ISTANBUL TECHNICAL UNIVERSITY ★ GRADUATE SCHOOL OF SCIENCE ENGINEERING AND TECHNOLOGY

EVOLUTIONARY ENGINEERING AND MOLECULAR CHARACTERIZATION OF ETHANOL-RESISTANT *Saccharomyces cerevisiae*

Ph.D. THESIS

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Department of Advanced Technologies

Molecular Biology – Genetics and Biotechnology Programme

JULY 2012

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ETANOLE DİRENÇLİ *Saccharomyces cerevisiae*'nin EVRİMSEL MÜHENDİSLİĞİ VE MOLEKÜLER KARAKTERİZASYONU

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FOREWORD

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ABBREVIATIONS

CDW : Cell dry weight
C_T : Threshold cycle
EMS : Ethyl methane sulfonate
HPLC : High performance liquid chromatography
GC : Gas chromatography
HOG : High osmolarity glycerol
HSP : Heat shock protein
MAT : Mating type
MPN : Most probable number
OD : Optical density
PCA : Principle component analysis
qRT-PCR : Quantitative real time polymerase chain reaction
ROS : Reactive oxygen species
RT-PCR : Reverse transcription polymerase chain reaction
YEPDG : Yeast extract - peptone - dextrose - glycerol
YMM : Yeast minimal medium
YPD : Yeast extract - peptone - dextrose (Yeast complex medium)

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EVOLUTIONARY ENGINEERING AND MOLECULAR CHARACTERIZATION OF ETHANOL-RESISTANT *Saccharomyces cerevisiae*

SUMMARY

Owing to its efficient ethanol production capacity, *Saccharomyces cerevisiae* has been the most widely utilized microorganism in both traditional and industrial ethanol-related bioprocesses. In addition to baking, brewing and wine-making industries, the importance of this organism for bio-ethanol production processes has significantly increased due to the rising trend of using ethanol as a bio-fuel in the recent years.

In order to increase the efficiency of ethanol production, several attempts have been made by the researchers for maximum improvement. Directly engineering the yeast ethanol metabolism for yielding higher levels of ethanol, has been the starting point of many approaches. In combination with the fact that the production levels can never exceed the resistance levels of the yeast cultures several research groups focused on the ethanol resistance mechanism in *S. cerevisiae* cells. As a result of these studies, some of the genes responsible in ethanol resistance mechanism have been introduced. However, the complexity of this trait limited the rational metabolic engineering strategies to enlighten the whole system in detail.

In the current study, an inverse metabolic engineering strategy has been adopted to obtain and characterize ethanol-resistant *S. cerevisiae* cells. This methodology starts with the selection of the resistant cells from a genetically diversified population by exposure to the stress condition of interest. The strategy favors the survival of the stress-resistant cells and is called evolutionary engineering. After isolating the cells with the desired characteristics, they are characterized to understand the genetic basis of the desired phenotypic traits. As the last step of this strategy, improved strains may be obtained by using the newly acquired information and the recombinant DNA technology tools.

In this study, the ethanol resistant mutant cultures have successfully been selected under constant (5%, v/v) and gradually increasing (5% to 11.4%,v/v) ethanol stress levels following EMS-mutagenesis. Gradually increasing ethanol stress application was found to be more effective in yielding high ethanol resistance. Thus, the individual mutants isolated from the final generation of gradually increasing ethanol stress application were used in further characterization studies. The individual mutants with highest ethanol resistance levels were shown to have cross-resistances to other industrially important stress conditions such as oxidative, osmotic, heat and freeze-thaw stresses. Growth behaviour of the mutants under non-stress and stress conditions were also analyzed and compared to that of the wild type. Growth physiology of the mutants were investigated by determination of macrokinetic parameters such as ethanol, glucose, glycogen, trehalose levels, biomass formation and viability during aerated fed-batch fermentation. During basic genetic

characterization experiments, the emergence of diploidy in the mutant cultures was realized. Microscopic observations after incubating on sporulation media and genome size determination studies by flow cytometry confirmed the preliminary results on diploidy. Screening of the intermediate populations of selection revealed that the diploidization emerged at around 7%-8% (v/v) ethanol stress level during gradually increasing ethanol stress selection. Moreover, it was demonstrated that diploidization occurred only under gradually increasing ethanol stress application, but not under other stress conditions including the constant ethanol stress selection at 5% (v/v) ethanol concentration. This case-specific feature of diploidization event was further investigated by PCR-based fingerprinting and the results proposed that the cultures were subjected to mating type switching and concomitant diploidization. The homothallic switching endonuclease, encoded by HO gene, is known to be responsible in initiating the mating type switching mechanism in yeast. This gene was sequenced with its -1000 bp and +200 bp flanking regions to detect any possible mutations. However, no mutations were observed. The expression levels of HO and MKT1 encoding its post-transcriptional regulator were analyzed by qRT-PCR. In addition, the *petite* colony frequency of the mutants together with the intermediate populations were investigated. No correlation was observed between diploidy and the tested parameters. Gradually increasing ethanol stress selection studies with *EMS-mutagenized ΔHO*, *EMS-mutagenized ΔMKT1*, *EMS-mutagenized X2180-2A* and *non-mutagenized CEN.PK* as the starting cultures were accomplished in order to identify the potential roles of the strain type, EMS-mutagenesis, HO and MKT1 genes in diploidization.

The acquired data pointed out a tendency to multi-ploidy in ethanol-resistant *S. cerevisiae* strains as a result of the evolutionary engineering application. It is also known that industrial strains of *Saccharomyces cerevisiae* have polyploidy and are generally more robust than haploid laboratory strains used for research purposes. Thus, this study constitutes a pioneering work on the induction of mating type switching in response to gradually increasing levels of ethanol stress in *Saccharomyces cerevisiae* cells and suggest a potential link between polyploidy and high ethanol resistance.

ETANOLE DİRENÇLİ *Saccharomyces cerevisiae*'NİN EVRİMSEL MÜHENDİSLİĞİ VE MOLEKÜLER KARAKTERİZASYONU

ÖZET

Saccharomyces cerevisiae verimli etanol üretim kapasitesi dolayısıyla etanolle ilişkili geleneksel ve endüstriyel biyo-proseslerde en sık kullanılan mikroorganizmadır. Ekmek, bira ve şarap üretim endüstrilerine ek olarak son yıllarda etanolün biyo-yakıt olarak kullanımındaki artışla birlikte bu organizmanın biyo-etanol üretim prosesleri açısından önemi de her geçen gün artmaktadır.

Etanol üretim süreçlerindeki verimin arttırılması için, araştırmacılar tarafından en üst düzeyde iyileştirmeye yönelik çok sayıda girişim gerçekleştirilmiştir. Daha yüksek düzeylerde etanol üretimi için doğrudan mayadaki etanol metabolizması üzerinde yapılacak mühendislik çalışmaları, çok sayıda yaklaşım için başlangıç noktası olarak kabul edilmiştir. Üretimde kullanılan maya kültürlerinin üreteceği alkol konsantrasyonunun hiçbir zaman dayanabildikleri en yüksek etanol düzeyinden daha yüksek olamayacağı gerçeği ile bağlantılı olarak araştırma grupları *S. cerevisiae* hücrelerindeki etanol direnç mekanizması üzerine odaklanmışlardır. Bu çalışmalar sonucunda etanole direnç mekanizmasında görevli bazı genler açıklanabilmiştir. Ancak, bu davranışın oldukça karmaşık bir genetik alt yapısı olması dolayısı ile rasyonel metabolizma mühendisliği uygulamaları sistemin tamamını ayrıntılı olarak aydınlatamamıştır.

Bu çalışmada, etanole dirençli *S. cerevisiae* hücreleri elde etmek ve karakterize etmek amacı ile bir tersine metabolizma mühendisliği stratejisi benimsenmiştir. Bu metot genetik çeşitliliği arttırılmış bir başlangıç kültüründen ilgili stres koşuluna maruz bırakılarak bu strese dirençli hücrelerin seçilimi ile başlamaktadır. Bu strateji dirençli hücrelerin hayatta kalmasının sağlanması temeline dayanmakta ve evrimsel mühendislik diye adlandırılmaktadır. İstenen özellikteki hücreler izole edildikten sonra fenotipik özelliğin genetik temelinin açıklanabilmesi için hücrelerin karakterizasyon işlemleri başlar. Stratejinin son aşaması olarak elde edilen yeni bilgiler ve rekombinant DNA teknolojileri uygulamaları ile özellikleri geliştirilmiş suşlar elde etmek mümkündür.

Bu çalışmada, etanole dirençli mutant kültürleri etil metan sulfonat (EMS)-mutasyonunu takiben sabit (hacmen %5) ve dereceli olarak artan (hacmen %5 - %11.4) etanol stres düzeyleri altında başarılı bir şekilde seçilmişlerdir. Kültürlerdeki direnç düzeylerinin belirlenmesinde stresli ve stressiz kontrol koşullarında büyümüş olan maya hücrelerinin sayıları en olası sayı (most probable number, MPN) adı verilen Poisson regresyonu temelli bir istatistiksel yöntem kullanılmıştır. %95 doğruluk aralığında sonuç veren bu yöntem 96-kuyucuklu *plate*'lerde beş tekrarlı olarak gerçekleştirilmektedir. Etanol direnci yüksek düzeylerde hücrelerin elde edilmesinde dereceli olarak artan etanol stresi uygulamasının, sabit stres uygulamasına kıyasla daha etkili olduğu görülmüştür. Bu sebeple, daha ileri incelemelerde dereceli olarak artan etanol stresi uygulamasından seçilen mutant

bireylere odaklanılmıştır. Yüksek etanol direnç düzeylerine sahip mutant bireylerin aynı zamanda oksidatif, sıcaklık, donma-erime gibi endüstriyel stres koşullarına da yüksek çapraz direnç gösterdikleri belirlenmiştir. Farklı stres koşullarına direnç durumları arasındaki bağlantı *principle component analizi* yöntemi ile incelenmiştir. Mutantların stresli ve stressiz kontrol koşullarındaki üreme eğrisi analizleri yaban tip ile kıyaslamalı olarak yapılmıştır. Etanol üretim düzeyleri kesikli kültivasyon koşullarında örneklenip gaz kromotografi yöntemi ile incelenmiştir. Etanol direnci yüksek mutant kültürlerin üreme fizyolojileri havalandırmalı yarı-kesikli fermentasyon koşullarında etanol, glukoz, glikojen, trehaloz düzeyleri, biyokütle oluşumu ve canlılık (*viability*) gibi makrokinetik parametreler incelenmiştir. Yüksek etanol direncine sahip mutant bireyler olan B2 ve B8 kültürlerin yaban tip ile kıyaslandığında aynı zamanda yüksek etanol üretim kapasitelerine de sahip oldukları belirlenmiştir.

Bir genel stres cevabı parametresi olarak stressiz ve hacmen %7'lik etanol stresi altında HSP104 geni ifade düzeyleri ters transkripsiyon PCR yöntemi ile incelenmiştir. Mutant kültürlerin yaban tip ile geri çaprazlanması deneyleri sırasında etanole dirençli mutant kültürlerin başlangıçta kullanılan yaban tip kültürün aksine haploit formda olmadıkları fark edilmiştir. Sporlanma ortamında inkübasyonun ardından yapılan mikroskobik incelemeler ve genom boyutunun tayini çalışmaları sonucunda kültürlerin diploit forma geçtikleri belirlenmiştir. Dereceli olarak artan etanol stresi koşulları altında seleksiyon protokolü uygulaması sırasında elde edilen ara populasyonlar üzerinde yapılan tarama çalışmaları diploit forma geçişin hacmen %7-%8 etanol stresi uygulama aşamasında gerçekleştiğini göstermiştir. Ayrıca, diploit forma geçişin sadece dereceli olarak artan etanol stresi altında gerçekleştiği; benzer stratejilerle uygulanan hacmen %5'lik sabit etanol stresi de dahil olmak üzere kobalt, demir, bor, donma-erime, ozmotik, oksidatif stresler çok sayıda farklı stres koşulu altında gözlenmediği dikkat çekicidir.

Diploit hale dönüşen kültürler PCR-tabanlı parmak izleme çalışması ile incelenmiş ve kültürlerin eşey tipi değişimi ve karşıt eşey tiplerinin birleşmesine bağlı diploit hale geçiş durumunun oluştuğu belirlenmiştir. Homotalik dönüşüm endonükleazını kodlayan HO geninin mayada eşey tipi değişimi mekanizmasını başlatmaktan sorumlu olduğu bilinmektedir. Bu gende olası bir mutasyonu belirlemek için, -1000 baz çifti ve +200 baz çifti komşu gen bölgelerini de kapsayacak şekilde DNA dizi analizi yapılmıştır. Ancak, etanole dirençli mutant kültürlerin HO genlerinde herhangi bir mutasyona rastlanmamıştır. HO geninin ve bu genin transkripsiyon sonrası regülatörü olan MKT1 geninin ifade düzeyleri hem etanole dirençli mutant kültürlerde hem de bu kültürlerin seçimi sırasında elde edilen ara popülasyonlarda qRT-PCR ile belirlenmiştir. Buna ek olarak, aynı kültürlerde petite koloni frekans analizi yapılarak mitokondriyel hasar durumu tesbit edilmiştir. Bu parametreler açısından incelenen kültürlerde herhangi bir korelasyona rastlanmamıştır. Dereceli olarak artan stres uygulaması ile EMS-mutasyonuna uğratılmış ΔHO, EMS-mutasyonuna uğratılmış ΔMKT1, EMS-mutasyonuna uğratılmış X2180-2A ve mutasyona uğratılmamış CEN.PK kültürleri başlangıç kültürü olarak kullanılarak aynı yöntemle dereceli olarak artan etanol stresine maruz bırakılmışlardır. Her birinin diploit forma dönüşüp dönüşmediği ara popülasyonlar oluştukça kontrol edilerek suş tipi, EMS mutasyonu ile HO ve MKT1 genlerinin diploit forma dönüşüme olan potansiyel etkileri incelenmiştir. Analiz sonucunda bu kültürlerden sadece EMS-mutasyonuna uğratılmış ΔHO kültürünün diploit forma dönüştüğü belirlenmiştir.

Elde edilen sonuçlar, uygulanan evrimsel mühendislik yönteminin etanole dirençli ve etanol üretim kapasiteleri yüksek kültürler elde etmede oldukça etkili bir yöntem olduğunu ortaya koymuştur. Ayrıca etanole dirençli kültürlerdeki birden fazla kromozom kopyası içermeye (*multi-ploidy*) yönelik bir eğilimin, laboratuvar koşullarında da dereceli olarak artan etanol stresi altında görüldüğünü göstermiştir. Bu çalışma mayada eşey dönüşümünün etanol stresi etkisi ile tetiklendiğini gösteren öncü sonuçları içermekte ve poliploid özellik ile yüksek etanol direnci arasında potansiyel bir ilişkiye dikkat çekmektedir.

1. INTRODUCTION

1.1 *Saccharomyces cerevisiae*: A Brief Introduction

Saccharomyces cerevisiae, also known as baker's yeast or budding yeast, is a unicellular eukaryote and particularly an ascomycetous fungus. *S. cerevisiae* cells are in oval shape with 5-10 μm diameters and the volume of a cell can be 70-120 μm^3. The cells are surrounded by a cell wall composed of mainly glucose, N-acetylglucoseamine and mannose residues (Alberts B. , 2002; Sherman, 2002).

S. cerevisiae is one of the most widely studied microorganisms and the first eukaryote to have its complete genome sequenced in 1996. The haploid yeast genome contains 16 chromosomes. The yeast genome is highly compact even when compared to other yeasts and fungi, with 6275 genes representing 72% of the total sequence. It can grow rapidly in 90 minutes of doubling time in complete media (YPD: 1% yeast extract, 2% peptone, and 2% glucose) at 30°C. It shares technical advantages of prokaryotes such as having low genetic complexity. In addition, ease of replica plating and mutant isolation, dispersed cells, a well-defined genetic system, nonpathogenicity, commercially availability, relatively easy and inexpensive culturing and a highly versatile DNA transformation system are other important properties of yeast cells. Due to these advantages, *S. cerevisiae* is accepted as one of the best model eukaryotic microorganisms for biological studies (Sherman, 1997; Geoffrey, 2000; Sherman, 2002; Madigan, 2006) .

S. cerevisiae cells can reproduce either vegetatively or sexually. Vegetative reproduction occurs via budding, during which the mother cell undergoes mitosis and emerges a small bud from the surface. The number of buds formed by each parent cell is about 20-30 during its lifetime. The age of a cell can be determined by the number of bud scars formed on the cell wall (Lodish H., 1995; Solomon E.P., 1999). A typical *S. cerevisiae* cell with the bud scars is shown in Figure 1.1.

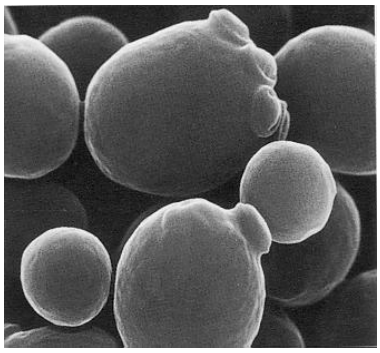

Figure 1.1: Scanning electron microscopy image of *S. cerevisiae* (Alan E. Wheals, PhD, University of Bath).

Sexual reproduction is the second reproduction system in *S. cerevisiae.* There are two different mating types in the mating process; a and α considered to be the analogous of the male and female gametes. Each mating type responds to the pheromones of the opposite mating type by being arrested in G1 phase of cell cycle and changes its shape by elongating and forming a pear-shaped non-dividing cell, which is called a ”shmoo” (Figure 1.2). Fusing of two shmoos results with formation of a single diploid cell. Diploid cells might undergo meiosis under nitrogen starvation conditions and form an ascus structure involving four ascospores, two of each mating type (Alberts B. , 2002; Madigan, 2006).

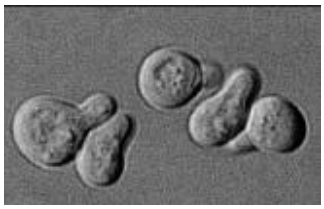

Figure 1.2: Shmoo formation in *S. cerevisiae* cultures (Alberts B. , 2002)

The mating type is determined by two alleles of mating type (MAT) locus on chromosome III. Regulatory proteins a1 and α1-α2 are encoded by MAT a and MAT α, respectively (Figure 1.3). Silent copies of either a1 or α1 and α2 genes are expressed in MAT locus by a process called cassette mechanism.

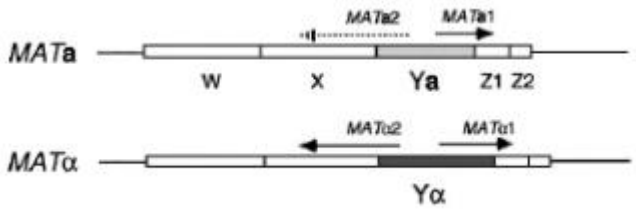

Figure 1.3: Structure of MAT a and MAT α alleles (Haber, 1998).

Switching of the mating type is possible by a gene conversion mechanism initiated by a double-strand break on MAT locus by the site-specific HO endonuclease. HO gene is post-transcriptionally regulated by MKT1 (Tadauchi et al., 2004). Following the double strand break, one strand of DNA is removed and Rad51 protein binds to the second strand to find a template to repair the break. One of the two loci, HML a or HMR α, located respectively at 200 kb upstream and 100 kb downstream of MAT, is used as a template for the repairing event resulting with replacement of the transcribed sequence in each case (Haber, 1998; Coic et al., 2006). The structure of the mating type loci on chromosome III is given in Figure 1.4.

