücre savunma virülans ana grupları ile ilişkili genler, kafein içermeyen stressiz koşullarda uyarılmışlardır. Bu ana işlevsel grupların altında, glikoliz ve glukoneojenez, pentoz fosfat sinyal yolağı (PPP), elektron taşıma sistemi ve zar bağlantılı enerji korunumu, enerji rezervlerinin (trehaloz, glikojen vb.) metabolizması, protein katlanması ve stabilizasyonu, oksijen ve radikal detoksifikasyonu alt grupları yer alır ve bunlar en az iki kat artmıştır. Bunun yanında, nükleotit ve amino asit metabolizmalarını içeren metabolizma, transkripsiyon, protein sentezi, protein bağlanma işlevi, ve hücre içi taşıma ana kategorileri ile ilişkili genler ise, yine kafein içermeyen stressiz koşulda baskılanmışlardır. Baskılanan genlerin içinde yer aldığı işlevsel grupların altında, pürin ve pirimidin metabolizması, RNA işlenmesi ve modifikasyonu, ribozom biyojenezi, translasyon, translasyonel kontrol, aminoaçil- tRNA-sentaz, RNA bağlanması, ağır metal iyon taşıması (Cu^{+}, Fe^{3+}, vb.), amin/poliamin taşıması, nükleotit taşıması, vitamin/kofaktör taşınması, taşıma vasıtaları alt grupları yer almaktadır ve bunlar da az iki kat zenginleştirilmişlerdir. Bunların yanı sıra, bu kategoriler içerisinde yer almayıp, bu çalışma için önemleri tüm genom tekrar dizileme analiz sonucunda ortaya çıkan genler, pleiotropik ilaç direnci ile ilişkili genlerdir ve ilaç/toksin taşıma kategorisinde yer almışlardır.

Referans suş ile kıyaslamalı olarak elde edilen tüm genom dizileme analiz sonuçları, kafeine yüksek düzeyde dirençli Caf905-2 suşunda, yalnızca üç tane tek nükleotit varyasyonu (SNV) olduğunu ortaya çıkarmıştır. Bu SNV'lerin ise *PDR1*, *PDR5* ve *RIM8* adlı üç farklı gende oldukları görülmüştür. *PDR1* geninde A2456T, *PDR5* geninde G2008T ve *RIM8* geninde A1097G şeklinde SNV'ler mevcuttur ve bu SNV'ler, sırasıyla V819D, A670S ve Q366R amino asit değişikliklerine karşılık

gelmektedir. Yapılacak ilerki çalışmalar ile, bu genlerin mayadaki kafein tepki ve direnç mekanizmasındaki rollerinin detaylı olarak incelenmesi uygun olacaktır.

1. INTRODUCTION

1.1 A Brief History of Caffeine

Caffeine has been originated from Kaffee and café as German and French words, respectively. The oldest historian records have shown that caffeine was being consumed in 2737 BC. According to this record, Chinese emperor Shen Nung has boiled tea leaves around bush with water (Arab & Blumberg, 2008). It is assumed that the history of coffee as a caffeine source began around 850 AD, based on a legend. According to this, a herder named Kaldi or Khalid in Ethiopia or Yemen realized that when his goats ate wild coffee berries, it increased their energy (Url-1). Caffeine was, for the first time, isolated from green coffee beans in 1820 and its name was included as “cofeina” in 1823 in the "Dictionnaire des termes de médecine". In the nineteenth century, the presence of caffeine was detected in the tea, maté and kola nut (Dews, 1984). Recently, it is known that caffeine is produced by different plants, over 60 species, such as coffee, tea, kola nuts, guarana berries, Yerba mate and cocoa bean plants (Heckman et al., 2010).

1.2 Caffeine (1,3,7-trimethylxanthine)

Caffeine (1,3,7-trimethylxanthine), a major purine alkaloid, is a secondary metabolite (Heckman et al., 2010). Caffeine production is carried out by young leaves and immature fruits of tea and coffee plants (Ashihara et al., 1996) through xanthosine, which is an intermediate molecule in the caffeine biosynthesis pathway. Among four ways, which are responsible for xanthosine production, the most important route is the production from inosine 5′-monophosphate, derived from de novo purine nucleotide biosynthesis. Purine ring of caffeine is synthesized by this route (Ashihara & Crozier, 1999; Ito & Ashihara, 1999; Koshiishi et al., 2001). Xanthosine, the first purine molecule for the caffeine biosynthesis pathway, is sequentially converted into caffeine by four steps and in these steps, three methyl groups are donated to caffeine via *N*-methyl transferase enzymes by S-adenosyl-L-methionine (SAM) molecule

which is produced via SAM cycle, also called as the activated-methyl cycle (Koshiishi et al., 2001) (Figure 1.1).

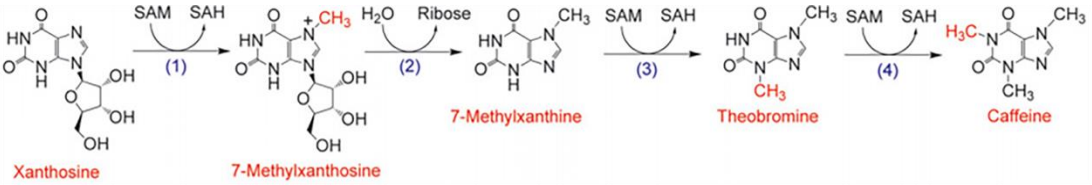

Figure 1.1 : Major pathway for caffeine biosynthesis from the main intermediate xanthosine, adapted from Ashihara et al. (2008).

In caffeine catabolism, caffeine is degraded into xanthine which can be further catabolized into amino group or amino-derived molecule. Caffeine is degraded into theophylline in coffee plants in the first step, which is rate-limiting (Ashihara & Crozier, 1999). Bacterial caffeine degradation shows a difference from caffeine catabolism in higher plants. In caffeine-degrading bacteria including *Pseudomonas cepacia, Pseudomonas putida* and *Serratia marcescens,* the first two steps include a conversion of caffeine into initially theobromine, then 7-methylxanthine and xanthine, whereas it is sequentially converted into theophylline, 3-methylxanthine and xanthine in plants (Ashihara & Crozier, 1999; Asano et al., 1994) (Figure 1.2). In human, caffeine is absorbed by gastrointestinal system, following caffeine intake and reaches maximum concentration plasma after about one hour. Since it has adequate hydrophobic properties to pass through the cell membrane, its distribution quickly occurs throughout human body (Bonati et al., 1984). Although caffeine does not accumulate in any body parts, it seems to exist in all body fluids including saliva, breast milk, semen and bile (Zylber-Katz et al., 1984). The human polycyclic aromatic hydrocarbon-inducible cytochrome P450 (CYP1) subfamily enzymes are involved in major steps of caffeine metabolism, starting from demethylation (Carrillo & Benitez, 2000).

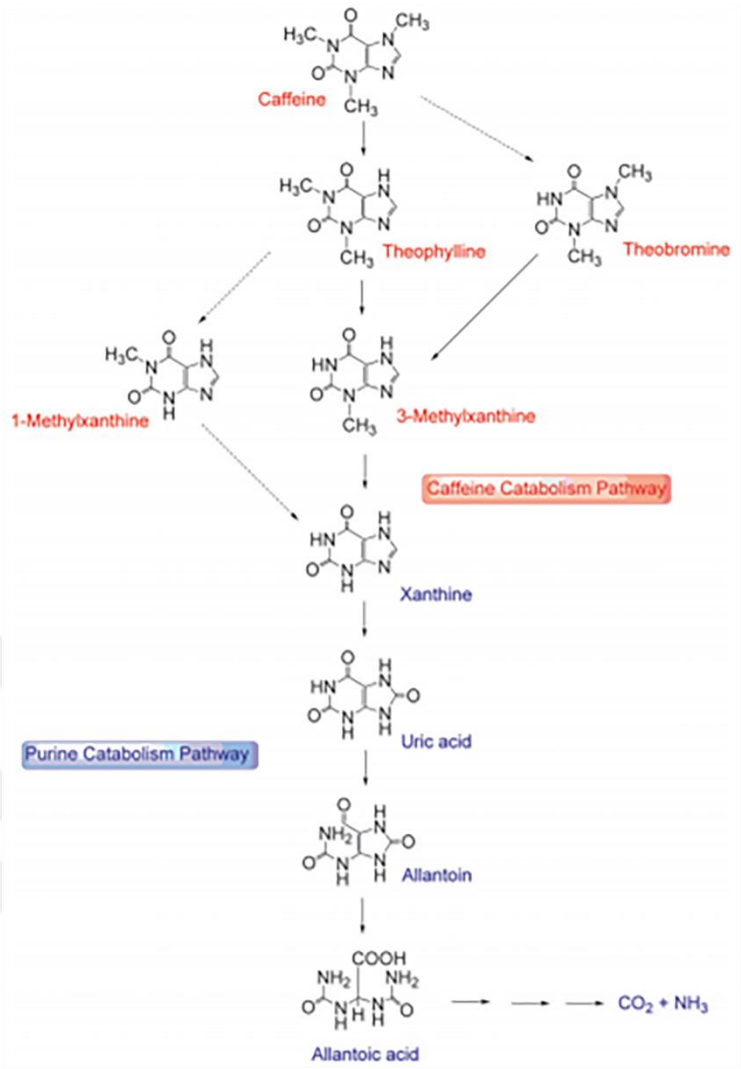

Figure 1.2 : Caffeine catabolism pathway to obtain xanthine destined to ammonia through purine catabolism pathway, adapted from Ashihara et al. (2008).

Two hypotheses 'allelopathic theory' and 'chemical defence theory' have been suggested to explain the role of high caffeine concentrations, which are found in coffee, tea and some other plant species. According to allelopathic theory, caffeine is released from some plant parts such as seed coats to the soil and it blocks the germination of other plant seeds (Chou & Waller, 1980). Additionally, caffeine prevents that other plants reach water and nutrients by inhibiting mitosis of their roots (Friedman & Waller, 1983). This theory is supported by a previous report about which *Arabidopsis* and tobacco plants show incomplete development of seedling size in terms of root and shoot, fast yellowing and decline of chlorophyll amount in seedlings, thus reaching senescence earlier, when they are exposed to caffeine (Mohanpuria & Yadav, 2009). Another 'chemical defence theory' suggests that

caffeine plays a pesticide role reinforcing plant defense against some beetles, insects and herbivores (Juddy et al., 2014).

1.3 Pleiotropic Effect of Caffeine on Organisms

Caffeine which is an alkaloid with odourless, white color and an intense bitter taste, acting as a natural pesticide (Preedy, 2012; Fisone et al., 2004; Nathanson, 1984), is the most widely used psychoactive drug throughout the world (Ferré, 2013). 80% of people have become caffeine consumers (Ogawa & Ueki, 2007). It is an ingredient of some diets including beverages such as coffee, tea, soft drinks, energy drinks and soda, and foods like chocolate and cocoa. Additionally, caffeine is also found in some prescription drugs and in over-the-counter (OTC) medications including stimulants, pain relievers, diuretics and cold remedies (Carrillo & Benitez, 2000). Caffeine is globally used up in the range of 80-400 mg by each person per day even though it changes from region to region (Fredholm et al., 1999; Gokulakrishnan et al., 2005). It is well known that caffeine has a dose-dependent effect on people. For instance, it has been shown that 20-200 mg referring to low-moderate doses of caffeine has psychostimulant impact such as increasing the felicity, bliss, awareness, energy and sociability, while higher doses of it lead to anxiety and tension (Griffiths et al., 2003; Rogers et al., 2010).

Caffeine pharmacologically influences some organ systems including central nervous system (CNS), cardiovascular system, renal system, and muscles in human body (Carrillo & Benitez, 2000). Accordingly, caffeine is a nonselective adenosine receptor antagonist and inhibits A1 and A2A adenosine receptors (Daly, 1993). Thus, it stimulates the human central nervous system and leads to an increase in blood pressure, heart rate and physical activity, as during sport. Also, it causes smooth muscle relaxation, sleep disturbances, and urine discharge. Additionally, caffeine has effects on human body because of paraxanthine, theobromine and theophylline, the metabolites formed during caffeine degradation. They are related to an increase in blood sugar levels, and some hormones such as cortisol and epinephrine. Moreover, they boost the excretion of pepsin and gastric acid, and cause a rise in plasma fatty acid levels and intraocular pressure. They also result in bone loss due to the loss of calcium (Klang et al., 2002; Higginbotham et al., 1989).

Caffeine affects major processes of many cellular and molecular events (Figure 1.3). For example, caffeine inhibits DNA repair and arrests cell cycle checkpoints (Sabisz & Skladanowski, 2008), mediating its mutagenic effect (Moser et al., 2000; Saiardi et al., 2005). In addition, this small compound inhibits cell growth in a wide range of organisms, including bacteria, yeast and higher organisms (e.g. human cell lines) and increases chronological life span (CLS), retarding aging (Wanke et al., 2008). These phenomena are associated with the inhibition of the central regulator TORC1 of growth and metabolism (Loewith & Hall 2011; de Virgilio & Loewithi 2006; Wullschleger et al., 2006); thus, it prevents tumour formation and prompts the apoptosis of existing tumours (Lu et al., 2002; Hashimoto et al., 2004). Additionally, relatively low doses of caffeine induce antioxidation processes (Lacorte et al., 2013); and give rise to cell wall damage in fission and budding yeast (Calvo et al., 2009; Kuranda et al., 2006).

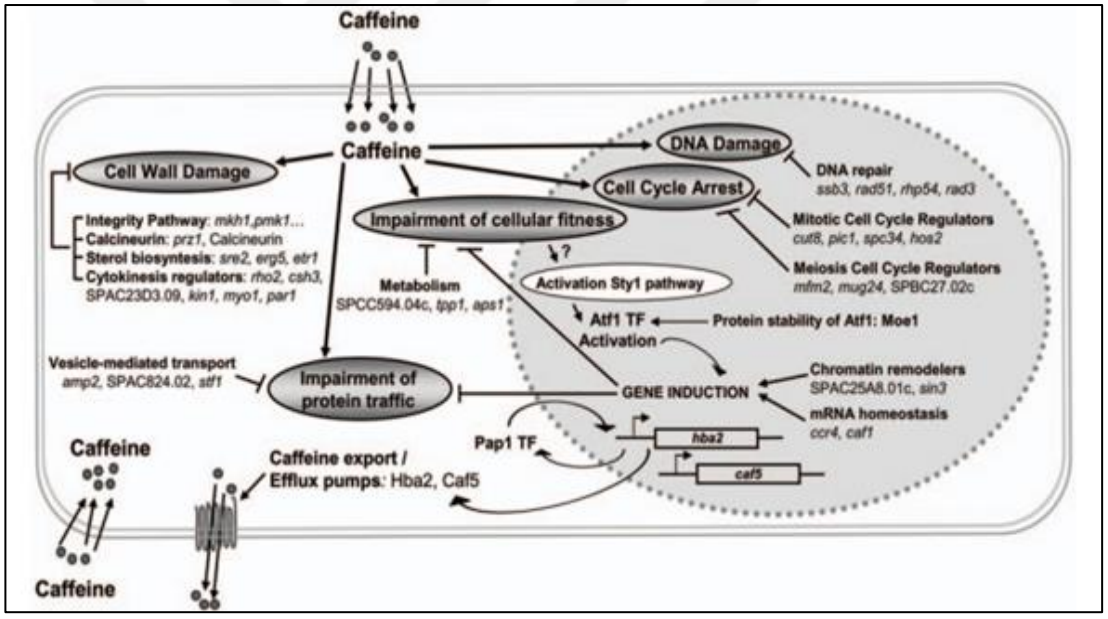

Figure 1.3 : Caffeine pleiotropic effect at molecular and cellular levels, adapted from Calvo et al. (2009).

1.4 Molecular Factors Underlying Caffeine Resistance and Response on *Saccharomyces cerevisiae*

In literature, some molecular factors underlying caffeine resistance and response have been reported, based on a variety of gene deletion and overexpression studies. Accordingly, Schmitz and colleagues (2002) have shown that *rho5*Δ *S. cerevisiae* mutants conferred resistance against caffeine, calcofluor white and congo red; thus,

protein kinase C-dependent signal transduction pathway was stimulated (Schmitz et al., 2002). Also, the overexpression of *RTS3*, *SDS23* or *SDS24*, associating with the phosphatase 2A-like Sit4 pathway, becomes resistant to caffeine in *S. cerevisiae*. Hse1p/Vps27p, a membrane protein in ubiquitin-mediated protein sorting pathway, confers caffeine resistance when overexpressed in budding yeast (Hood-DeGrenier, 2011). Furthermore, *rts3*Δ mutant, which is sensitive to caffeine, was associated with the repression of the target of rapamycin (TOR)C1 kinase complex (Hood-DeGrenier 2011). A protein kinase TOR, which is highly conserved among many organisms, regulates cell growth and is linked to aging (Loewith & Hall, 2011). Investigation on *S. cerevisiae*, to great extent, made a contribution to the discovery of TOR, which is an important signaling network in eukaryotes, thereby associating with crucial roles in basic and clinical biology (Loewith & Hall, 2011; de Virgilio & Loewith, 2006; Wullschleger et al., 2006). Caffeine plays role as a small substance inhibitor for TORC1 in *S. cerevisiae.* Also, the yeast Ras-like small GTPases Gtr1p and Gtr2p, which may be involved in nutrient-responsive TOR signaling pathway, are necessary for conferring caffeine resistance (Wang et al., 2009). Transcriptomic analysis outputs with caffeine response in *S. cerevisiae* indicated that caffeine causes the activation of the Pkc1p-Mpk1p pathway through TOR1p-mediated signaling and the inhibition of the Ras/cAMP-dependent protein kinase signaling pathway via Rom2p, which is a GDP/GTP exchange factor (Kuranda et al., 2006). Beside above-mentioned studies, one recent report has shown that caffeine resistance could be obtained by its efflux as a result of induction of *SNQ2* and/or *PDR5*, which are ATP-binding cassette (ABC) transporters (Tsujimoto et al., 2015). The fact that many molecular factors were responsible for caffeine resistance, as revealed in these reports that are mainly based on gene disruption or overexpression, explicitly points out the complexity of caffeine resistance at the molecular and cellular level.

1.5 *Saccharomyces cerevisiae* : a Model Organism

Saccharomyces cerevisiae, the well-known baker's/brewer's yeast, is a unicellular eukaryotic organism and a type of fungus, placed in ascomycota phylum. It can thrive in a wide range of natural habitats including flowers and leaves in plants, and soil (Kurtzman & Fell, 1998). Its diameter is approximately 5 μm in size (Duina et al., 2014). A budding *S. cerevisiae* cell has an oval shape (Walker et al., 2002),

which is mainly designated by its cell wall structure composed of inner and outer layer. The inner layer of a budding yeast cell wall encompasses mechanically stable polysaccharides like branched 1,3-β-glucan, whereas the outer layer contains mannoproteins (Klis et al., 2006).

The budding yeast *S. cerevisiae* has the ability to reproduce at high rates, with a doubling time of about 90 min under optimum conditions. Vegetative reproduction of *S. cerevisiae* involves the formation of a small daughter bud cell from the mother by mitosis. Every parent forms approximately 20-30 buds throughout its lifetime. The number of bud scars, which form on the cell wall, determine cell age (Lodish, 1995; Solomon, 1999). Also, *S. cerevisiae* cells can exist as haploid and diploid forms, and have a life cycle as sexual reproduction. Here, a haploid cell, which can be of 'a' or 'α' mating type, naturally has an ability to switch its mating type to the opposite type by sexual reproduction (Duina et al., 2014). Mating types are assigned by MAT locus, including the alleles MATa and MATα, which enable the cell to produce 'a factor' and 'α factor' as pheromone, respectively. These pheromones are, in turn, secreted by MATa and MATα cells to mate with each other so that a diploid cell occurs (Duina et al., 2014; Esslinger, 2009).

The minimum and maximum growth temperatures of *S. cerevisiae* are 4-13^{o}C and 38-39^{o}C, respectively (Jermini et al., 1987). This yeast has intrinsic resistance against very low pH; it can survive below pH 1.6 of hydrochloric acid (HCl) (Bergman, 2001). The budding yeast *S. cerevisiae* is easy to grow under laboratory conditions. The storage of yeast cells is also relatively easy in glycerol stocks at -80°C or in freeze-dried form at room temperature (Duina et al., 2014).

The first eukaryotic genome which has been completely sequenced belongs to *S. cerevisiae* S288c strain (Goffeau et al., 1996). A haploid yeast cell has about 12 Mb genome placed in 16 chromosomes, which encompasses 6,604 genes. The 78% of those genes is verified based on *Saccharomyces* Genome Database (SGD), as of June 18, 2019 (Url-2).

S. cerevisiae is classified as a single-celled eukaryotic organism, and it has been utilized for various industrial purposes such as baking, beer and wine production by human beings. In addition, it has been extensively used as a model organism for molecular biology and genetic studies. There are several reasons for this use: firstly, it is economically feasible to work with *S.cerevisiae*. Also, it exhibits rapid growth in

solid and/or liquid media with a generation time of about 1.5 hours. In addition, since it can be haploid, genetic and/or cellular manipulations can be easily carried out. Moreover, it has common ortholog genes and proteins with mammalian and plant cells, and its whole genome sequence has already been determined. Eventually, it can be utilized as a model organism for human health and plant studies (Mell & Burgess, 2002).

1.6 Evolutionary Engineering : a Promising Strategy for Obtaining Genetically Complex Phenotypes

In 1991, Jay Bailey defined metabolic engineering as 'the improvement of cellular activities by manipulation of enzymatic, transport, and regulatory functions of the cell with the use of recombinant DNA technology' (Bailey, 1991). There are two approaches in metabolic engineering: the first one is rational metabolic engineering and the second one is inverse metabolic engineering (Bailey et al., 1996). Rational metabolic engineering encompasses the defined genetic manipulations, which are applied on a described metabolic system for the aim of activation in desired route of metabolic flux or phenotypic trait. In 1996, Jay Bailey and colleagues defined inverse metabolic engineering as "the elucidation of a metabolic engineering strategy by: first, identifying, constructing, or calculating a desired phenotype; second, determining the genetic or the particular environmental factors conferring that phenotype; and third, endowing that phenotype on another strain or organism by directed genetic or environmental manipulation" (Bailey et al., 1996). The major advantage of inverse metabolic engineering over rational metabolic engineering is that there is no need for extensive information about genetic and biochemical mechanisms of the organism or metabolic pathway of interest. Another advantage is that the improved phenotype may not be evaluated as a Genetically Modified Organism (GMO), since a vector and/or foreign DNA is not necessarily used (Bailey et al., 1996). A combinatorial concept can be performed using these two approaches synergistically in strain improvement studies. To this end, output knowledge of molecular factors deciphering a desired trait, which is obtained by inverse metabolic engineering, can be transferred using rational methods, or a rationally constructed

strain can be additionally developed using inverse metabolic engineering (Oud et al., 2012) (Figure 1.4).

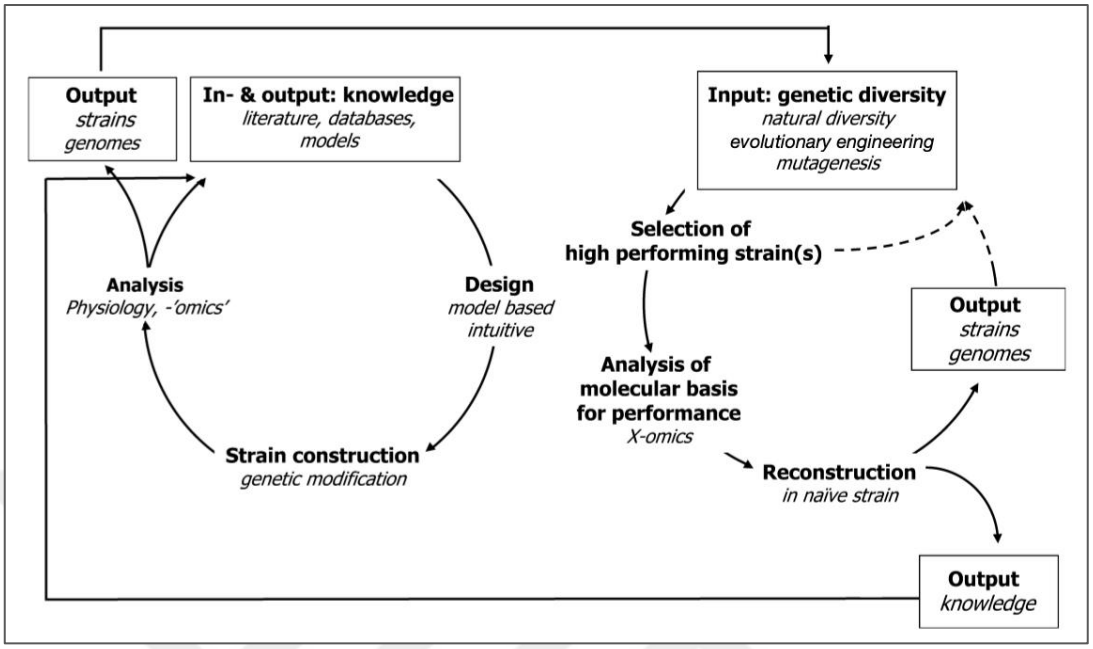

Figure 1.4 : A combinatorial concept of inverse and rational metabolic engineering approaches, adapted from Oud et al. (2012).