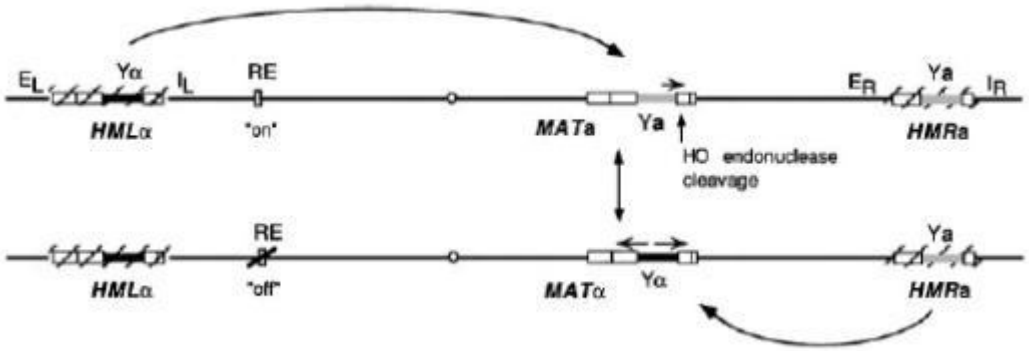

Figure 1.4: Structure of the mating type loci on chromosome III (Haber, 1998).

According to the capability of mating type switching, *S. cerevisiae* cells are classified as either heterothallic or homothallic. Homothallic strains are only found in diploid form whereas; heterothallic strains have a life cycle involving both diploid and haploid forms through mating type switching and self-diploidization due to mating with the opposite type. Conversion to diploid state appears to endow an evolutionary advantage to heterothallic strains (Haber, 1998; Madigan, 2006).

S. cerevisiae cells are able to carry out two modes of chemoorganotrophic metabolism. When O_2 is present in the environment, aerobic respiration takes place and results with increased biomass and CO_2 production through citric acid cycle. Under anoxic conditions, they reduce biomass formation and produce ethanol and CO_2 via anaerobic fermentation. Unlike many other yeast cultures, *S. cerevisiae* cells are facultative aerobic fermenters which means that they can carry on fermentation under both aerobic and anaerobic conditions (Madigan, 2006).

1.2 Industrial Importance of *Saccharomyces cerevisiae*

Saccharomyces cerevisiae is one of the earliest domesticated organisms in human civilization history. Owing to its capability for rapid and efficient conversion of sugars into ethanol, efficient glucose repression and tolerance to high ethanol concentrations, *S. cerevisiae* has been a popular microorganism for both traditional and industrial production processes (Piskur et al., 2006).

In baking, *S. cerevisiae* is used as a leavening agent. CO_2 production as a result of the fermentation process, leads to a dough rise in bread with a lighter and finer texture (Black G., 2002). In wine production, *S. cerevisiae* carries out the primary fermentation by converting glucose into ethanol. There are a number of yeasts that are used for the primary fermentation of wine production. However, *S. cerevisiae* cells are reported to be the only ones that can pursue functioning as the ethanol levels increase during the processes due to their higher resistance to ethanol (Piper, 1995; Madigan, 2006). In brewing, it is used as the ale yeast for brewing from malted barley by a fermentation process. Moreover, in addition to its consumption as active dry yeast, it is also used as a nutritional supplement as vitamin B source especially for low-meat and vegetarian diets (Ratledge C., 2001).

Apart from its importance for nutritional requirements, the use of *S. cerevisiae* in bioethanol production from biomass resources such as corn, maize, wheat crops, reed canary grass, cord grasses, jerusalem artichoke, myscanthus and sorghum plants, has been increasing all over the world in the recent years. By milling and hydrolysis steps, the starch is broken down into simple C6 monomers to be used for fermentation pathways. Recent advances focusing on the use of lignocellulosic and waste-based sources for bioethanol production are considered to be expensive and technically more demanding due to requirements of several pretreatment processes. There are ongoing research and development on diversifying the feedstock range. Bioethanol industry serving for pharmacology, cosmetics, medicine, food sectors as well as for biofuel for transportation and fuel cell systems to be used for heating and power generation renewed the interest on bio-ethanol production and ethanol stress resistance in recent years (Shimokawa et al., 2009; Zhao and Bai, 2009; Babu et al., 2011; Han et al., 2011; Nigam and Singh, 2011).

1.3 Ethanol Stress Response in *Saccharomyces cerevisiae*

Induction of stress response mechanisms provides the cell to sense the alterations in the environment and to survive under severe conditions. In order to broaden the industrial applications of microorganisms, it is noteworthy to remark the importance of stress response studies. Yeast has been used as a model system to study the regulation networks under stress conditions for years. It has been pointed out to be one of the most suitable organisms to study stress tolerance (Lewis J.G., 1997; Estruch, 2000; Yale and Bohnert, 2001).

S. cerevisiae cells are being exposed to several stress conditions such as osmotic, heat, oxidative, starvation, low pH and organic solvents such as ethanol during bio-processes. They have the ability for rapid synthesis of protective molecules and activation of signal transduction pathways for sensing and tolerating the stress conditions (Ruis and Schuller, 1995). The cellular responses might be specific to a stress condition or more commonly might be overlapping so that the cell might trigger cross-resistance to multiple stressing factors. Genome wide studies based on DNA-microarray, deletion mutants and proteome analysis experiments have been carried out to target the responsible elements of these complex stress resistance mechanisms (Boy-Marcotte et al., 1999; Gasch and Werner-Washburne, 2002; Higgins et al., 2002; Trabalzini et al., 2003; Fujita et al., 2006; Auesukaree et al., 2009).

Being in association with several overlapping pathways, ethanol resistance mechanism constitutes one of the most complex regulatory networks of signal transduction pathways and transcription factors (Dickinson J.R., 2004; Ma and Liu, 2010). Ethanol is a well-known inhibitor of growth. Increasing ethanol levels have been reported to affect growth, cell viability and fermentation rates negatively during batch fermentation. The limiting stress level of ethanol was shown to be 6% (v/v) for *S. cerevisiae* cells (Piper, 1995; Kubota et al., 2004; Ma and Liu, 2010). The adverse effects of ethanol cause crucial problems for the yeast-based economy. Thus, the ethanol stress response of yeast has been investigated with great interest and, obtaining mutant yeast strains with a high level of ethanol tolerance to maintain high efficiency of fermentation and a high yield of ethanol has been a desire for several research groups. In this section, the steps taken to enlighten the ethanol resistance

mechanism in yeast are detailed.

Previous studies aiming to explain the ethanol-resistance mechanisms, focused on specific heat shock proteins. Hsp104p and Hsp12p were shown to be responsible in the tolerance mechanism (Sanchez et al., 1992; Sales et al., 2000). Ethanol stress, causing a selective mRNA export, was shown to lead to the accumulation of bulk poly(A)$^+$ mRNA in the nucleus, while heat shock protein mRNA was being exported (Takemura et al., 2004). In addition, alterations in membrane lipid composition, plasma membrane H^+-ATPase action and trehalose accumulation were shown to be emerged by exposure to ethanol stress (Piper, 1995; You et al., 2003; Aguilera et al., 2006; Dinh et al., 2008; Mannazzu et al., 2008). Moreover, it was demonstrated that increasing intracellular accumulation of trehalose by deletion or by expression of the antisense for acid trehalase gene *ATH1*, resulted with improved ethanol stress tolerance (Kim et al., 1996; Jung and Park, 2005).

Another approach was to increase ethanol tolerance by increasing the intracellular proline accumulation. Takagi and his colleagues showed that L-Proline protected yeast cells from damage caused by freezing or oxidative stress. (Takagi et al., 2005). Du and Takagi reported N-Acetyltransferase Mpr1 of *S.cerevisiae* as a promising potential for developing ethanol-resistant yeast strains. It was pointed out that Mpr1 reduces intracellular ROS levels, compensates H_2O_2-detoxifying enzymes, and by this way protects the cells from ethanol stress (Du and Takagi, 2007). A novel gene, ETP1 (ethanol tolerance protein 1) was introduced which was reported to be needed for adapting to ethanol either as a sole carbon source or a stressor (Snowdon et al., 2009). Puria and his colleagues conferred a novel role to RPI1 (Ras-cAMP pathway inhibitor 1), stating that disruption of this gene caused a drastic reduction in viability during fermentation while overexpression resulted with retained viability at the end of fermentation. They proposed the reuse of RPI1 overexpressing strains in subsequent rounds of bioprocesses for a more economical ethanol production technology (Puria et al., 2009).

Optimization of the culturing conditions and medium composition yielded with improvements in ethanol production. Xue and his colleagues studied the effects of media optimization on the ethanol tolerance of a self-flocculating yeast. The optimized combination of $MgSO_4$, $CaCl_2$ and $ZnSO_4$ was reported to yield the highest viability after shock ethanol stress exposure (Xue et al., 2008). In another

study, the optimization of cultivation conditions were compared and an optimal condition in terms of growth, viability and ethanol production was proposed (Alfenore et al., 2004). Zhao and his colleagues demonstrated that zinc sulfate supplementation in the growth media increased ethanol tolerance of self-flocculating yeast strain and resulted with an increased ethanol production (Zhao et al., 2009).

An alternative approach for detecting the genes that are responsible for ethanol resistance was to focus on the strains that are already used in wine production (Osho, 2005; Zuzuarregui et al., 2006; Cardona et al., 2007). An example to this group of studies is the transcriptomic analysis on 14 commercial wine yeast strains which indicated that each strain has a unique pattern of gene expression (Carrasco et al., 2001). Proteomic analysis of a wine strain isolated from a grape must showed the presence of fermentation stress-induced autoproteolysis directed mainly towards specific isoforms of glycolytic enzymes (Trabalzini et al., 2003).

Izawa and his colleagues worked on Asr1p (alcohol sensitive ring/PHD finger 1 protein) which was previously reported to be important for ethanol tolerance (Betz et al., 2004). However, the results could not be reconfirmed by a latter study (Izawa et al., 2006). On the other hand, it was once more suggested that Asr1 was involved in response to ethanol, based on analysis of expression profiles after 7% ethanol exposure and monitoring the subcellular localization by fluorescence microscopy (Ding et al., 2010). These conflicts seem to be inevitable results of an understanding that takes snapshots of transient expression responses into consideration to explain complex networks as previously reviewed by Ma and Liu (Ma and Liu, 2010).

The combination of technological developments and the insufficiency in the former methodologies to explain the complete tolerance mechanism exhibited a need for more comprehensive approaches. A DNA microarray study was performed to investigate the expression profile of yeast genes in response to ethanol. The data showed that, in addition to the previously identified ethanol-induced genes, a very large number of genes were involved in ionic homeostasis, trehalose synthesis, antioxidant defense and protection against heat were also responsible in ethanol stress tolerance (Alexandre et al., 2001). Another gene array analysis was achieved to determine the alterations in gene expression of yeast at 5% (v/v) ethanol stress. A shock ethanol stress exposure of one hour was shown to result with an increase of more than 3-fold in transcription level of 100 genes and 274 gene transcripts were

reported to decrease more than 3-fold. Clustering of the up-regulated genes were shown to be associated with general stress responses, energy utilization, transport mechanisms, cell surface interactions and lipid metabolism. Similarly, three hours of ethanol stress exposure resulted with 14 up-regulated genes associated with energy utilization, general stress responses and vacuolar functions. Only 7 of these 14 genes were common to that of one-hour stress application. The number of down-regulated genes at three hours decreased to 99 (Chandler et al., 2004). A genome-wide identification of genes that are required for growth at 6% ethanol containing medium was conducted to screen the complete 'EUROSCARF' *Saccharomyces cerevisiae* deletion collection. Genes encoding proteins involved in vacuolar function, cell integrity pathway, mitochondrial function were shown to be important for growth under ethanol stress (Van Voorst et al., 2006).

Another approach included the development of ethanol-tolerant strains by altering the key proteins regulating the global transcriptome via global transcription machinery engineering (Alper et al., 2006). For identification of target genes for constructing ethanol tolerant strains, Hirasawa and his colleagues adopted a DNA microarray analysis based selection protocol on a laboratory strain and a sake strain at 5% ethanol stress. Their results indicated that overexpression of the tryptophan biosynthesis genes or tryptophan supplementation in the growth media led to increased ethanol tolerance (Hirasawa et al., 2007).

The quantitative trait loci (QTL) analysis, which is able to map the genes associated with a polygenic trait, seemed to be promising for a better characterization in ethanol tolerance studies (Hu et al., 2007; Marullo et al., 2007; Katou et al., 2009).

Despite the fact that the ethanol resistance of yeast is considered to be one of the major areas of research for both science and industry, previous studies on this subject including the genome-wide analyses are not more than introducing some of the genes responsible for ethanol tolerance rather than illuminating the whole mechanism and thus, the complete mechanism of yeast ethanol tolerance is still not well known.

1.4 Evolutionary Engineering: A Promising Approach for Investigation of Complex Traits

In parallel to the technological innovations, strain improvement methods evolved from selective breeding to induced mutagenesis and recombinant DNA technology applications. Development in recombinant DNA technology has been crucial for strain improvement in recent decades. Metabolic engineering approach, conferring directed improvements in cellular properties, uses recombinant DNA technology applications for modification of biochemical pathways (Bailey et al., 1996; Stephanopoulos, 1999). In this approach, a deep insight into the genetic basis is required in order to achieve manipulations on the specific points of interest. However, due to limited background information on the regulation systems of the metabolic networks, rational metabolic engineering applications often eventuates with unpredicted results because the cell activates secondary counteracting regulation systems as a response to genetic alterations. Moreover, it is challenging to manipulate the genomes of industrial microorganisms because of their genetic complexity (Çakar et al., 2012). Such limitations directed the scientists to develop an alternative strategy called inverse metabolic engineering.

In contrast to the rational metabolic engineering approach, inverse metabolic engineering starts with the identification of a desired phenotype. Secondly, the genetic basis of this phenotype is determined and then re-engineering the behaviour in the organism of interest is achieved (Bailey et al., 1996; Yang Y.T., 1998).

In order to successfully isolate the desired phenotype, a heterogeneous population is obtained via random mutations applied on the starting culture either physically by UV mutagenesis or chemically by alkylating agents such as ethyl methane sulphonate. The genetically diversified initial culture is then exposed to a selective pressure to select the mutants with desired characteristics. As a consequence, by following the evolution-based selection strategy, strains with improved functions are obtained rapidly in laboratory conditions. Moreover, the derived strains are regarded as safe and approved by the public acceptance since the methodology is a natural and universal selection strategy that does not involve the introduction of foreign genes (Bailey et al., 1996; Arnold and Volkov, 1999; Gill, 2003).

Evolutionary engineering strategy was successfully applied to develop multiple-stress resistant (Çakar et al., 2005) and cobalt hyper-resistant (Çakar et al., 2009) mutants of *Saccharomyces cerevisiae.* Wei and his colleagues confirmed the efficiency of these previous studies on successive cycles of freeze-thaw stress treatment (Çakar et al., 2005) by improving multiple-stress tolerance of an ethanologenic *S. cerevisiae* strain (Wei et al., 2007).

Scientists adopted strategies similar to evolutionary engineering such as adaptation to ethanol stress by repetitive culturing under ethanol stress (Dinh et al., 2008), and yielding increased ethanol production by adaptation to high ethanol concentrations by using Dinh's protocol with some modifications (Fiedurek et al., 2011). In another study, following long term culturing on gluconate, cells were directed from glycolysis to pentose phosphate pathway, and displayed higher fermentation rates and enhanced aroma production characteristics (Cadiere et al., 2011).

A group of studies involved alternative techniques such as transposon mutagenesis (Takahashi et al., 2001), genome shuffling (Shi et al., 2009; Hou, 2010) and over-expression of a proofreading-deficient DNA polymerase δ (Abe et al., 2009) for broadening the pool prior to selection of the desired phenotype.

It is now predicted that the complexity of ethanol-resistance mechanism will be enlightened by evolution-based approaches (Stanley et al., 2010).

1.5 The Scope of the Current Study

In this study, evolutionary engineering methodology was adopted to obtain ethanol-resistant *Saccharomyces cerevisiae* strains. For this purpose, the cells were chemically mutagenized by ethyl methane sulfonate (EMS) to increase the genetic diversity of the culture. Following EMS mutagenesis, cells were exposed to a series of continuous ethanol stress applications. Stress conditions were designed for enrichment of ethanol resistant mutants in the culture. Two selection strategies were used which were selection at increasing and constant levels of ethanol stress. Two strategies were carried out in parallel. Increasing levels of ethanol stress application resulted with highly ethanol-resistant mutant individuals not only under ethanol stress but also under various other industrially important stress conditions. Growth physiology studies by aerated fed-batch cultivations verified the ethanol resistance by high viability and concomitantly high ethanol production in mutants, compared to

wild type. As an unexpected outcome of ethanol stress exposure throughout batch selection protocols, the haploid wild type strain used in selection was found to switch its mating type and form a diploid, mutant culture. Referring to the commercial polyploid yeast strains used for industrial ethanol production, the tendency of the laboratory strain used in this study to polyploidy demonstrated that it might be a prerequisite to have multiple copies of specific genes in the genome for efficient ethanol production. When taken together, the present results revealed significant clues on ethanol resistance mechanism in *S. cerevisiae* which is promising for improving related industrial bioprocesses.

2. MATERIALS AND METHODS

2.1 Materials

2.1.1 Yeast strain

Saccharomyces cerevisiae CEN.PK113-7D (*MATa, MAL2-8c, SUC2*), wild type strain, was kindly provided by Dr. P. Kötter from Johann Wolfgang Goethe University, Frankfurt, Germany. *Saccharomyces cerevisiae* CEN.PK113-7D *ΔHO* and *Saccharomyces cerevisiae* CEN.PK113-7D *ΔMKT1* deletion mutants were constructed by C. Alkım at INSA Toulouse, France. X2180-2A strain was provided by Prof. Dr. J.M. François from INSA Toulouse, France.

2.1.2 Yeast culture media and cultivation conditions

The composition of yeast minimal medium (YMM) is given in Table 2.1. Unless otherwise stated, this medium was used for stress application cultivations throughout the study.

Table 2.1: Yeast minimal medium

Medium component	**Weight (**per liter of distilled water)
Yeast Nitrogen Base without amino acids	6.7 g
Dextrose	20 g
Agar (for solid media)	20 g

The composition of yeast complex medium (YPD) is given in Table 2.2.

Table 2.2: Yeast complex medium.

Medium component	**Weight (**per liter of distilled water**)**
Bacto Yeast Extract	10 g
Dextrose	20 g
Bacto Peptone	20 g
Agar (for solid media)	20 g

The composition of yeast sporulation medium is given in Table 2.3.

Table 2.3: Yeast sporulation medium

Medium component	**Concentration (**w/v, in distilled water**)**
Potassium acetate	1%
Agar	2%

The composition of YEPDG medium, which was used in petite frequency assay, is given in Table 2.4.

Table 2.4: YEPDG medium

Medium component	**Concentration (**%, in distilled water**)**
Yeast Extract	1% (w/v)
Peptone	0.1% (w/v)
Dextrose	0.1% (w/v)
Glycerol	3% (v/v)
Agar	2% (w/v)

Unless otherwise stated, for growth physiological experiments, cells were grown in 50 mL culture tubes with 10 mL culture volume at 30ºC, 150 rpm for 12-15 hours.

2.1.3 Chemicals

Chemicals used in this study are listed in Table 2.5.

Table 2.5: Chemicals.

Chemical	**Producer**
Agar	Difco
Agarose	Applichem
Ampicillin	Sigma Aldrich
Bacto peptone	Difco
D-(+)-Trehalose dihydrate	Sigma Aldrich
Dextrose	Riedel-de Haen
Ethanol absolute	J.T.Baker
Ethyl methane sulphonate	Alpha-Aeasar
Glycogen	Sigma Aldrich
High Pure RNA Isolation Kit	Roche
Hydrogen peroxide(% 35, v/v)	Merck
IPTG (isopropyl-β-d-thiogalactopyranoside)	Promega
Light Cycler 480 SYBR Green I Master Kit	Roche
Methylene blue	Sigma Aldrich

Table 2.5 (Continued): Chemicals.

Chemical	Producer
pGEM-T Easy Vector System	Promega
2-Phenyl-ethanol	Sigma Aldrich
Phusion High Fidelity DNA polymerase	New England Biolabs
Potassium acetate	Carlo Erba Reagents
Propidium iodide	Sigma Aldrich
QIA-Quick PCR purification kit	Qiagen
Quant-iT RNA assay kit	Invitrogen
Sodium thiosulphate	J.T.Baker
Sorbitol	Merck
SuperScript III One Step RT-PCR System	Invitrogen
Transcriptor High Fidelity cDNA Synthesis Kit	Roche
X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside)	Promega
Yeast extract	Merck
Yeast nitrogen base without aminoacids	Difco
β-glucuronidase/arylsulfatase from *Helix pomatia*	Roche

2.1.4 Buffers and solutions

Buffers and solutions that were used in this study are listed in Table 2.6.

Table 2.6: Buffers and solutions.

Buffer / Solution	Concentration
Acetate buffer (pH 4.2)	0.1M
DEPC (diethylene pyrocarbonate)	0.1 % (v/v)
DNase free RNase	0.25 g/l
Glycerol	30% (v/v)
H_2O_2 solution	5 M
H_2SO_4 solution	5 mM
Phosphate buffered saline (pH 7.4)	50 mM
Potassium phosphate buffer (pH 7.0)	50 mM
Proteinase K	20 g/l
Sodium citrate (pH 7.5)	50 mM
Sodium thiosulfate solution	10 % (w/v)
TAE (Tris-acetate-EDTA)	Stock solution: 10X, Working solution: 0.5X

2.1.5 Laboratory equipments

Laboratory equipments that were used in this study are listed in Table 2.7.

Table 2.7: Laboratory equipment.

Laboratory Equipment	Producer
Autoclave	TOMY SX-700E High Pressure Steam Sterilizer
Balance	Precisa XB220A, Precisa XB620C
Deep freezers and refrigerators	-80˚C Heto Ultrafreeze 4410
	-20˚C Arçelik
	+4˚C Arçelik
Electrophoresis tank	Midicell PrimoTM EC 330
Fermentor	Sartorius ® Biostat B Plus
Flow cytometer	FACS Calibur (Becton Dickinson)
Fluorometer	Qubit Invitrogen
Fluorospectrometer	Nanodrop 3300 Thermo Fisher Scientific
Gas chromatography system	6890 N Network Agilent
GC column	122-5711, DB-5HT, Length:15m, Internal diameter: 0.25mm, Film: 0.1 µm, Agilent Technologies
High performance liquid chromatography (HPLC) system	Shimadzu
HPLC Column	Aminex© HPX-87H (300mm x 7.8mm) Bio-Rad
Incubator	Nüve EN400, Nüve EN500
Laminar flow cabinet	Faster BH-EN 2003
Light microscope	Olympus CH30
Microfuge	Beckman®Coulter Microfuge
	Eppendorf Centrifuge 5424
Micropipettes	Eppendorf Research, Gilson Pipetman
Orbital shaker incubators	Certomat S II Sartorius
pH meter	Mettler Toledo MP220
Power supply for electrophoresis	Powerpack Basic (Bio-Rad)
Real time PCR	LightCycler 480 II Roche
Thermomixer	Eppendorf, Thermomixer Compact
Ultrapure water system	USF-Elga UHQ
UV-Visible spectrophotometer	Shimadzu UV-1700
Water baths	Memmert wb-22

2.2 Methods:

2.2.1 EMS mutagenesis

Saccharomyces cerevisiae CEN.PK 113-7D culture, approximately at a concentration of $1X10^6$ cells/ml; was inoculated into 10 mL YPD, and incubated overnight at 30ºC and 150 rpm to reach a cell concentration of approximately $2X10^8$ cells/ml. 2.5 mL of this culture was washed twice with 50 mM potassium phosphate buffer (pH 7) and resuspended in the same buffer to obtain a final concentration of $5x10^7$ cells/ml. 300 µL of EMS was added into each 10 mL of cell suspension in a screw-cap glass tube. The tube was vortexed and then incubated for 30 minutes at 30º C. In order to stop EMS mutagenesis, an equal volume of freshly made and filter-sterilized sodium thiosulfate solution (10%, w/v) was added into the tube. The solution was mixed well by vortexing and the cells were centrifuged at 10,000 rpm for 10 min (Beckman Coulter, JA 30.50i rotor). The supernatant was discarded and the cells were washed twice with yeast minimal medium without dextrose. The mutated cells were then cultured in YPD and glycerol stock solutions were prepared in 30% (v/v) glycerol for long term preservation at -80°C.

2.2.2 Evolutionary selection strategy

The chemically mutagenized culture with increased genetic diversity was used in batch selection protocols as previously described (Çakar et al., 2005). The technique is simply based on transferring the survivors of a stress application step to the next stress application step. In this study the stress condition was the continuous ethanol content in the growth medium (YMM).

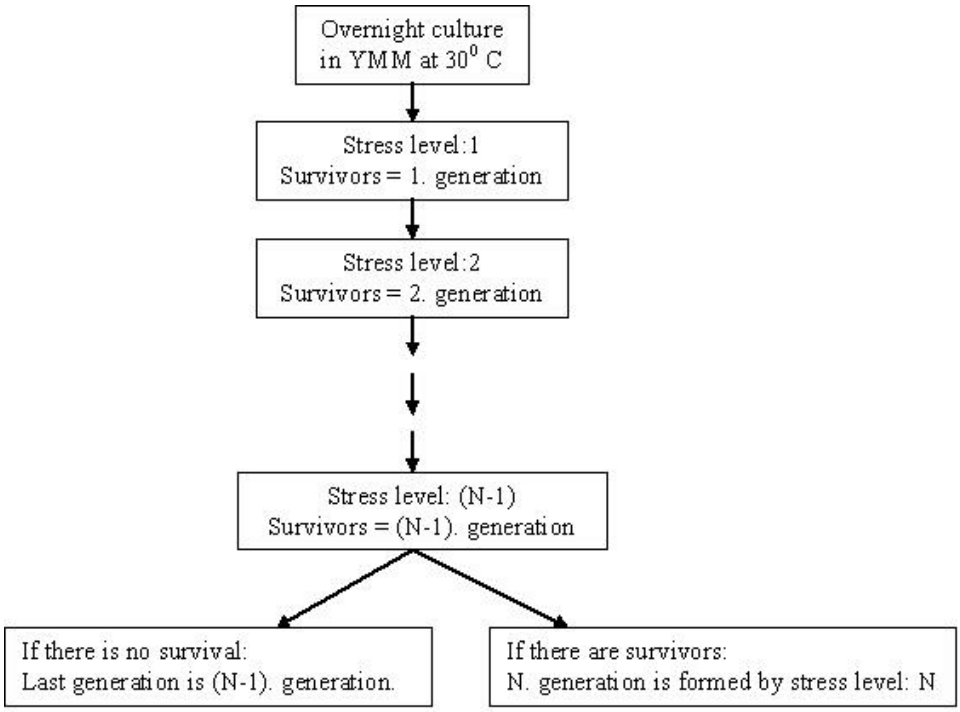

Figure 2.1: Evolutionary selection algorithm.

Two different selection strategies were adopted in parallel: increasing and constant stress selection. In increasing stress selection strategy, the cells were exposed to a gradually increasing level of ethanol stress at each step. The initial stress level was 5% (v/v) ethanol and this stress level was increased to obtain the following generations: 5%, 5.5%, 6%, 6.5% and so on. In constant stress selection, cells were exposed to a constant level of stress at each step. Initial stress level (5% ethanol, v/v) was applied to the cells repeatedly from 1st generation to the last generation. Either constant or increasing, stress selection was carried out continuously, which means that the stress condition was continuously present throughout the cultivation. Optical density values at 24, 48 and 72nd hours of cultivations were reported and used for survival ratio calculations. Survival ratio was calculated by dividing the final optical density value at stress condition to that of non-stress condition.

2.2.3 Selection of individual mutants

As a result of exposure to ethanol stress for several generations, cells with higher resistance levels towards ethanol were selected. In order to obtain the individual mutants from the final mutant population, random selection of individuals was performed. For this purpose, overnight liquid cultures of the final populations obtained either at constant or increasing stress conditions were inoculated into agar

plates. By diluting the culture using streaking or spreading method, individual colonies were randomly selected and isolated. The individuals were transferred to liquid media by using sterile toothpicks and glycerol stock solutions were prepared to be used for further investigation.

2.2.4 Determining the number of cells by most probable number (MPN) method

Determination of cell numbers at stress and non-stress conditions were performed in 96-well plates with five repeats by most probable number (MPN) method which is a statistical, high-throughput technique with 95% confidence (Russek and Colwell, 1983; Çakar et al., 2005). According to this protocol, cells were inoculated into YMM with and without stress conditions in 96-well plates with 5 replicates and serially diluted up to 10^8-fold dilution. The growth in the wells was recorded and the MPN score was converted to cell numbers by using MPN table (Appendix A.1) based on Poisson regression analysis.

2.2.5 Stress application protocols

Stress applications were carried out in liquid minimal media unless otherwise stated. Stress response of wild type and evolved mutants to ethanol (5-12%, v/v), oxidative stress agent 0.3 M H_2O_2 and osmotic stress (2 M sorbitol) was determined by measuring the survival ratios after 72 h of incubation in 96-well plates by the MPN method. The freeze-thaw stress was performed by freezing in either liquid nitrogen for 25 min or at -20°C for 90 min. For this purpose, a one mL sample of exponentially growing yeast culture in YMM was centrifuged, resuspended in 1 mL fresh YMM prior to the stress exposure. The frozen culture was then thawn at 30°C for 20 min. Following stress treatment, cultures were transferred into 96-well-plates and cultivated at 30°C for 72 h for survival rate estimation by MPN assays. Temperature stress was carried out similarly as for the freeze-thaw stress except that the exposure was for 10 min at 60°C. Phenyl-ethanol stress was applied on solid media containing 0 g/L, 3 g/L, 4 g/L and 5 g/L phenyl-ethanol.

2.2.6 Principle component analysis

The resistance levels of the individual mutants selected by INCR strategy under continuous ethanol, pulse ethanol, oxidative, osmotic, cold, freeze-thaw stresses were compared by using principle component analysis (PCA) to analyze the stress

response behaviour of the mutants and the wild type. Analysis was performed by MATLAB software (version 12).

2.2.7 Analysis of viability

Viability of ethanol-resistant mutants were analyzed in reference to wild type as described previously (Hu et al., 2007). Briefly, cells were incubated in YPD medium until they reached stationary phase (OD_{600} ~ 6). They were then centrifuged at 14000 rpm for 5 min in a benchtop centrifuge (Eppendorf) and the supernatant was removed. Cell pellets were then washed in 2 mL sterile water and resuspended in water to a final cell concentration of 10^5-10^6 cells/μL. The cell suspension of 10 μL was added into 5 mL ethanol stress medium containing 0.1 M acetate buffer (pH 4.2), 1% glucose and ethanol (at varying concentrations). The medium was incubated at 30°C, 280 rpm for 72 h. A cell suspension of 5 μL was transferred onto a YPD plate and incubated at 30°C for 48 h. The phenotype of ethanol tolerance was scored as the ethanol concentration at which the cells grew visually to the same cell density on YPD plates as the cells with 10^{-2} dilution factor at 0% ethanol concentration. The cells were then incubated for 72 h at 30°C after which the viabilities were recorded.

2.2.8 Determination of ethanol concentrations by gas chromatography

Ethanol standards were prepared by using absolute ethanol (Sigma) and double distilled water in 0.5-4% (v/v) concentrations. Cells were grown in 50 mL shake flasks containing 10 mL of YMM for 48 hours at 30°C, 150 rpm. They were harvested in a benchtop centrifuge at 14000 rpm for 5 minutes and the supernatants were filtered through 0.2 μm syringe filters (Millipore, USA). The ethanol contents of the filtered supernatants were determined using a gas chromotography system (6890N network, Agilent Technologies, USA) with a flame ionization detector. The filtrate was run on a fused-silica capillary column (DB-5HT, 15 m X 0.25 mm I.D., 0.1 μm film thickness; J & W, USA). The mobile phase was He/N_2.

2.2.9 Macrokinetic analysis by aerated fed-batch fermentation

Cultivations were carried out in a 2 L fermentor SARTORIUS®, Biostat B Plus. Growth medium composition, cultivation conditions and the step-wise glucose feeding procedure were specified as described previously (Alfenore et al., 2002) The air flow was 60 $l.h^{-1}$. Glucose concentration was controlled to be kept above 1g L^{-1}

by feeding with glucose solution in a stepwise manner. Biomass formation and production of ethanol, glycogen and trehalose were monitored during 60h of incubation. Glucose, glycerol and ethanol concentrations were determined by HPLC using an Aminex HPX-87H+ column (300 mm x 7.8 mm) with the following conditions: temperature 40°C, eluent 5 mM H_2SO_4 with a flow rate of 0.5 $ml.min^{-1}$, and dual detection (refractometer and UV at 210nm). Intracellular trehalose and glycogen contents were determined enzymatically as previously described (Parrou et al., 1997). Briefly, 2-5 x $l0^8$ cells were collected by centrifugation at 3800 x*g* for 6 minutes. After washing in 1 mL cold water, it was resuspended in 250 µL 0.25 M Na_2CO_3 and heated to 95°C for 2 h with stirring. The cell suspension was adjusted to pH 5.2 by using acetic acid and sodium acetate buffer. Freshly prepared 100 µg of α-amyloglucosidase from *Aspergillus niger* (Boehringer) from 10 mg mL^{-l} stock solution prepared in 0.2 M sodium acetate buffer (pH 5.2) was added to half of the cell suspension. It was incubated overnight at 57°C with continuous shaking. The second half of the cell suspension was incubated overnight at 37°C in the presence of 3 mU trehalase (Sigma, 0.25 U mL^{-l}). The glucose content obtained after trehalase treatment was determined by using the glucose oxidase assay kit (Sigma). Cell viability (%) was detected by methylene blue staining procedure as previously described by Alfenore et al., 2002.

2.2.10 Analysis of expression levels of HSP104 by multiplex reverse transcription PCR

Expression levels of HSP104 gene were determined by multiplex reverse transcription (RT)-PCR protocol. Total RNA isolation was performed by using High Pure RNA Isolation Kit (Roche) at mid-log phase (OD600 2.0-3.0) of the cells grown under stress (7% v/v ethanol) and non-stress conditions in YMM. Determination of RNA concentrations following RNA isolation was done by using Qubit® Fluorometer and Quant-iT™ RNA assay kit (Invitrogen). To amplify HSP104 gene, 5′-AGCAGGCTCGTCAAGGTAAA-3′ (forward) and 5′-TAGTGGGAACGTCATCGTCA-3′ (reverse) primers were designed by using "Primer 3" and "Amplify 3" softwares. Reverse transcription of mRNA samples and amplification steps were performed by SuperScript™ III One-Step RT-PCR System involving SuperScript™ III RT/ Platinum® Taq DNA polymerase enzyme complex (Invitrogen). Beta-actin gene was used as the internal control. PCR amplification was

conducted as follows: 55^0 C (30 min) for cDNA synthesis, 94^0 C (2 min) for initial denaturation and then 36 cycles at 94^0 C (15 sec) / 50^0 C (90 sec) / 68^0 C (50 sec) and a final extension at 68^0 C (5 min).

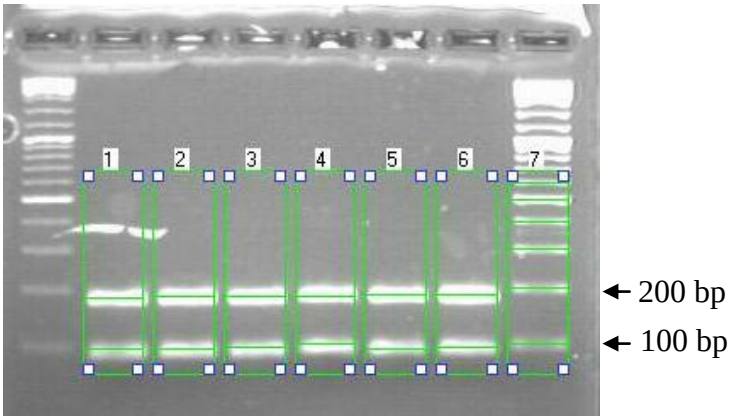

Figure 2.2: Densitometric analysis. Lane 1: wild type at 0%, v/v ethanol, Lane 2: wild type at 7%, v/v ethanol, Lane 3: mutant B2 at 0%, v/v ethanol, Lane 4: mutant B2 at 7%, v/v ethanol, Lane 5: mutant B8 at 0%, v/v ethanol, Lane 6: mutant B8 at 7%, v/v ethanol, Lane 7: DNA ladder (Gene Ruler™ #SM0333).

Densitometric analysis of the PCR products was performed by using "Biocapt Software" (Vilber-Lourmat, Marne La Valle, France). The software output is given in Figure 2.2. Densitometric profiles of the PCR products of wild type, mutant B2 and mutant B8 at 0%, v/v ethanol and 7%, v/v ethanol were analyzed in comparison with that of the DNA ladder and the expression levels of HSP104 were semi-quantitatively determined.

2.2.11 Backcrossing and dissection of ascospores

The mating of the yeast cultures was performed by incubating the opposite mating types of cell cultures together on YMM plates. After 24 hours of incubation, cultures were transferred to sporulation medium with a metal loop and incubated for 24 hours at 30°C. The cultures were analyzed under microscope for detection of tetrad formation. The asci walls were digested with β-Glucuronidase/Arylsulfatase from *Helix pomatia* (Roche) and incubated at 37°C for 1 hour. The culture was transferred to a YPD plate with a metal loop and dissected by using Singer MSM series 200 System micromanipulator (Figure 2.3).

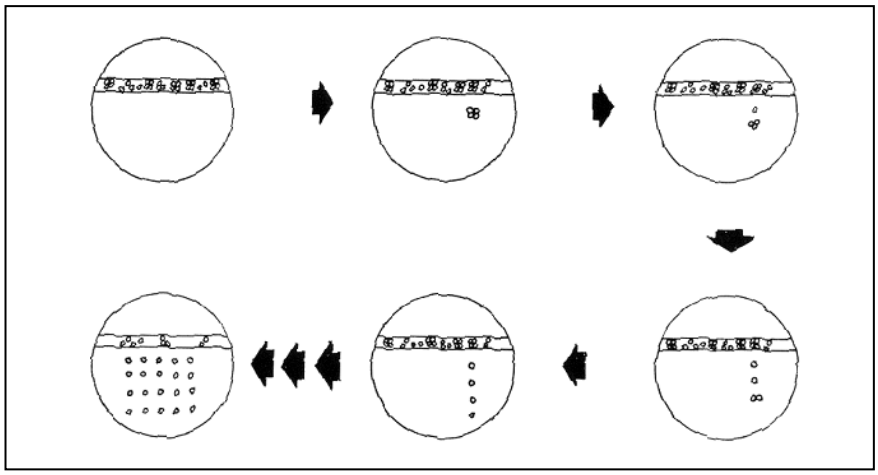

Figure 2.3: Dissection methodology via micromanipulator (Sherman, 2002).

The colonies of the haploid segregants were observed on YPD medium after incubation at 30°C for 1-2 days.

2.2.12 Analysis of diploidy on sporulation medium

The cultures were grown on YPD agar plates and the cells were inoculated onto sporulation agar (KAc) plates by a metal loop and incubated at 30°C. The cells were visualized under light microscope for detecting the presence of tetrads after 24 hours of incubation. Observations were repeated each day up to 72 hours.

2.2.13 Analysis of genome size by flow cytometer

The protocol of Bradbury and his colleagues was adopted for sample preparation for flow cytometry analysis (Bradbury et al., 2006). Cultures were cultivated for 4 days in YPD at 30°C to assure that the cells reached stationary phase. Approximately, 15 x 10^6 cells were collected by centrifugation. Supernatants were discarded and the pellets were placed in 70% (v/v) ethanol for 1 hour for fixation. Cells were centrifuged again and washed with 1 mL 50mM sodium citrate (pH 7.5) and resuspended in 1 mL 50mM sodium citrate (pH 7.5) with 0.25 g/L DNase free RNase. The suspensions were incubated for 1 hour at 55°C. After addition of 10 µl 20g/l proteinase K, they were incubated at 55°C for 1 hour. 50 µL of cell suspensions were sonicated and stained with 4 µL of 50g/l propidium iodide. After overnight incubation at 4°C in dark, analyses were performed by using FACS Calibur (Becton Dickinson) and CellQuest software.