Evolutionary engineering is a strategy of inverse metabolic engineering to obtain a desired phenotype. It is based on random mutations, which can be spontaneous or inducible, and systematic selection of desired characters. Applying the systematic technique under a constant or increasing selective pressure confers the isolated individual variants a genetic background with higher adaptation ability to the specific challenge or stress used in selection (Figure 1.5). Owing to evolutionary engineering, multi-stress resistant (Çakar et al., 2005), cobalt-resistant (Çakar et al., 2009), nickel-resistant (Küçükgöze et al., 2013), ethanol-tolerant (Turanlı-Yıldız et al., 2017), chronologically long-lived (Arslan et al., 2018), and coniferyl aldehyde-resistant (Hacısalihoğlu et al., 2019) *S. cerevisiae*, were successfully obtained. Caffeine studies on *S. cerevisiae*, however, particularly focus on gene deletion or transposon mutagenesis methods; and there are no studies in the literature with a *S. cerevisiae* strain that was made highly caffeine-resistant by inverse metabolic or evolutionary engineering approaches, and investigated by –omic approaches, to gain insight into the complex molecular mechanisms of caffeine resistance.

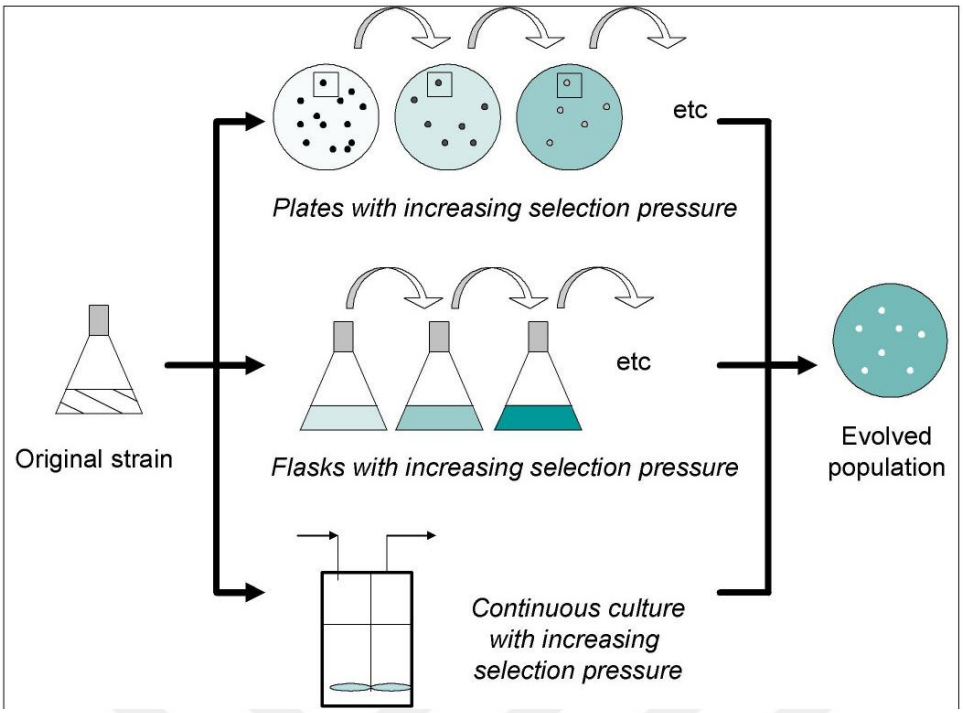

Figure 1.5 : Evolutionary engineering strategy to obtain a desired phenotype, as an inverse metabolic engineering approach, adapted from Hahn-Hägerdal et al. (2005).

1.7 Aim of the Study

The aim of this study was to obtain caffeine-resistant *S. cerevisiae* by evolutionary engineering, and to investigate in detail the molecular factors underlying caffeine resistance in *S. cerevisiae* by using genomic, transcriptomic and physiological analyses. For this purpose, *S. cerevisiae* was systematically subjected to successive batch selection in the presence of gradually increased caffeine stress levels, with and without using a chemical mutagen (EMS) before selection. One of the highly caffeine-resistant mutants obtained from the selection without prior EMS mutagenesis was characterized at the physiological, transcriptomic and genomic levels to investigate the molecular mechanisms underlying caffeine-hyperresistance.

2. MATERIALS AND METHODS

2.1 Materials

2.1.1 Strains

S. cerevisiae CEN.PK 113-7D *(MATa, MAL2-8c, SUC2)* was used as the reference strain and it was kindly provided by Prof. Dr. Jean Marie François and Dr. Laurent Benbadis (University of Toulouse, France).

2.1.2 Growth media

Yeast extract-peptone-dextrose (YPD) medium was used for the revival of *S. cerevisiae* strains from -80^{o}C stocks. Yeast minimal medium (YMM) was generally used for cultivations, including precultures of *S. cerevisiae* strains. The ingredients of each media were dissolved in distilled water (dH_2O) and sterilization was performed at 121 °C for 15 min in an autoclave. Agar (2 % (w v^{-1})) was used for solid YMM and YPD media. The ingredients of YPD and YMM media are shown in Tables 2.1 and 2.2, respectively.

Table 2.1 : Yeast extract-Peptone-Dextrose (YPD) contents.

Ingredient	Percentage (w v^{-1})
Yeast extract	1%
Peptone	1%
Glucose	2%
Agar (for solid media)	2%

Table 2.2 : Yeast Minimal Medium (YMM) contents.

Ingredient	Percentage (w v^{-1})
Yeast nitrogen base without amino acids	0.67%
Dextrose	2%
Agar (for solid media)	2%

2.1.3 Chemicals and equipment

Chemicals and equipment listed in Appendix A (Table A.1 and Table A.2) were used in this study.

2.2 Methods

2.2.1 Yeast culture conditions

The reference and the mutant strains from the glycerol stocks (30 %; v v^{-1}) were revived using 10 mL of YPD and precultured using 10 mL of YMM in 50 mL culture tubes at 30^{o}C, 150 rpm in a rotary shaker for 24 h. The absorbance of the cultures was measured at 600 nm wavelength (OD_{600}) using a spectrophotometer (Shimadzu UV-1700, Japan). The overnight precultures were inoculated into fresh YMM media using Erlenmeyer flasks or 50 mL culture tubes to an initial OD_{600} of 0.2-0.25 (about $3.5x10^{6}$ cells mL^{-1}).

2.2.2 The effect of caffeine on various stress-resistant strains previously obtained by evolutionary engineering strategy

The effect of caffeine on various stress-resistant strains previously obtained using evolutionary engineering strategy (Table 2.3) was performed using the semi-quantitative spot assay method. For this purpose, the overnight cultures of the reference and stress-resistant strains were grown in 10 mL YMM in 50 mL culture tubes, initiating at OD_{600} of 0.25 (approximately $3.5x10^{6}$ cells mL^{-1}) until reaching on OD_{600} of 2.0. Each culture was centrifuged at 10000 g for 3 min, and then adjusted to 4 OD_{600} per mL. Each culture was serially diluted from 10^{-1} to 10^{-5} (180 µL of YMM + 20 µL of culture). Five µL of each diluted sample was spotted onto YMM agar containing 10 mM and 15 mM caffeine, as well as onto the control plates (without caffeine). The growth was monitored for three days and images were taken at the 72nd h of incubation.

Table 2.3 : Previously obtained *S.cerevisiae* mutant strains, their characteristics and sources.

Strains	Characteristics	Source
REF (reference strain)	CEN.PK 113-7D (haploid)	Entian and Kötter (2007)
B2	Ethanol-resistant	Turanlı-Yıldız et al. (2017)
B8	Ethanol-resistant	Turanlı-Yıldız et al. (2017)
F3	Sulphur dioxide-resistant	Çakar's Lab collection
8C	Iron-resistant	Çakar's Lab collection
T8	Salt-resistant	Çakar's Lab collection
H7	Hydrogen peroxide-resistant	Çakar's Lab collection
CI25E	Cobalt-resistant	Çakar et al. (2009)
M9	Nickel-resistant	Küçükgöze et al. (2013)
F1	Freeze thaw-resistant	Çakar's Lab collection
2E	Silver-resistant	Çakar's Lab collection
C9	Phenylethanol-resistant	Çakar's Lab collection
FD11	Propolis-resistant	Çakar's Lab collection

2.2.3 Screening of the reference strain and EMS-mutagenized population against various caffeine stress levels

The screening of the reference strain and EMS-mutagenized population was performed by exposure to caffeine stress in a range of 0-20 mM. In doing this, the overnight cultures of the reference strain and EMS- mutagenized population were inoculated in 10 mL of YMM in 50 mL culture tubes containing caffeine stress as well as control cultures without caffeine, at an initial OD_{600} of 0.25 (about $3.5x10^6$ cells mL^{-1}). Cell growth was then measured at the 24^{th} hour. The percent survival rate was calculated by division of the OD_{600} value of each culture with caffeine stress to that of its control culture for the reference strain and EMS-mutagenized cultures, and multiplying by 100. This calculation was carried out for each caffeine concentration.

2.2.4 Evolutionary engineering strategy for the selection of caffeine-resistant mutants

The reference strain and the EMS-mutagenized population were simultaneously used for the selection of caffeine-resistant mutants using two parallel selections as the evolutionary engineering strategy. For systematic selection using serial batch cultivation, both reference strain and the EMS-mutagenized population were exposed to gradually increased levels of caffeine stress. For this purpose, the overnight cultures of the reference strain and the EMS-mutagenized population were precultured and grown for 24 h in 10 mL YMM using 50 mL culture tubes, at an initial OD_{600} of 0.25 (about $3.5x10^6$ cells mL^{-1}). Each culture included 7.5 mM caffeine and their counterparts without caffeine as the control cultures. This was the first batch passage of the reference strain and the EMS-mutagenized population. Similarly, the following caffeine-resistant populations were obtained along 48 passages. The gradual increase of caffeine concentration reached to 50 mM (9.74 g mL^{-1}) at the 48^{th} population which was the final population. For every passage, the percent survival rate was calculated as the division of the OD_{600} value of caffeine-exposed culture to that of the control culture. The culture of each passage was stored as aliquots in 1.5 mL microcentrifuge tubes at -80^{o} C, using 30% (v v^{-1}) glycerol. The final populations obtained from the reference strain and the EMS-mutagenized population were named as Caf905 and Caf906, respectively. Twelve individual colonies were randomly chosen from each culture of Caf905 and Caf906. To do this, the overnight cultures of Caf905 and Caf906 were grown in 10 mL YMM using 50 mL culture tubes for 24 h. Each culture was then diluted from 10^{-1} to 10^{-6} and spread onto YMM agar including 50 mM caffeine. The growth of populations was grown at 30^{o}C and monitored at 24^{th}, 48^{th}, 72^{nd} and 96^{th} h.

2.2.5 Estimation of caffeine resistance of the evolved strains

Caffeine resistance of the randomly chosen 24 evolved strains was evaluated, using spot assay and the five-tube MPN analysis method. The spot assay was performed as described in Section 2.2.2. In this assay, YMM agar supplemented with 15 mM and 50 mM caffeine, and the control plate (YMM) were used. In addition, the high throughput, MPN analysis method was carried out to estimate the cell number of each of the 24 caffeine-resistant *S.cerevisiae* mutant strains and the reference strain, the EMS-mutagenized population and the final populations. The overnight cultures

were precultured in 10 mL of YMM, initiating from an OD_{600} of 0.1 until 1.0, and each culture was adjusted to 1 OD_{600} mL^{-1}. Each well in 96-well plates involved culture dilutions ranging from 10^{-3} to 10^{-10} and each dilution of the cultures was studied with five repetitions. Each culture was grown in media including 15 mM caffeine, as well as in control media without caffeine. Thus, they were observed at 24^{th}, 48^{th} and 72^{nd} h of cultivation, using the 5-tubes MPN table.

2.2.6 Estimation of cross-resistance against various stress types by spot assay

The cross-resistance of the selected caffeine-resistant individual strains obtained from Caf905 and Caf906 was also tested by spot assay. The following stress conditions were applied during spot assay: 15 mM caffeine, 150 µg mL^{-1} propolis, 1 mM coniferyl aldehyde, 4 mM vanillin, 50 nM antimycin, 20 ng mL^{-1} rapamycin, 1000 ng mL^{-1} cycloheximide, 8% (v v^{-1}) ethanol, 2 g L^{-1} phenylethanol, 40 mM $(NH_4)_2Fe(SO_4)_2$, 1 mM $CoCl_2$, 0.5 mM $NiCl_2$, 7.5 mM $AlCl_3$, 0.5 M NaCl, 25 mM LiCl, 0.4 M sodium acetate (NaAc), 50 mM H_3BO_4, 17.5 mM $MnCl_2$, pH 7.5, heat stress (growth at 38^oC), 1 M NH_4Cl, 1 M sorbitol, 2.25 mg mL^{-1} glyphosate, and 8% (v v^{-1}) DMSO.

2.2.7 Genetic stability test

The genetic stability test was applied to selected caffeine-resistant strains, based on cell growth performance, to determine the genetic stability of the gained caffeine resistance. Spot assay procedure was performed as explained above on day 1, 3, 5, 7, 9 and 10. On each day, this experiment was applied on one stress plate including 10 and 15 mM caffeine, and one control plate. The growth was monitored at 72 h for each day.

2.2.8 Physiological analysis of the caffeine-resistant *S. cerevisiae* mutant

2.2.8.1 Growth profile

The growth profile was determined based on turbidimetric measurements using spectrophotometry (Shimadzu UV-1700, Japan) and cell dry weight (cdw) analysis. Simply, the overnight cultures were initiated from 0.2 of OD_{600} using 200 mL of YMM in 1-L Erlen flasks. For each culture, regular spectrophotometric measurements at OD_{600} were performed until 30 h of cultivation in nonstress conditions, and until 48 h of cultivation in 10 mM caffeine stress conditions. Also, 2

mL of each culture was withdrawn at specific time intervals, centrifuged for cell dry weight analysis and transferred into preweighed microcentrifuge tubes.The pellets were then dried in an oven for 48 h and weighed using an analytical balance. At last, cell dry weights of the reference strain and the evolved strain Caf905-2 were calculated. In addition, doubling time was calculated, based on spectrophotometric measurements of the reference strain and the evolved strain Caf905-2 cultures. Those analyses were carried out as three biological repeats.

2.2.8.2 Metabolite analyses

The metabolite analyses included metabolite production (ethanol and glycerol) and residual glucose measurements, using HPLC (Shimadzu Series 10A HPLC, Shimadzu Co., Kyoto, Japan). For this purpose, 1 mL of supernatant samples were collected until 30 h of cultivation in nonstress conditions, and until 48 h of cultivation in 10 mM caffeine stress conditions. The HPLC-analyses were carried out as three biological repeats. The HPLC system was integrated with an RID-10A refractive-index detector and an Aminex HPX-87H ion exclusion column (300 mm×7.8 mm, Bio-Rad Laboratories, CA, USA). The system was hold at 65° C with a flow rate of 0.6 mL min^{-1} throughout analyses and the mobile phase was 5 mM H_2SO_4. The standard calibration curves (Appendix D) were obtained at the initial step of the analysis, using standard solution mixtures that contained different concentrations of each metabolite (Table 2.4).

Table 2.4 : Contents of the standard solutions for metabolite analysis by HPLC.

Solution Number	1	2	3	4	5	6
Glucose (g L^{-1})	20	15	10	5	2.5	1.25
Glycerol (g L^{-1})	1	0.75	0.5	0.25	0.125	0.0625
Acetate (g L^{-1})	2	1.50	1.0	0.50	0.250	0.1250
Ethanol (g L^{-1})	15	11.25	7.5	3.75	1.875	0.9375

2.2.8.3 Storage carbohydrate (intracellular trehalose) accumulation

Intracellular trehalose was measured as described previously (Divate et al. 2016), with slight modifications. Briefly, the overnight culture was inoculated to an initial OD_{600} of 0.2, using 200 mL of YMM. For each culture, 2 mL samples were withdrawn for intracellular trehalose (storage carbohydrate) measurements, and 1.5

mL samples for cell dry weight (cdw) analyses, at 6, 12, 15, 21, and 30 h of cultivation under nonstress condition, whereas the sampling was done at 12, 15, 21, 30, and 48 h of cultivation under 10 mM caffeine stress condition. The samples were centrifuged at 10000 g for 3 min, the pellets were washed by dH_2O and the samples were again harvested at 10000 g for 3 min. Following the washing step, each pellet was dissolved in 1 mL of dH_2O and incubated at 95^oC for one hour. The samples were then centrifuged at 10000 g for 3 min. The supernatant containing intracellular trehalose was measured by HPLC (Shimadzu Series 10A HPLC, Shimadzu Co., Kyoto, Japan) as described for the metabolite analyses. The HPLC analyses were carried out as three biological repeats. Intracellular trehalose content was calculated by dividing the measured trehalose concentration to cell dry weight (cdw).

2.2.9 Lyticase susceptibility assay

The lyticase susceptibility assay was adapted from Kuranda et al. (2006). For this purpose, overnight cultures of the reference strain and caffeine-resistant mutant Caf905-2 were cultivated in 100 mL YMM using 500 mL flasks and grown at 30^oC, 150 rpm, starting with an initial OD_{600} of 0.2 (approximately $5.6x10^6$ cells mL^{-1}) under 10 mM caffeine stress and nonstress (control) conditions, until early exponential phase (0.4-0.6 OD_{600}) and the stationary phase. The cultures were harvested by centrifugation at 5500 g for 10 min and the pellets were dissolved in 10 mL of 10 mM Tris/HCl buffer (pH 7.4) to an OD_{600} of 0.9 mL^{-1}, including 40 mM β-mercaptoethanol. After the samples were incubated at 25^oC for 30 min, 2 U mL^{-1} of lyticase (β-1,3 glucanase) from *Arthrobacter luteus* (Sigma-Aldrich, St. Louis, MO, USA) (10000 U/mL) was added into each sample, as well as the blank solution (10 mM Tris/HCl buffer (pH 7.4)) and incubated at 30 ^oC, 300 rpm. The decrease in OD_{600} of the samples was monitored with time. The analyses were carried out as three biological repeats.

2.2.10 Comparative whole genome transcriptomic analysis of the evolved strain

This work is fully MIAME-compliant and has been deposited at Gene Expression Omnibus (GEO) website. The accession number is GSE124452.

2.2.10.1 Total RNA extraction and RNA quality assessment

The caffeine-resistant strain and the reference strain were precultured as five biological repeats at 150 rpm and 30°C in 100 mL YMM, using 500 mL flasks and at an initial OD_{600} of 0.1. After reaching an OD_{600} of 1.0 ± 0.1 (approximately $1.4x10^7$ cells mL^{-1}), total RNA isolation was carried out by RNeasy Total RNA Isolation Kit (Qiagen), according to manufacturer's instructions. The RNA concentration was detected by NanoDrop 2000 UV–Vis spectrophotometer (Thermo Scientific, USA) and the RNA quality was checked using Agilent RNA 6000 Nano kit and 2100 Bioanalyzer (Agilent Technologies, USA). RNA samples having an RNA Integrity Number (RIN) higher than 8 were successfully prepared for the RNA labelling step and they were stored at -80° C until RNA labelling.

2.2.10.2 RNA labelling and hybridization

cRNA labelling with Cy3 was prepared by using One-Color Microarray-Based Gene Expression Analysis kit (Low Input Quick Amp Labelling; Agilent Technologies) according to manufacturer's instructions. For doing so, at first cRNA was obtained from RNA, and the amplification and labelling of cRNA was performed using T7 RNA Polymerase Blend. Cy3-labelled samples were obtained in pure form by Absolutely RNA Nanoprep Kit (Agilent) and their concentrations were measured using NanoDrop ND-1000 UV–Vis Spectrophotometer. The hybridization of the samples as four biological replicates was carried out to Yeast (V2) Gene Expression Microarray, 8x15 K arrays (G4813A; Agilent Technologies) onto a rotary hybridization chamber at 65°C for 17 h, following the fragmentation. Finally, the slides with the samples were scanned by using the Agilent DNA Microarray Scanner (G2505B).

2.2.10.3 Data analysis process

Microarray data composed of the probe signals were analysed by GeneSpring GX Software (v14.5) (Agilent Technologies). The normalization of the probe signals was performed by Quantile algorithm with Bonferroni FWER (Family Wise Error Rate) correction. The categorization of the statistically significant ($p < 0.05$) and differentially expressed genes (at least two-fold change) was carried out using *FunCat* database. Transcription factors of the whole transcriptomic data were determined by YEASTRACT database (Teixeira et al., 2018).

2.2.11 Whole genome re-sequencing of the evolved strain

Genomic DNA isolation for the whole genome re-sequencing of the reference strain and the caffeine-resistant evolved strain was performed using MasterPure DNA Purification Kit (Epicentre), following overnight growth in 100 mL of YPD in 500 mL flasks at 30^{o}C, 150 rpm. DNA concentrations and qualities were checked using UV-Vis spectrophotometer (NanoDrop 2000). The library construction of genomic DNA was performed by Ion Xpress Plus Fragment Library Kit (ThermoFisher) and Ion 540™ Chip Kit, according to the manufacturer's protocols, following adjustment to 100 ng. The sequencing of DNA samples was carried out on an integration system of Ion S5 next-generation sequencing platform (ThermoFisher) and library prep platform Ion Chef (ThermoFisher). The raw data quality was assessed by FastQC v.0.11.5 (Babraham Bioinformatics). Low-quality sequence and remnant adapters were trimmed using Trimmomatic v.0.32 (Bolger et al., 2014). DNA sequence reads of the evolved strain and the reference strain were aligned to the DNA sequence of *S. cerevisiae* CEN.PK113-7D (GCA_000269885.1) (Nijkamp et al., 2012), using the Burrows-Wheeler aligner MEM v.0.7.1 (Li & Durbin, 2009). The differences in DNA sequences were detected and reviewed by Genome Analysis Toolkit (GATK) v.3.8.0 (DePristo et al., 2011) and GenomeBrowse v2.1.2 (GoldenHelix), respectively. The filtration of low quality differences in DNA was performed using in-house R scripts. Differences in the DNA sequence of the caffeine-resistant mutant strain were annotated using Variant Effect Predictor v.90 and *S. cerevisiae* CEN.PK113-7D, ASM26988v1 template. Whole-genome re-sequencing data have been deposited in the NCBI Sequence Read Archive (SRA) under BioProject PRJNA514961.

3. RESULTS

3.1 The Effect of Caffeine on Various Stress-resistant *Saccharomyces cerevisiae* Strains obtained by Evolutionary Engineering Strategy

The effect of caffeine on a variety of stress-resistant strains was determined, using spot assay under 10 mM and 15 mM caffeine stress conditions, as well as the nonstress (control) condition. The analysis results showed that, among all resistant strains tested, the most caffeine-resistant strain was the propolis-resistant FD11 strain, compared to the reference strain. Its resistance against 10 mM and 15 mM caffeine stresses were clearly observed, compared to the other strains. Also, oxidative stress (H_2O_2)-resistant H7 and ethanol-resistant B8 strains were slightly resistant to 10 mM caffeine stress. Besides, ethanol-resistant B2, sulphur dioxide-resistant F3 and iron-resistant 8C were, to some extent, resistant to 15 mM caffeine stress. On the other hand, cobalt-resistant CI25E and nickel-resistant M9 strains were clearly sensitive to 10 mM caffeine stress. The remaining strains (freeze thaw-resistant F1, silver-resistant 2E, phenyl ethanol-resistant C9 and salt-resistant T8 strains) were neither resistant nor sensitive against caffeine stress (Figure 3.1).

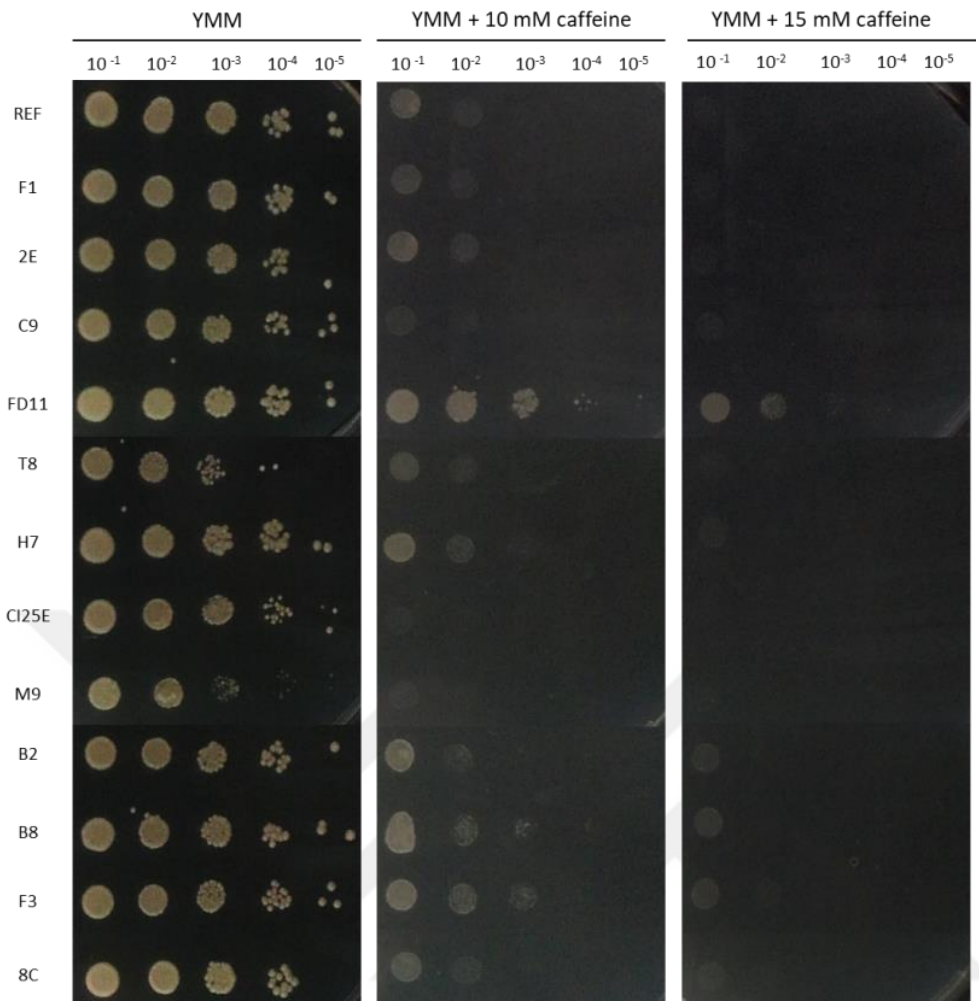

Figure 3.1 : The effect of caffeine stress (10 mM and 15 mM) on sulphur dioxide-resistant F3, iron-resistant 8C, salt-resistant T8, hydrogen peroxide-resistant H7, cobalt-resistant CI25E, nickel-resistant M9, freeze thaw-resistant F1, silver-resistant 2E, phenyl ethanol-resistant C9, propolis-resistant FD11, and two ethanol-resistant B2 and B8 strains, as well as the reference strain (REF).