2.2.14 PCR-fingerprinting by using microsatellite primers

PCR-based fingerprinting protocol using microsatellite primers was conducted according to the method described previously (Da Silva et al., 2005). Cell cultures were grown overnight in YPD medium. DNA extraction was performed by using MasterPure Yeast DNA Purification Kit (Epicentre Biotechnologies) with 1.5 mL overnight cell cultures. Isolated DNA was used for fingerprinting by PCR with microsatellite primer $(GTG)_5$. Amplification reaction, which was catalyzed by Taq polymerase, contained 2.5 µM primer and 2.5 µM of each dNTP in 1x Taq buffer in 25 µL final volume. The amplification tubes were hot started at 95°C for 5 min followed by 30 cycles of 95°C for 30 sec, 50°C for 90 sec and 72°C for 3 min, with final extension at 72°C for 7 min. The amplification products were separated in 1.5% agarose gels in 0.5x TAE at 50V for 90 min.

2.2.15 Cloning and sequencing of homothallic switching endonuclease (HO) gene

Previously, DNA isolations from CEN.PK wild type, B2 and B8 cultures were performed by using MasterPure Yeast Purification Kit (Epicentre Biotechnologies). Forward 5'-attgctattgagtaagttcgatc-3' and reverse 5'-tgaggaaagttgatcaagacc-3' primers were designed to amplify HO gene with -1000 bp and +200 bp flanking regions. Amplification was performed by using Phusion high-fidelity DNA polymerase under these conditions: 98°C for 30 sec followed by 30 cycles of 98°C for 10 sec, 50°C for 90 sec and 72°C for 3 min, with final extension at 72°C for 7 min. The amplification products were separated in 0.8% agarose gels in 0.5x TAE at 135V for 20 min. The purification of PCR products were performed via QIAQuick PCR purification kit. The purified PCR products were Poly(A)-tailed according to the technical manual of pGEM-T Easy vector system A-tailing protocols to obtain sticky ends. After A-tailing, ligation was performed again according to pGEM-T Easy vector system ligation protocols. Five µL of ligation solutions were used for transformation. Transformed cells were incubated in LB plates containing ampicillin, IPTG and X-gal. After overnight incubation at 37°C, colonies with the inserts were selected by blue-white screening and cultured in liquid LB containing ampicillin. After overnight incubation, plasmid DNA isolation was performed and visualized on agarose gel. For confirmation of the inserts, restriction enzyme digestion was

performed by using SphI and MfeI enzymes. Plasmid DNA concentrations were quantified by using Nanodrop and the samples were sequenced.

2.2.16 Determination of HO and MKT1 expression levels by qRT-PCR

Total RNA isolations were performed by using High Pure RNA Isolation Kit Protocol (Roche). RNA concentrations were measured by using Quant-iT™ RNA Assay Kit and Qubit® Florometer (Invitrogen). cDNA synthesis from isolated total RNA was performed according to Transcriptor High Fidelity cDNA Synthesis Kit protocol (Roche). Total RNA concentration was adjusted to 500 ng/reaction. qRT-PCR analysis was performed according to protocol of DNA SYBR Green I Master (Roche) kit by using Roche-LightCycler® 480. ACT1 gene was used as the internal control group. The gene expression levels were calculated according to the $2^{(-(\Delta Ct))}$ method (Pfaffl, 2005).

2.2.17 Petite frequency assay

Cultures were streaked onto YPD plates from the glycerol freezer stock to provide growth as single colonies for 2 days. Before spreading on YEPDG, single colonies of the chosen mutant were suspended in phosphate buffered saline (PBS) with different dilution factors; 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5} for optimizing the suitable dilution ratio (Figure 2.2). Four independent colonies for each streaking plate were diluted in sterile 1 mL of PBS as 10^{-4} to spread onto large YEPDG plates. The *petite* and *grande* colonies were counted after growth at 30°C for 5 days. The reported petite frequency was the ratio of small colonies to total number of colonies.

2.2.18 Proteome analysis via electrospray ionization quadrupole time-of-flight (TOF) mass spectrometry

The protein expression levels of the ethanol resistant mutant B8 was analyzed in reference to wild type by electrospray ionization quadrupole time-of-flight (TOF) mass spectrometry in TÜBİTAK Research Institute for Genetic Engineering and Biotechnology (RIGEB) Laboratories by A. Gökçe, Y. Öztürk and T. Baykal, according to the protocol mentioned by Callister et al., 2006.

3. RESULTS

3.1 Chemical Mutagenesis

Wild type culture was chemically mutagenized by using ethyl methane sulphonate as previously described in Methods section. After exposure to EMS for 10 min, 20 min and 30 min, cells were transferred to Petri plates in order to determine the survival ratios of the cultures by counting the number of colony forming units in comparison with the control group. The optimal exposure time was determined to be 30 min that resulted with 90% death ratio. The culture obtained by 30 min of exposure to EMS was used as the initial culture for stress selection strategies.

3.2 Determination of the Inhibitory Ethanol Concentration

To observe the effect of ethanol concentration in the growth medium on final OD_{600} values of wild type and EMS-mutagenized *S. cerevisiae* and to determine a mild stress level for the selection procedure, fresh cultures were incubated in YMM containing 0-15% (v/v) ethanol. The growth results of 48 h incubated cultures are shown in Figure 3.1.

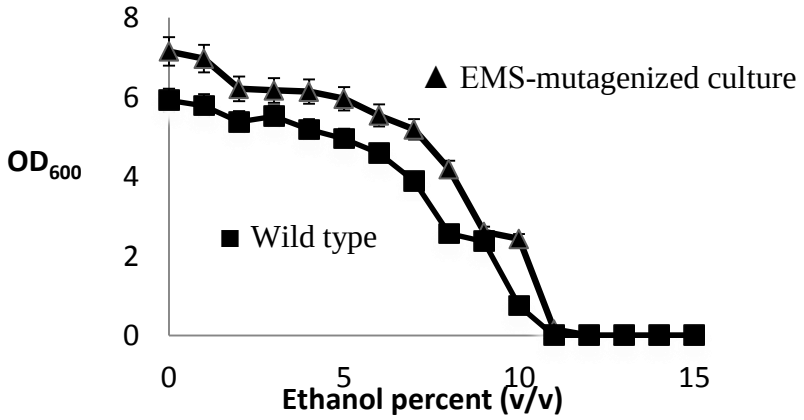

Figure 3.1: The effect of ethanol concentration in the growth medium on final OD_{600} values of wild type (■) and EMS-mutagenized (▲) *S. cerevisiae.*

The results given as the mean values of three independent experiments, revealed a slight difference between these two cultures. Apparently, mutagenesis did not result with a significant change in ethanol resistance. In addition, it was observed that both cultures resulted with 50% reduction in growth under ethanol stress levels higher than 7% (v/v). Five % ethanol stress level was determined to be the initial stress level for the stress selection strategy.

3.3 Selection Strategies under Continuous Ethanol Stress Conditions

After determining the initial stress level, batch selection protocols were applied by simply transferring the survivors of a stress level to the next stress level. Ethanol stress was applied continuously throughout the cultivations. Continuous stress selection strategy is summarized in Figure 3.2.

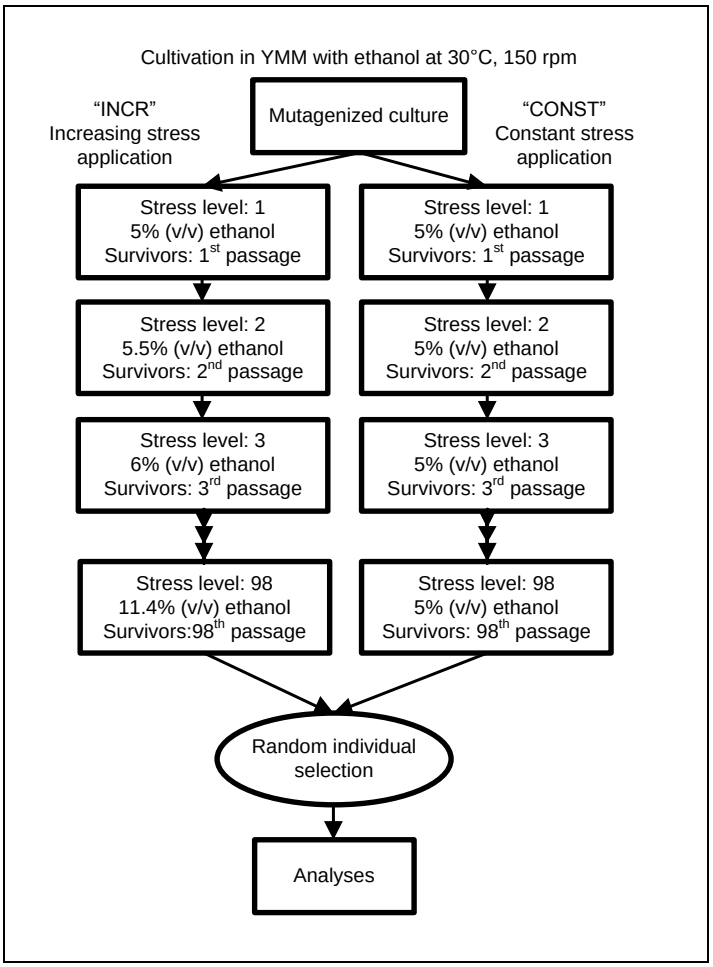

Figure 3.2: Evolutionary selection algorithm based on continuously applied increasing and constant ethanol stress application.

Two selection strategies were adopted in parallel: “INCR” strategy where increasing concentrations of ethanol exposure was applied at each passage starting from 5 % up to a final stress level that the population can no longer tolerate higher values; and “CONST” strategy where a constant application of the same concentration of ethanol of 5 % (v/v) was carried out throughout the selection protocol. For both constant and increasing ethanol stress selections, 98 passaging steps were accomplished. The final stress level of increasing stress selection strategy was 11.4% (v/v) ethanol.

3.3.1 Selection strategy under constant ethanol stress condition

In parallel to increasing stress selection, cells were exposed to a constant stress level of 5% (v/v) ethanol for 98 passaging steps. Table 3.1 shows the optical density and survival ratio values of the constant stress generations.

Table 3.1: Optical density values and the survival ratio values of constant stress generations.

Constant stress passages	% ethanol (v/v)	OD_{600} control	OD_{600} generations	Survival ratio	Incubation time (h)
1	5	5.24	4.34	0.83	48
2	5	6.43	5.35	0.83	48
3	5	6.05	4.36	0.72	48
4	5	5.82	4.40	0.76	48
5	5	6.13	4.58	0.75	48
6	5	6.10	4.00	0.66	48
7	5	6.72	5.01	0.75	48
8	5	5.21	4.66	0.89	48
9	5	6.37	4.66	0.73	48
10	5	6.19	5.72	0.92	48
11	5	6.54	5.38	0.82	40
12	5	5.85	5.11	0.87	48
13	5	6.63	5.46	0.82	48
14	5	6.43	5.46	0.85	48
15	5	5.88	5.09	0.87	48
16	5	5.37	5.10	0.95	48
17	5	5.92	5.29	0.89	48
18	5	5.32	5.25	0.99	48
19	5	5.74	5.66	0.99	48
20	5	6.49	5.53	0.85	48
21	5	6.26	5.24	0.84	48
22	5	6.06	5.84	0.96	48
23	5	6.12	4.64	0.76	48
24	5	5.28	4.82	0.91	48
25	5	5.53	5.25	0.95	48
26	5	5.87	4.44	0.76	48
27	5	4.87	4.85	1.00	48
28	5	5.15	4.08	0.79	72
29	5	5.63	4.42	0.79	51
30	5	4.83	3.93	0.81	48
31	5	5.31	5.16	0.97	72
32	5	6.00	5.46	0.91	48
33	5	4.47	4.17	0.93	72

Table 3.1 (Continued): Optical density values and the survival ratio values of constant stress generations.

Constant stress passages	% ethanol (v/v)	OD_{600} control	OD_{600} generations	Survival ratio	Incubation time (h)
34	5	5.09	4.64	0.91	60
35	5	5.01	3.28	0.65	48
36	5	4.59	4.43	0.97	72
37	5	6.17	4.50	0.73	72
38	5	4.42	4.37	0.99	72
39	5	13.51	4.87	0.36	72
40	5	7.10	4.61	0.65	72
41	5	5.82	5.72	0.98	72
42	5	5.54	4.51	0.81	64
43	5	5.15	4.04	0.78	72
44	5	5.81	4.58	0.79	72
45	5	5.53	5.00	0.90	51
46	5	6.52	4.63	0.71	69
47	5	5.73	4.22	0.74	72
48	5	5.65	5.03	0.89	72
49	5	5.92	4.94	0.83	89
50	5	5.80	4.87	0.84	70
51	5	5.97	5.21	0.87	72
52	5	6.10	5.67	0.93	90
53	5	5.13	4.92	0.96	72
54	5	5.89	5.48	0.93	68
55	5	5.78	5.21	0.90	52
56	5	4.20	3.10	0.74	44
57	5	6.48	5.09	0.79	75
58	5	5.99	5.02	0.84	72
59	5	5.79	5.51	0.95	72
60	5	4.79	4.30	0.90	48
61	5	5.56	4.94	0.89	48
62	5	5.24	5.14	0.98	48
63	5	5.78	5.40	0.93	50
64	5	6.35	5.16	0.81	72
65	5	11.06	10.22	0.92	48
66	5	5.02	4.01	0.80	48
67	5	5.09	4.75	0.93	72
68	5	5.36	5.10	0.95	48
69	5	5.62	4.71	0.84	90
70	5	4.45	4.08	0.92	65
71	5	6.05	4.32	0.71	96
72	5	4.96	4.06	0.82	96
73	5	5.33	4.95	0.93	84
74	5	5.75	3.51	0.61	72
75	5	5.78	5.60	0.97	48
76	5	5.69	4.85	0.85	96
77	5	4.89	4.60	0.94	50
78	5	3.96	3.51	0.89	29
79	5	4.27	3.97	0.93	48
80	5	5.02	4.51	0.90	48
81	5	5.17	5.10	0.99	72
82	5	5.65	5.61	0.99	72
83	5	5.27	4.56	0.87	48
84	5	4.98	4.52	0.91	48
85	5	5.19	5.15	0.99	72
86	5	5.33	4.89	0.92	72
87	5	5.42	3.90	0.72	72
88	5	5.40	4.84	0.90	96

Table 3.1 (Continued): Optical density values and the survival ratio values of constant stress generations.

Constant stress passages	% ethanol (v/v)	OD_{600} control	OD_{600} generations	Survival ratio	Incubation time (h)
89	5	4.92	4.91	1.00	48
90	5	5.39	5.35	0.99	48
91	5	4.88	4.77	0.98	70
92	5	6.21	5.85	0.94	72
93	5	6.95	5.04	0.73	96
94	5	5.32	6.72	1.26	72
95	5	5.47	5.83	1.07	48
96	5	4.87	4.36	0.90	120
97	5	6.36	6.25	0.98	72
98	5	6.11	5.59	0.91	120

Figure 3.3 shows the survival ratio values of each step under constant stress.

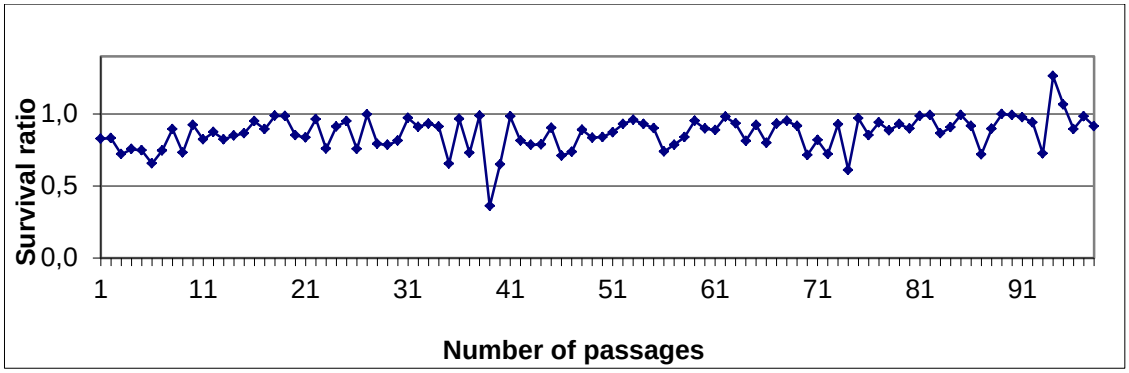

Figure 3.3: Survival ratio values of constant stress selection strategy throughout passages.

The survivors of 98th constant stress selection step was chosen to be the final population.

3.3.2 Selection strategy under increasing ethanol Ssress conditions

Starting from 5% (v/v) ethanol stress, the stress level was increased up to 11.4% (v/v) ethanol at the end of 98 passaging steps. The optical density and the corresponding survival ratio values of each step are given in Table 3.2.

Table 3.2: Optical density values and survival ratio values of increasing stress generations.

Increasing stress passages	% ethanol (v/v)	OD_{600} control	OD_{600} generation	Survival ratio	Incubation time (h)
1	5.0	5.24	4.34	0.83	48
2	5.5	6.42	4.71	0.73	48
3	6.0	6.05	4.01	0.66	48
4	6.5	5.83	3.93	0.67	48
5	6.6	6.70	2.65	0.40	48
6	6.7	6.57	4.83	0.74	48
7	6.8	6.35	4.65	0.73	48

Table 3.2 (Continued): Optical density values and survival ratio values of increasing stress generations.

Increasing stress passages	% ethanol (v/v)	OD_{600} control	OD_{600} generation	Survival ratio	Incubation time (h)
8	6.9	5.87	3.50	0.60	48
9	7.0	6.91	4.38	0.63	48
10	7.1	6.42	5.25	0.82	48
11	7.2	6.85	2.65	0.39	40
12	7.3	5.81	5.03	0.87	48
13	7.4	5.84	2.02	0.35	48
14	7.5	5.26	3.02	0.57	48
15	7.6	4.60	2.79	0.61	48
16	7.7	4.86	3.72	0.77	48
17	7.8	5.00	3.06	0.61	48
18	7.9	4.75	2.39	0.50	48
19	8.0	5.19	3.20	0.62	48
20	8.1	5.22	1.65	0.32	48
21	8.2	4.61	2.09	0.45	48
22	8.3	5.47	3.68	0.67	48
23	8.4	5.04	3.44	0.68	48
24	8.4	4.84	1.07	0.22	48
25	8.4	7.15	1.53	0.21	48
26	8.4	4.73	1.49	0.32	48
27	8.4	4.72	1.70	0.36	48
28	8.4	5.12	3.28	0.64	72
29	8.4	4.67	2.00	0.43	51
30	8.4	4.38	1.98	0.45	48
31	8.4	4.73	3.30	0.70	72
32	8.4	5.14	3.23	0.63	48
33	8.5	4.11	3.03	0.73	72
34	8.6	4.45	3.04	0.68	60
35	8.7	4.77	1.29	0.27	48
36	8.8	4.76	3.43	0.72	72
37	8.8	5.16	3.87	0.75	72
38	8.8	4.64	2.55	0.55	72
39	8.9	5.02	1.52	0.30	72
40	8.9	4.68	1.35	0.29	72
41	9.0	4.48	3.11	0.58	72
42	9.0	4.59	2.58	0.48	64
43	9.1	4.83	2.20	0.46	72
44	9.2	4.54	1.53	0.34	72
45	9.3	4.99	1.82	0.36	51
46	9.4	4.93	2.37	0.48	69
47	9.5	4.70	2.16	0.46	72
48	9.6	5.12	2.03	0.40	72
49	9.7	4.84	3.85	0.80	89
50	9.7	5.10	3.57	0.70	70
51	9.7	4.96	2.36	0.48	72
52	9.7	4.43	3.19	0.72	90
53	9.7	4.73	3.09	0.65	72
54	9.8	5.12	2.58	0.50	68
55	9.8	5.14	3.09	0.60	52
56	9.8	4.30	3.44	0.80	44
57	9.9	5.10	3.21	0.63	75
58	9.9	5.78	2.99	0.52	72
59	9.9	5.01	2.80	0.56	72
60	10.0	4.28	1.30	0.30	48

Table 3.2 (Continued): Optical density values and survival ratio values of increasing stress generations.

Increasing stress passages	% ethanol (v/v)	OD_{600} control	OD_{600} generation	Survival ratio	Incubation time (h)
61	10.0	3.90	1.84	0.47	48
62	10.0	4.89	2.93	0.60	48
63	10.1	4.50	2.88	0.64	50
64	10.1	5.40	2.99	0.55	72
65	10.1	8.70	5.98	0.69	48
66	10.2	4.37	2.03	0.46	48
67	10.3	4.34	1.77	0.41	72
68	10.4	4.55	1.82	0.40	48
69	10.4	3.86	2.72	0.70	90
70	10.4	4.65	1.00	0.22	65
71	10.4	3.94	2.39	0.61	96
72	10.4	5.35	3.01	0.56	96
73	10.4	4.76	1.59	0.33	84
74	10.4	5.58	3.02	0.54	72
75	10.4	5.50	3.67	0.67	48
76	10.5	5.61	2.65	0.47	96
77	10.5	5.59	2.42	0.43	50
78	10.5	3.22	1.70	0.53	29
79	10.6	3.75	1.08	0.29	48
80	10.6	3.64	1.69	0.46	48
81	10.7	4.13	2.76	0.67	72
82	10.7	5.17	3.13	0.61	72
83	10.8	3.98	1.55	0.39	48
84	10.8	4.04	1.82	0.45	48
85	10.9	4.58	3.49	0.76	72
86	10.9	4.42	2.53	0.57	72
87	10.9	2.22	1.56	0.70	72
88	10.9	4.34	2.92	0.67	96
89	11.0	4.65	2.86	0.62	48
90	11.0	4.26	2.55	0.60	48
91	11.1	4.56	2.16	0.47	70
92	11.2	4.23	1.16	0.27	72
93	11.2	3.76	1.33	0.35	96
94	11.2	4.05	2.95	0.73	72
95	11.3	4.66	3.79	0.81	48
96	11.4	3.63	3.60	0.99	120
97	11.4	5.29	3.43	0.65	72
98	11.4	5.61	1.45	0.26	120

Figure 3.4 shows the survival ratio values of the increasing generations.

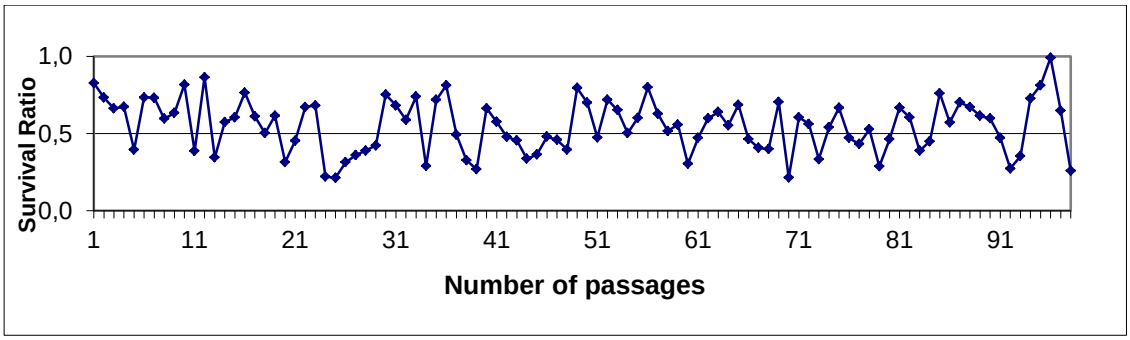

Figure 3.4: Survival ratio values of the increasing stress selection strategy throughout the passages.

The survivors of 98th increasing stress application step corresponding to 11.4 % (v/v) ethanol stress level was chosen to be the final population.

3.4 Analysis of Ethanol Resistance of Intermediate Populations

Ethanol resistance analysis was performed using the statistical high-throughput most probable number (MPN) assay, as described previously. The method is based on serial dilution of cells in the range of 10^{-1} to 10^{-8} in each well containing 180 µl YMM with 2 % glucose. The number of viable cells was determined by using MPN tables with 95% confidence limits (Lindquist, 2012). The survival rate values were obtained by dividing number of viable cells after the stress treatment to that of non-treated cells.

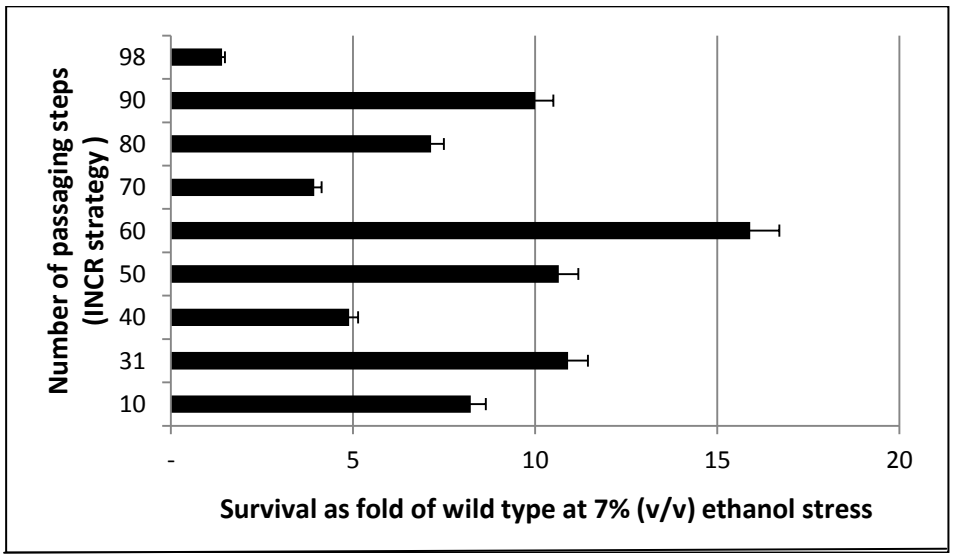

Figure 3.5: Survival values of the intermediate populations obtained by passaging.

The survival values of the intermediate populations obtained through the stress selection studies displayed a randomized pattern rather than gradually increasing resistance level in parallel to the increasing number of passages at ethanol stress (Figure 3.5). The heterogeneity of the culture might be responsible for the non-linear pattern of resistance levels.

3.5 Isolation of Individual Mutants

As a result of exposure to ethanol stress for several generations, cells with higher resistance levels towards ethanol were selected. However, the final culture was a heterogeneous population involving separate cells with varying resistance characteristics. In order to obtain the individual mutants from the heterogeneous population, random selection of individuals was performed.

Overnight liquid cultures of the final populations that were selected under constant and increasing levels of ethanol stress were inoculated into agar plates. Either diluting the culture by streaking method or spreading 100 µl of the overnight culture in a dilution ratio of $1:10^5$ was applied for selection of individual mutant colonies. Following this strategy, 5 mutant individuals named as *B1*, *B2*, *B5*, *B6* and *B8* were selected from the final population obtained by increasing stress selection strategy, and 12 mutant individuals named as *C1*, *C2*, *C3*, *C4*, *C5*, *C6*, *C7*, *C8*, *C9*, *C10*, *C11* and *C12* were selected from the final population obtained by constant stress selection strategy. The individuals were transferred into fresh 10 mL of YMM by using sterile toothpicks and used for characterization studies.

3.6 Characterization of the Individual Mutants

3.6.1 Analysis of ethanol resistance

Ethanol resistance analysis was performed using the statistical high-throughput most probable number (MPN) assay. The number of cells was determined by using MPN tables with 95% confidence limits. The survival rate values were obtained by dividing the number of cells after the stress treatment by that of non-treated cells.

Individual colonies that were obtained by using constant stress selection strategy were cultivated in the presence of 5% (v/v) ethanol in 96-well plates. MPN method was used to determine the ethanol resistance levels. The arithmetic mean resistance

value of the mutant individuals that were analyzed in this experiment was calculated and shown as "I" in Figure 3.6. The results showed that they exhibited less than two-fold improvement of ethanol resistance when compared to the wild type culture (Figure 3.6).

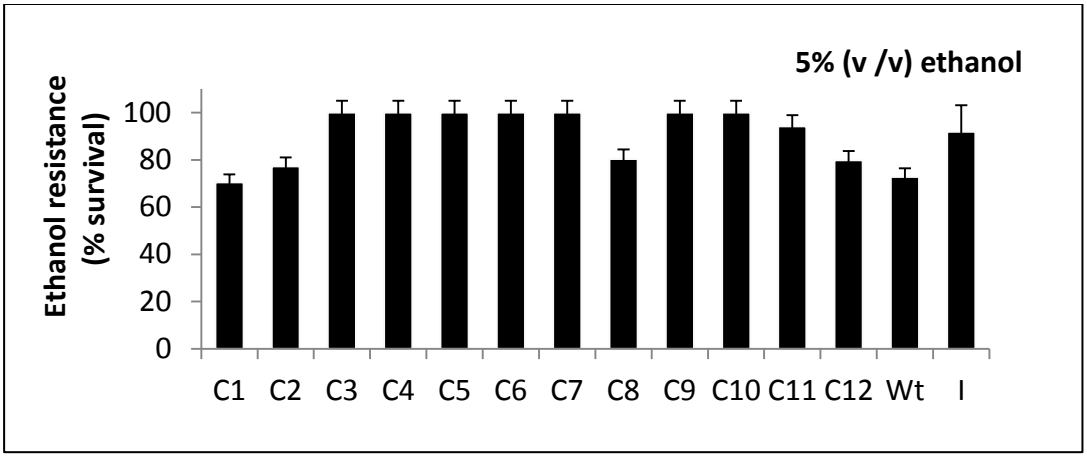

Figure 3.6: Ethanol resistance levels of mutant individuals isolated from continuously applied, constant ethanol stress selection (CONST) strategy.

Ethanol resistances of mutant individuals selected from increasing stress selection strategy were determined in 96-well plates by MPN method in the presence of 10, 11 and 12 % (v/v) ethanol and compared with that of the wild type.

The number of cells/ml and percent survival values of overnight cultures of mutant individuals B1, B2, B5, B6, B8 and wild type under continuous ethanol stress were determined by 5 tube-MPN method. The cultures without ethanol stress were used as control groups. The results at 72nd hour of incubation are shown in Table 3.3.

Table 3.3: Number of cells / mL and percent survival values under continuous ethanol stress at 72nd hour of incubation in YMM.

Sample name	Percent Survival				Survival as fold of wild type			
	8%	10%	11%	12%	8%	10%	11%	12%
B1	54.17	458.33	0.20	0.29	2.2	313.3	31.4	44.9
B2	44.44	64.81	29.63	2.59	1.8	44.3	4558.4	398.9
B5	218.18	154.55	0.03	0.06	9.1	105.6	4.9	9.8
B6	31.76	54.12	4.65	0.13	1.3	37.0	714.9	19.9
B8	145.83	14.58	7.08	0.20	6.1	10.0	1089.7	31.4
Wt	24.07	1.46	0.01	0.01	-	-	-	-

The survival ratio values normalized to wild type are shown in Figure 3.7.

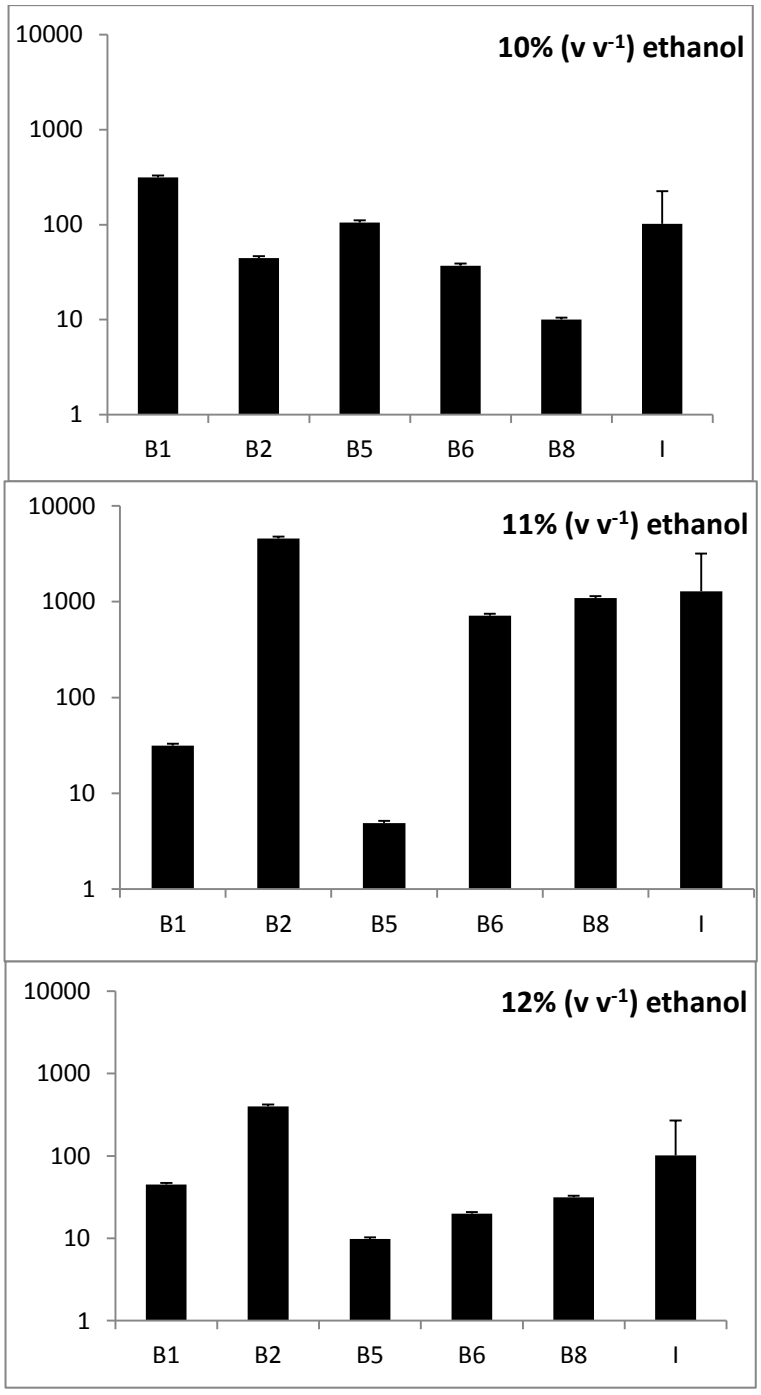

Figure 3.7: Ethanol resistance levels of individuals isolated from the increasing stress levels selection (INCR) strategy. The resistance tests were performed at 10, 11 and 12% (v/v) ethanol.

In contrast to the results of the mutants obtained from "CONST" selection strategy, ethanol resistance results of mutant individuals selected from "INCR" selection strategy were significantly higher than those of the wild type. The survival rates of individual ethanol-resistant mutants obtained by "INCR" strategy were ranging

between 5 to 4558-fold of the wild type at 11% (v/v) ethanol and between 5 to 500-fold of the wild type at 12 % (v/v) ethanol.

In order to analyze whether the mutants retain their resistance towards ethanol stress during growth in complex medium (YPD) as well as minimal medium, overnight cultures of B1, B2, B5, B6, B8 mutants and the wild type were inoculated into YPD medium with 0%, 10%, 11% and 12% ethanol (v/v). The number of cells /ml after 72 hours of incubation were determined by 5 tube-MPN method. The results are given in Table 3.4.

Table 3.4: Number of cells / mL after 72 hours of incubation in YPD.

	Percent survival			**Survival as fold of wild type**		
	10%	**11%**	**12%**	**10%**	**11%**	**12%**
B1	239.4	48.5	48.5	1.1	14.7	228.6
B2	51.5	163.6	1.6	0.2	49.6	7.7
B5	60.8	84.6	0.5	0.3	25.6	2.5
B6	130.8	100.0	0.4	0.6	30.3	1.8
B8	245.5	159.1	109.1	1.2	48.2	514.3
Wt	212.1	3.3	0.2	-	-	-

The increase in survival rates of the mutants normalized to wild type is depicted in Figure 3.8.

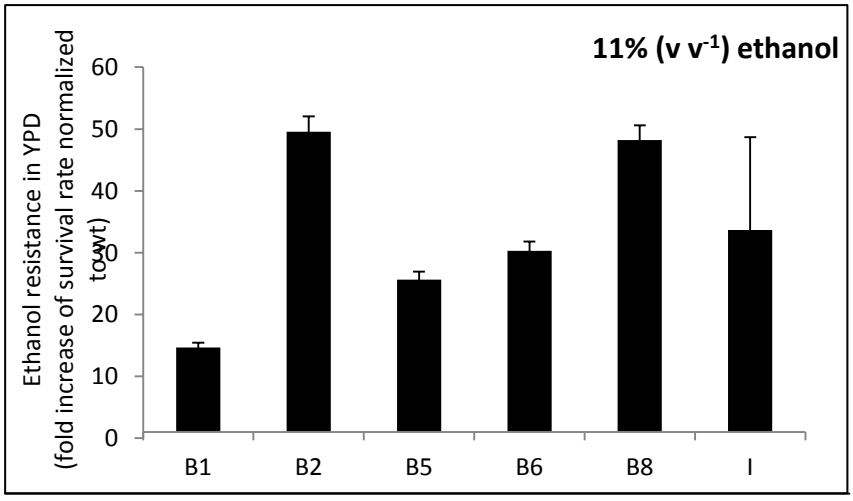

Figure 3.8: Ethanol resistance levels (as fold of wild type resistances) of mutant individuals selected from final evolved population of increasing ethanol stress (INCR) selection strategy, when grown on YPD medium including 11% (v/v) ethanol.

All of the mutants had higher survival values under ethanol stress when compared to wild type in YPD medium. B2 and B8 had the highest resistance levels with approximately 50-fold survival rate normalized to wild type at 11% (v/v) ethanol stress.

3.6.2 Ethanol pulse stress application

To determine the resistances of the mutants to shock exposure to ethanol stress, ethanol stress was applied to the mutants and the wild type as a pulse stress. One mL of overnight cultures of mutant individuals *B1, B2, B5, B6, B8* and wild type cultures were washed with dextrose-free YMM and exposed to 20% (v/v) ethanol stress for 90 minutes. After this pulse stress application, the number of cells per mL and the percent survival values were determined by 5 tube- MPN method. The cultures without ethanol stress exposure were used as control groups. The results at 72^{nd} hours of incubation are given in Table 3.5.

Table 3.5: Number of cells / mL and percent survival values at 20% (v/v) pulse ethanol stress at 72^{nd} hour of incubation.

Sample name	% Survival (20% EtOH)	Survival as fold of wild type (20% EtOH)
B1	0.0073	6.6
B2	0.0007	0.7
B5	0.0042	3.8
B6	0.0029	2.6
B8	0.0015	1.3
905	0.0011	-

The most resistant mutants under continuous ethanol stress conditions (B2 and B8) were shown to be the least resistant under pulse ethanol stress conditions (Table 3.5). This result might be based on the possible differences between the molecular mechanisms of cellular response to pulse and continuous stress.

3.6.3 Analysis of viability

Viability of ethanol-resistant mutants was analyzed in reference to wild type according to the procedure previously explained by Hu *et al*, 2007: Briefly, cells were incubated in YPD medium until the stationary phase (OD_{600} ~ 6). The cells were centrifuged at 14000 rpm for 5 minutes and the supernatants were removed. Cell pellets were washed in 2 mL sterilized water and resuspended in water with the final cell concentration of 10^5-10^6 cells/μl. The cell suspension of 10 μl was added into 5 mL ethanol stress medium with 0.1 M acetate buffer (pH 4.2), 1% glucose and ethanol (with varying concentrations). The medium was incubated at 30°C with 280 rpm shaking for 72 hours. A cell suspension of 5 μl was transferred onto YPD plate and incubated at 30°C for 48 hours. Phenotype of ethanol tolerance was scored as the

ethanol concentration at which the cells grew visually to the same cell density on YPD plates as the cells with 10^{-2} dilution ratio under 0% ethanol concentration.

In Figure 3.9, the growth of the viable colonies on YPD plates following 10%, 11% and 12% ethanol stress exposure are given in reference to control groups for the wild type and the evolved mutants B1, B2, B5, B6 and B8.