3.2 Determination of the Initial Caffeine Stress Level for the Evolutionary Engineering Experiments

Reference strain (CEN.PK113-7D) and EMS-mutagenized population were screened at various caffeine stress levels, to determine the Minimal Inhibitory Concentration (MIC) of caffeine. The cultures were incubated for growth at various caffeine concentrations (0-20 mM) for 24 h. The screening study showed that caffeine caused a similar inhibition profile in the reference strain and the EMS-mutagenized population. Both cultures were slightly inhibited up to 7.5 mM caffeine; however, the inhibition dramatically increased at higher caffeine concentrations and the percent survival rate decreased below 20% in the presence of 20 mM caffeine. Thus, the

MIC value was estimated to be between 7.5 to 10 mM caffeine (Figure 3.2). Finally, 7.5 mM caffeine with a survival rate higher than 50% was chosen as the initial, mild caffeine stress level of the evolutionary selection procedure.

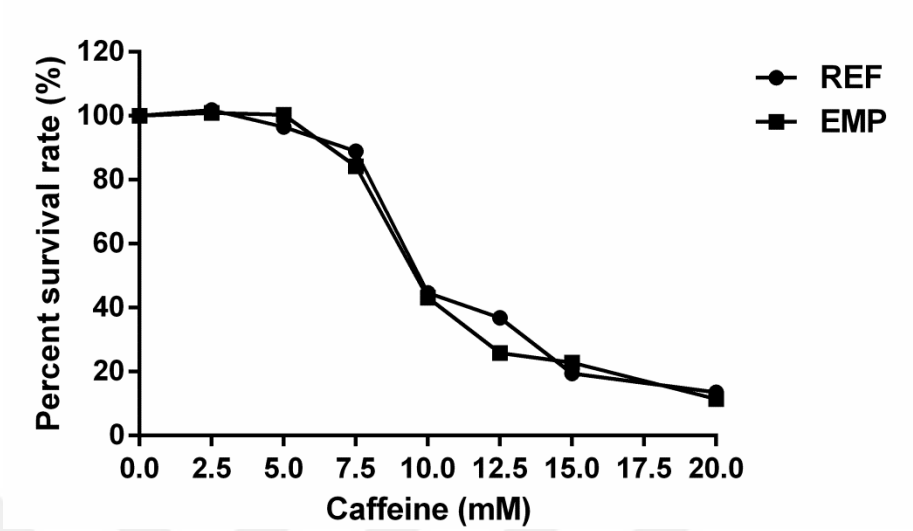

Figure 3.2 : Percent survival rates of the EMS-mutagenized population (EMP) and the reference strain (REF) at different concentrations of caffeine.

3.3 Evolutionary Engineering of Caffeine-resistant *S. cerevisiae* Mutants

Successive batch cultivation was applied to the reference strain and its EMS-mutagenized population for the parallel selection of caffeine-resistant *S. cerevisiae* evolved strains in the presence of 7.5 mM caffeine as the initial stress level. To determine the caffeine inhibition effect on the reference strain and the EMS-mutagenized population, cultures grown in the presence of caffeine were compared to the control cultures grown without caffeine. Eventually, 48 populations were obtained from each selection and the final caffeine concentration reached 50 mM (9.74 g L^{-1}) (Figure 3.3). Caffeine concentration was gradually increased along 48 passages. Accordingly, an increase by 0.5 mM, 1 mM, and 2 mM caffeine was performed; from the 1^{st} to 28^{th} population, 28^{th} to 41^{st} population, and 41^{st} to 48^{th} population, respectively. The cultures of the reference strain and the EMS-mutagenized population had similar survival profiles along their populations. Also, each culture had fluctuations in survival rates at increasing caffeine concentrations and none had percent survival rates below 50%. The final populations of both reference strain and the EMS-mutagenized population selections had nearly similar

percent survival (56.6% and 58.7%, respectively) at 50 mM caffeine concentration (Tables B.1 and B.2).

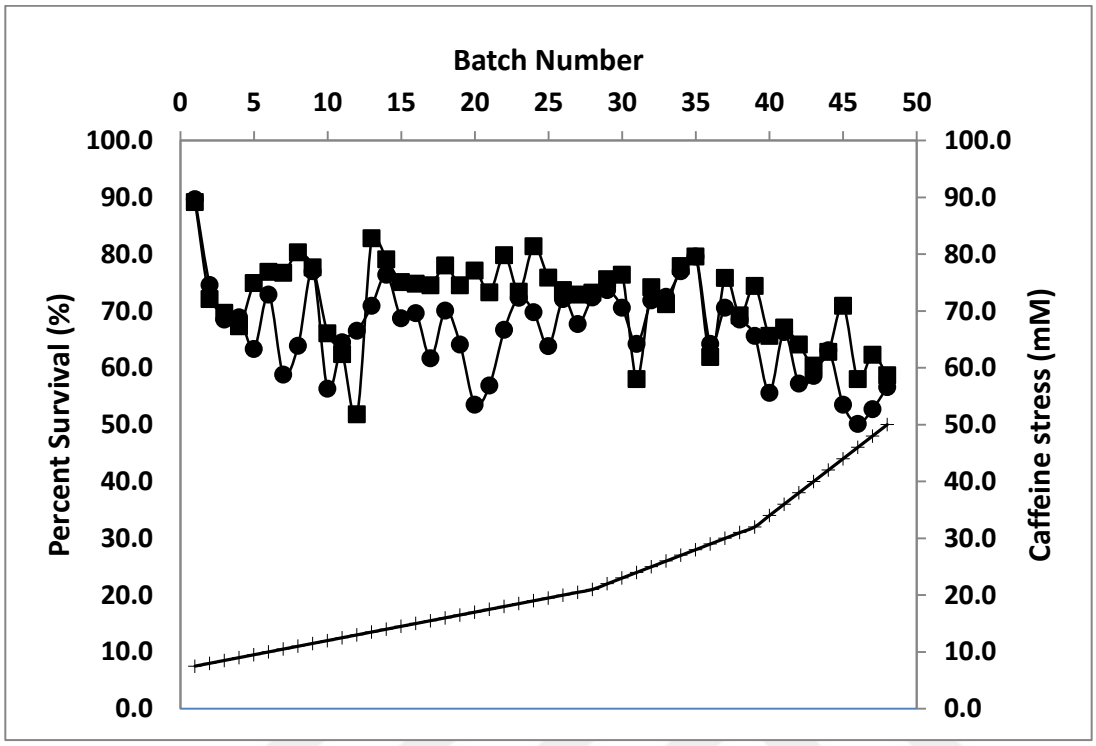

Figure 3.3 : Percent survival rate of each of the 48 populations obtained during two parallel successive batch cultivations, beginning with EMS-mutagenized population (EMP) and the reference strain under gradually increased caffeine stress levels, by evolutionary engineering. '■' indicates each population obtained from EMP as the initial population of selection '●' indicates each population obtained from the reference strain as the initial population of selection. '+' indicates caffeine concentration.

The final populations of both reference strain and the EMS-mutagenized population were named as Caf905 and Caf906, respectively. To isolate caffeine-resistant individuals, following evolutionary engineering of both reference strain and EMS-mutagenized population at high caffeine concentrations, each final population was grown on YMM solid media containing 50 mM caffeine, at 30^{o}C for 96 h. Twenty four caffeine-resistant *S. cerevisiae* mutant strains were randomly chosen from the final populations grown on these media. Twelve caffeine-resistant *S. cerevisiae* mutant strains obtained from the Caf905 population were named as Caf905-1 to Caf905-12, while twelve evolved strains obtained from the Caf906 population were named as Caf906-1 to Caf906-12.

3.3.1 Estimation of the caffeine resistance levels of the evolved strains

The caffeine-resistance levels of 24 individuals obtained from Caf905 and Caf906 final populations were determined using spot assay, upon growth at 30^{o}C for 72 h. All of the caffeine-resistant *S. cerevisiae* strains grew well on YMM medium including 15 mM caffeine and control medium. Also, all exhibited growth patterns similar to each other. On the other hand, 15 mM caffeine had a strong inhibitory effect on the reference strain and its EMS-mutagenized population, compared to the caffeine-resistant *S. cerevisiae* evolved strains. However, no growth difference was observed between the caffeine-resistant *S. cerevisiae* strains in YMM including 15 mM caffeine (Figure 3.4a and 3.4b).

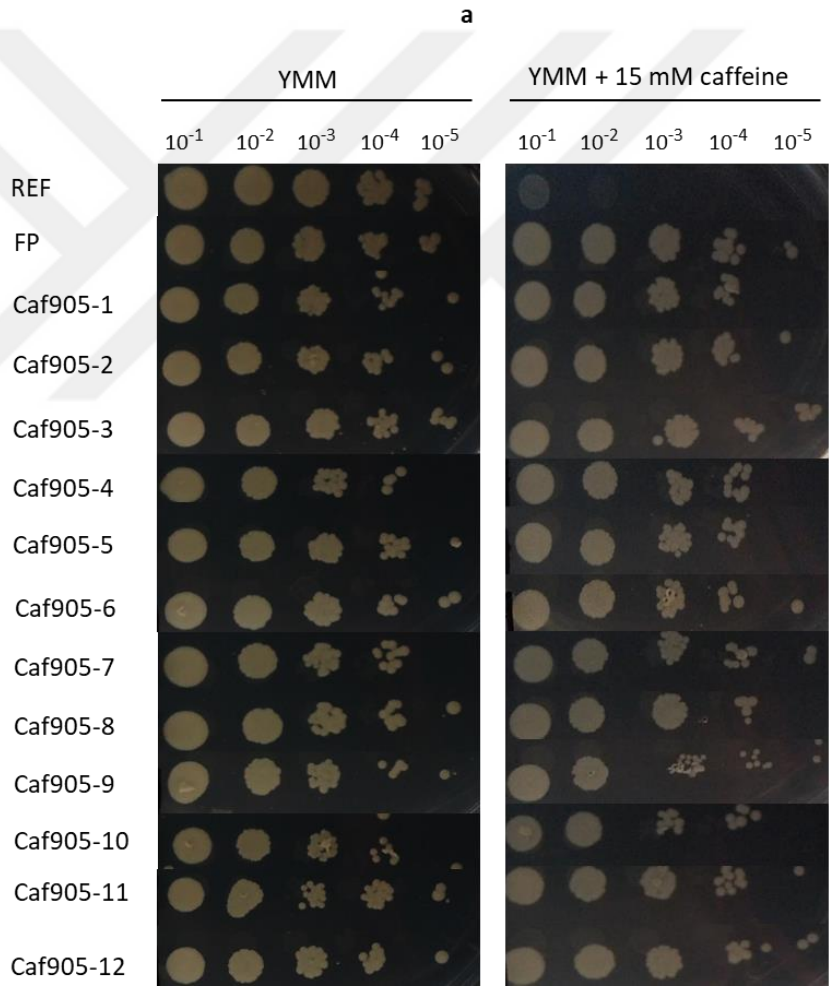

Figure 3.4 : Caffeine resistance of the randomly chosen evolved strains under 15 mM caffeine stress and control conditions, upon 72 h incubation. **a** includes 12 of the caffeine-resistant mutants (Caf905-1 to Caf905-12), final population Caf905(FP) and the reference strain (REF) and **b** includes 12 of the caffeine-resistant mutants (Caf906-1 to Caf906-12), final population Caf906(FP) and the EMS-mutagenized population (EMP).

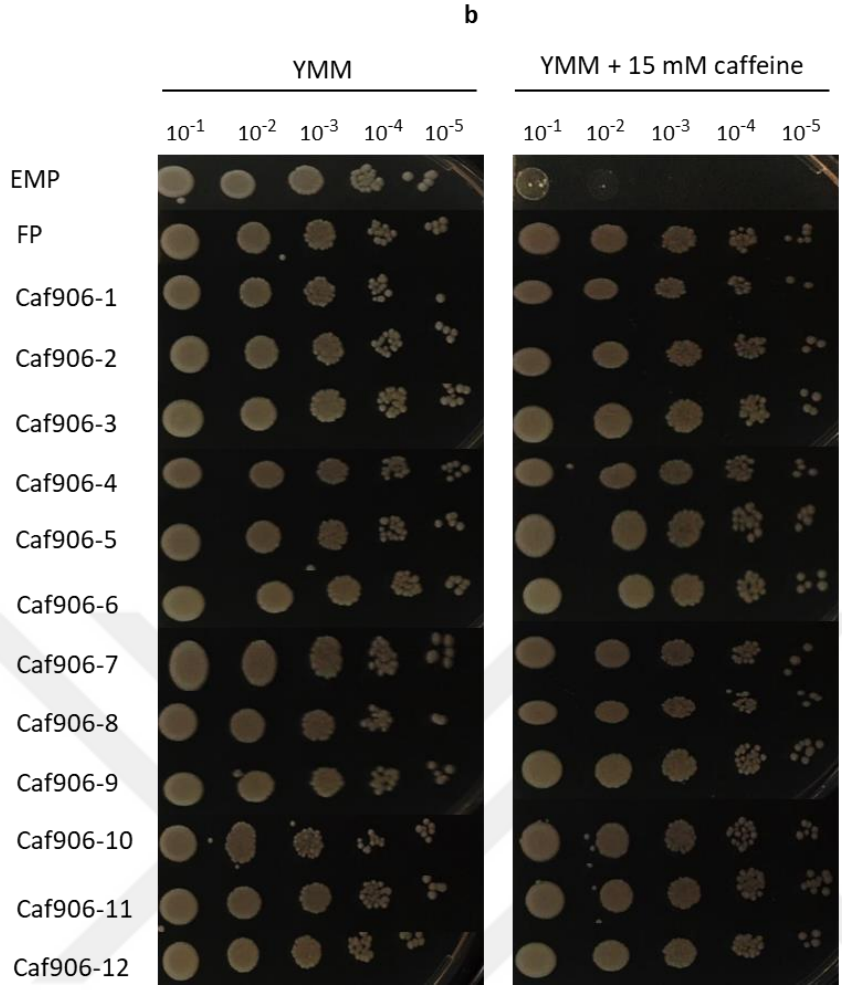

Figure 3.4 (continued) : Caffeine resistance of the randomly chosen evolved strains under 15 mM caffeine stress and control conditions, upon 72 h incubation. **a** includes 12 of the caffeine-resistant mutants (Caf905-1 to Caf905-12), final population Caf905(FP) and the reference strain (REF) and **b** includes 12 of the caffeine-resistant mutants (Caf906-1 to Caf906-12), final population Caf906(FP) and the EMS-mutagenized population (EMP).

In addition, the resistance of the caffeine-resistant strains isolated from the Caf905 final population was also determined under 50 mM caffeine stress. Accordingly, all gave spots, at least, at 10^{-4} diluted sample, even though the reference strain did not grow at all, under 50 mM caffeine stress condition (Figure 3.5).

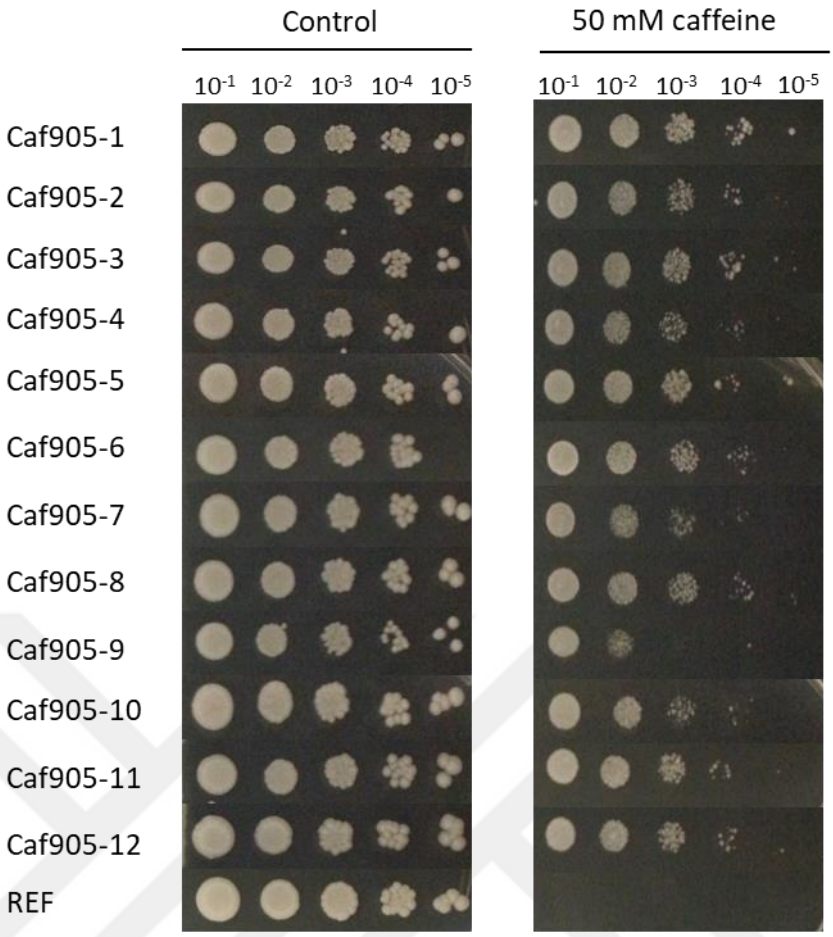

Figure 3.5 : Caffeine resistance of selected mutants obtained from the Caf905 final population (Caf905-1 to Caf905-12), relative to the reference strain (REF), under 50 mM caffeine stress and control conditions upon 72 h incubation (Sürmeli et al., 2019).

To reveal the growth differences, cell growth or survival was determined quantitatively at 24th, 48th, and 72nd h under 15 mM caffeine stress, using five-tube MPN method. MPN analysis results showed that the mutant individuals obtained from the Caf906 population had significantly higher resistance than those of the Caf905 population. At each time point, Caf905-2 exhibited the highest percent survival rate among the mutant individuals obtained from Caf905. Caf906-11 and then Caf906-12 also grew better than the other individual mutants obtained from both Caf905 and Caf906 populations. Caf905-2, Caf906-11, and Caf906-12 were chosen for genetic stability test, because each had higher percent survival at 24th, 48th and 72nd h, compared to the other evolved strains tested (Figure 3.6a and 3.6b), indicating that they have a higher caffeine resistance than the other evolved strains.

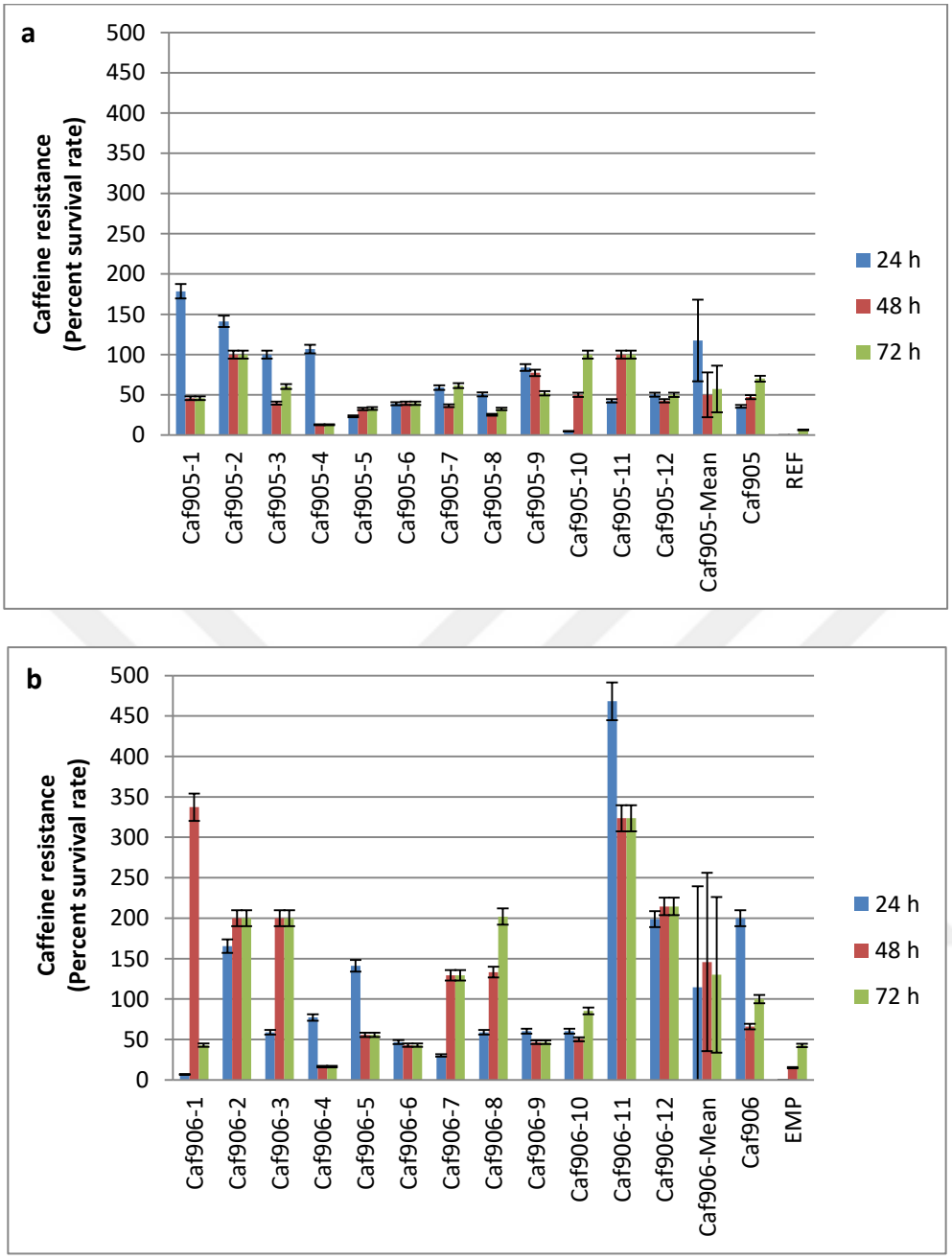

Figure 3.6 : MPN results of caffeine-resistant *S. cerevisiae* mutant strains in YMM with 15 mM caffeine at 24th, 48th and 72nd **a.** It includes Caf905-1 to Caf905-12, Caf905 (final population), calculated Caf905-arithmetic mean, as well as the reference strain, **b.** It includes Caf906-1 to Caf906-12, Caf906 (final population), calculated Caf906- arithmetic mean, as well as the EMS-mutagenized population (EMP).

3.3.2 Genetic stability of the evolved strains with the highest caffeine resistance

Genetic stability test of the three mutants (Caf905-2, Caf906-11, and Caf906-12), exhibiting the highest caffeine stress survival based on five-tube MPN results, was carried out as ten repetitive batch passages under 10 mM and 15 mM caffeine, and control (no caffeine) conditions. The results showed that Caf905-2, Caf906-11, and

Caf906-12 were genetically stable (Figure 3.7a, 3.7b and 3.7c). Thus, these strains were chosen for cross-resistance analyses against a variety of stress types.