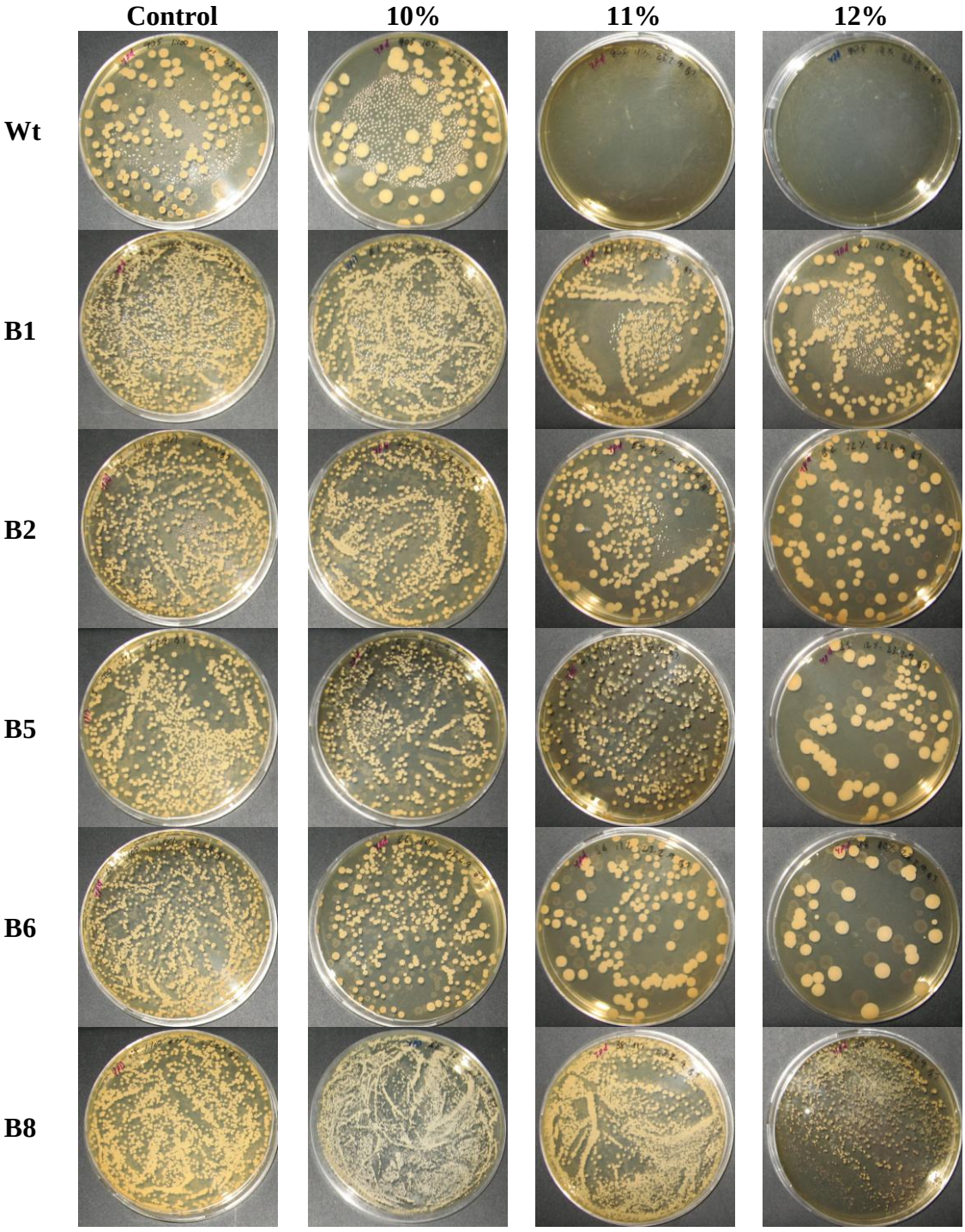

Figure 3.9: Growth of the viable colonies on YPD plates following 10%, 11% and 12% ethanol stress exposure.

The results showed that the evolved mutants (B1, B2, B5, B6 and B8) were fully viable, whereas the wild type cells could not grow on the YPD plate even after 72 h of incubation, following exposure to ethanol concentrations higher than 10% (v/v). The phenotype scoring test results showed that B8 resulted with the highest viability under 11-12% ethanol (v/v).

3.6.4 Cross-resistance analyses

3.6.4.1 Oxidative stress

The number of cells/ml and percent survival values of overnight cultures of mutant individuals *B1, B2, B5, B6, B8* and wild type cultures under continuously applied 0.3M H_2O_2 stress were determined by 5 tube-MPN method. The cultures without H_2O_2 stress were used as control groups. The results at 72nd hour of incubation are given in Table 3.6 and Figure 3.10. "I" represents the arithmetic mean of the resistances of five individual mutants B1-B8 in Figure 3.10, as fold increases normalized to wild type.

Table 3.6: Number of cells / mL and percent survival values under 0.3M H_2O_2 stress at 72nd hour of incubation.

Sample name	% Survival (0.3M H_2O_2)	Survival as fold of wild type (0.3M H_2O_2)
B1	0.0002	0.4
B2	0.0005	0.9
B5	0.0010	1.9
B6	0.0010	1.9
B8	0.0010	1.9
Wt	0.0005	-

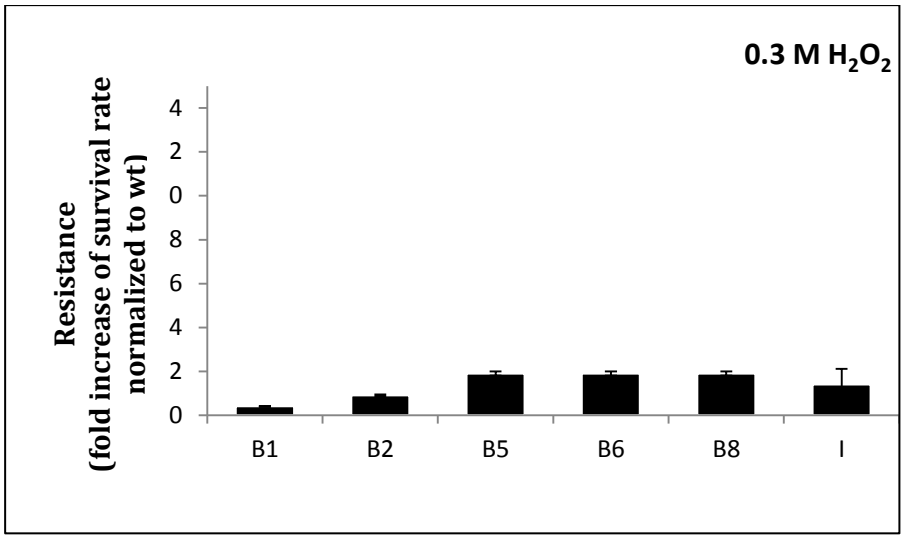

Figure 3.10: Cross-resistance of ethanol-resistant mutant individuals obtained by INCR strategy to continuous oxidative (0.3M H_2O_2) stress.

The most resistant mutants under continuous 0.3M H_2O_2 stress were determined to be B5, B6 and B8. However, the resistance levels of mutants are not higher than 2 fold of wild type levels.

3.6.4.2 Sorbitol stress

The number of cells/ml and percent survival values of overnight cultures of mutant individuals *B1, B2, B5, B6, B8* and wild type cultures under continuously applied 2M sorbitol stress were determined by 5 tube-MPN method. The cultures without sorbitol stress were used as control groups. The results at 72nd hours of incubation are given in Table 3.7 and Figure 3.11. "I" represents the arithmetic mean of the resistances of five individual mutants B1-B8 in Figure 3.11, as fold increases normalized to wild type.

Table 3.7: Number of cells / mL and percent survival values under 2M sorbitol stress at 72nd hour of incubation in YMM.

Sample name	% Survival (2M sorbitol)	Survival as fold of wild type (2M sorbitol)
B1	18.5	1.3
B2	16.1	1.1
B5	329.2	23.0
B6	7.1	0.5
B8	54.2	3.8
Wt	14.3	-

The most resistant mutants under 2M continuous sorbitol stress were found to be B5 and B8. In addition, all of the mutants analyzed had higher resistance values in terms of fold increase in survival rate normalized to wild type.

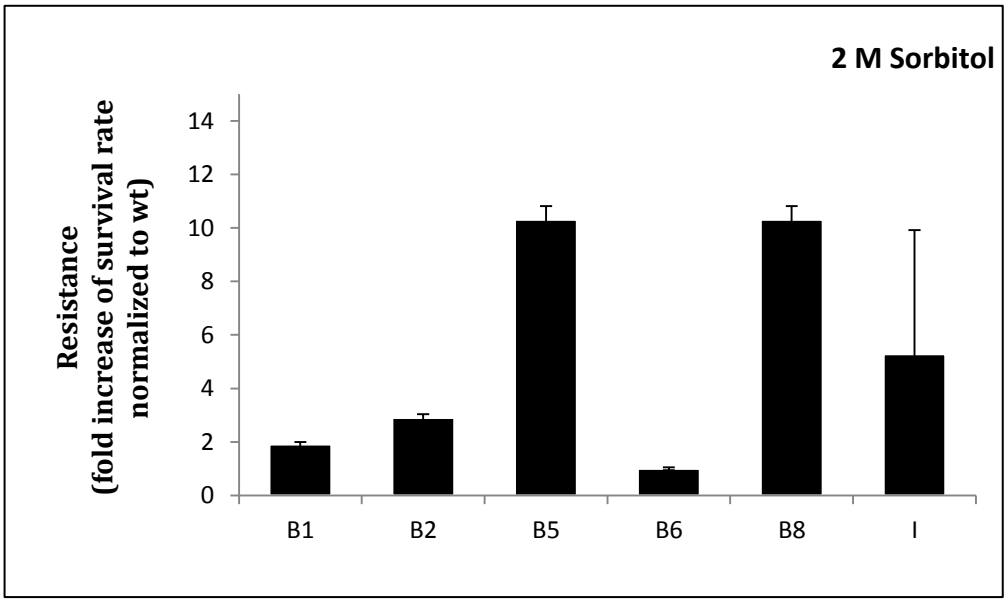

Figure 3.11: Cross-resistance of ethanol-resistant mutant individuals obtained by INCR strategy to 2M continuous sorbitol stress.

In order to investigate whether the mutants were resistant to sorbitol stress in complex medium as well as in minimal medium, overnight cultures of B1, B2, B5, B6, B8 mutants and the wild type were inoculated into YPD medium with 0M and 2M sorbitol. The number of cells /ml after 24, 48 and 72 hours of incubation were determined by 5 tube-MPN method. The results are given in Table 3.8.

Table 3.8: Number of cells / mL at 72nd hour of incubation in YPD.

	Percent survival 2M Sorbitol	**Survival as fold of wild type 2M Sorbitol**
B1	60.77	2.0
B2	67.35	2.2
B5	23.94	0.8
B6	424.24	14.0
B8	376.92	12.4
Wt	30.38	-

The most resistant mutants under sorbitol stress in YPD medium were B6 and B8 with survival as fold of wild type values of 14.0 and 12.4, respectively. The results showed that B8 was resistant to sorbitol stress in both minimal and rich media.

3.6.4.3 Heat stress

One mL of overnight cultures of mutant individuals *B1, B2, B5, B6, B8* and wild type cultures were washed with dextrose-free YMM and exposed to 60^0C temperature stress for 10 minutes. After this pulse stress application, the cells were harvested by centrifugation at 14,000 rpm for 5 minutes. The number of cells per mL and the percent survival values were determined by 5 tube-MPN method. The cultures with 30^0C exposure were used as control groups. The results at 72^{nd} hour of incubation are given in Table 3.9.

Table 3.9: Number of cells / mL and percent survival values under 60^0C pulse heat stress at 72^{nd} hour of incubation in YMM.

Sample name	Survival ratio at 60^0C	% Survival at 60^0C	Survival as fold of wild type at 60^0C
B1	0.025	2.5	2.5
B2	0.004	0.4	0.4
B5	0.010	1.0	1.0
B6	0.003	0.3	0.3
B8	0.061	6.1	6.1
Wt	0.010	1.0	-

B1 and B8 were shown to have the highest resistance levels under 60^0C pulse heat stress.

In order to investigate whether the mutants were resistant to heat stress in complex medium as well as in minimal medium, one mL of overnight cultures of mutant individuals *B1, B2, B5, B6, B8* and wild type cultures were washed with dextrose-free YMM and exposed to 60^0C temperature stress for 10 minutes. After this pulse stress application, the cells were harvested by centrifugation at 14,000 rpm for 5 minutes. The number of cells per mL and the percent survival values in YPD medium were determined by 5 tube-MPN method. The cultures with 30^0C exposure were used as control groups. The results at 72^{nd} hour of incubation are given in Table 3.10 and Figure 3.12. "I" represents the arithmetic mean of the resistances of five individual mutants B1-B8 in Figure 3.12, as fold increases normalized to wild type.

Table 3.10: Number of cells / mL and percent survival values under 60^0C temperature stress at 72^{nd} hour of incubation in YPD.

	% Survival at 60^0C	Survival as fold of wild type at 60^0C
B1	0.28	9.3
B2	0.00	0.1
B5	0.01	0.5
B6	0.01	0.5
B8	0.03	0.9
Wt	0.03	-

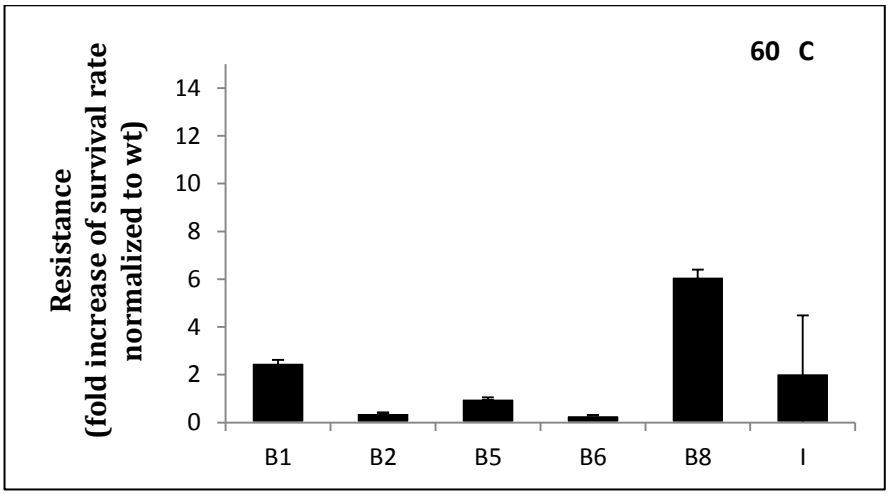

Figure 3.12: Cross-resistance of ethanol-resistant mutant individuals obtained by INCR strategy to pulse heat stress.

According to 24 hour results, B8 has the highest survival value under 60^0C heat stress. It is followed by B1. However, at 48^{th} and 72^{nd} hours of incubation, mutant B1 has the highest survival value under 60^0C heat stress in YPD medium. These results showed that resistance characteristics of the mutants did not change with medium composition.

3.6.4.4 Freeze-thaw stress

1.5 mL of overnight cultures of mutant individuals *B1, B2, B5, B6, B8* and wild type were frozen in liquid nitrogen (-196°C) for 25 minutes. This freezing step was followed by thawing at 30^0C for 20 minutes. The cells were washed and resuspended with YMM-without dextrose and the number of survivors was determined by MPN-based method in reference to control groups (no exposure to freezing-thawing stress). The results at 72^{nd} hour of incubation are given in Table 3.11 and Figure 3.13. "I" represents the arithmetic mean of the resistances of five individual mutants B1-B8 in Figure 3.13, as fold increases normalized to wild type.

Table 3.11: Number of cells/ml and the survival values after freezing thawing stress application at 72nd hour of incubation.

	% Survival	Survival as fold of wild type
B1	83.6	139.4
B2	1.4	2.4
B5	5.4	9.0
B6	3.8	6.4
B8	3.2	5.3
Wt	0.6	-

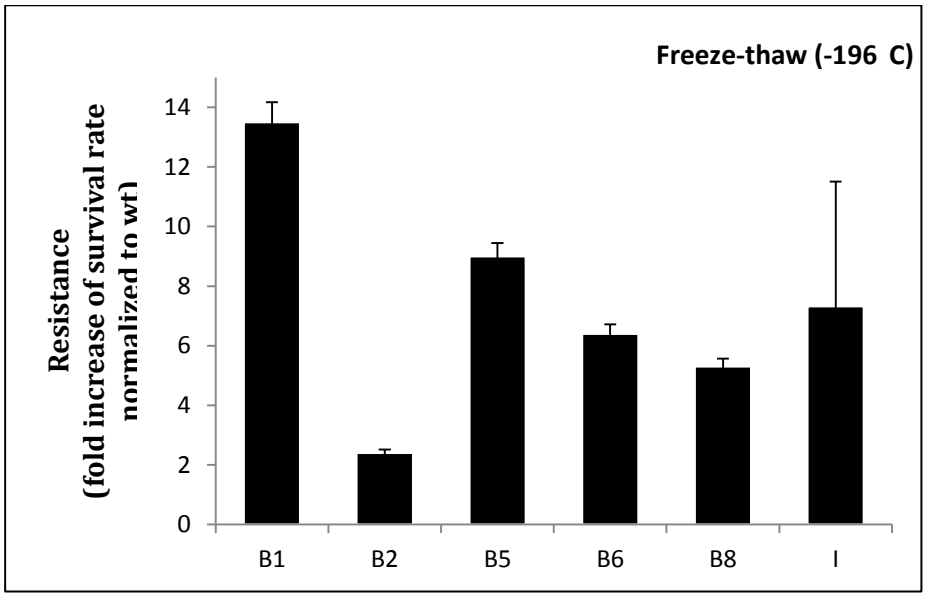

Figure 3.13: Cross-resistance of ethanol-resistant mutant individuals obtained by INCR strategy to freeze-thaw (-196°C) stress.

All mutants tested were more resistant to freezing-thawing stress when compared to wild type. B1 was found to be the most resistant mutant under this stress condition.

3.6.4.5 Cold stress (-20°C)

One mL of overnight cultures of mutant individuals *B1, B2, B5, B6, B8* and wild type *905* were washed with dextrose-free YMM and exposed to -20°C cold stress for 90 minutes. After this pulse stress application, the cells were thawed at room temperature. The number of cells per mL and the percent survival values were determined by 5 tube-MPN method. The cultures with 30°C exposure were used as control groups. The results at 72nd hour of incubation are given in Table 3.12 and Figure 3.14. "I" represents the arithmetic mean of the resistances of five individual mutants B1-B8 in Figure 3.14, as fold increases normalized to wild type.

Table 3.12: Number of cells/ml and the survival values after cold (-20°C) stress application at 72nd hour of incubation.

	% Survival	Survival as fold of wild type
B1	10.0	0.4
B2	53.8	2.0
B5	32.9	1.2
B6	100.0	3.8
B8	100.0	3.8
Wt	26.5	-

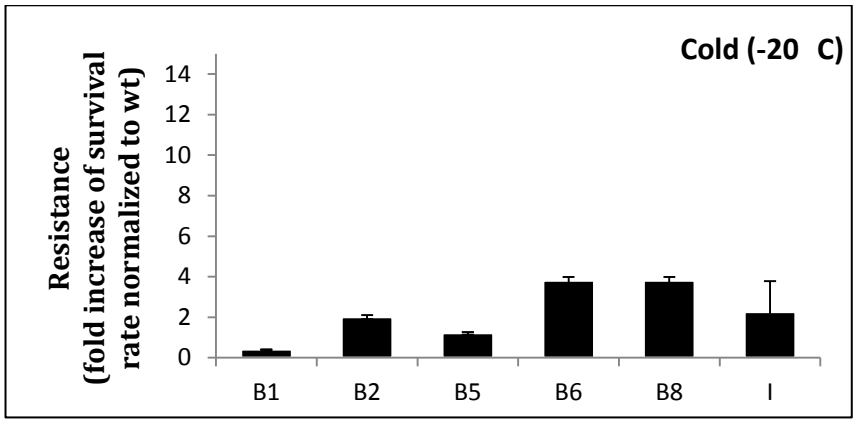

Figure 3.14: Cross-resistance of ethanol-resistant mutant individuals obtained by INCR strategy to cold (-20°C) stress.