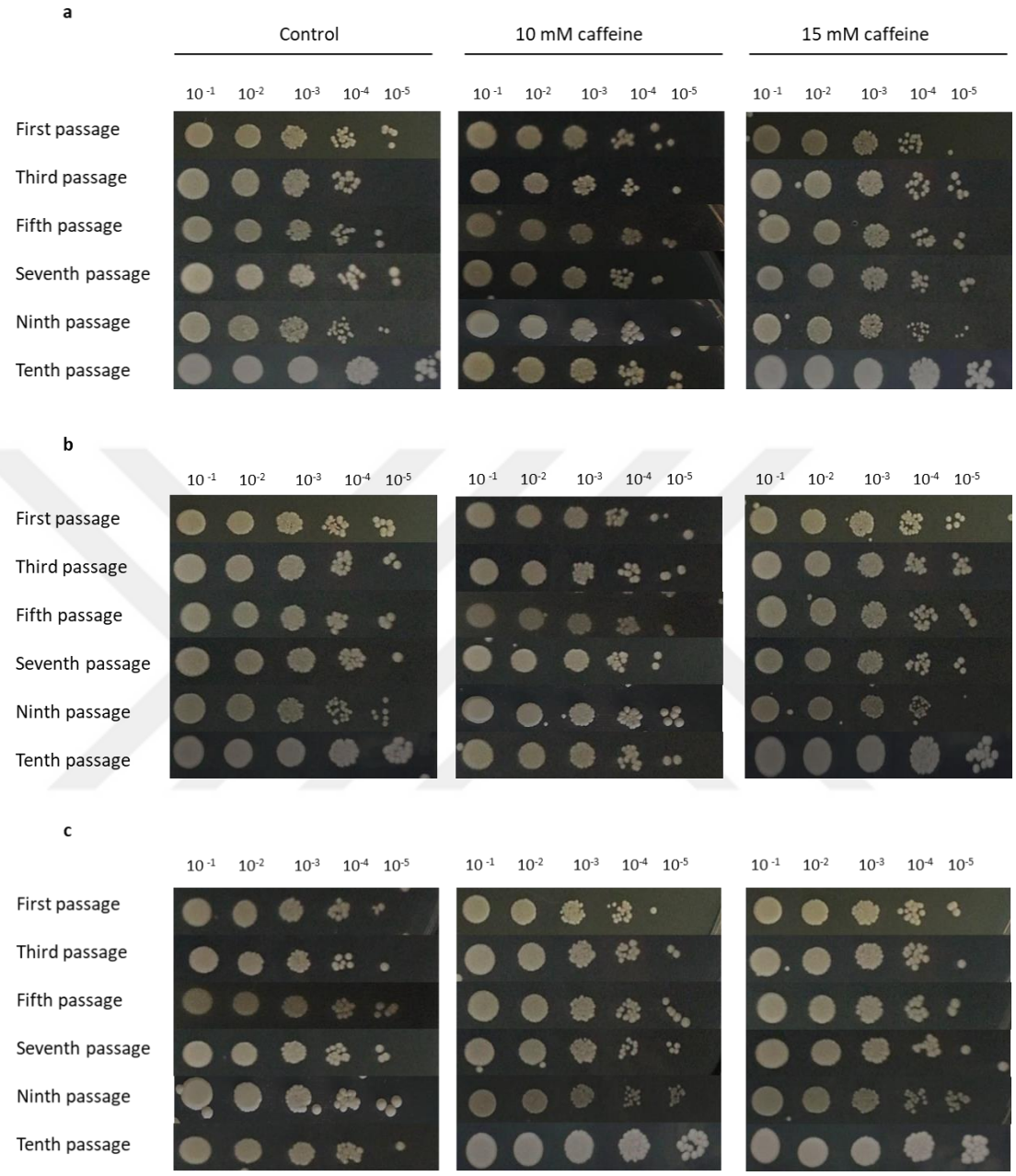

Figure 3.7 : Growth of the caffeine-resistant strains on 10 mM and 15 mM caffeine-containing YMM agar and YMM agar (control) after 1,3,5,7,9 and 10 successive cultivations in caffeine-free media at 72h of incubation. **a.** Caf905-2, **b.** Caf906-11, **c.** Caf906-12.

3.3.3 Analysis of cross-resistance or sensitivity of selected evolved strains against various stress types

Caffeine-resistant strains (Caf905-2, Caf906-11, and Caf906-12) were grown in the presence of different stress factors. The results showed that all three strains were highly resistant to the tested antibiotics (50 nM antimycin A, 20 ng mL^{-1} rapamycin), and phenolics (150 µg mL^{-1} propolis, 1 mM coniferyl aldehyde). However, the three strains behaved differently when exposed to another phenolic, 1 mM vanillin, such that Caf906-11 was slightly resistant, Caf906-12 was sensitive, and Caf905-2 was neither sensitive nor resistant. In addition, Caf905-2 and Caf906-12 were highly susceptible to all tested metals (1 mM $CoCl_2$, 7.5 mM $AlCl_3$, 17.5 mM $MnCl_2$, 40 mM $NH_4Fe(SO_4)_2$ and 0.5 mM $NiCl_2$). Caf906-11 was also sensitive to 1 mM $CoCl_2$, and 7.5 mM $AlCl_3$; however, it was resistant to 17.5 mM $MnCl_2$ and 40 mM $NH_4Fe(SO_4)_2$, and neither resistant nor sensitive to 0.5 mM $NiCl_2$. All three strains were highly sensitive to 50 mM boric acid, 0.5 mM hydrogen peroxide, and slightly sensitive to 0.5 M NaCl. Caf905-2 was highly sensitive and the other mutants were slightly sensitive to 1 M NH_4Cl. Caf905-2 and Caf906-12 were sensitive to 2 g L^{-1} phenylethanol and heat stress (38^{o}C); but Caf906-11 was neither resistant nor sensitive to these stresses. Furthermore, Caf906-11 and Caf906-12 were neither sensitive nor resistant against 2.25 mg mL^{-1} glyphosate; however, Caf905-2 was highly susceptible to this stress. Caf906-12 was neither resistant nor sensitive, Caf906-11 was slightly sensitive and Caf905-2 was highly sensitive to ethanol 8% (v v^{-1}). All mutants were neither sensitive nor resistant against 1 M sorbitol. In addition, five stress factors (cycloheximide, mild alkaline pH, lithium chloride, sodium acetate (NaAc) and DMSO) were applied only to Caf905-2. According to these results, Caf905-2 was highly resistant to 1000 ng mL^{-1} cycloheximide and slightly resistant to 8% (v v^{-1}) DMSO; however, it was susceptible to pH 7.5, LiCl and NaAc (Figure 3.8).

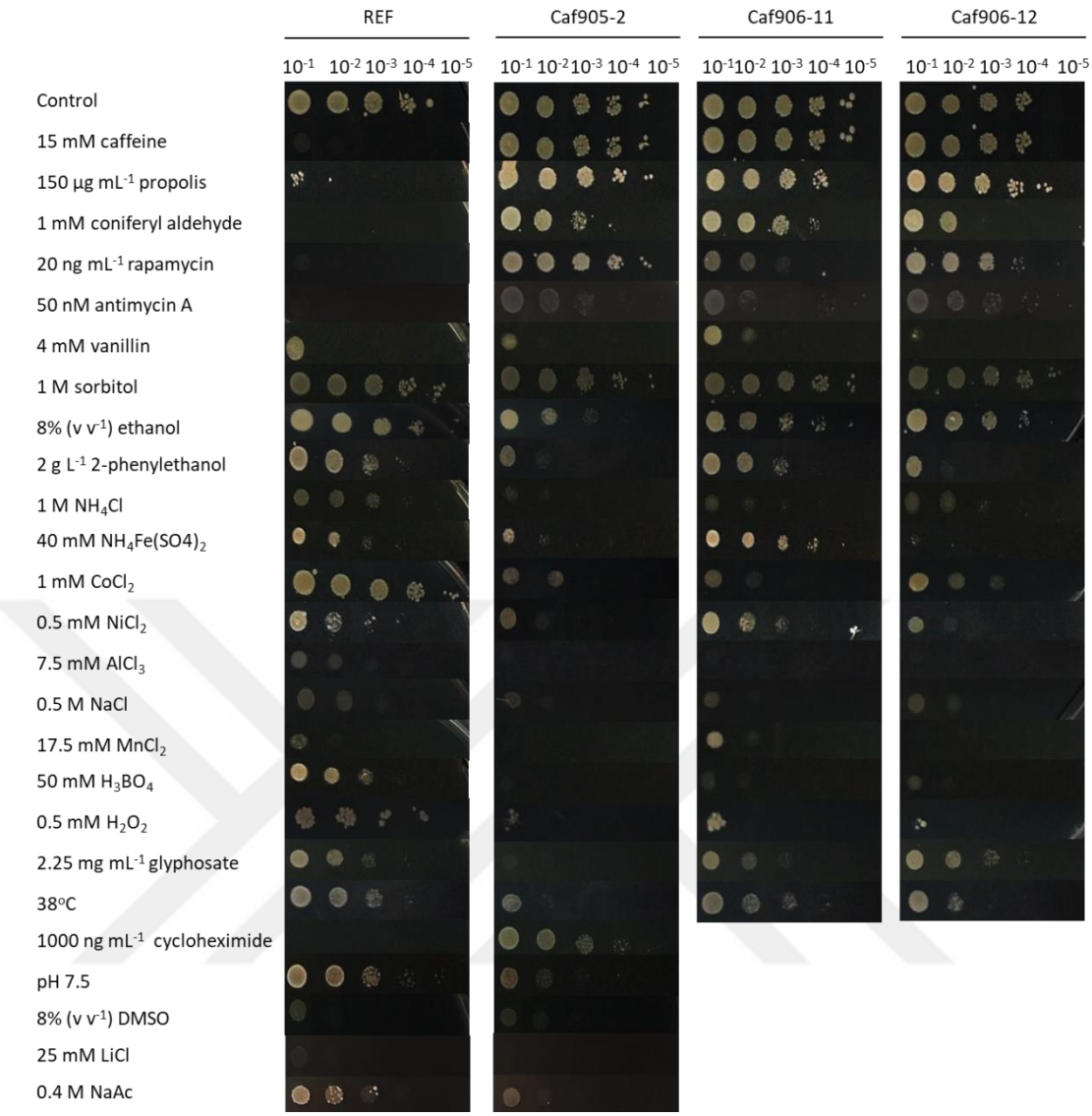

Figure 3.8 : Cross-resistance/sensitivity of selected genetically stable, evolved strains against different stress factors, relative to the reference strain (REF). Control: YMM, caffeine, propolis, coniferyl aldehyde, rapamycin, antimycin A, vanillin, sorbitol, ethanol, 2-phenyl ethanol, ammonium chloride (NH_4Cl), ammonium iron(III) sulfate ($NH_4Fe(SO_4)_2$), cobalt chloride ($CoCl_2$), nickel chloride ($NiCl_2$), aluminium chloride ($AlCl_3$), salt (NaCl), Manganese chloride ($MnCl_2$), boric acid (H_3BO_4), hydrogen peroxide (H_2O_2), glyphosate, heat, cycloheximide, mild alkaline pH (pH 7.5), lithium chloride (LiCl), sodium acetate (NaAc) and dimethyl sulfoxide (DMSO) at 72 h of incubation.

3.4 Physiological Analyses of the Caffeine-resistant Evolved Strain Caf905-2

3.4.1 Growth physiology, glucose consumption and metabolite production

Growth physiology and metabolite profiles of the caffeine-resistant evolved strain Caf905-2 and the reference strain were analyzed under 10 mM caffeine stress and nonstress conditions (Figure 3.9). (μ_{max}) of Caf905-2 (0.27 ± 0.01 h^{-1}) was about 3-fold of that of the reference strain (0.10 ± 0.01 h^{-1}) in the presence of 10 mM caffeine. Additionally, Caf905-2 entered the stationary phase of growth at 12 h; however, the reference strain entered after 30 h. On the other hand, μ_{max} values were 0.30 ± 0.01 h^{-1} and 0.31 ± 0.01 h^{-1} for Caf905-2 and the reference strain, respectively, under nonstress condition. Thus, there was no significant difference between the growth rates of the two strains under control conditions. At 30 h, OD_{600} for Caf905-2 and the reference strain reached their maximum values of 4.89 ± 0.05 and 5.98 ± 0.05, respectively, under nonstress condition. On the other hand, the maximum OD_{600} values for Caf905-2 and the reference strain at 48 h were 4.97 ± 0.14 and 5.25 ± 0.16, respectively, under 10 mM caffeine stress (Table 3.1 and Table C.1). The final biomass concentration or cell dry weight (cdw (mg mL^{-1})) of the Caf905-2 culture at 30 h of growth was, to some extent, higher than that of the reference strain, in the presence of 10 mM caffeine. Caffeine stress caused an increase in the biomass concentration in the reference strain and Caf905-2, respectively, at 30 h of growth. At this time, biomass concentrations of Caf905-2 and the reference strain were 1.88 ± 0.06 and 2.18 ± 0.03 in the absence of caffeine, and they both increased to more than 1.5-fold and 2-fold, in the presence of caffeine, respectively (Table 3.1). Metabolite analyses showed that the caffeine-resistant Caf905-2 did not reduce its glucose consumption and metabolite production (glycerol and ethanol) rates under 10 mM caffeine stress; however, the reference strain seemed to be strongly inhibited under caffeine stress (Figure 3.9a, 3.9b, and 3.9c). The ethanol yield was also reduced in the reference strain under caffeine stress, however, it did not change in Caf905-2. Surprisingly, caffeine stress did not cause any change in the final glycerol yield of the reference strain; however, it significantly reduced the final glycerol yield of Caf905-2. Under nonstress condition, the final glycerol yield in caffeine-resistant Caf905-2 was two-fold of that of the reference strain (Table 3.1).

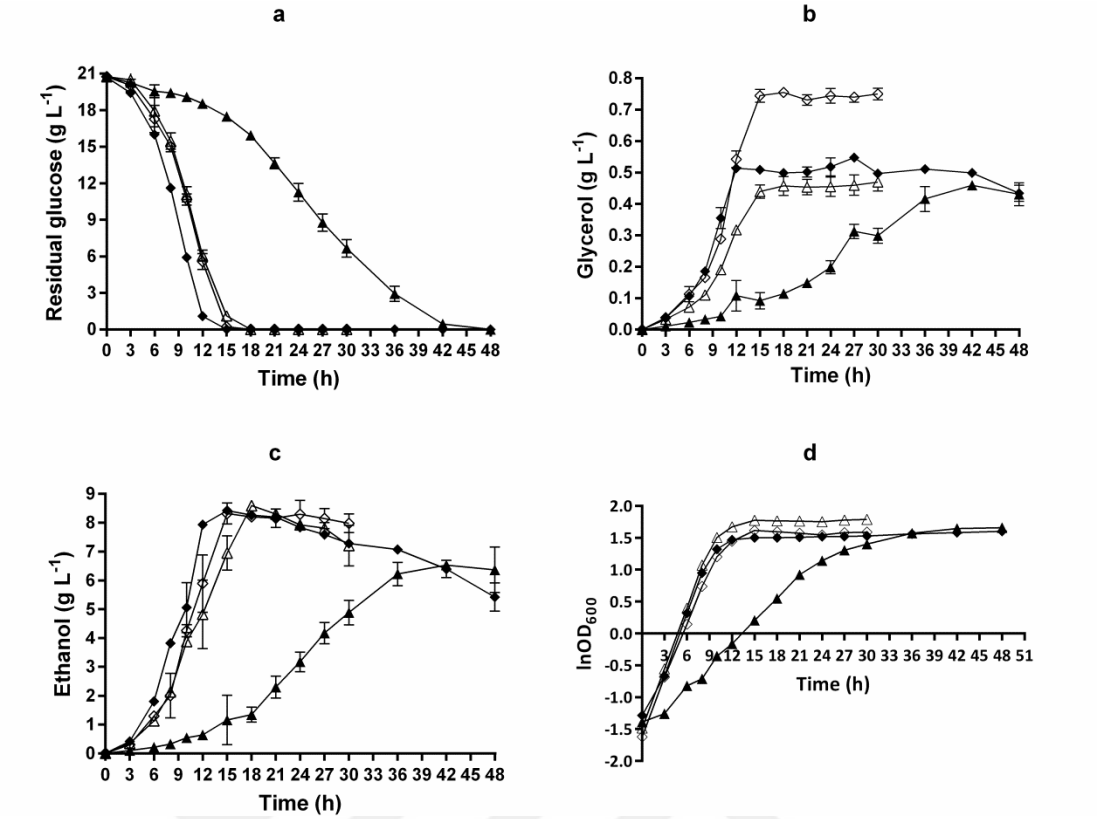

Figure 3.9 : Metabolite (a: residual glucose, b: glycerol, c: ethanol) and growth profiles (d) of the reference strain and the evolved strain Caf905-2 in the presence of 10 mM caffeine stress condition, as well as control (nonstress) condition. '◊' indicates caffeine-resistant Caf905-2 (no stress), '♦' indicates caffeine-resistant Caf905-2 under 10 mM caffeine stress, 'Δ' indicates the reference strain (no stress), and '▲' indicates the reference strain under 10 mM caffeine stress (Sürmeli et al., 2019).

Table 3.1 : Physiological parameters (maximum specific growth rate (μ_{max}), cell dry weight (cdw), ethanol and glycerol yields) during cell growth in 2% (w v^{-1}) glucose YMM, under caffeine-free and 10 mM caffeine stress conditions.

Strain	μ_{max} (h^{-1})	Biomass (cell dry weight) at 30h (mg cdw mL^{-1})	Ethanol yield (g g^{-1}glucose consumed)	Glycerol yield (g g^{-1} glucose consumed)
0mM caffeine				
Reference	0.31 ± 0.01	2.18 ± 0.03	0.430 ± 0.004	0.020 ± 0.002
Caf905-2	0.30 ± 0.01	1.88 ± 0.06	0.430 ± 0.020	0.040 ± 0.001
10 mM caffeine				
Reference	0.10 ± 0.01	3.53 ± 0.38	0.320 ± 0.040	0.020 ± 0.002
Caf905-2	0.27 ± 0.01	4.03 ± 0.25	0.420 ± 0.010	0.020 ± 0.001

3.4.2 Intracellular trehalose accumulation

The analysis of intracellular trehalose accumulation indicated that the reference strain accumulated more trehalose than Caf905-2, especially after 15 h of cultivation under caffeine-free condition (Figure 3.10a). Under 10 mM caffeine stress, the reference strain and Caf905-2 had similar levels of intracellular trehalose at about 12 and 15 h. However, Caf905-2 accumulated significantly higher amounts of trehalose than the reference strain at 21 and 30 h. Interestingly, the reference strain accumulated significantly higher amounts of trehalose than Caf905-2 after 30 h, until 48 h of cultivation (Figure 3.10b).

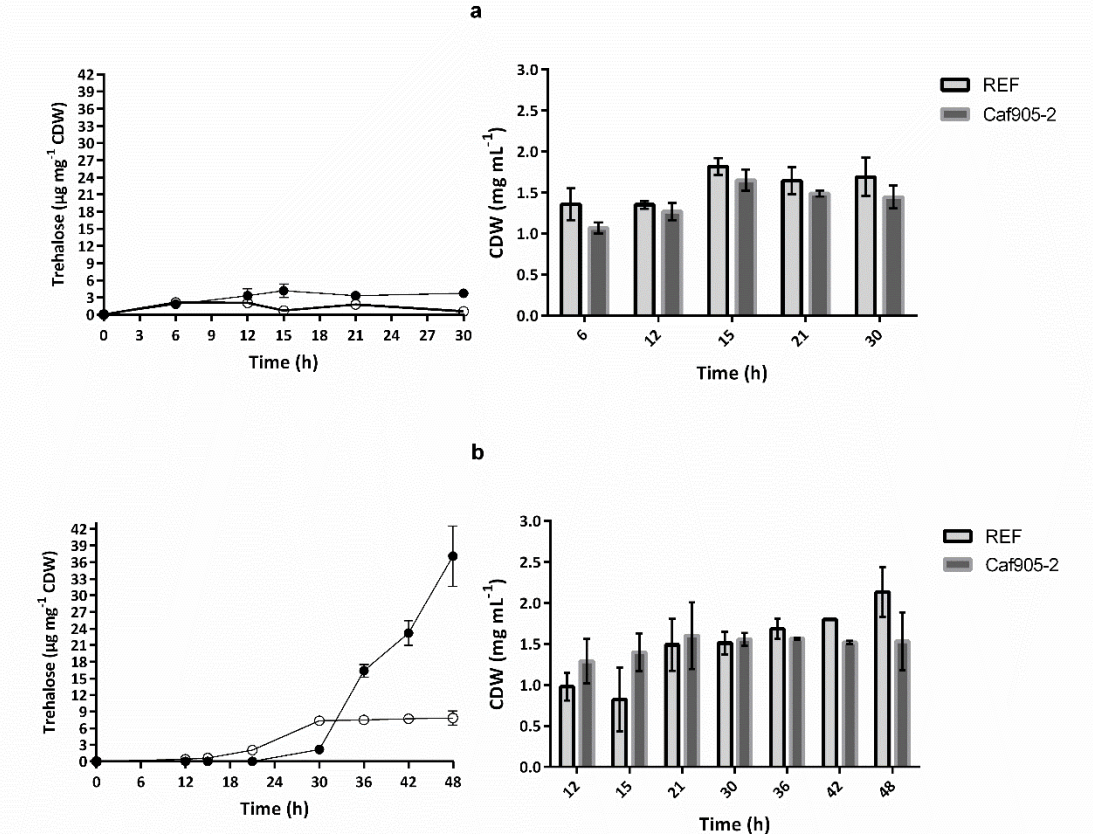

Figure 3.10 : Intracellular trehalose amounts and the cell dry weights (CDW) of caffeine-resistant Caf905-2 and the reference strain. **a.** under nonstress (control) condition. **b.** under 10 mM caffeine stress condition. '○' indicates caffeine-resistant Caf905-2, '●' indicates the reference strain (Sürmeli et al., 2019).

3.5 Lyticase susceptibility of the evolved strain Caf905-2

The cell wall integrity can be tested using the cell wall β-1,3-glucan-degrading enzymes such as lyticase or zymolyase, to investigate the effects of the cell wall damage agents like caffeine (Aguilar-Uscanga and François 2003; Kuranda et al.,

2006). The lysis of the cells can be followed spectrophotometrically, by the decrease in OD_{600} with time (Kuranda et al., 2006). The results of this assay indicated that stationary phase and early exponential phase cells of caffeine-resistant Caf905-2 resisted lyticase more than those of the reference strain, under nonstress condition. With the addition of 10 mM caffeine, lyticase resistance of the stationary phase and early exponential phase cells of the reference strain significantly increased, to a comparable level to that of Caf905-2. Different from the reference strain, the presence of 10 mM caffeine did not change the lyticase resistance of the evolved strain Caf905-2, compared to the nonstress condition (Figure 3.11a and 3.11b).

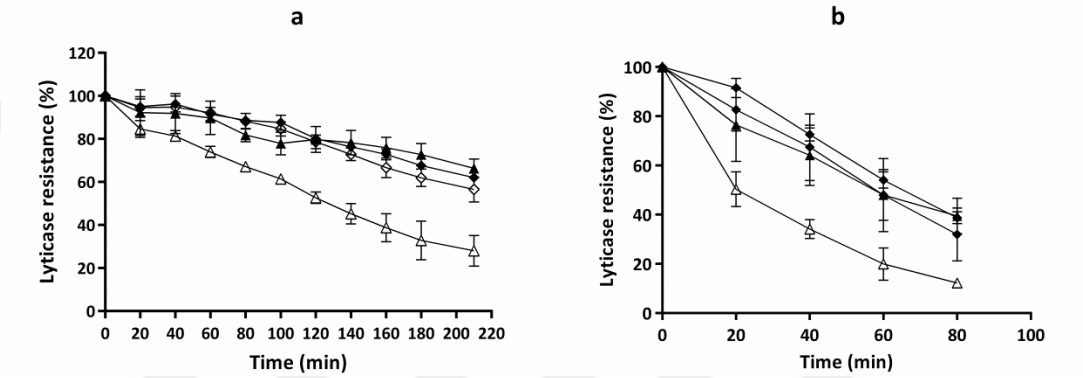

Figure 3.11 : Lyticase susceptibility of caffeine-resistant Caf905-2 and the reference strain in the presence of 10 mM caffeine stress and nonstress conditions. **a.** Lyticase susceptibility of stationary phase cells, **b.** Lyticase susceptibility of early exponential phase cells '◊' indicates caffeine-resistant Caf905-2 (no stress), '♦' indicates caffeine-resistant Caf905-2 under 10 mM caffeine stress, 'Δ' indicates the reference strain (no stress), and '▲' indicates the reference strain under 10 mM caffeine stress.

3.6 Whole Genome Transcriptomic Analysis Results

Comparative whole genome transcriptomic analysis was carried out for the caffeine-resistant mutant Caf905-2 and the reference strain by DNA microarray to investigate the molecular mechanisms underlying caffeine resistance. The analysis results elucidated that, among genes with at least two-fold expression change, 745 Open Reading Frames (ORF) were upregulated and 741 ORF were downregulated in Caf905-2, compared to the reference strain, under nonstress conditions. *FunCat* database (Ruepp et al., 2004) was used to categorize the functional classes of the upregulated and downregulated genes of caffeine-resistant Caf905-2 and to assess their biological significance**.**

3.6.1 Functional categories of the upregulated genes in Caf905-2

The significantly upregulated genes were highlighted in the main functional categories of 'Metabolism', 'Energy', 'Protein Fate', 'Protein with Binding Function or Cofactor Requirement', and 'Cell Rescue, Defence and Virulence'. Each main functional category of at least two-fold upregulated genes was divided into different numbers of categories which are biologically significant. Within the 'Metabolism' category, there were two subcategories as "C-compound and carbohydrate metabolism" and "Secondary metabolism". The former included 16.7% of at least two-fold upregulated genes, whereas the latter category contained 4.93%. Both were 1.4-fold enrichment categories. 'Energy' category was divided into five different subcategories called "Glycolysis and gluconeogenesis" (3.42% of the upregulated genes), "Pentose-phosphate pathway" (1.23% of the upregulated genes), "Electron transport and membrane-associated energy conservation" (3.83% of of the upregulated genes), "Aerobic respiration" (3.01% of of the upregulated genes), and "Metabolism of energy reserves (e.g. glycogen, trehalose)" (3.01% of of the upregulated genes). Except "Aerobic respiration", all had at least two-fold enriched genes. In addition, the 'Protein Fate' main category was composed of "Protein folding and stabilization" (4.24%), "Modification by ubiquitination, deubiquitination" (3.28%), "Modification by ubiquitin-related proteins" (1.36%), "Protein processing (proteolytic)" (3.42%), and "cytoplasmic and nuclear protein degradation" (5.89%). Among these, only "Protein folding and stabilization" subcategory had at least two-fold enriched genes. 'Protein with Binding Function or Cofactor Requirement' category contained just one biological significance subcategory which is "Complex cofactor/cosubstrate/vitamin binding", including 4.93% of the upregulated genes and with 1.5-fold enrichment. The final main category of the upregulated genes, 'Cell Rescue, Defence and Virulence', was composed of "Stress response" and "Oxygen and radical detoxification" subcategories, which correspond to 18.4% and 1.36% of the upregulated genes, respectively. The latter contained at least two-fold enriched genes (Table 3.2).

Table 3.2 : Main functional categories of at least two-fold upregulated genes in the evolved strain Caf905-2, relative to the reference strain, based on *FunCat* analysis outputs (Sürmeli et al., 2019).

	Functional category	Count[a]	%[b]	Fold Enrichment[c]	p-value
Metabolism	C-compound and carbohydrate metabolism	122	16.7	1.4	$1.34x10^{-4}$
	secondary metabolism	36	4.93	1.4	$3.29x10^{-2}$
Energy	glycolysis and gluconeogenesis	25	3.42	2.1	$1.75x10^{-4}$
	pentose-phosphate pathway	9	1.23	2.5	$8.16x10^{-3}$
	electron transport and membrane-associated energy conservation	27	3.83	2.1	$7.91x10^{-5}$
	aerobic respiration	21	3.01	1.5	$2.73x10^{-2}$
	metabolism of energy reserves (e.g. glycogen, trehalose)	22	3.01	2.4	$5.06x10^{-5}$
Protein Fate	protein folding and stabilization	31	4.24	2	$1.34x10^{-4}$
	modification by ubiquitination, deubiquitination	24	3.28	1.5	$3.16x10^{-2}$
	modification by ubiquitin-related proteins	10	1.36	1.9	$2.85x10^{-2}$
	protein processing (proteolytic)	25	3.42	1.6	$1.02x10^{-2}$
	cytoplasmic and nuclear protein degradation	43	5.89	1.5	$5.77x10^{-3}$
Protein with Binding Function or Cofactor Requirement	complex cofactor/cosubstrate/vitamin binding	36	4.93	1.5	$1.08x10^{-2}$
Cell Rescue, Defense and Virulence	stress response	135	18.4	1.4	$2.06x10^{-6}$
	oxygen and radical detoxification	10	1.36	2.5	$4.98x10^{-3}$

[a] number of query genes found in each functional category .
[b] percentage of the involved genes among the total genes in the query.
[c] ratio of frequencies of the query and the reference gene set.

3.6.1.1 At least two-fold enriched upregulated genes in Caf905-2

At least two-fold enriched upregulated genes were analyzed, according to the results obtained from the *FunCat* database. Based on this analysis, 26% of the genes in "Electron transport and membrane-associated energy conservation" were upregulated. This category mainly contains the subunits of cytochrome c oxidase (*COX20*, *COX9*, *COX13*, *COX5b*, *COX12*, *COX7*), subunits of the ubiqunol-cytochrome-c reductase (Complex III) (*QCR10*, *QCR7*, *QCR9*, *QCR8*), subunits of the F0 sector of mitochondrial F1F0 ATP synthase (*ATP17*, *ATP19*), and cytochrome

isoforms (*CYC1* and *CYC7*). *CYC7* and *TMA10* genes in "Electron transport and membrane-associated energy conservation" were the two most upregulated genes among all genes that were at least two-fold upregulated, (approximately 222-fold and 153-fold, respectively) in Caf905-2. Also, 30% of the genes from the "Pentose-phosphate pathway", category were upregulated. The highly overexpressed *NQM1* and *PGM2* genes (about 27-fold and 20-fold, respectively) are placed in this functional category. It also includes *SOL4* and *GND2* associated with the oxidative branch of the pentose phosphate pathway. In addition, 30% of the genes placed in the "Metabolism of energy reserves" functional category were induced. This functional category consists of the genes from trehalose (*NTH1, ATH1, TSL1, TPS3*), glycogen metabolism (*GSY1, GSY2, GLC3, GPH1, GDB1*), and maltose metabolism (*MAL32, MAL12, IMA1, IMA2, IMA3, IMA4, IMA5*). 26% of the genes from "Glycolysis and gluconeogenesis" category were also induced. This includes the highly overexpressed gene encoding hexokinase isoenzyme 1 (*HXK1*). Furthermore, 30.3% of the genes were within the functional category of "Oxygen and radical detoxification". This functional category contains the genes related to glutathione (*GPX1, GPX2* and *GRX1*) and the genes associated with thioredoxin (*PRX1, TRX2* and *TSA2*). Within the "Protein folding and stabilization" functional category, heat shock protein (HSP)-related genes are found. Some genes are placed in more than one functional categories. For example, *UGP1* gene encoding UDP-glucose pyrophosphorylase (UGPase) is found in "Metabolism of energy reserve" and "Glycolysis and gluconeogenesis" functional categories, whereas *PGM2* encoding phosphoglucomutase is placed in "Pentose phosphate pathway (PPP)", in addition to the above-mentioned two categories. *HSP82* encoding Hsp90 chaperon is placed within the functional categories of "Glycolysis and gluconeogenesis" and "Protein folding and stabilization". *ZWF1* encoding glucose-6-phosphate dehydrogenase (G6PD), *YPL113C* encoding glyoxylate reductase, and *TKL2* encoding transketolase are found in "Glycolysis and gluconeogenesis" and "Pentose phosphate pathway (PPP)" categories. Additionally, *SOD1* encoding cytosolic copper-zinc superoxide dismutase is placed in "Oxygen and radical detoxification" and "Protein folding and stabilization" categories (Table E.1).

The pleiotropic drug resistance (PDR) family genes were upregulated in the category of "Drug/toxin transport", although the category was not indicated within the

enriched functional categories. This category contains four ATP-binding cassette (ABC) transporters placed in plasma membrane (*SNQ2, PDR5, PDR15,* and *YOR1*) as well as two transcription factors (*YRM1* and *YRR1*) implicated in multidrug resistance (Table E.1).

3.6.2 Functional categories of the downregulated genes in Caf905-2

The significantly downregulated genes were highlighted in the main functional categories of 'Metabolism', 'Transcription, 'Protein Synthesis, 'Protein with Binding Function or Cofactor Requirement', and 'Cellular Transport, Transport Facilities and Transport Routes'. Each main functional category of at least two-fold downregulated genes was divided into different numbers of categories which are biologically significant. Within the 'Metabolism' category, there were three subcategories called "Amino acid metabolism", "Purine nucleotide/nucleoside/nucleobase metabolism", and "Pyrimidine nucleotide/nucleoside/nucleobase metabolism". They included 7.47%, 4.15%, and 3.32% of the downregulated genes, respectively. The latter two subcategories contained at least two-fold enriched genes. 'Transcription' category was divided into three different subcategories as "RNA synthesis" (18.5% of the downregulated genes), "RNA processing" (22.2% of the downregulated genes), and "RNA modification" (5.4% of the downregulated genes). The latter two subcategories had at least two-fold enriched genes. In addition, 'Protein Synthesis' main category was composed of "Ribosome biogenesis" (17.7%), "Translation" (11%), "Translational control" (3.46%), and "Aminoacyl tRNA-synthetases" (2.21%) subcategories. All had at least two-fold enriched genes. 'Protein with Binding Function or Cofactor Requirement' category contained two biologically significant subcategories which are "RNA binding" and "Nucleotide/nucleoside/nucleobase binding", that include 14.8% and 17% of the downregulated genes, respectively. The "RNA binding" subcategory had at least 1.5-fold enrichment. The final main category of the downregulated genes in Caf905-2 was 'Cellular Transport, Transport Facilities and Transport Routes'. It consisted of "Ion transport" (5.81%), "Heavy metal ion transport (Cu^{+}, Fe^{3+}, etc.)" (2.49%), "Amino acid/amino acid derivatives transport" (1.93%), "Amine/polyamine transport" (0.96%), "Nucleotide/nucleoside/nucleobase transport" (1.24%), "Vitamin/cofactor transport" (1.52%), and "Transport facilities" (7.75%) subcategories. Among these, four subcategories (Heavy metal ion transport (Cu^{+},

Fe^{3+}, etc.), Amine/polyamine transport, Nucleotide/nucleoside/nucleobase transport, Vitamin/cofactor transport) contained at least two-fold enriched genes (Table 3.3).

Table 3.3 : Main functional categories of at least two-fold downregulated genes in the evolved strain Caf905-2, relative to the reference strain, based on *FunCat* analysis outputs (Sürmeli et al., 2019).

	Functional category	Count[a]	%[b]	Fold Enrichment[c]	p-value
Metabolism	amino acid metabolism	54	7.47	1.4	6.38×10^{-3}
	purine nucleotide/nucleoside/nucleobase metabolism	30	4.15	2	1.43×10^{-4}
	pyrimidine nucleotide/nucleoside/nucleobase metabolism	24	3.32	2.3	3.95×10^{-5}
Transcription	RNA synthesis	134	18.5	1.2	5.61×10^{-3}
	RNA processing	161	22.2	2.7	4.96×10^{-37}
	RNA modification	39	5.4	4.5	6.16×10^{-16}
Protein Synthesis	ribosome biogenesis	128	17.7	2.9	1.80×10^{-33}
	Translation	80	11	2.5	5.08×10^{-16}
	translational control	25	3.46	2	4.76×10^{-4}
	aminoacyl-tRNA-synthetases	16	2.21	3.1	2.24×10^{-5}
Protein with Binding Function or Cofactor Requirement	RNA binding	107	14.8	2.5	1.17×10^{-21}
	nucleotide/nucleoside/nucleobase binding	123	17	1.5	7.80×10^{-7}
Cellular Transport, Transport Facilities and Transport Routes	ion transport	42	5.81	1.7	2.96×10^{-4}
	heavy metal ion transport (Cu^{+}, Fe^{3+}, etc.)	18	2.49	2.2	9.88×10^{-4}
	amino acid/amino acid derivatives transport	14	1.93	1.6	4.09×10^{-2}
	amine / polyamine transport	7	0.96	2.4	2.10×10^{-2}
	nucleotide/nucleoside/nucleobase transport	9	1.24	2.5	7.59×10^{-3}
	vitamin/cofactor transport	11	1.52	2.8	1.27×10^{-3}
	transport facilities	56	7.75	1.3	1.47×10^{-2}

[a] number of query genes found in each functional category.
[b] percentage of the involved genes among the total genes in the query.
[c] ratio of frequencies of the query and the reference gene set.

3.6.2.1 At least two-fold enriched downregulated genes in Caf905-2

At least two-fold enriched downregulated genes were analyzed, according to the results obtained from the *FunCat* database. Based on the results of this analysis, many genes were placed in RNA processing, RNA modification, "Ribosome biogenesis", "Translation", "Translational control", "Aminoacyl tRNA-synthetases", and "RNA binding". In addition, 24% and 29% of the genes associated with purine and pyrimidine metabolism were downregulated, respectively. Those categories include *URA* gene clusters (*URA1*, *URA3*, *URA5*, *URA7*), *DAL* gene clusters (*DAL1*, *DAL2*, *DAL3*, and DAL7) related to urea metabolism, the genes (*PRS1* and *PRS3*) responsible for the synthesis of 5-phospho-ribosyl-1(alpha)-pyrophosphate (PRPP), and adenine metabolism-related genes (*ADE1*, *ADE12* and *AAH1*). In addition, 26% of the genes from the functional category of "Heavy metal ion transport" were highly repressed. This category includes the genes (*ARN1*, *SIT1*, *FTR1*, *AFT1*, *FET3*, *FET4*, *FRE4* and *FSF1*) associated with iron utilization and transport. Within this functional category, *PHO84* encoding the high-affinity inorganic phosphate (P_i) transporter was the most downregulated (by approximately 154-fold) gene among all at least two-fold downregulated genes. Furthermore, 29% of the genes placed in the functional category of "Amine/polyamine transport" were also repressed. This category contains *TPO2* and *TPO3* genes encoding polyamine transporter of the major facilitator superfamily. Within the functional category of "Nucleotide transport", 30% of the genes were repressed. In addition, 33% of the genes from the functional category of "Vitamin/cofactor transport" were highly downregulated. Within this category, *PHO3* and *PHO12*, encoding acid phosphatases, were found. *FUR4*, and *FUI1* genes were placed in "Amine/polyamine transport" and "Nucleotide transport" categories, whereas *FCY21*, *FCY2*, and *TPC1* genes were found in "Nucleotide transport" and "Vitamin/cofactor transport". Additionally, *THI7* gene encoding the plasma membrane transporter responsible for the uptake of thiamine, was found in three categories; "Amine/polyamine transport", "Nucleotide transport", and "Vitamin/cofactor transport" (Table E.2)**.**

3.6.3 Transcription factors with significant expression changes in Caf905-2

There were 38 transcription factors in Caf905-2 with expression level changes of at least two-fold of the reference strain. Of these, 22 were upregulated and 16 were downregulated, based on the YEASTRACT analysis results. Among the upregulated

transcription factors, there were *CIN5*, *YAP5* and *YAP6* belonging to yAP-1 family, and *SIP4* and *CAT8* genes related to carbon source responsive elements (CSREs). *HAP4* and *RPN4* were also among the upregulated transcription factors which are the global regulator of the respiratory gene expression, and the inducer of the proteasome gene expression, respectively. Some of the downregulated transcription factors were *GCN4*, an activator of amino acid biosynthetic genes, *GAT1*, an activator of nitrogen catabolite repression genes, *PPR1*, responsible for the positive regulation of *URA* family genes, acting on *de novo* pyrimidine biosynthesis, and *RPI1* which blocks the Ras-cAMP pathway.

3.7 Whole Genome Re-sequencing of the Caffeine-resistant Strain Caf905-2

To determine the variations in the genome of caffeine-resistant strain Caf905-2 relative to the reference strain, whole genome re-sequencing was carried out, using the integrated system of Ion S5 next-generation sequencing platform with the library prep platform Ion Chef. This generated 21.1 and 22.1 million reads for the genome of the reference strain and caffeine-resistant Caf905-2, respectively. The raw sequence reads of Caf905-2 and the reference strain had nearly 178× and 192× depth coverage, respectively. The genome of the caffeine-resistant mutant was aligned to the genome sequence of CEN.PK113-7D (Nijkamp *et al.* 2012), and a total of only three single nucleotide variations (SNVs) were found in the genome of Caf905-2, relative to the reference strain. These SNVs were located in three different genes (*PDR1*, *PDR5*, and *RIM8*), and each of them was intragenic, missense SNVs. The transcription factor *PDR1* had a transversion SNV (A2456T), where valine was replaced by aspartic acid (V819D). In addition, another transversion SNV was G2008T in the *PDR5* gene which encodes an ABC transporter. This transversion corresponded to A670S amino acid substitution, replacing alanine with serine. The last missense SNV was found in *RIM8*, as a single transition SNV (A1097G). It corresponded to Q366R amino acid substitution, replacing glutamine with arginine.

4. DISCUSSION

In this study, highly caffeine-resistant *Saccharomyces cerevisiae* mutants (Caf905-2, Caf906-11, and Caf906-12) were obtained using evolutionary engineering strategy, which is based on systematic application of successive batch cultivation under a selection pressure, namely the caffeine stress. The reference strain and the EMS-mutagenized population were used as the initial populations for selection, to obtain the evolved strains, Caf905-2, Caf906-11 and Caf906-12. To determine the cross-resistance of the evolved strains against various stress factors, semi-quantitative spot assay was applied to the evolved mutants and the reference strain CEN.PK 113-7D. To gain insight into the molecular mechanisms underlying caffeine resistance; comparative physiological, genomic and transcriptomic analyses were performed with the caffeine-resistant mutant Caf905-2.

To increase the genetic diversity of the initial population prior to selection experiments, random mutagenesis is usually applied to the reference strain (Sauer, 2001; Çakar et al., 2012). In general, chemical mutagens like ethyl methanesulfonate (EMS) are used to speed up, and increase the performance of the evolutionary selection process. This usually enables the evolved final populations at the end of the selection process to tolerate a higher stress level, as compared to the early populations of selection, or when no EMS mutagenesis was performed. By doing so, final *S. cerevisiae* populations with higher percent survival rates are usually obtained, rather than non-mutagenized populations, after completion of selection (Çakar et al., unpublished data). Surprisingly, the final population obtained from selection with the reference strain without EMS mutagenesis could tolerate very high levels of caffeine (50 mM), at a relatively high survival rate of about 60%. This population had a similar survival performance (about 60%) to that of the final population obtained from the EMS-mutagenized initial population under 50 mM caffeine stress (Tables B.1 and B.2). This finding could be an evidence for the mutagenic effect of high caffeine levels, as indicated in previous studies with bacteria, fungi, plants, and mammalian cells (Nehlig & Debry, 1994; Kihlman et al., 1971). In line with this

information, Kumar and Keserwani (2016) showed that caffeine leads to abnormality on chromosomes of root meristems of grass pea (*Lathyrus sativus* L.) (Kumar & Keserwani 2016). In addition, Saiardi et al. (2005) and Bentley et al. (1996) reported that caffeine blocks Rad3/Mec1 from fission yeast and Tel1 from budding yeast (Saiardi et al., 2005; Bentley et al., 1996). These phosphoinositide 3-kinase-related protein kinases are implicated in cell cycle checkpoints related to DNA damage. Therefore, their inhibition and/or disruption leads to susceptibility to cell to DNA-damaging agents (Pennaneach & Kolodner 2004; Sanchez et al., 1996; Vialard et al., 1998). ATM and ATM-related ATR in human, homologous to Tel1 and Mec1/Rad3, are blocked by caffeine, and this brings about susceptibility to DNA damage (Sanchez et al., 1996; Blasina et al., 1999; Sarkaria et al., 1999). Thus, the findings of this thesis work seem to confirm the mutagenic effect of caffeine at high concentrations. These findings indicate for the first time three specific mutations in *S. cerevisiae* that result from prolonged exposure to high caffeine levels using whole genome re-sequencing analysis.

It is well-documented that caffeine inhibits the cell growth of many organisms including human (Sandlie et al., 1980; Reinke et al., 2006; Kuranda et al., 2006; Rallis et al., 2013; Sasamoto et al., 2015; Merighi et al., 2007; Chen & Hwang, 2016). Accordingly, Kuranda et al. (2006) have shown that 8 mM caffeine is sufficient to decrease the maximum specific growth rate (μ_{max}) of *S. cerevisiae* by 50% (Kuranda et al., 2006). However, μ_{max} of the evolved strain Caf905-2 did not decrease under 10 mM caffeine stress, whereas the μ_{max} of the reference strain decreased by about three-fold (Table 3.1). Also, the evolved strain could maintain growth even in the presence of 50 mM caffeine, approximately exhibiting a survival rate of 60% (data not shown). Many studies about the improvement of caffeine resistance in yeast have been documented in the literature, by mutation or overexpression of different genes. For example, *tor1*Δ cells conferring *TOR1* (I1954V/W2176R) double mutant could grow in the presence of 20 mM caffeine (Reinke et al., 2006). The *rho5*Δ mutant could also resist 20 mM caffeine stress and is implicated in an increased activity of the protein kinase C (Pkc1p)-dependent signal transduction pathway (Schmitz *et al.* 2002). Furthermore, the mutants with the overexpression of *HSE1, RTS3, SDS23* and *SDS24* genes could grow under 15 mM caffeine stress condition (Hood-DeGrenier, 2011). Nevertheless, so far, there have

not been any studies reporting a yeast mutant that could resist very high caffeine concentrations, such as 50 mM (Figure 3.5).

According to our comparative genome re-sequencing analysis results, three single nucleotide polymorphisms (SNVs) were detected in only three genes in the evolved strain Caf905-2, which were *PDR1*, *PDR5* and *RIM8*. Pdr1p, a zinc-finger transcription factor (Akache & Turcotte, 2002), is present in homo- and heterodimer forms of Pdr1p/Pdr3p (Balzi et al., 1987; Delaveau et al., 1994; Mamnun et al., 2002), which are implicated in the promoter regulation of PDRE (pleiotropic drug resistance elements) genes through cis-acting elements (Wolfger et al., 1997; Mahé et al., 1996; Katzmann et al., 1994). *PDR1* gene in the evolved strain Caf905-2 conferred a transversion SNV of T2456A, corresponding to an amino acid substitution of V819D, which replaces valine with aspartic acid at the position of 819. So far, there have not been any report about this SNV in *PDR1*, even though many studies have been reported on SNVs in *PDR1* at proximate locations. For instance, Carvajal et al. (1997) have documented that yeast with a single amino acid substitution of F815S in *PDR1* had enhanced resistance against cycloheximide which inhibits protein synthesis, oligomycin which inhibits mitochondrial oxidative phosphorylation, ketoconazole which inhibits ergosterol biosynthesis and 4-nitroquinoline-N-oxide which is a chemical mutagen (Carvajal et al., 1997). Likewise, caffeine-resistant mutant Caf905-2 had, to a great extent, cross-resistance against cycloheximide, as well as to antimycin A that has a similar function and structure with oligomycin) (Figure 3.8). Surprisingly, it has been recently reported that an evolved *S. cerevisiae* mutant resistant to coniferyl aldehyde had a single amino acid substitution of C862Y in the *PDR1* gene, which was also found to be upregulated. This mutant also had cross-resistance to caffeine in addition to its resistance to various phenolic compounds (Hacısalihoğlu et al., 2019). Other cases of SNVs, with similar position to V819D of *PDR1* in Caf905-2, are involved with the resistance to different substances, including C10 alkane biofuels (F815S in *PDR1*) (Ling et al., 2015), hydrophilic like DMSO (R821S in *PDR1*) (Nishida et al., 2013) and hydrophobic like n-nonane and isooctane (R821S in *PDR1*) (Matsui et al., 2008) organic solvents. All SNVs in *PDR1* that have been documented previously, along with V819D in Caf905-2, are located on multiple inhibitory domains, which are extended between N-terminal DNA-binding and C-terminal activation domains. The

large multiple inhibitory domains repress Pdr1p activation (Kolaczkowska et al., 2002; Johnston et al., 1986; Balzi et al., 1987). By this way, the documented SNVs may have reduced this repression effect, induced *PDR1* and *PDR1*-regulated processes, and enhanced tolerance to various substances.

YEASTRACT enrichment analysis (Teixeira et al., 2018) results revealed that *PDR1* regulates about 31.4% of at least two-fold upregulated genes in Caf905-2, 17 of which were transcription factors. The results also showed that the hypoxic gene *CYC7* was upregulated approximately as high as 222-fold in the evolved Caf905-2 mutant (Table E.1). The *CYC7* gene, located in the chromosomes of the nucleus, encodes a protein of the terminal region of the electron transport chain in mitochondria. *CYC7*, a hypoxic gene, is well expressed under microaerophilic or anoxic conditions, and not transcribed below 0.5 mM O_2 concentration (Burke et al., 1997). Antimycin A (complex III inhibitor in mitochondrial respiration system) exposure gave rise to an increase in the transcription of the hypoxic gene *CYC7* in *S. cerevisiae* (Liu & Barrientos, 2013). Given that the evolved strain Caf905-2 had significant cross-resistance to antimycin A stress (Figure 3.8), the highly upregulated hypoxic gene *CYC7* may play a key role in caffeine and antimycin A resistance, influencing the energy metabolism in mitochondria. In addition, *PDR1* regulates many genes of 'Metabolism of energy reserves' category in the transcriptomic data of the caffeine-resistant Caf905-2 (Table E.1). They are *GLC7, GLC3*, *GSY2* and *GAC1* genes associated with glycogen synthesis, and *GDB1* and *GPH1* implicated in glycogen degradation (François & Parrou, 2001). Likewise, the *TSL1* and *TPS3* genes related to trehalose synthesis and *ATH1* and *NTH1* implicated in trehalose degradation (François & Parrou, 2001) were also upregulated in Caf905-2. Those results are correlated with previous studies about other evolved *S. cerevisiae* strains, such as coniferyl aldehyde-resistant (Hacısalihoğlu et al., 2019), chronologically long-lived (Arslan et al., 2018), ethanol-resistant (Turanlı-Yıldız et al., 2017), and nickel-resistant (Küçükgöze et al., 2013) mutants, where a great number of the genes related to trehalose and glycogen metabolism were also found to be upregulated. This indicates that a highly active glycogen and trehalose metabolism may be considered as a common critical factor in yeast tolerance to different stress types. Also, previous reports have shown that caffeine and rapamycin commonly activate genes associated with trehalose and glycogen metabolism (Kuranda et al., 2006; Hardwick et al.,

1999). Those previous reports were supported by the findings of this thesis work, where the evolved strain Caf905-2 had, to great extent, cross resistance against rapamycin (Figure 3.8). YEASTRACT analysis results of Caf905-2 revealed that *PDR1* controls many pleiotropic drug resistance (PDR) genes, which are placed in 'Drug/toxin transport' subcategory (Table E.1). It is well documented that ABC proteins are implicated in pleiotropic drug resistance (PDR) (Kolaczkowski et al., 1998; Mahé et al., 1996; Egner et al., 1998; Cui et al., 1998; Lucau-Danila et al., 2003). Accordingly, Tsujimoto et al. (2015) have demonstrated that *SNQ2* and *PDR5* are the genes related to caffeine resistance in *S. cerevisiae*, pumping caffeine out of the cell by the action of their products which are the multidrug resistance transporters Snq2p and Pdr5p, respectively (Tsujimoto et al., 2015). Previous reports have documented that *SNQ2* gene is overexpressed by the exposure of *S. cerevisiae* to caffeine (Kuranda et al., 2006), coniferyl aldehyde (Sundström et al., 2010), and propolis (de Castro et al., 2012) stress. Given that the evolved strain Caf905-2 showed cross-resistance against propolis and coniferyl aldehyde stress (Figure 3.8), and a coniferyl aldehyde-resistant *S. cerevisiae* mutant was also, to a great extent, cross-resistant against caffeine stress, along with the upregulated *SNQ2* gene (Hacısalihoğlu et al., 2019); *SNQ2* may commonly be a factor that is implicated in resistance to caffeine, coniferyl aldehyde, and propolis.