B6 and B8 had the highest survival values under cold (-20°C) stress.

3.6.4.6 Methanol stress

Mutant individuals *B1, B2, B5, B6, B8* and wild type cultures were exposed to 5%, 8% and 12% (v/v) methanol stress in 10 mL YMM in 50ml culture tubes. Optical density values were recorded and the percent survival values were calculated after 48 hours of incubation. The cultures at non-stress conditions were used as control groups. The results are shown in Table 3.13.

Table 3.13: Number of cells/ml and the survival values after methanol stress application at 72nd hour of incubation.

	Percent survival values		
	5% methanol	8% methanol	12% methanol
B1	100.00	79.73	48.73
B2	100.00	63.69	30.50
B5	96.37	70.94	41.88
B6	100.00	74.42	47.15
B8	100.00	91.38	44.61
Wt	99.69	66.35	10.22

The results showed that the mutants selected by evolutionary engineering strategy under ethanol stress conditions were 3-5 fold more resistant to 12% (v/v) methanol stress than the wild type.

3.6.4.7 Phenyl-ethanol stress

The growth results of the highly ethanol resistant mutants B2, B8 and the wild type cultures on YPD plates with increasing phenyl-ethanol concentrations are shown in Figure 3.15. The cultures were grown in YPD plates containing 0, 3, 4 and 5 g/L phenyl-ethanol at 30°C for 24 hours. Each culture was diluted within a range of 10^{0}-10^{-4}.

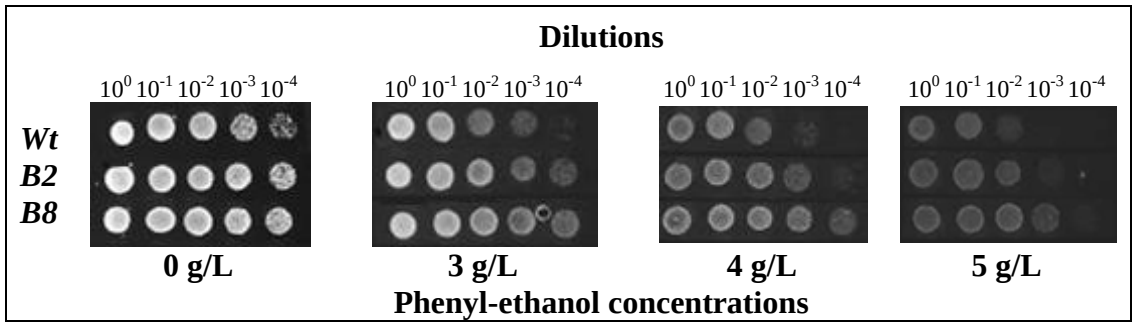

Figure 3.15: Growth of cultures in YPD with 0, 3, 4 and 5 g/L phenyl-ethanol.

Results demonstrated that the highly ethanol-resistant mutants B2 and B8 obtained by evolutionary engineering strategy were slightly more tolerant to phenyl-ethanol when compared to wild type. B8 was found to be more tolerant than B2 at phenyl-ethanol concentrations of 4 g/L and higher.

3.6.5 Principle component analysis

The data sets involving the resistance levels under continuous ethanol, pulse ethanol, oxidative, osmotic, cold and freeze-thaw stresses of the individual mutants selected by INCR strategy were used for principle component analysis. In Figure 3.16, the results of the analysis are given.

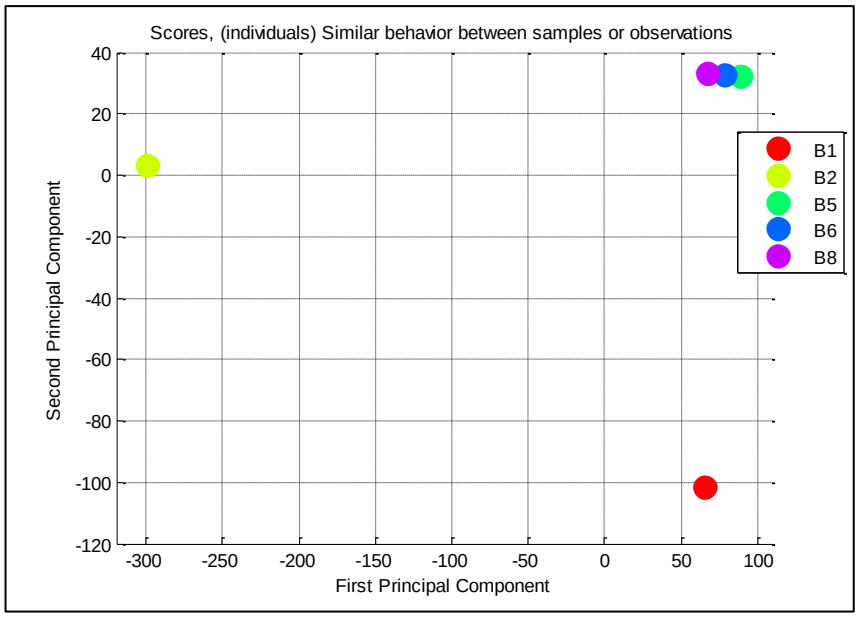

Figure 3.16: Principle component analysis results of the mutants selected by INCR strategy.

According to the PCA results, it was observed that the mutants B1 and B2 had distinct responses under stress conditions when compared with other mutants.

The resistance levels of the individual mutants under continuous ethanol, pulse ethanol, oxidative, osmotic, cold and freeze-thaw stresses were used as a group for principle component analysis. In Figure 3.17, the results of the analysis are given.

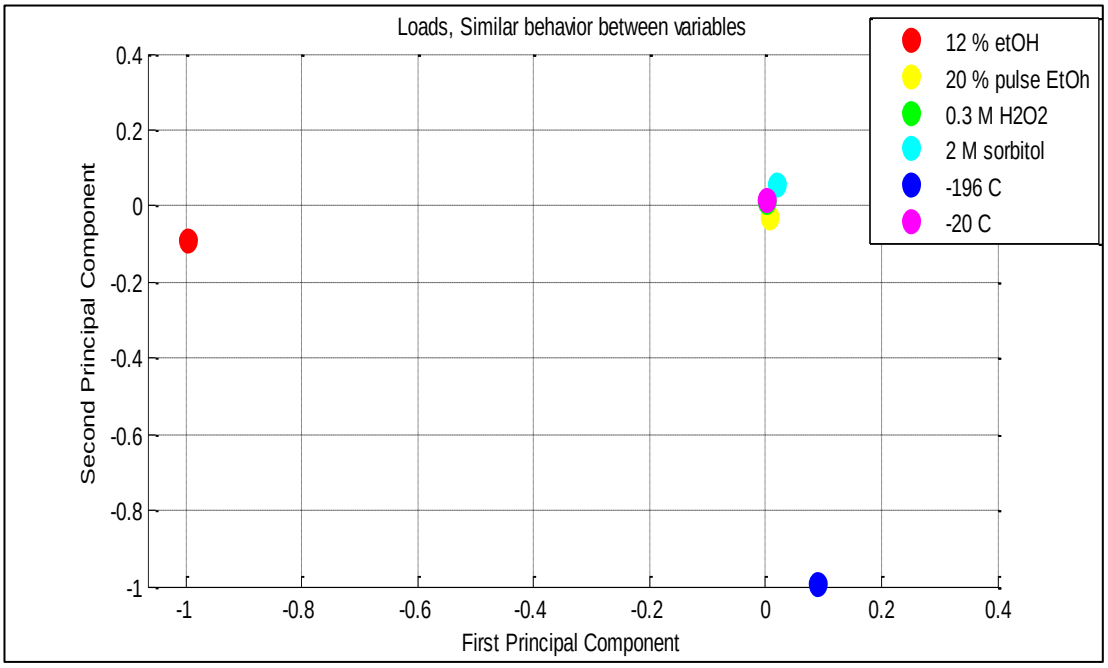

Figure 3.17: Principle component analysis results of the mutants selected by INCR strategy, based on response to different stress types.

When the PCA results of the selected individual mutants were taken into consideration as a group, the stress responses towards continuous ethanol stress under 12% (v/v) ethanol and freezing-thawing stress in liquid nitrogen (-196°C) were shown to have distinct behaviours.

3.6.6 Analysis of ethanol production levels

To determine the ethanol production levels of the mutants, overnight cultures of B1, B2, B5, B6, B8 and the wild type were inoculated in 10 mL YMM in 50 mL culture tubes with an initial optical density value of 0.2 at 600 nm. The cultures were incubated at 150 rpm and 30°C for 48 h. The optical density values and cell dry weight values (mg/ml) were determined and ethanol analysis was performed by gas chromatography (GC).

The standard solutions and their peak areas used to determine the ethanol concentrations of the samples by GC are shown in Table 3.14, and Figure 3.18 shows the standard curve.

Table 3.14: Standard ethanol solutions and their corresponding peak areas obtained by gas chromatography.

% Ethanol (v/v)	Peak Area (GC)
0.5	236.0
1	450.7
2	974.1
3	1375.2
4	1789.1

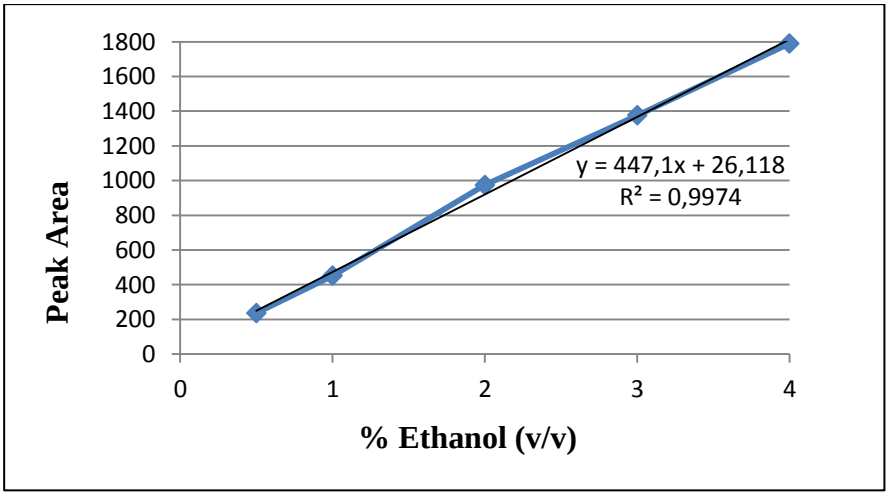

Figure 3.18: Linear calibration curve for ethanol measurements by GC.

The ethanol production levels of the mutants are given in Table 3.15.

Table 3.15: Optical density values, cell dry weight values as mg/ml, % ethanol values as mg cdw/ml.

Sample	OD_{600}	CDW (mg/ml)	Peak Area	EtOH %	% EtOH /[mg cdw /ml]
B1	6.18	1.85	557.0	1.19	0.64
B2	8.62	1.58	1205.3	2.64	1.67
B5	4.96	0.90	515.5	1.09	1.22
B6	6.84	1.70	496.6	1.05	0.62
B8	5.58	1.48	1120.3	2.45	1.66
Wt	5.74	1.30	508.0	1.08	0.83

The results showed that the highest ethanol production levels belonged to the mutants with the highest ethanol resistance (B2 and B8).

3.6.7 Growth physiology under ethanol stress and non-stress conditions

Growth physiology of two ethanol resistant mutants B2, B8 and the wild type cultures was studied under 10% (v/v) ethanol stress and non-stress (0% ethanol, v/v) conditions. The optical density values (OD_{600}) of the samples under non-stress conditions were determined during various time points of incubation (Table 3.16).

Table 3.16: Optical density values of the cultures under non-stress conditions.

Incubation time (h)	Optical density (OD_{600})		
	Wt-0%	B2-0%	B8-0% ethanol
0	0.050	0.050	0.050
2.5	0.076	0.085	0.078
6	0.263	0.266	0.269
9	0.868	0.999	0.906
12	4.545	5.205	4.625
31	12.620	11.180	10.090

In Figure 3.19, the growth behaviour of the wild type and the mutants cultures B2 and B8 is shown under non-stress conditions, during exponential phase.

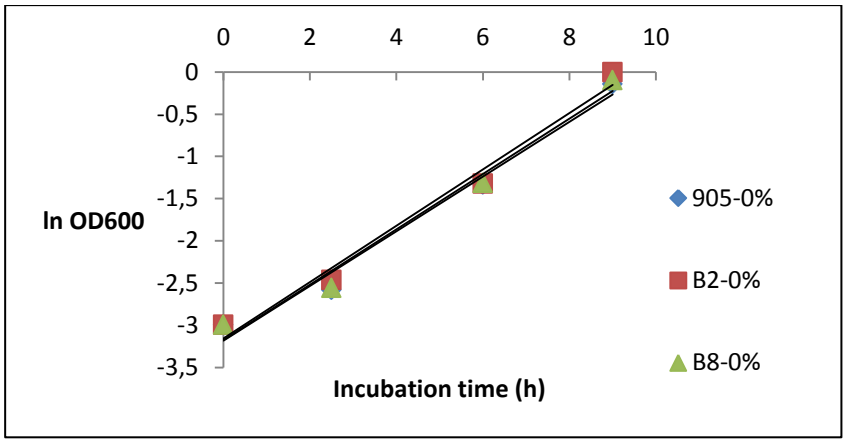

Figure 3.19: Growth behaviour of each culture during exponential phase under non-stress conditions.

The optical density values (OD_{600}) of the samples at 10% (v/v) continuously applied ethanol stress were also determined throughout the cultivation (Table 3.17).

Table 3.17: Optical density values of the cultures under 10 % ethanol stress conditions.

Incubation time (h)	Optical density (OD_{600})		
	Wt-10%	B2-10%	B8-10% ethanol
0	0.250	0.250	0.250
10	0.464	0.420	0.576
12	0.510	0.486	0.640
15	0.588	0.604	0.800
17	0.648	0.636	0.964
20	0.752	0.808	1.328
23	0.868	0.972	1.732
36	2.680	3.860	7.570

In Figure 3.20, the growth behaviour of each culture is shown under 10% (v/v) continuously applied ethanol stress conditions, during exponential growth phase.

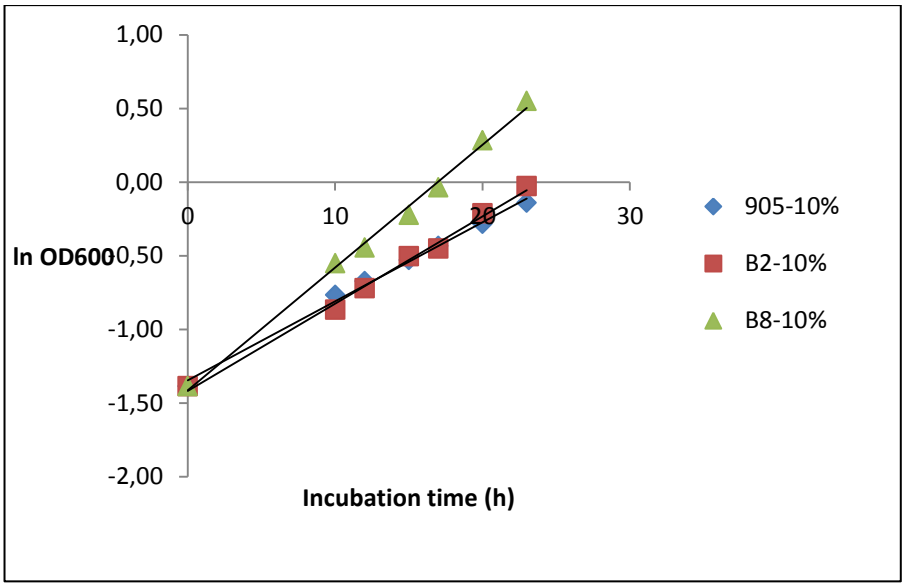

Figure 3.20: Growth behaviour of each culture under 10% (v/v) ethanol stress condition.

In Table 3.18, the calculated maximum specific growth rates of each culture are given under both non-stress and 10% (v/v) ethanol stress conditions.

Table 3.18: Maximum specific growth rates of the cultures.

Sample name	Maximum specific growth rate (μ_{max})	
	0% ethanol	10% ethanol
Wt	0.32	0.05
B2	0.33	0.06
B8	0.33	0.08

The results showed that, under non-stress conditions, the maximum specific growth rates of the mutants were not significantly different from that of the wild type. However, under 10% (v/v) ethanol stress, B8 had a significantly higher maximum specific growth rate than the wild type.

3.6.8 Macrokinetic analysis of ethanol resistant mutants grown in bioreactors

Growth physiology of the two highly ethanol-resistant individual mutants (B2 and B8) was investigated and compared to that of the wild type cells in aerated bioreactors, under fed-batch cultivation conditions. Macrokinetic parameters throughout the cultivation were reported. The results are shown in Figure 3.21. The vertical dashed line in Figure 3.21 indicates the initiation of the uncoupling phase where cell growth stopped but ethanol production continued.

Maximum specific growth rates (μ_{max}) and ethanol production values of B2, B8 and the wild type cultures at the end of the fed-batch process are shown in Table 3.19. It

was observed that both maximum specific growth rates and the maximum ethanol production levels of the mutants were higher than that of the wild type. Ethanol productivity levels of the mutants B2 and B8 were shown to be significantly higher than that of the wild type.

Table 3.19: Maximum specific growth rates, maximum ethanol production and productivity of wild type and ethanol resistant mutants B2 and B8.

	Maximum specific growth rate (h^{-1})	**Maximum ethanol production ($g\ l^{-1}$)**	**Productivity ($g\ l^{-1}\ h^{-1}$)**
Wild type	0.28	125	3.57
Mutant B2	0.36	136	5.25
Mutant B8	0.32	134	4.80

Ethanol production of the mutants continued even after the growth of the culture had stopped. In addition, viability values of the mutants were significantly higher than that of the wild type throughout the cultivation. Another important result was the 5-fold higher accumulation of glycogen by mutant B2 than by mutant B8. However, the trehalose levels were similar for all cultures.