The second SNV in the caffeine-resistant evolved strain was another transversion SNV G2008T in *PDR5* gene, replacing alanine with serine at the position of 670 (A670S). *PDR5* gene plays a role in yeast multidrug resistance, and it is controlled via Pdr1p transcription factor encoded by *PDR1* gene (Balzi et al., 1987; Balzi et al., 1994). Balzi et al. (1994) have reported that mutations in *PDR1* caused multidrug resistance and a highly increased expression of Pdr5p, a cytoplasmic membrane protein in *S. cerevisiae* (Balzi et al., 1994). A recent study has shown that *PDR5* and *SNQ2* genes are determined as two important factors to gain caffeine resistance. According to this study, based on the screening of the genomic library, *SNQ2* had a stronger effect than *PDR5* in conferring caffeine resistance. Also, *S. cerevisiae* strains with the overexpression of *PDR5* and *SNQ2* showed resistance against 20 mM caffeine (Tsujimoto et al., 2015). A670S in the caffeine-resistant mutant Caf905-2 is located in the transmembrane helix 5 (TMH5), belonging to the transmembrane domain 1 (TMD1) in Pdr5p protein (Golin & Ambudkar, 2015;

Rutledge et al., 2011). Surprisingly, a previous study reported three cycloheximide-tolerant *S. cerevisiae* strains each of which had a single amino acid substitution in *PDR5* gene, and one of them was A670S (Downes et al., 2013). This is the same amino acid substitution with that in the evolved strain Caf905-2 conferring, to a great extent, cycloheximide resistance (Figure 3.8). Thus, the A670S amino acid substitution found in *PDR5* gene may be implicated in cycloheximide and caffeine resistance. Also, Sauna et al. (2008) reported that amino acid substitution of S558Y in transmembrane helix 2 (TMH2) of Pdr5p gives rise to susceptibility to drugs like cycloheximide, and eliminates cycloheximide resistance mediated by Pdr5p.

The evolved strain Caf905-2 also had a transition SNV (A1097G) in the *RIM8* gene, replacing glutamine with arginine at the position of 366 (Q366R). This amino acid substitution is located in the C-terminal domain (residue 259 to 394) of Rim8p protein, which is an α-arrestin family protein that plays a critical role in the Rim101 pathway (Smardon & Kane, 2014; Velivela & Kane, 2018). Some documents have shown that a variety of *RIM8* mutations cause salt susceptibility. In this regard, double (P506A/Y508A), single (K521R), or deletion *rim8* mutants confer sensitivity to sodium acetate (Watcharawipas et al. 2017). Furthermore, deletion of *RIM8* has been also associated with sensitivity to sodium chloride and lithium chloride. In fact, this mutant was neither resistant nor sensitive to sorbitol; however, it was susceptible to alkaline pH (Marques et al. 2015). In addition, two other studies revealed lithium chloride sensitivity in the case of the deletion of *RIM8* and S76P mutation in *RIM8*, respectively (Hayashi et al. 2005; Herrador et al. 2015). In line with the literature, Caf905-2 was susceptible to lithium chloride, sodium chloride, sodium acetate, as well as mild alkaline pH stresses, also neither resistant nor sensitive to sorbitol stress (Figure 3.8).

It is well known that the Rim101 signaling pathway is implicated in cell wall assembly (Li & Mitchell 1997; Castrejon et al. 2006). In the Rim101 pathway, Rim21p, which interacts with Rim8p, plays a role as transmembrane helix protein, whereas Rim101p acts on *RIM8* as transcriptional repressor. In this regard, deletion of Rim101 and Rim21 give rise to zymolyase and caffeine susceptibilities. Each deletion brings about deficiency of cell wall in yeast, resulting in caffeine and zymolyase sensitivities (Castrojen et al. 2006). The present study indicated that the evolved strain Caf905-2 was also lyticase-resistant (Figure 3.11). Caffeine and

zymolyase are cell wall-disrupting compounds, as documented in literature (Ferreira et al. 2006; Kuranda et al. 2006); thus, the zymolyase or lyticase resistance of the caffeine-resistant strain Caf905-2 is not surprising.

5. CONCLUSION

This thesis study showed that evolutionary engineering is a strong strategy to develop or improve complex phenotypes including stress resistance. This strategy allowed the isolation of three genetically stable, caffeine-hyperresistant *S. cerevisiae* strains, both with and without using physical or chemical mutagens, prior to the selection procedure. Among the three caffeine-resistant strains selected, Caf905-2 has been evolved without using a mutagen, due to the mutagenic effect of high caffeine concentrations. All of the isolated caffeine-resistant strains could survive very high concentrations of caffeine (50 mM) that have not been documented so far for *S. cerevisiae*. The evolved strain Caf905-2 did not have any decrease in its growth rate under 10 mM caffeine stress, usually an inhibitory concentration for yeast. The results of the comparative whole genome transcriptome and re-sequencing analyses identified key molecular factors, especially the pleiotropic drug resistance and cell wall assembly/integrity-linked genes (*PDR1, PDR5* and *RIM8*) that may play a critical role in caffeine-hyperresistance. Even though the importance of *PDR5*, regulated by Pdr1p, has been shown in yeast caffeine resistance (as high as 20 mM) using genomic library screening (Tsujimoto *et al.* 2015), this is the first and only report so far on a caffeine hyper-resistant, evolved yeast strain (50 mM), with only three SNVs in three genes: *PDR1, PDR5* and *RIM8*. Three SNVs belonging to this caffeine hyper-resistant mutant that has been obtained by evolutionary engineering, and probably by the mutagenic effect of prolonged caffeine stress at high levels during selection, are yet to be investigated in detail as a future work. In this respect, functional genomic analyses could help reveal the combined and individual roles of *PDR1, PDR5* and especially of *RIM8* in caffeine-hyperresistance trait.

REFERENCES

Aguilar-Uscanga B. & François J. (2003) A study of the yeast cell wall composition and structure in response to growth conditions and mode of cultivation. *Lett. Appl. Microbiol. 37*:268-274.

Akache B. & Turcotte B. (2002). New regulators of drug sensitivity in the family of yeast zinc cluster proteins. *J. Biol. Chem*. *277*(24):21254-21260.

Arab J.B. & Blumberg L. (2008). Introduction to the proceedings of the fourth international scientific symposium on tea and human Health. *J. Nutr*. 138:1526-1528

Arslan M., Holyavkin C., Kısakesen H.I., Topaloğlu A., Sürmeli Y. & Çakar Z.P. (2018). Physiological and transcriptomic analysis of a chronologically long-lived *Saccharomyces cerevisiae* strain obtained by evolutionary engineering. *Mol. Biotechnol*. *60*:468-474.

Asano Y., Komeda T. & Yamada H. (1994). Enzymes involved in theobromine production from caffeine by *Pseudomonas putida* No. 352. *Biosci. Biotechnol. Biochem*. *58*(12):2303-2304

Ashihara H., Monteiro A.M., Gillies F.M. & Crozier A. (1996). Biosynthesis of caffeine in leaves of coffee. *Plant Physiol*. *111*(3):747-753.

Ashihara H. & Crozier A. (1999). Biosynthesis and metabolism of caffeine and related purine alkaloids in plants. *Adv. Bot. Res. 30*:118-205.

Ashihara H., Sano H. & Crozier A. (2008). Caffeine and related purine alkaloids: Biosynthesis, catabolism, function and genetic engineering. *Phytochemistry*. *69*(4):841-856.

Bailey J.E. (1991). Toward a science of metabolic engineering. *Science*. *252*(5013):1668-1675.

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

Balzi E., Chen W., Ulaszewski S., Capieaux E. & Goffeau A. (1987). The multidrug resistance gene *PDR1* from *Saccharomyces cerevisiae*. *J. Biol. Chem. 262*(35):16871-16879.

Balzi E., Wang M., Leterme S., Van Dyck L. & Goffeau A. (1994). *PDR5*, a novel yeast multidrug resistance conferring transporter controlled by the transcription regulator *PDR1*. *J. Biol. Chem. 269*(3):2206-2214

Bentley N.J., Holtzman D.A., Flaggs G., Keegan K.S., DeMaggio A., Ford J.C., … & Jarr A.M. (1996). The *Schizosaccharomyces pombe rad3* checkpoint gene. *EMBO J*. *15*(23):6641-6651.

Bergman L.W. (2001). Growth and maintenance of yeast. *Methods Mol. Biol. 177*:9-14.

Blasina A., de Weyer I.V., Laus M.C., Luyten W.H., Parker A.E. & McGowan C.H. (1999). A human homologue of the checkpoint kinase Cds1 directly inhibits Cdc25 phosphatase. *Curr. Biol. 9*(1):1-10.

Bolger A.M., Lohse M. & Usadel B. (2014). Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics.30*:2114-2120.

Bonati M., Latini R., Tognoni G., Young J.F. & Garattini S. (1984). Interspecies comparison of in vivo caffeine pharmacokinetics in man, monkey, rabbit, rat, and mouse. *Drug. Metab. Rev. 15*(7):1355-1383

Burke P.V., Raitt D.C., Allen L.A., Kellogg E.A. & Poyton R.O. (1997). Effects of oxygen concentration on the expression of cytochrome c and cytochrome c oxidase genes in yeast. *J. Biol. Chem. 272*(23):14705-14712.

Calvo I.A., Gabrielli N., Iglesias-Baena I., García-Santamarina S., Hoe K.L., Kim D.U., … Hidalgo E. (2009). Genome-wide screen of genes required for caffeine tolerance in fission yeast. *PLoS One. 4*(8):e6619.

Carrillo J.A. & Benitez J. (2000). Clinically significant pharmacokinetic interactions between dietary caffeine and medications. *Clin. Pharmacokinet. 39*:127-153.

Carvajal E., van den Hazel H.B., Cybularz-Kolaczkowska A., Balzi E. & Goffeau A. (1997). Molecular and phenotypic characterization of yeast *PDR1* mutants that show hyperactive transcription of various ABC multidrug transporter genes. *Mol. Gen. Genet. 256*(4):406-415.

Castrejon F., Gomez A., Sanz M., Duran A. & Roncero C. (2006). The RIM101 pathway contributes to yeast cell wall assembly and its function becomes essential in the absence of mitogen-activated protein kinase Slt2p. *Eukaryot. Cell. 5*(3):507-517

Chen J.-C. & Hwang J.-H. (2016). Effects of caffeine on cell viability and activity of histone deacetylase 1 and histone acetyltransferase in glioma cells. *Ci Ji Yi Xue Za Zhi. 28*(3):103-108.

Chou C.H. & Waller G.R. (1980). Possible allelopathic constituents of *Coffea arabica*. *J. Chem. Ecol. 6*:643-654.

Cui Z., Shiraki T., Hirata D. & Miyakawa T. (1998). Yeast gene *YRR1*, which is required for resistance to 4-nitroquinoline N-oxide, mediates transcriptional activation of the multidrug resistance transporter gene *SNQ2*. *Mol. Microbiol. 29*(5):1307-1315.

Çakar Z.P., Alkım C., Turanlı B., Tokman N., Akman S., Sarıkaya M., … & Francois J.M. (2009). Isolation of cobalt hyper-resistant mutants of *Saccharomyces cerevisiae* by *in vivo* evolutionary engineering approach. *J. Biotechnol. 143*:130-138.

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

Çakar Z.P., Turanlı-Yıldız B., Alkım C. & Yılmaz U. (2012). Evolutionary engineering of *Saccharomyces cerevisiae* for improved industrially important properties. *FEMS Yeast Res.12*:171-182.

Daly J.W. (1993). Mechanism of action of caffeine. In: Garattini S, editor. *Caffeine, coffee, and health.* New York: Raven Press Ltd., 97-150

De Castro P.A., Savoldi M., Bonatto D., Malavazi I., Goldman M.H., Berretta A.A. & Goldman G.H. (2012). Transcriptional profiling of *Saccharomyces cerevisiae* exposed to propolis. *BMC Complement Altern. Med. 12*:194.

De Virgilio C. & Loewith R. (2006). Cell growth control: little eukaryotes make big contributions. *Oncogene*. *25*:6392-6415.

Delaveau T., Delahodde A., Carvajal E., Subik J. & Jacq C. (1994). *PDR3*, a new yeast regulatory gene, is homologous to *PDR1* and controls the multidrug resistance phenomenon. *Mol. Gen. Genet. 244*(5):501-511.

DePristo M.A., Banks E., Poplin R., Garimella K.V., Maguire J.R., Hartl C., … & Daly M.J. (2011). A framework for variation discovery and genotyping using next-generation DNA sequencing data. *Nat. Genet. 43*:491-498.

Dews P.B. (1984). *Caffeine: perspectives from recent research*. 1st edition. Springer-Verlag Berlin Heidelberg**.**

Divate N.R., Chen G.H., Wang P.M., Ou B.R. & Chung Y.C. (2016). Engineering *Saccharomyces cerevisiae* for improvement in ethanol tolerance by accumulation of trehalose. *Bioengineered. 7*:445-458.

Downes M., Mehla J., Ananthaswamy N., Wakschlag A., LaMonde M., Dine E., … & Golin J. (2013). The transmission interface of the *Saccharomyces cerevisiae* multidrug transporter Pdr5: Val-656 located in intracellular loop 2 plays a major role in drug resistance. *Antimicrob. Agents Chemother*. *57*:1025-1034.

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

Egner R., Rosenthal F.E., Kralli A., Sanglard D. & Kuchler K. (1998). Genetic separation of FK506 susceptibility and drug transport in the yeast Pdr5 ATP-binding cassette multidrug resistance transporter. *Mol. Biol. Cell. 9*(2):523-543.

Entian K.D. & Kötter P. (2007). Yeast genetic strain and plasmid collections. *Methods Microbiol*. *36*:629-666.

Esslinger H.M. (2009). *Handbook of brewing: processes, technology, markets*. Weinheim, Wiley-VCH.

Ferré S. (2013). Caffeine and substance use disorders. *J. Caffeine Res*. *3*(2):57-58.

Ferreira C., Silva S., van Voorst F., Aguiar C., Kielland-Brandt M.C., Brandt A. & Lucas C. (2006). Absence of Gup1p in *Saccharomyces cerevisiae* results in defective cell wall composition, assembly, stability and morphology. *FEMS Yeast Res. 6*(7):1027-1038.

Fisone G., Borgkvist A. & Usiello A. (2004). Caffeine as a psychomotor stimulant: mechanism of action. *Cell. Mol. Life Sci. 61*:857-872.

François J. & Parrou J.L. (2001). Reserve carbohydrates metabolism in the yeast *Saccharomyces cerevisiae*. *FEMS Microbiol. Rev. 25*:125-145.

Fredholm B.B., Battig K., Holmen J., Nehlig A. & Zvartau E.E. (1999). Actions of caffeine in the brain with special reference to factors that contribute to its widespread use. *Pharmacol. Rev. 51*:83-133.

Friedman, J. & Waller G.R. (1983). Caffeine hazards and their prevention in germinating seeds of coffee (*Coffea arabica L.*). *J. Chem. Ecol*. *9*:1099-1106.

Goffeau A., Barrell B.G., Bussey H., Davis R.W., Dujon B., Feldmann H., … & Oliver S.G. (1996). Life with 6000 genes. *Science*. *274*(5287):546-567.

Gokulakrishnan S., Chandraraj K. & Gummadi S.N. (2005). Microbial and enzymatic methods for the removal of caffeine. *Enzyme Microb. Technol*. *37*:225-232.

Golin J. & Ambudkar S.V. (2015). The multidrug transporter Pdr5 on the 25th anniversary of its discovery: an important model for the study of asymmetric ABC transporters. *Biochem. J. 467*(3):353-363.

Griffiths R.R., Juliano L.M. & Chausmer A.L. (2003). *Caffeine pharmacology and clinical effects*. In: Graham A.W., Schultz T.K., Mayo-Smith M., Ries R.K. & Wilford B.B. (eds). *Principles of Addiction Medicine*. 3rd edn. American Society of Addiction Medicine, Chevy Chase, pp 193-224.

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

Hahn-Hägerdal B., Karhumaa K., Larsson C.U., Gorwa-Grauslund M., Görgens J., van Zyl W.H. (2005). Role of cultivation media in the development of yeast strains for large scale industrial use. *Microb. Cell Fact*. *4*:31.

Hardwick J.S., Kuruvilla F.G., Tong J.K., Shamji A.F. & Schreiber S.L. (1999). Rapamycin-modulated transcription defines the subset of nutrient-sensitive signaling pathways directly controlled by the Tor proteins. *Proc. Natl. Acad. Sci. 96*:14866-14870.

Hashimoto T., He Z., Ma W.-Y., Schmid P.C., Bode A.M., Yang C.S. & Dong Z. (2004). Caffeine inhibits cell proliferation by G_0/G_1 phase arrest in JB6 cells. *Cancer Res*. *64*(9):3344-3349.

Hayashi M., Fukuzawa T., Sorimachi H. & Maeda T. (2005). Constitutive activation of the pH-responsive Rim101 pathway in yeast mutants defective in late steps of the MVB/ESCRT pathway. *Mol. Cell Biol*. *25*(21):9478-9490.

Heckman M.A., Weil J. & De Mejia E.G. (2010). Caffeine (1, 3, 7-trimethylxanthine) in foods: a comprehensive review on consumption, functionality, safety, and regulatory matters. *J. Food Sci. 75*(3):77-87.

Herrador A., Livas D., Soletto L., Becuwe M., Léon S. & Vincent O. (2015). Casein kinase 1 controls the activation threshold of an α-arrestin by multisite phosphorylation of the interdomain hinge. *Mol. Biol. Cell. 26*(11):2128-2138.

Higginbotham E.J., Kilimanjaro H.A., Wilensky J.T., Batenhorst R.L & Herman D. (1989). The Effect of caffeine on intraocular pressure in glaucoma patients. *Ophthalmology*. *96*(5):624-626.

Hood-DeGrenier J.K. (2011). Identification of phosphatase 2A-like Sit4-mediated signaling and ubiquitin-dependent protein sorting as modulators of caffeine sensitivity in *S. cerevisiae*. *Yeast. 28*(3):189-204.

Ito E. & Ashihara H. (1999). Contribution of purine nucleotide biosynthesis de novoto the formation of caffeine in young tea (*Camellia sinensis*) leaves. *J. Plant Physiol*. *254*:145-151.

Jermini M.F.G., Geigen O. & Schmidt-Lorenz W. (1987). Detection, isolation and identification of osmotolerant yeasts from high- sugar products. *J. Food Prot. 50*:468-472.

Johnston S., Zavortink M., Debouck C. & Hopper J. (1986). Functional domains of the yeast regulatory protein GAL4. *Proc. Natl. Acad. Sci. 83*:6553-6557.

Juddy A.U., Esenwah E.C., Ikoro N.C., Azuamah Y.C., George G.O., Okorie M.E., … & Nwakamma G. (2014). Effect of caffeinated coffee on tear production. *Int. J. Res*. *1*(9):1264-1268.

Katzmann D.J., Burnett P.E., Golin J., Mahé Y. & Moye-Rowley W.S. (1994). Transcriptional control of the yeast *PDR5* gene by the *PDR3* gene product. *Mol. Cell Biol. 14*(7):4653-61.

Kihlman B.A., Odmark G., Norlen K. & Karlsson M.-B. (1971). Caffeine, caffeine derivatives and chromosomal aberrations, I. The relationship between ATP-concentration and the frequency of 8-ethoxy-caffeine-induced chromosomal exchanges in *Vicia faba*. *Hereditas*. *68*:291-304.

Klang M.J., Wang N. & Meoni L.A. (2002). Coffee intake and risk of hypertension: the john hopkins precursors study. *Arch. Intern. Med*. *62*:657-662.

Klis F.M., Boorsma A. & De Groot P.W.J. (2006) Cell wall construction in *Saccharomyces cerevisiae*. *Yeast*. *23*:185-202.

Kolaczkowski M., Kolaczowska A., Luczynski J., Witek S. & Goffeau A. (1998). *In vivo* characterization of the drug resistance profile of the major ABC transporters and other components of the yeast pleiotropic drug resistance network. *Microb. Drug Resist. 4***:**143-158.

Kolaczkowska A., Kolaczkowski M., Delahodde A. & Goffeau A. (2002). Functional dissection of Pdr1p, a regulator of multidrug resistance in *Saccharomyces cerevisiae*. *Mol. Genet. Genomics. 267*:96-106.

Koshiishi C., Kato A., Yama S., Crozier A. & Ashihara H. (2001). A new caffeine biosynthetic pathway in tea leaves: utilisation of adenosine released from the S-adenosyl-L-methionine cycle. *FEBS Lett*. *499*:50-54.

Kumar G. & Keserwani S. (2016). Mitotraumatism triggered by alkaloids (caffeine and nicotine) in root meristems of *Lathyrus sativus* L. (grass pea). *International Journal of Research in Botany. 6*(1):5-9.

Kuranda K., Leberre V., Sokol S., Palamarczyk G. & François J. (2006). Investigating the caffeine effects in the yeast *Saccharomyces cerevisiae* brings new insights into the connection between TOR, PKC and Ras/cAMP signaling pathways. *Mol. Microbiol. 61*(5):1147-1166.

Kurtzman C.P. & Fell J.W. (1998). *The yeasts, a taxonomic study*. 4th Edition, Elsevier, Amsterdam.

Küçükgöze G., Alkım C., Yılmaz U., Kısakesen H.İ., Gündüz S., Akman S. & Çakar Z.P. (2013). Evolutionary engineering and transcriptomic analysis of nickel-resistant *Saccharomyces cerevisiae*. *FEMS Yeast Res. 13*:731-746.

Lacorte L.M., Seiva F.R.F., Rinaldi J.C., Delellaa F.K., Moroza A., Sarobo C., … & Felisbino S.L. (2013). Caffeine reduces cadmium accumulation in the organism and enhances the levels of antioxidant protein expression in the epididymis. *Reprod. Toxicol*. *35*:137-143.

Li H. & Durbin R. (2009). Fast and accurate short read alignment with Burrows-Wheeler transform. *Bioinformatics. 25*:1754-1760.

Li W. & Mitchell A.P. (1997). Proteolytic activation of Rim1p, a positive regulator of yeast sporulation and invasive growth. *Genetics*. *145***:**63-73.

Ling H., Juwono N.K.P., Teo W.S., Liu R., Leong S.S.J. & Chang M.W. (2015). Engineering transcription factors to improve tolerance against alkane biofuels in *Saccharomyces cerevisiae*. *Biotechnol. Biofuels. 8*:231.

Liu J. & Barrientos A. (2013). Transcriptional regulation of yeast oxidative phosphorylation hypoxic genes by oxidative stress. *Antioxid. Redox Signal*. *19*(16):1916-1927.

Lodish H.B.D., Berk A., Zipursky S.L., Matsudaira P. & Darnell J. (1995). *Molecular cell biology*. 3rd edition USA: Scientific American Books.

Loewith R & Hall M.N. (2011). Target of rapamycin (TOR) in nutrient signaling and growth control. *Genetics*. *189*(4):1177-1201.

Lu Y.-P., Lou Y.-R., Xie J.-G., Peng Q.-Y., Liao J., Yang C.S., … & Conney A.H. (2002). Topical applications of caffeine or (−)-epigallocatechin gallate (EGCG) inhibit carcinogenesis and selectively increase apoptosis in UVB-induced skin tumors in mice. *Proc. Natl. Acad. Sci*. *99*(19):12455-12460.

Lucau-Danila A., Delaveau T., Lelandais G., Devaux F. & Jacq C. (2003). Competitive promoter occupancy by two yeast paralogous transcription factors controlling the multidrug resistance phenomenon. *J. Biol. Chem. 278*(52):52641-52650.

Mahé Y., Lemoine Y. & Kuchler K. (1996). The ATP binding cassette transporters Pdr5 and Snq2 of *Saccharomyces cerevisiae* can mediate transport of steroids *in vivo*. *J. Biol. Chem. 271*:25167-25172.

Mamnun Y.M., Pandjaitan R., Mahé Y., Delahodde A. & Kuchler K. (2002). The yeast zinc finger regulators Pdr1p and Pdr3p control pleiotropic drug resistance (PDR) as homo- and heterodimers in vivo. *Mol. Microbiol. 46*(5):1429-1440.

Marques M.C., Zamarbide-Fores S., Pedelini L., Llopis-Torregrosa V., & Yenush L. (2015). A functional Rim101 complex is required for proper accumulation of the Ena1 Na+-ATPase protein in response to salt stress in *Saccharomyces cerevisiae*. *FEMS Yeast Res. 15*(4):fov017

Matsui K., Teranishi S., Kamon S., Kuroda K. & Ueda M. (2008). Discovery of a modified transcription factor endowing yeasts with organic-solvent tolerance and reconstruction of an organic-solvent-tolerant *Saccharomyces cerevisiae* strain. *J. Appl. Environ. Microbiol. 74*(13):4222-4225.

Mell J.C. & Burgess S.M. (2002). Yeast as a Model Genetic Organism. *Encyclopedia of Life Sciences*. 1-8.

Merighi S., Benini A., Mirandola P., Gessi S., Varani K., Simioni C., … & Borea P.A. (2007). Caffeine inhibits adenosine-induced accumulation of hypoxia-inducible factor 1α, vascular endothelial growth factor, and interleukin-8 expression in hypoxic human colon cancer cells. *Mol. Pharmacol. 72*(2):395-406.

Mohanpuria P. & Yadav S.K. (2009). Retardation in seedling growth and induction of early senescence in plants upon caffeine exposure is related to its negative effect on rubisco. *Photosynthetica. 47*:293-297.

Moser B.A., Brondello J.M., Baber-Furnari B. & Russell P. (2000). Mechanism of caffeine-induced checkpoint override in fission yeast. *Mol. Cell Biol. 20*(12):4288-4294

Nathanson J.A. (1984). Caffeine and related methylxanthine possible naturally occurring pesticides. *Science*. *226*:184-187.

Nehlig A. & Debry G. (1994). Potential genotoxic, mutagenic and antimutagenic effects of coffee: a review. *Mutat. Res. 317*(2):145-162.

Nijkamp J.F., von den Broek M., Datema E., de Kok S., Bosman L., Luttik M.A., … & Daran J.-M. (2012). *De novo* sequencing, assembly, and analysis of the genome of the laboratory strain *Saccharomyces cerevisiae* CEN.PK113-7D, a model for modern industrial biotechnology. *Microb. Cell Fact. 11*:36.

Nishida N., Ozato N., Matsui K., Kuroda K. & Ueda M. (2013). ABC transporters and cell wall proteins involved in organic solvent tolerance in *Saccharomyces cerevisiae*. *J. Biotechnol. 165*(2):145-152.

Ogawa H. & Ueki N. (2007). Clinical importance of caffeine dependence and abuse. *Psychiatry Clin. Neurosci*. *61*:263-268.

Oud B., van Maris A.J., Daran J. M. & Pronk J. T. (2012). Genome-wide analytical approaches for reverse metabolic engineering of industrially relevant phenotypes in yeast. *FEMS Yeast Res*. *12*(2):183-196.

Pennaneach V. & Kolodner R.D. (2004). Recombination and the Tel1 and Mec1 checkpoints differentially effect genome rearrangements driven by telomere dysfunction in yeast. *Nat. Genet. 36*(6):612-617.

Preedy V.R. (2012). *Caffeine: chemistry, analysis, function, and effects*. The Royal Society of Chemistry, Cambridge, UK.

Rallis C., Codlin S. & Bähler J. (2013). TORC1 signaling inhibition by rapamycin and caffeine affect lifespan, global gene expression, and cell proliferation of fission yeast. *Aging Cell. 12*(4):563-573.

Reinke A., Chen J.-C.-Y., Aronova S. & Powers T. (2006). Caffeine targets TOR complex I and provides evidence for a regulatory link between the FRB and kinase domains of Tor1p. *J. Biol. Chem. 281*(42):31616-31626.

Rogers P.J., Hohoff C., Heatherley S.V. Mullings E.L., Maxfield P.J., Evershed R.P., … & Nutt D.J. (2010). Association of the anxiogenic and alerting effects of caffeine with *ADORA2A* and *ADORA1* polymorphisms and habitual level of caffeine consumption. *Neuropsychopharmacology*. *35*:1973-1983.

Ruepp A., Zollner A., Maier D., Albermann K., Hani J., Mokrejs M., … & Mewes H.W. (2004). The *FunCat*, a functional annotation scheme for systematic classification of proteins from whole genomes. *Nucleic Acids Res. 32*:5539-5545

Rutledge R.M., Esser L., Ma J. & Xia D. (2011). Toward understanding the mechanism of action of the yeast multidrug resistance transporter Pdr5: a molecular modeling study. *J. Struct. Biol. 173*:333-344.

Sabisz M. & Skladanowski A. (2008). Modulation of cellular response to anticancer treatment by caffeine: inhibition of cell cycle checkpoints, DNA repair and more. *Curr. Pharm. Biotechnol. 9*(4):325-338.

Saiardi A., Resnick A.C., Snowman A.M., Wendland B. & Snyder S.H. (2005). Inositol pyrophosphates regulate cell death and telomere length through phosphoinositide 3-kinase-related protein kinases. *Proc. Natl. Acad. Sci. 102*:1911-1914.

Sanchez Y., Desany B.A., Jones W.J., Liu Q., Wang B. & Elledge S.J. (1996). Regulation of RAD53 by the ATM-like kinases MEC1 and TEL1 in yeast cell cycle checkpoint pathways. *Science*. *271*(5247):357-360.

Sandlie I., Solberg K. & Kleppe K. (1980). The effect of caffeine on cell growth and metabolism of thymidine in *Escherichia coli*. *Mutat. Res. 73*:29-41.

Sarkaria J.N., Busby E.C., Tibbetts R.S., Roos P., Taya Y., Karnitz L.M. & Abraham R.T. (1999). Inhibition of ATM and ATR kinase activities by the radiosensitizing agent, caffeine. *Cancer Res. 59*(17):4375-4382.

Sasamoto H., Fujii Y. & Ashihara H. (2015). Effect of purine alkaloids on the proliferation of lettuce cells derived from protoplasts. *Nad. Prod. Commun. 10*(5):751-754.

Sauer U. (2001). Evolutionary engineering of industrially important microbial phenotypes. *Adv. Biochem. Eng. Biotechnol. 73*:130-166.

Sauna Z.E., Bohn S.S., Rutledge R., Dougherty M.P., Cronin S., May L., … & Golin J. (2008). Mutations define cross-talk between the N-terminal nucleotide-binding domain and transmembrane helix-2 of the yeast multidrug transporter Pdr5: possible conservation of a signaling interface for coupling ATP hydrolysis to drug transport. *J. Biol. Chem. 283*(50):35010-35022.

Schmitz H.-P., Huppert S., Lorberg A. & Heinisch J.J. (2002). Rho5p downregulates the yeast cell integrity pathway. *J. Cell Sci.* 115:3139-3148.

Smardon A.M. & Kane P.M. (2014). Loss of vacuolar H+-ATPase activity in organelles signals ubiquitination and endocytosis of the yeast plasma membrane proton pump Pma1p. *J. Biol. Chem. 289*(46):32316-32326.

Solomon E.P., Berg. L. R. & Martin D.W. (1999). *Biology*. 5th edition USA: Saunders College Publishing.

Sundström L., Larsson S. & Jönsson L.J. (2010). Identification of *Saccharomyces cerevisiae* genes involved in the resistance to phenolic fermentation inhibitors. *Appl. Biochem. Biotechnol. 161*:106-115.

Sürmeli Y., Holyavkin C., Topaloglu A., Arslan M., Kısakesen H.İ. & Cakar Z.P. (2019). Evolutionary engineering and molecular characterization of a caffeine-resistant *Saccharomyces cerevisiae* strain. *World J. Microbiol. Biotechnol. 35*:183.

Teixeira M.C., Monteiro P.T., Palma M., Costa C., Godinho C.P., Pais P., … & Sá-Correia I. (2018). YEASTRACT, an upgraded database for the analysis of transcription regulatory networks in *Saccharomyces cerevisiae*. *Nucl. Acids Res. 46* (Issue D1): D348-D353.

Tsujimoto Y., Shimizu Y., Otake K., Nakamura T., Okada R., Miyazaki T. & Watanabe K. (2015). Multidrug resistance transporters Snq2p and Pdr5p mediate caffeine efflux in *Saccharomyces cerevisiae*. *Biosci. Biotechnol. Biochem. 79*(7):1103-1110.

Turanlı-Yıldız B., Benbadis L., Alkım C., Sezgin T., Akşit A., Gökçe A., … & François J.M. (2017). *In vivo* evolutionary engineering for ethanol-tolerance of *Saccharomyces cerevisiae* haploid cells triggers diploidization. *J. Biosci. Bioeng. 124*(3):309-318.

Url-1. https://en.wikipedia.org/wiki/Kaldi, retrieved on 02.11.2019.

Url-2. The *Saccharomyces cerevisiae* Database. https://www.yeastgenome.org, retrieved on 02.11.2019.

Velivela S.D. & Kane P.M. (2018). Compensatory internalization of Pma1 in V-ATPase mutants in *Saccharomyces cerevisiae* requires calcium- and glucose-sensitive phosphatases. *Genetics. 208*(2):655-672

Vialard J.E., Gilbert C.S., Green C.M. & Lowndes N.F. (1998). The budding yeast Rad9 checkpoint protein is subjected to Mec1/Tel1-dependent hyperphosphorylation and interacts with Rad53 after DNA damage. *EMBO J. 17*(19):5679-5688.

Walker K., Skelton H. & Smith K. (2002). Cutaneous lesions showing giant yeast forms of *Blastomyces dermatitidis*. *J. Cutan. Pathol. 29*(10):616-618.

Wang Y., Kurihara Y., Sato T., Toh H., Kobayashi H. & Sekiguchi T. (2009). Gtr1p differentially associates with Gtr2p and Ego1p. *Gene. 437*:32-38.

Wanke V., Cameroni E., Uotila A., Piccolis M., Urban J., Loewith R. & De Virgilio C. (2008). Caffeine extends yeast lifespan by targeting TORC1. *Mol. Microbiol*. *69*(1):277-285.

Watcharawipas A., Watanabe D., & Takagi H. (2017). Enhanced sodium acetate tolerance in *Saccharomyces cerevisiae* by the Thr255Ala mutation of the ubiquitin ligase Rsp5. *FEMS Yeast Res. 17*(8):fox083.

Wolfger H., Mahé Y., Parle-McDermott A., Delahodde A. & Kuchler K. (1997). The yeast ATP binding cassette (ABC) protein genes *PDR10* and *PDR15* are novel targets for the Pdr1 and Pdr3 transcriptional regulators. *FEBS Lett***.** *418*(3):269-274.

Wullschleger S., Loewith R. & Hall M.N. (2006). TOR signaling in growth and metabolism. *Cell. 124*(3):471-484.

Zylber-Katz E., Granit L. & Levy M. (1984). Relationship between caffeine concentrations in plasma and saliva. *Clin. Pharmacol. Ther. 36*(1):133-137.

APPENDICES

APPENDIX A : Chemicals and laboratory equipment used in this study.

APPENDIX B : The percent survival rate and caffeine concentration during selection by evolutionary engineering.

APPENDIX C : OD_{600} values of Caf905-2 and the reference strain during growth in the presence of 10 mM caffeine, and under nonstress condition.

APPENDIX D : Standard calibration curve for glucose, glycerol and ethanol.

APPENDIX E : Particular functional classes of at least two-fold enriched upregulated and downregulated genes in the evolved strain Caf905-2.

APPENDIX A : Chemicals and laboratory equipment used in this study.

Table A.1 : Chemicals.

Chemical	Company
Agarose low EEO (Agarose Standard)	Applichem
Aluminium chloride hexahydrate	Merck
Ammonium chloride	Riedel-de Haen
Boric Acid	Merck
Buffered Peptone Water	Biorad
Yeast extract granulated	Merck
Cobalt(II) sulfate heptahydrate	Sigma
D-Sorbitol	Sigma
Sodium chloride	Merck
Nickel(II) chloride hexahydrate	Merck
Iron(II) sulfate-7-hydrate (Extra pure)	Riedel-de Haen
Manganase(II) chloride tetrahydrate	Merck
Ethanol	J.T Baker
2-phenylethanol	Sigma-Aldrich
Caffeine	MERCK
Acetic acid	MERCK
Glycerol	Duchefa Biochemie
Agar	BD Difco™
Yeast nitrogen base without amino acids	BD Difco™
Ethyl methane sulfonate (EMS)	Alpha-Aeasar
Dextrose	Riedel-de Haen
Coniferyl aldehyde	Sigma-Aldrich
Vanillin	Sigma-Aldrich
4-hydroxybenzaldehyde	Sigma-Aldrich

Table A.2 : Laboratory equipment.

Laboratory Equipment	Brand/ Model
Microcentrifuge	Eppendorf Microcentrifuge 5424
Vortex mixer	Nuve NM 110
Deep freezer	-80^{o}C SANYO UltraLow
Laminar Flow Cabinet	Faster BH-EN 2003
Autoclave	GR 110 GF-Zealway
UV-Visible Spectrophotometer	Shimadzu UV-1700
Nanodrop 2000	Thermo Fisher Scientific
Orbital Shaker	Certomat S II Sartorius
Ultrapure water systems	USF-Elga UHQ
HPLC	Shimadzu Series 10A
HPLC column	Bio-Rad Aminex HPX-87H
Balance	Precisa XB220A

APPENDIX B : The percent survival rate and caffeine concentration during selection by evolutionary engineering.

Table B.1 : The percent survival rate and caffeine concentration of each population obtained using the reference strain as the initial population (without EMS mutagenesis) during selection by evolutionary engineering.

Population	Caffeine concentration (mM)	OD_{600} (control)	OD_{600} (stress)	Percent survival rate*
1	7.5	6.071	5.448	89.7
2	8.0	5.287	3.942	74.6
3	8.5	5.565	3.810	68.5
4	9.0	4.905	3.379	68.9
5	9.5	5.282	3.344	63.3
6	10.0	4.913	3.583	72.9
7	10.5	5.318	3.128	58.8
8	11.0	5.327	3.401	63.9
9	11.5	5.372	4.136	77.0
10	12.0	5.585	3.144	56.3
11	12.5	5.048	3.257	64.5
12	13.0	5.139	3.419	66.5
13	13.5	5.170	3.665	70.9
14	14.0	5.041	3.853	76.4
15	14.5	5.376	3.691	68.7
16	15.0	4.912	3.420	69.6
17	15.5	4.652	2.868	61.7
18	16.0	4.996	3.503	70.1
19	16.5	5.347	3.426	64.1
20	17.0	5.285	2.829	53.5
21	17.5	5.228	2.976	56.9
22	18.0	5.257	3.509	66.7
23	18.5	5.157	3.730	72.3
24	19.0	5.020	3.502	69.8
25	19.5	5.146	3.285	63.8
26	20.0	4.992	3.602	72.1
27	20.5	5.295	3.584	67.7
28	21.0	4.679	3.387	72.4
29	22.0	4.712	3.470	73.7
30	23.0	4.671	3.297	70.6

*Percent survival rate = (OD_{600} (stress)) x 100/(OD_{600} (control))

Table B.1 (continued) : The percent survival rate and caffeine concentration of each population obtained using the reference strain as the initial population (without EMS mutagenesis) during selection by evolutionary engineering.

Population	Caffeine concentration (mM)	OD_{600} (control)	OD_{600} (stress)	Percent survival rate*
31	24.0	4.790	3.077	64.2
32	25.0	4.526	3.254	71.9
33	26.0	4.442	3.222	72.5
34	27.0	4.995	3.847	77.0
35	28.0	4.360	3.470	79.6
36	29.0	4.755	3.055	64.2
37	30.0	4.673	3.301	70.6
38	31.0	4.366	2.990	68.5
39	32.0	4.580	3.006	65.6
40	34.0	4.572	2.542	55.6
41	36.0	4.443	2.947	66.3
42	38.0	4.494	2.570	57.2
43	40.0	4.438	2.600	58.6
44	42.0	4.750	2.998	63.1
45	44.0	4.525	2.420	53.5
46	46.0	4.535	2.270	50.1
47	48.0	4.582	2.416	52.7
48	50.0	4.670	2.641	56.6

Table B.2 : The percent survival rate and caffeine concentration of each population obtained using the EMS-mutagenized population as the initial population during selection by evolutionary engineering.

Population	Caffeine concentration (mM)	OD_{600} (control)	OD_{600} (stress)	Percent survival rate*
1	7.5	5.972	5.329	89.2
2	8.0	5.478	3.949	72.1
3	8.5	5.254	3.662	69.7
4	9.0	5.034	3.390	67.3
5	9.5	5.273	3.950	74.9
6	10.0	4.926	3.788	76.9
7	10.5	5.399	4.144	76.7
8	11.0	5.095	4.091	80.3
9	11.5	5.359	4.163	77.7
10	12.0	5.255	3.473	66.1
11	12.5	5.239	3.268	62.4
12	13.0	4.988	2.584	51.8
13	13.5	5.026	4.163	82.8
14	14.0	5.201	4.112	79.1
15	14.5	5.374	4.034	75.1

*Percent survival rate = (OD_{600} (stress)) x 100/(OD_{600} (control))

Table B.2 (continued) : The percent survival ratio and caffeine concentration of each population obtained using the EMS-mutagenized population as an initial population during selection by evolutionary engineering.

Population	Caffeine concentration (mM)	OD_{600} (control)	OD_{600} (stress)	Percent survival rate*
16	15.0	4.929	3.686	74.8
17	15.5	4.904	3.653	74.5
18	16.0	5.071	3.956	78.0
19	16.5	5.028	3.744	74.5
20	17.0	5.254	4.048	77.1
21	17.5	5.353	3.923	73.3
22	18.0	4.975	3.971	79.8
23	18.5	5.084	3.730	73.4
24	19.0	5.145	4.190	81.4
25	19.5	4.943	3.754	75.9
26	20.0	4.941	3.643	73.7
27	20.5	4.916	3.582	72.9
28	21.0	4.837	3.543	73.2
29	22.0	4.936	3.734	75.6
30	23.0	4.966	3.795	76.4
31	24.0	5.154	2.992	58.0
32	25.0	4.848	3.598	74.2
33	26.0	4.705	3.348	71.2
34	27.0	5.079	3.958	77.9
35	28.0	4.706	3.745	79.6
36	29.0	5.455	3.377	61.9
37	30.0	4.876	3.697	75.8
38	31.0	4.614	3.191	69.2
39	32.0	4.809	3.577	74.4
40	34.0	4.958	3.250	65.6
41	36.0	4.694	3.148	67.1
42	38.0	4.787	3.067	64.1
43	40.0	4.862	2.936	60.4
44	42.0	5.085	3.195	62.8
45	44.0	4.747	3.366	70.9
46	46.0	4.807	2.786	58.0
47	48.0	4.817	3.000	62.3
48	50.0	4.950	2.904	58.7

APPENDIX C : OD_{600} values of Caf905-2 and the reference strain during growth in the presence of 10 mM caffeine, and under nonstress condition.

Table C.1 : OD_{600} values of Caf905-2 and the reference strain during growth in the presence of 10 mM caffeine, and under nonstress condition.

Time	OD_{600} at 0 mM Caffeine		OD_{600} at 10 mM Caffeine	
(h)	Reference strain	Caf905-2	Reference strain	Caf905-2
0	0.23 ± 0.01	0.20 ± 0.01	0.25 ± 0.00	0.28 ± 0.00
3	0.57 ± 0.01	0.50 ± 0.01	0.28 ± 0.00	0.51 ± 0.01
6	1.49 ± 0.02	1.15 ± 0.07	0.44 ± 0.01	1.37 ± 0.05
8	2.92 ± 0.02	2.10 ± 0.08	0.49 ± 0.02	2.58 ± 0.07
10	4.51 ± 0.05	3.32 ± 0.11	0.70 ± 0.02	3.75 ± 0.05
12	5.32 ± 0.09	4.24 ± 0.12	0.85 ± 0.06	4.34 ± 0.08
15	5.91 ± 0.04	5.03 ± 0.06	1.23 ± 0.15	4.49 ± 0.06
18	5.84 ± 0.23	4.92 ± 0.03	1.73 ± 0.13	4.50 ± 0.03
21	5.82 ± 0.04	4.81 ± 0.11	2.51 ± 0.13	4.52 ± 0.04
24	5.76 ± 0.02	4.68 ± 0.07	3.13 ± 0.20	4.56 ± 0.08
27	5.91 ± 0.08	4.86 ± 0.16	3.69 ± 0.11	4.59 ± 0.09
30	5.98 ± 0.05	4.89 ± 0.05	4.06 ± 0.10	4.60 ± 0.04
36			4.81 ± 0.16	4.77 ± 0.01
42			5.18 ± 0.09	4.86 ± 0.06
48			5.25 ± 0.16	4.97 ± 0.14

APPENDIX D : Standard calibration curve for glucose, glycerol and ethanol.

Retention times for glucose, glycerol and ethanol found in the standard solution mixtures were, 8.88 min, 12.93 min, and 20.17 min, respectively, in ion exclusion chromatographic analysis. Standard calibration curves for glucose, glycerol and ethanol are, shown in Figures D.1, D.2, and D.3, respectively.

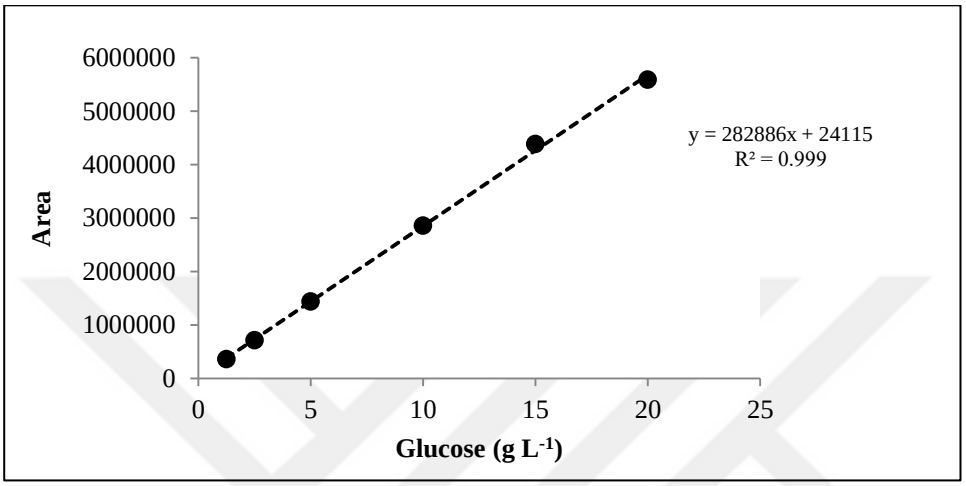

Figure D.1 : Standard calibration curve for glucose.

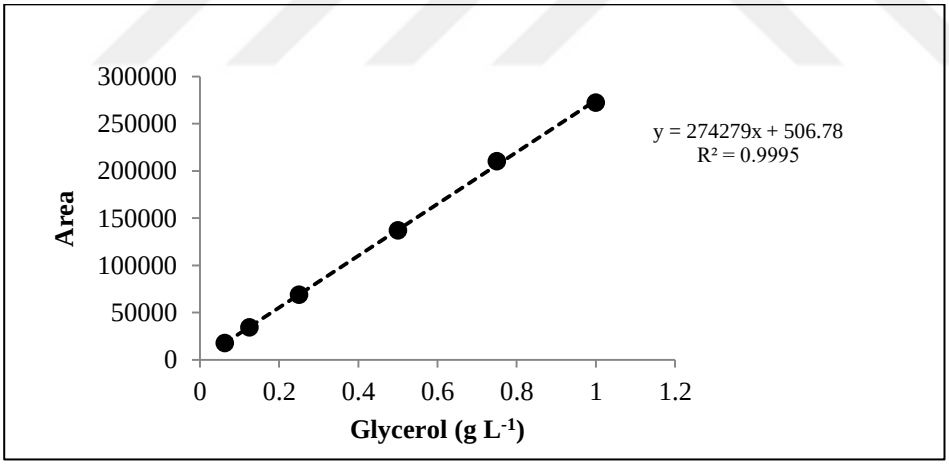

Figure D.2 : Standard calibration curve for glycerol.

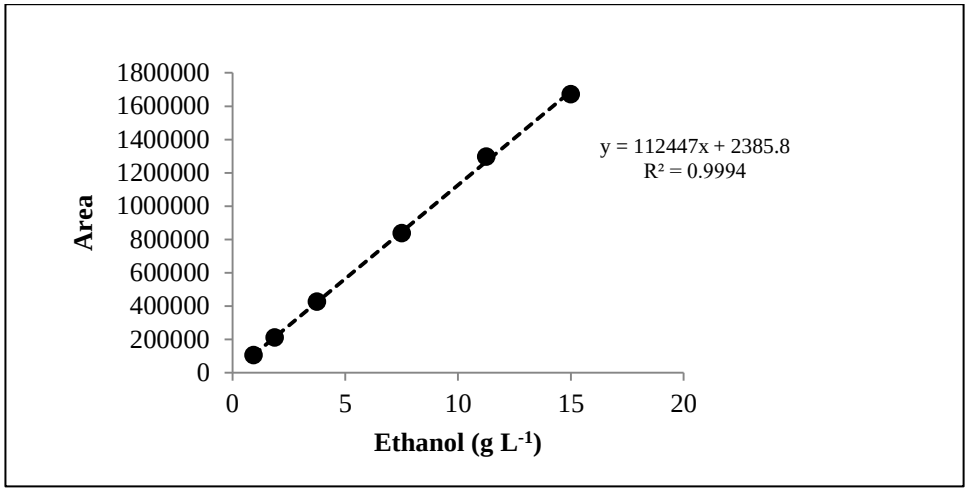

Figure D.3 : Standard calibration curve for ethanol.

APPENDIX E : Particular functional classes of at least two-fold enriched upregulated and downregulated genes in the evolved strain Caf905-2.

Table E.1 : Particular functional classes of at least two-fold enriched upregulated genes in the evolved strain Caf905-2, relative to the reference strain, obtained by *FunCat* analysis.

	Systematic name	Gene name[a]	Fold change	Description
Metabolism of energy reserves	YHR060W	*VMA22*	4.570	Protein that is required for vacuolar H+-ATPase (V-ATPase) function
	YBR299W	*MAL32*	14.626	Maltase (alpha-D-glucosidase)
	YDR001C	*NTH1*	2.270	Neutral trehalase, degrades trehalose
	YER133W	*GLC7*	2.418	Type 1 S/T protein phosphatase (PP1) catalytic subunit
	YEL011W	*GLC3*	9.359	Glycogen branching enzyme, involved in glycogen accumulation
	YFR015C	*GSY1*	10.964	Glycogen synthase
	YGR292W	*MAL12*	16.281	Maltase (alpha-D-glucosidase)
	YGR287C	*IMA1*	5.013	Major isomaltase (alpha-1,6-glucosidase/alpha-methylglucosidase)
	YIL172C	*IMA3*	4.179	Alpha-glucosidase; weak, but broad substrate specificity for alpha-1,4- and alpha-1,6-glucosides
	YJL216C	*IMA5*	7.240	Alpha-glucosidase; specificity for isomaltose, maltose, and palatinose, but not alpha-methylglucoside
	YJL221C	*IMA4*	4.005	Alpha-glucosidase; weak, but broad substrate specificity for alpha-1,4- and alpha-1,6-glucosides
	YKL035W	***UGP1***	3.645	UDP-glucose pyrophosphorylase (UGPase)
	YLR258W	*GSY2*	4.616	Glycogen synthase
	YML100W	*TSL1*	8.893	Large subunit of trehalose 6-phosphate synthase/phosphatase complex
	YMR261C	*TPS3*	2.116	Regulatory subunit of trehalose-6-phosphate synthase/phosphatase
	YMR105C	***PGM2***	20.352	Phosphoglucomutase
	YOL157C	*IMA2*	3.589	Isomaltase (alpha-1,6-glucosidase/alpha-methylglucosidase)
	YOR178C	*GAC1*	17.329	Regulatory subunit for Glc7p type-1 protein phosphatase (PP1)

[a] Bold gene names were classified into at least two functional categories.

Table E.1 (continued) : Particular functional classes of at least two-fold enriched upregulated genes in the evolved strain Caf905-2, relative to the reference strain, obtained by *FunCat* analysis.

	Systematic name	Gene name[a]	Fold change	Description
	YPR184W	*GDB1*	2.889	Glycogen debranching enzyme
	YPR160W	*GPH1*	38.171	Glycogen phosphorylase required for the mobilization of glycogen
	YPR026W	*ATH1*	2.476	Acid trehalase required for utilization of extracellular trehalose
	YPL240C	***HSP82***	7.993	Hsp90 chaperone
Glycolysis and gluconeogenesis	YBR117C	***TKL2***	5.774	Transketolase
	YCL040W	*GLK1*	3.938	Glucokinase
	YDR255C	*RMD5*	2.382	Component of GID Complex that confers ubiquitin ligase (U3) activity
	YDR516C	*EMI2*	11.274	Non-essential protein of unknown function
	YDL021W	*GPM2*	14.250	Nonfunctional homolog of Gpm1p phosphoglycerate mutase
	YEL012W	*UBC8*	3.756	Ubiquitin-conjugating enzyme that regulates gluconeogenesis
	YFR053C	*HXK1*	82.787	Hexokinase isoenzyme 1
	YGR066C		4.934	Putative protein of unknown function
	YGL227W	*VID30*	2.922	Central component of GID Complex, involved in FBPase degradation
	YIL155C	*GUT2*	3.119	Mitochondrial glycerol-3-phosphate dehydrogenase
	YIL107C	*PFK26*	2.526	6-phosphofructo-2-kinase
	YIL097W	*FYV10*	2.634	Subunit of GID complex
	YJL052W	*TDH1*	3.343	Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), isozyme 1
	YJL089W	*SIP4*	10.786	C6 zinc cluster transcriptional activator
	YJL155C	*FBP26*	4.897	Fructose-2,6-bisphosphatase, required for glucose metabolism
	YKL035W	***UGP1***	3.645	UDP-glucose pyrophosphorylase (UGPase)

Table E.1 (continued) : Particular functional classes of at least two-fold enriched upregulated genes in the evolved strain Caf905-2, relative to the reference strain, obtained by *FunCat* analysis.

	Systematic name	Gene name[a]	Fold change	Description
	YLR345W		3.389	Similar to 6-phosphofructo-2-kinase enzymes
	YMR280C	*CAT8*	4.537	Zinc cluster transcriptional activator
	YMR105C	***PGM2***	20.352	Phosphoglucomutase
	YNL241C	***ZWF1***	4.793	Glucose-6-phosphate dehydrogenase (G6PD)
	YOR393W	*ERR1*	2.395	Putative phosphopyruvate hydratase
	YOL151W	*GRE2*	4.831	3-methylbutanal reductase and NADPH-dependent methylglyoxal reductase
	YOR347C	*PYK2*	3.602	Pyruvate kinase
	YPR006C	*ICL2*	3.181	2-methylisocitrate lyase of the mitochondrial matrix
	YPL113C		6.729	Glyoxylate reductase
Electron transport and membrane-associated energy conservation	YHR001W-A	*QCR10*	6.954	Subunit of the ubiqunol-cytochrome c oxidoreductase complex
	YDR529C	*QCR7*	3.447	Subunit 7 of ubiquinol cytochrome-c reductase (Complex III)
	YDR231C	*COX20*	2.057	Mitochondrial inner membrane protein; required for proteolytic processing of Cox2p and its assembly into cytochrome c oxidase
	YDR377W	*ATP17*	6.016	Subunit f of the F0 sector of mitochondrial F1F0 ATP synthase
	YDL149W	*ATG9*	2.347	Transmembrane protein involved in forming Cvt and autophagic vesicles
	YDR322C-A	*TIM11*	8.729	Subunit e of mitochondrial F1F0-ATPase
	YDL067C	*COX9*	4.007	Subunit VIIa of cytochrome c oxidase (Complex IV)
	YDL130W-A	*STF1*	9.063	Protein involved in regulation of the mitochondrial F1F0-ATP synthase
	YEL039C	*CYC7*	221.806	Cytochrome c isoform 2
	YGR183C	*QCR9*	4.906	Subunit 9 of ubiquinol cytochrome-c reductase (Complex III)
	YGL191W	*COX13*	4.825	Subunit VIa of cytochrome c oxidase
	YGR008C	*STF2*	17.944	Protein involved in resistance to desiccation stress
	YIL111W	*COX5b*	10.938	Subunit Vb of cytochrome c oxidase

Table E.1 (continued) : Particular functional classes of at least two-fold enriched upregulated genes in the evolved strain Caf905-2, relative to the reference strain, obtained by *FunCat* analysis.

	Systematic name	Gene name[a]	Fold change	Description
	YJL166W	*QCR8*	5.594	Subunit 8 of ubiquinol cytochrome-c reductase (Complex III)
	YJL045W	*SDH1b*	8.806	Minor succinate dehydrogenase isozyme
	YJR048W	*CYC1*	2.740	Cytochrome c, isoform 1
	YJR033C	*RAV1*	2.290	Subunit of RAVE complex (Rav1p, Rav2p, Skp1p)
	YKL150W	*MCR1*	2.003	Mitochondrial NADH-cytochrome b5 reductase
	YLR038C	*COX12*	2.993	Subunit VIb of cytochrome c oxidase
	YLR327C	*TMA10*	152.870	Protein of unknown function that associates with ribosomes
	YMR256C	*COX7*	13.665	Subunit VII of cytochrome c oxidase (Complex IV)
	YMR118C	*SHH3*	2.395	Putative mitochondrial inner membrane protein of unknown function
	YML054C	*CYB2*	3.162	Cytochrome b2 (L-lactate cytochrome-c oxidoreductase)
	YOL077W-A	*ATP19*	5.499	Subunit k of the mitochondrial F1F0 ATP synthase
	YOR374W	*ALD4*	10.022	Mitochondrial aldehyde dehydrogenase
	YPL171C	*OYE3*	5.283	Conserved NADPH oxidoreductase containing flavin mononucleotide (FMN)
	YPL061W	*ALD6*	5.041	Cytosolic aldehyde dehydrogenase
Pentose-phosphate pathway	YBR117C	***TKL2***	5.774	Transketolase
	YCR036W	*RBK1*	3.175	Putative ribokinase
	YGR043C	*NQM1*	26.870	Transaldolase of unknown function
	YGR256W	*GND2*	2.633	6-phosphogluconate dehydrogenase (decarboxylating)
	YGR248W	*SOL4*	10.304	6-phosphogluconolactonase
	YMR105C	***PGM2***	20.352	Phosphoglucomutase
	YNL241C	***ZWF1***	4.793	Glucose-6-phosphate dehydrogenase (G6PD)
	YNR034W	*SOL1*	3.377	Protein with a possible role in tRNA export
	YPL113C		6.729	Glyoxylate reductase

Table E.1 (continued) : Particular functional classes of at least two-fold enriched upregulated genes in the evolved strain Caf905-2, relative to the reference strain, obtained by *FunCat* analysis.

	Systematic name	Gene name[a]	Fold change	Description
Oxygen and radical detoxification	YBL064C	*PRX1*	3.550	Mitochondrial peroxiredoxin with thioredoxin peroxidase activity
	YBR244W	*GPX2*	2.989	Phospholipid hydroperoxide glutathione peroxidase
	YCL035C	*GRX1*	3.564	Glutathione-dependent disulfide oxidoreductase
	YDR453C	*TSA2*	3.731	Stress inducible cytoplasmic thioredoxin peroxidase
	YGR088W	*CTT1*	5.507	Cytosolic catalase T
	YGR209C	*TRX2*	12.848	Cytoplasmic thioredoxin isoenzyme
	YJR104C	***SOD1***	2.333	Cytosolic copper-zinc superoxide dismutase
	YJL068C		2.426	Esterase that can function as an S-formylglutathione hydrolase
	YKL086W	*SRX1*	3.671	Sulfiredoxin
	YKL026C	*GPX1*	3.972	Phospholipid hydroperoxide glutathione peroxidase
Protein folding and stabilization	YAL005C	*SSA1*	3.754	ATPase involved in protein folding and NLS-directed nuclear transport
	YBR169C	*SSE2*	6.522	Member of Hsp110 subclass of the heat shock protein 70 (HSP70) family
	YBR072W	*HSP26*	36.555	Small heat shock protein (sHSP) with chaperone activity
	YDR002W	*YRB1*	3.023	Ran GTPase binding protein
	YDR258C	*HSP78*	8.959	Oligomeric mitochondrial matrix chaperone;
	YDR171W	*HSP42*	4.812	Small heat shock protein (sHSP) with chaperone activity
	YDR214W	*AHA1*	2.477	Co-chaperone that binds Hsp82p and activates its ATPase activity
	YDR519W	*FPR2*	2.708	Membrane-bound peptidyl-prolyl cis-trans isomerase (PPIase)
	YER103W	*SSA4*	17.461	Heat shock protein that is highly induced upon stress
	YFL016C	*MDJ1*	2.457	Co-chaperone that stimulates Hsp70 protein Ssc1p ATPase activity
	YGR249W	*MGA1*	4.536	Protein similar to heat shock transcription factor

Table E.1 (continued) : Particular functional classes of at least two-fold enriched upregulated genes in the evolved strain Caf905-2, relative to the reference strain, obtained by *FunCat* analysis.

	Systematic name	Gene name[a]	Fold change	Description
	YGR249W	*MGA1*	4.536	Protein similar to heat shock transcription factor
	YJR045C	*SSC1*	2.740	Hsp70 family ATPase
	YJR104C	***SOD1***	2.333	Cytosolic copper-zinc superoxide dismutase
	YJL179W	*PFD1*	5.324	Subunit of heterohexameric prefoldin
	YJR097W	*JJJ3*	2.385	Protein of unknown function
	YJL034W	*KAR2*	6.647	ATPase involved in protein import into the endoplasmic reticulum
	YJL073W	*JEM1*	2.494	DnaJ-like chaperone required for nuclear membrane fusion during mating
	YJR135W-A	*TIM8*	9.903	Mitochondrial intermembrane space protein
	YKL073W	*LHS1*	3.068	Molecular chaperone of the endoplasmic reticulum lumen
	YLR200W	*YKE2*	3.890	Subunit of the heterohexameric Gim/prefoldin protein complex
	YLR216C	*CPR6*	6.573	Peptidyl-prolyl cis-trans isomerase (cyclophilin)
	YLL026W	*HSP104*	5.382	Disaggregase
	YLL009C	*COX17*	6.060	Copper metallochaperone that transfers copper to Sco1p and Cox11p
	YML130C	*ERO1*	6.263	Thiol oxidase required for oxidative protein folding in the ER
	YNL135C	*FPR1*	2.984	Peptidyl-prolyl cis-trans isomerase (PPIase)
	YNL007C	*SIS1*	2.282	Type II HSP40 co-chaperone that interacts with the HSP70 protein Ssa1p
	YNL077W	*APJ1*	5.529	Chaperone with a role in SUMO-mediated protein degradation
	YNL281W	*HCH1*	2.042	Heat shock protein regulator
	YOR027W	*STI1*	3.701	Hsp90 co-chaperone
	YOR020C	*HSP10*	4.078	Mitochondrial matrix co-chaperonin
	YPL240C	***HSP82***	7.993	Hsp90 chaperone
Drug/toxin transport	YDR011W	*SNQ2*	8.339	Plasma membrane ATP-binding cassette (ABC) transporter
	YDR406W	*PDR15*	2.685	Plasma membrane ATP-binding cassette (ABC) transporter
	YGR281W	*YOR1*	4.746	Plasma membrane ATP-binding cassette (ABC) transporter

Table E.1 (continued) : Particular functional classes of at least two-fold enriched upregulated genes in the evolved strain Caf905-2, relative to the reference strain, obtained by *FunCat* analysis.

	Systematic name	Gene name[a]	Fold change	Description
	YOR153W	*PDR5*	24.044	Plasma membrane ATP-binding cassette (ABC) transporter
	YOR172W	*YRM1*	2.457	Zinc finger transcription factor involved in multidrug resistance
	YOR162C	*YRR1*	3.179	Zn2-Cys6 zinc-finger transcription factor; activates genes involved in multidrug resistance

Table E.2 : Particular functional classes of at least two-fold enriched downregulated genes in the evolved strain Caf905-2, relative to the reference strain, obtained by *FunCat* analysis.

	Systematic name	Gene name[a]	Fold change	Description
Purine and pyrimidine nucleotide/nucleoside/ nucleobase metabolism	YAR015W	*ADE1*	2.274	N-succinyl-5-aminoimidazole-4-carboxamide ribotide synthetase
	YBR248C	*HIS7*	2.019	Imidazole glycerol phosphate synthase
	YBR084W	*MIS1*	3.365	Mitochondrial C1-tetrahydrofolate synthase
	YLR432W	*IMD3*	3.720	Inosine monophosphate dehydrogenase
	YMR217W	*GUA1*	3.443	GMP synthase
	YML056C	*IMD4*	6.007	Inosine monophosphate dehydrogenase
	YNL220W	*ADE12*	2.185	Adenylosuccinate synthase
	YHR128W	*FUR1*	5.432	Uracil phosphoribosyltransferase; synthesizes UMP from uracil
	YHL011C	*PRS3*	3.632	5-phospho-ribosyl-1(alpha)-pyrophosphate synthetase; synthesizes PRPP
	YBL039C	*URA7*	8.447	Major CTP synthase isozyme (see also URA8)
	YDR399W	*HPT1*	2.828	Dimeric hypoxanthine-guanine phosphoribosyltransferase
	YDR441C	*APT2*	2.155	Potential adenine phosphoribosyltransferase
	YEL021W	*URA3*	2.390	Orotidine-5'-phosphate (OMP) decarboxylase
	YKL181W	*PRS1*	3.362	5-phospho-ribosyl-1(alpha)-pyrophosphate synthetase; synthesizes PRPP
	YLR014C	*PPR1*	3.663	Zinc finger transcription factor
	YML106W	*URA5*	4.597	Major orotate phosphoribosyltransferase (OPRTase) isozyme
	YKL216W	*URA1*	3.238	Dihydroorotate dehydrogenase
	YLR175W	*CBF5*	3.584	Pseudouridine synthase catalytic subunit of box H/ACA snoRNPs
	YNL292W	*PUS4*	3.275	Pseudouridine synthase
	YNR015W	*SMM1*	2.497	Dihydrouridine synthase
	YNR012W	*URK1*	4.001	Uridine/cytidine kinase

[a] Bold gene names were classified into at least two functional categories.

Table E.2 (continued) : Particular functional classes of at least two-fold enriched downregulated genes in the evolved strain Caf905-2, relative to the reference strain, obtained by *FunCat* analysis.

	Systematic name	Gene name[a]	Fold change	Description
	YOR209C	*NPT1*	2.282	Nicotinate phosphoribosyltransferase
	YOR243C	*PUS7*	5.716	Pseudouridine synthase
	YOR303W	*CPA1*	3.291	Small subunit of carbamoyl phosphate synthetase
	YPL212C	*PUS1*	2.747	tRNA:pseudouridine synthase
	YBR263W	*SHM1*	3.269	Mitochondrial serine hydroxymethyltransferase
	YCR027C	*RHB1*	2.133	Putative Rheb-related GTPase
	YDR226W	*ADK1*	2.400	Adenylate kinase, required for purine metabolism
	YDR454C	*GUK1*	2.133	Guanylate kinase
	YDR020C	*DAS2*	7.712	Putative protein of unknown function
	YER031C	*YPT31*	3.165	Rab family GTPase
	YEL042W	*GDA1*	2.559	Guanosine diphosphatase located in the Golgi
	YGR152C	*RSR1*	2.663	GTP-binding protein of the Ras superfamily
	YIR031C	*DAL7*	4.827	Malate synthase
	YIR029W	*DAL2*	2.486	Allantoicase
	YIR032C	*DAL3*	6.010	Ureidoglycolate lyase
	YIR027C	*DAL1*	3.556	Allantoinase
	YLR180W	*SAM1*	2.790	S-adenosylmethionine synthetase
	YLR058C	*SHM2*	2.826	Cytosolic serine hydroxymethyltransferase
	YNL141W	*AAH1*	17.757	Adenine deaminase (adenine aminohydrolase)
	YNL180C	*RHO5*	2.662	Non-essential small GTPase of the Rho/Rac family of Ras-like proteins
	YOR212W	*STE4*	2.926	G protein beta subunit
Nucleotide/nucleoside/ nucleobase transport	YBR021W	***FUR4***	4.598	Plasma membrane localized uracil permease
	YBL030C	*PET9*	3.025	Major ADP/ATP carrier of the mitochondrial inner membrane
	YBL042C	***FUI1***	14.250	High affinity uridine permease, localizes to the plasma membrane

Table E.2 (continued) : Particular functional classes of at least two-fold enriched downregulated genes in the evolved strain Caf905-2, relative to the reference strain, obtained by *FunCat* analysis.

	Systematic name	Gene name[a]	Fold change	Description
	YER060W	***FCY21***	4.246	Putative purine-cytosine permease
	YER056C	***FCY2***	12.878	Purine-cytosine permease
	YGL225W	*VRG4*	3.563	Golgi GDP-mannose transporter
	YGR096W	***TPC1***	2.071	Mitochondrial membrane transporter
	YIL114C	*POR2*	2.577	Putative mitochondrial porin (voltage-dependent anion channel)
	YLR237W	***THI7***	2.131	Plasma membrane transporter responsible for the uptake of thiamine
Heavy metal ion transport (Cu^{+}, Fe^{3+}, etc.)	YHL040C	*ARN1*	4.452	ARN family transporter for siderophore-iron chelates
	YHR175W	*CTR2*	3.181	Low-affinity copper transporter of the vacuolar membrane
	YEL065W	*SIT1*	3.890	Ferrioxamine B transporter
	YER145C	*FTR1*	7.141	High affinity iron permease
	YFL050C	*ALR2*	2.931	Probable Mg^{2+} transporter
	YGL255W	*ZRT1*	10.934	High-affinity zinc transporter of the plasma membrane
	YGL071W	*AFT1*	2.301	Transcription factor involved in iron utilization and homeostasis
	YGR191W	*HIP1*	2.305	High-affinity histidine permease; also involved in the transport of manganese ions
	YKR039W	*GAP1*	5.197	General amino acid permease
	YLR130C	*ZRT2*	2.832	Low-affinity zinc transporter of the plasma membrane
	YMR319C	*FET4*	7.237	Low-affinity Fe(II) transporter of the plasma membrane
	YMR058W	*FET3*	12.753	Ferro-O_2-oxidoreductase
	YML123C	*PHO84*	154.430	High-affinity inorganic phosphate (P_i) transporter; also low-affinity manganese transporter
	YMR243C	*ZRC1*	5.923	Vacuolar membrane zinc transporter
	YNR060W	*FRE4*	3.089	Ferric reductase
	YOR271C	*FSF1*	2.899	Putative protein; predicted to be an alpha-isopropylmalate carrier; likely to play a role in iron homeostasis

Table E.2 (continued) : Particular functional classes of at least two-fold enriched downregulated genes in the evolved strain Caf905-2, relative to the reference strain, obtained by *FunCat* analysis.

	Systematic name	Gene name[a]	Fold change	Description
	YOL130W	*ALR1*	3.434	Plasma membrane Mg^{2+} transporter
	YPL274W	*SAM3*	18.504	High-affinity S-adenosylmethionine permease
Amine/polyamine transport	YBR021W	***FUR4***	4.598	Plasma membrane localized uracil permease
	YBL042C	***FUI1***	14.250	High affinity uridine permease, localizes to the plasma membrane
	YDL210W	*UGA4*	2.277	GABA (gamma-aminobutyrate) permease
	YGL077C	***HNM1***	3.138	Plasma membrane transporter for choline, ethanolamine, and carnitine
	YGR138C	*TPO2*	3.706	Polyamine transporter of the major facilitator superfamily
	YLR237W	***THI7***	2.131	Plasma membrane transporter responsible for the uptake of thiamine
	YPR156C	*TPO3*	4.390	Polyamine transporter of the major facilitator superfamily
Vitamin/cofactor transport	YHR215W	*PHO12*	2.837	One of three repressible acid phosphatases
	YAL067C	*SEO1*	4.815	Putative permease
	YBR220C		3.359	Putative protein of unknown function
	YBR092C	*PHO3*	2.646	Constitutively expressed acid phosphatase similar to Pho5p
	YER060W	***FCY21***	4.246	Putative purine-cytosine permease
	YER056C	***FCY2***	12.878	Purine-cytosine permease
	YGR260W	*TNA1*	2.174	High affinity nicotinic acid plasma membrane permease
	YGR065C	*VHT1*	4.630	High-affinity plasma membrane H^+-biotin (vitamin H) symporter
	YGL077C	***HNM1***	3.138	Plasma membrane transporter for choline, ethanolamine, and carnitine
	YGR096W	***TPC1***	2.071	Mitochondrial membrane transporter
	YLR237W	***THI7***	2.131	Plasma membrane transporter responsible for the uptake of thiamine

CURRICULUM VITAE

Name Surname **:** Yusuf Sürmeli

Place and Date of Birth **:** Antakya, Hatay- 13.01.1985

E-Mail **:** ysurmeli1985@gmail.com

EDUCATION

- **B.Sc.** **:** 2008, İstanbul University, Faculty of Science, Molecular Biology & Genetics Department
- **M.Sc.** **:** 2013, İzmir Institute of Technology, Graduate School of Biotechnology&Bioengineering

PUBLICATIONS, PRESENTATIONS AND PATENTS ON THE THESIS:

- **Sürmeli Y.,** Holyavkin C., Topaloglu A., Arslan M., Kısakesen H.İ. & Cakar Z.P. (2019). Evolutionary engineering and molecular characterization of a caffeine-resistant *Saccharomyces cerevisiae* strain. *World J. Microbiol. Biotechnol. 35*:183.
- **Sürmeli Y.,** Arslan M., Topaloğlu A., Çakar Z.P. Evolutionary engineering of caffeine-resistant *Saccharomyces cerevisiae*. 6th Conference on Physiology of Yeasts and Filamentous Fungi, July 11-14, 2016, Lisbon, Portugal.

OTHER PUBLICATIONS, PRESENTATIONS AND PATENTS:

- **Sürmeli Y.,** İlgü H. & Şanlı-Mohamed G. (2019). Improved activity of α-L-arabinofuranosidase from *Geobacillus vulcani* GS90 by directed evolution: Investigation on thermal and alkaline stability. *Biotechnol. Appl. Biochem*. *66*:101-107.
- İlgü H., **Sürmeli Y.** & Şanlı-Mohamed G. (2019). A thermophilic alpha-L-Arabinofuranosidase from *Geobacillus vulcani* GS90: heterologous expression, biochemical characterization, and its synergistic action in fruit juice enrichment. *Eur. Food Res. Technol. 244*(9): 1627-1636.
- Arslan M., Holyavkin C., Kısakesen H.İ., Topaloglu A., **Sürmeli Y.** & Cakar Z.P. (2018). Physiological and transcriptomic analysis of a chronologically long-lived *Saccharomyces cerevisiae* strain obtained by evolutionary engineering. *Mol. Biotechnol*. *60*(7): 468-484.

- Günel T., Kalelioğlu I., **Sürmeli Y.,** Türken B., Ermiş H., Aydınlı K. (2011). Comparison of real-time polymerase chain reaction assay methods for detection of RHD gene in amniotic fluid. *J. Nat. Sc. Biol. Med*. *2*(2): 193-197.
- **Sürmeli Y.,** İlgü H. & Şanlı-Mohamed G. (2011). Application of protein engineering on α-l-arabinofuranosidase (abfa) from Thermophilic Bacterium. *Curr. Opin. Biotech*. *22*:S86-S87.
- Arslan M., **Sürmeli Y.,** Topaloğlu A., Çakar Z.P. Longevity of stress-resistant yeast mutants obtained by evolutionary engineering. Cell Symposia: aging and metabolism, July 10-12, 2016, Melia Sitges, Spain.