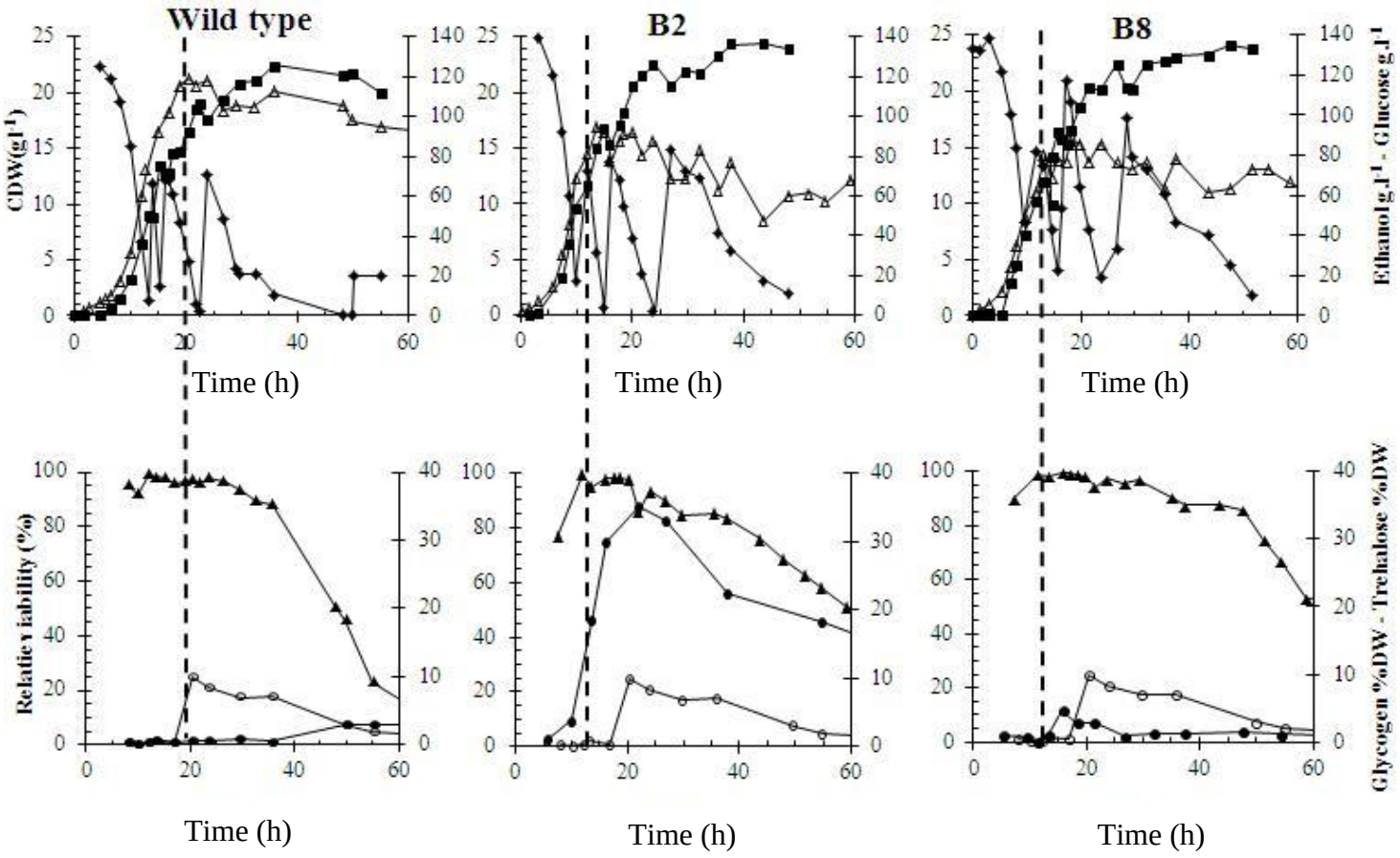

Figure 3.21: Macrokinetic parameters of the mutants B2, B8 and the wild type during aerated-fed batch fermentation process. Ethanol (■), biomass (△), glucose (◆), glycogen (●), and trehalose (○) concentration and cell viability (▲) levels are indicated with corresponding signs.

3.6.9 Semi-quantitative analysis of expression levels of HSP104 by reverse transcription-PCR

Expression levels of *HSP104* gene of the wild type and ethanol-resistant mutants B2 and B8 were determined semi-quantitatively by multiplex RT-PCR (reverse transcription-PCR). For this purpose, RNA isolation from wild type and two mutant cultures B2 and B8 was performed after cultivation under non-stress (0% v/v ethanol) and stress (7% v/v ethanol) conditions. RNA concentrations were determined by using Qubit® Flourometer and Quant-iT™ RNA assay kit (Invitrogen). In order to determine the optimal cycle number for analysis of *HSP104* expression levels, samples at 22, 24, 26, 28, 30, 33, 36th cycles of PCR protocol were taken and analyzed in 1.5% agarose gel. DNA ladder #SM0333 (Fermentas) was used as the size marker and ACT1 gene was used as an internal control. The results are shown in Figure 3.22.

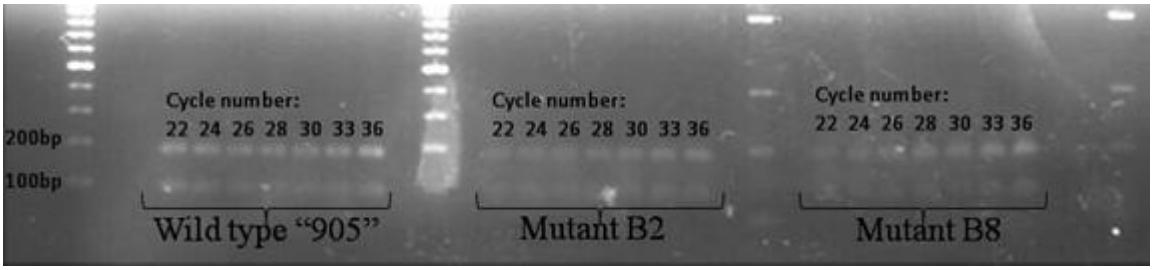

Figure 3.22: Cycle number optimization studies for RT-PCR experiments.

The optimal cycle number for the RT-PCR protocol was determined as 36. After determination of the optimal cycle number, the PCR products were visualized in 1.5% agarose gel for densitometric analysis (Figure 3.23).

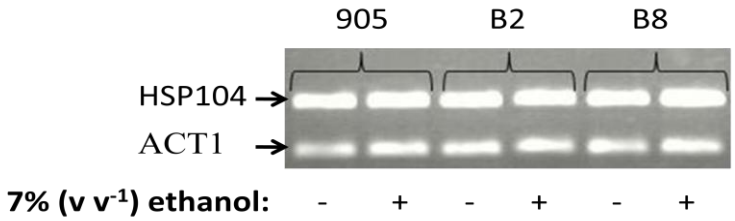

Figure 3.23: Amplification of HSP104 and ACT1 genes by RT-PCR under stress and non-stress conditions. Negative "-" and positive "+" signs indicate the absence and presence of 7% (v/v) ethanol stress, respectively.

The volumes of the bands determined by the PhotoCapt Software were used to calculate the concentrations of the PCR products in ng/µl. The same PCR products

were analyzed on two separate gels and the mean values were used to calculate the final concentrations. The ratio of gene expression levels under stress conditions to those under non-stress conditions are given in Table 3.20.

Table 3.20: Ratio of HSP104 gene expression levels, as determined by RT-PCR.

	PCR product concentrations		**Ratio**
	0% ethanol (ng/μl)	**7% ethanol** (ng/μl)	**(7%/0%) ethanol**
Wt	317.8	304.3	0.96
B2	322.6	301.5	0.93
B8	308.7	304.7	0.99

The ratio of the expression levels of HSP104 gene in the wild type under stress and non-stress conditions was slightly higher than that of mutant B2 and slightly lower than that of mutant B8. However, the differences in ratios are not significant.

3.6.10 Backcrossing experiments

The mutant cultures which were obtained by using EMS mutagenized MAT a type haploid wild type culture as the starting population, were incubated with MAT α type haploid wild type culture for mating and backcrossing experiments. The aim was to identify if ethanol-resistance phenotype had a simple genetic background such as a dominant/recessive character or a multi-genic nature. However, the mutants did not mate with the MAT α culture. For a haploid culture, it is not expected to show no response to the opposite mating type. Thus, it was suspected that the cultures might no longer be haploids.

3.6.11 Analysis of diploidy

The cultures were incubated in sporulation medium (1% potassium acetate and 2% agar, w/v) and were analyzed under the microscope for detection of tetrad formation. The tetrad formation results of the mutant cultures are shown in Table 3.21.

Table 3.21: Tetrad formation results. The presence of tetrad formation is shown with "+" sign and the cultures with no tetrads are shown with "-" sign.

Sample name	**Tetrad formation**
Mutant B2	+
Mutant B8	+
Haploid control	-
Diploid control	+

Both mutants formed tetrads on sporulation agar after incubation at 30°C for 72 hours. The tetrad structures were observed in B2 and B8 cultures. However, the haploid control culture composed of uniform, oval shaped single cells. This result showed that the mutant cultures were no longer haploid.

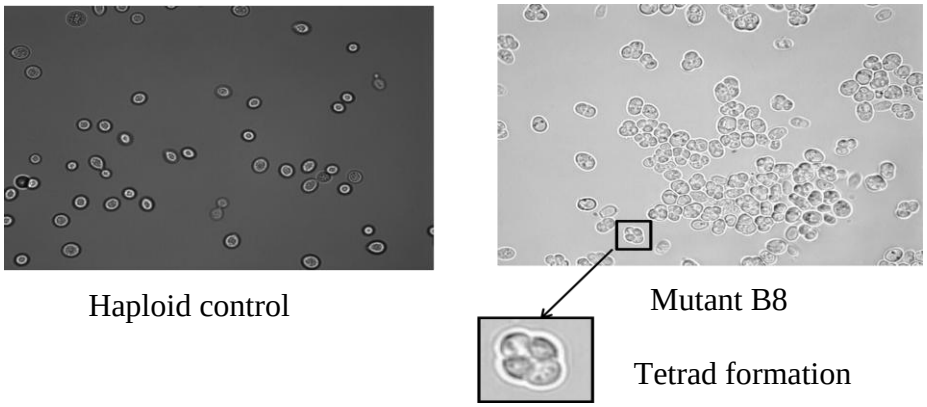

Figure 3.24: Microscopic images of haploid control and mutant B8 after incubation in sporulation media.

Figure 3.24 shows the microscopic view of the haploid control culture CEN.PK 113-7D and the mutant B8 after overnight incubation in sporulation medium.

In order to understand whether the diploidization was based on the type of the evolutionary selection stress, analysis of diploidy of mutants obtained by evolutionary engineering under a variety of stress conditions such as cobalt, iron, boron, H_2O_2, NaCl, sorbitol, citric acid, freeze-thaw at -80°C and freeze-thaw at -196°C, was performed on sporulation media, in addition to the mutants selected by constant ethanol stress application (Table 3.22). It was found that none of the stress selection strategies except for the increasing ethanol stress selection resulted with diploidization.

Table 3.22: Diploidization results for selections under various stress conditions.

Stress type	Selection strategy	Passage number	Diploidization
Ethanol	Constant continuous	98	-
	Increasing continuous	98	+
Cobalt	Increasing continuous	25	-
	Increasing pulse	25	-
Iron	Increasing continuous	15	-
Boron	Increasing continuous	28	-
H_2O_2	Increasing continuous	21	-
NaCl	Increasing continuous	40	-
Sorbitol	Increasing continuous	17	-
Citric acid	Increasing continuous	23	-
Freeze-thaw (-196°C)	Pulse	15	-
Freeze-thaw (-80°C)	Pulse	16	-

Diploidization results were confirmed by flow cytometry. In Figure 3.25, the histogram results of the mutants B2 and B8 in reference to haploid and diploid control groups are given.

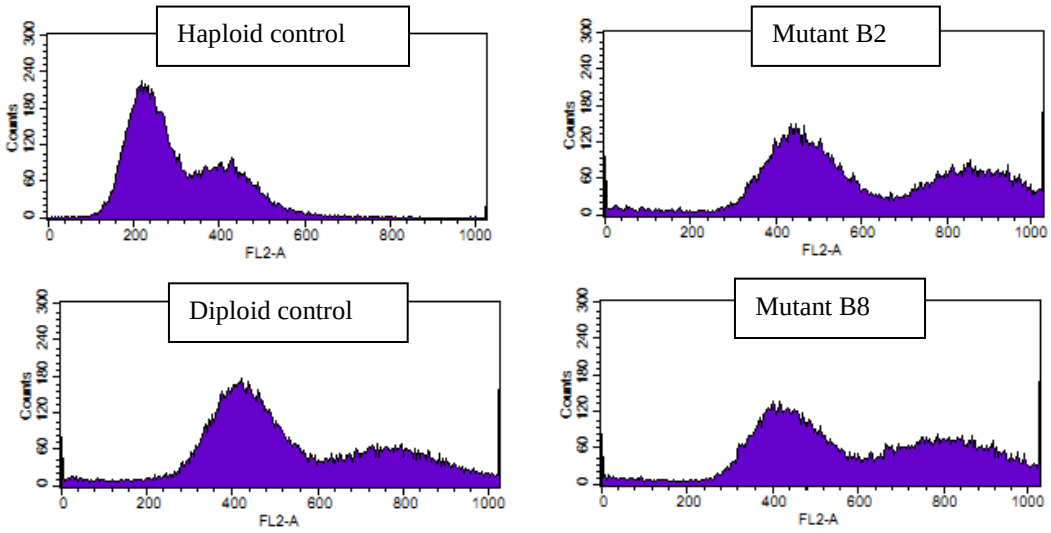

Figure 3.25: Flow cytometry results of the mutants B2 and B8 in comparison with the haploid and diploid control groups.

Flow cytometry results confirmed that B2 and B8 cultures were diploids. This is consistent with the previous results obtained by using sporulation medium.

3.6.12 Determination of the initiation time of diploidization

The initial culture used for the evolutionary selection strategy was originally a haploid wild type culture. However, the mutants selected from the final population obtained after application of increasing ethanol stress conditions were detected to be diploids. In order to understand at which stage of the selection these cultures became diploids, the intermediate populations that were obtained throughout the selection strategy were analyzed. The results are given in Figure 3.26.

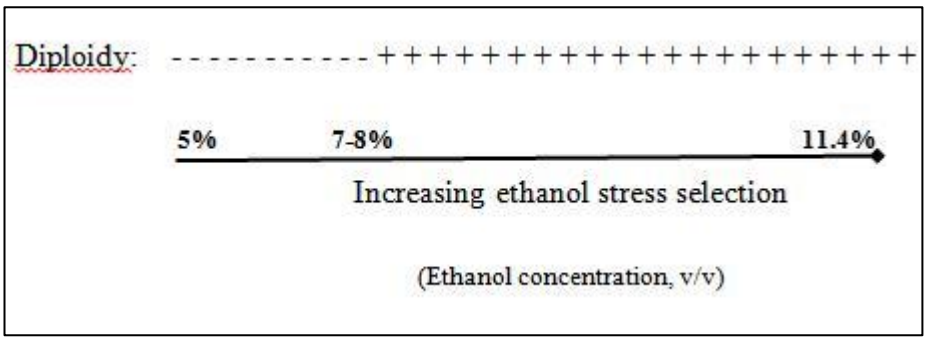

Figure 3.26: Analysis of initiation of diploidy during evolutionary stress selection strategy.

The results suggested that at approximately 7-8% (v/v) ethanol stress condition, the selection culture formed diploids. This result was confirmed by triple repeats of the evolutionary selection strategy under continuous exposure to increasing ethanol concentrations, where the initial culture was obtained by three independent EMS mutagenesis applications.

3.6.13 Fingerprinting of ethanol-resistant mutants by microsatellite primers

PCR-fingerprinting with microsatellite primer $(GTG)_5$ was performed to verify that there was no contamination. Figure 3.27 shows the fingerprinting results of the mutants and the wild type culture.

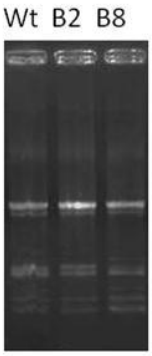

Figure 3.27: PCR-fingerprinting with $(GTG)_5$ microsatellite primer.

The fingerprinting patterns of the wild type and the mutants B2 and B8 were the same, implying that the diploidization of the culture was not a result of mating with a contaminating strain.

3.6.14 Analysis of segregants of diploid mutants

Segregants of diploid mutant cultures B2 and B8 were dissected and incubated in YPD medium. The growth of segregants after 2 days of incubation is shown in Figure 3.28.

Segregants of mutant B2 **Segregants of mutant B8**

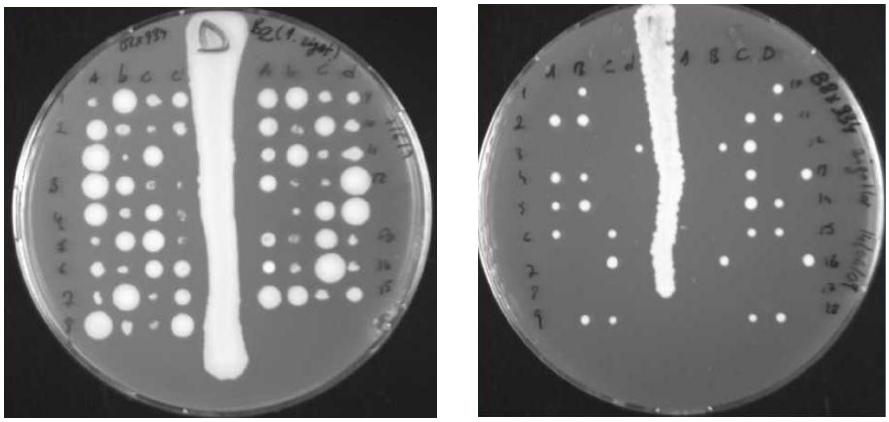

Figure 3.28: Segregants of mutants B2 and B8 obtained by dissection.

It was observed that only two out of four segregants of B8 could grow after disection. In addition, two segregants of B2 could grow slower than the other two segregants (Figure 3.28).

Overnight cultures of CEN.PK haploid wild type, CEN.PK diploid wild type, mutant B2, mutant B8 and two haploid segregants B8-2A, B8-2B were inoculated into YPD containing 0% and 10% (v/v) ethanol at an initial OD_{600} value of 0.25 and incubated at 30°C, 200 rpm in 250 mL shake flasks containing 50 mL growth media.

In Table 3.23, OD_{600} values of the cultures grown in the absence of ethanol stress are given.

Table 3.23: OD_{600} values of the mutant cultures, two segregants of mutant B8, haploid and diploid wild type strains at non-stress conditions.

	OD_{600} values					
Incubation time (h)	**CEN.PK (n)**	**CEN.PK (2n)**	**B2**	**B8**	**B8-2A**	**B8-2B**
0	0.25	0.25	0.25	0.25	0.25	0.25
2	0.68	0.34	0.35	0.34	0.44	0.39
4	0.88	0.70	0.82	0.75	1.12	0.92
5	1.77	1.40	1.69	1.42	2.17	1.74
6	2.63	2.16	2.52	2.28	3.32	2.75
7	3.52	2.96	3.34	3.21	4.61	3.56
22	15.16	12.52	13.36	13.54	12.56	16.64

Figure 3.29 shows the growth curves of the cultures at non-stress conditions.

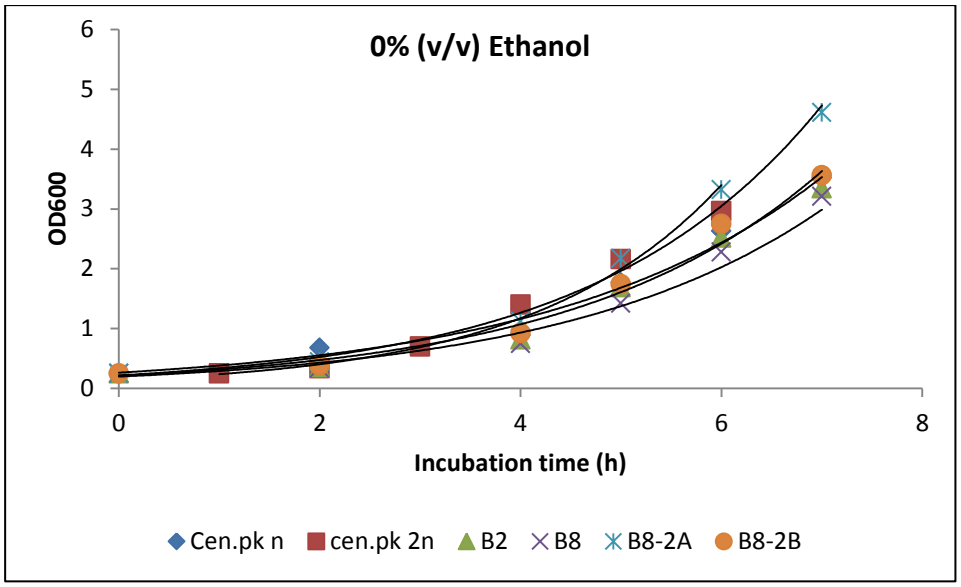

Figure 3.29: Growth curves of the cultures under non-stress conditions.

OD_{600} values of the cultures grown under 10% (v/v) ethanol stress are given in Table 3.24.

Table 3.24: OD_{600} values of the mutant cultures, two segregants of mutant B8, haploid and diploid wild type strains at 10 % (v/v) stress conditions.

Incubation time	CEN.PK (n)	CEN.PK (2n)	B2	B8	B8-2A	B8-2B
0	0.25	0.25	0.25	0.25	0.25	0.25
7	0.35	0.29	0.31	0.30	0.38	0.34
22	0.60	0.35	0.51	0.54	0.74	0.57
31	0.89	0.48	0.76	0.93	1.20	0.82
48	2.76	1.92	3.95	5.42	4.96	1.60

The growth curves of the cultures under 10% ethanol conditions are shown in Figure 3.30.

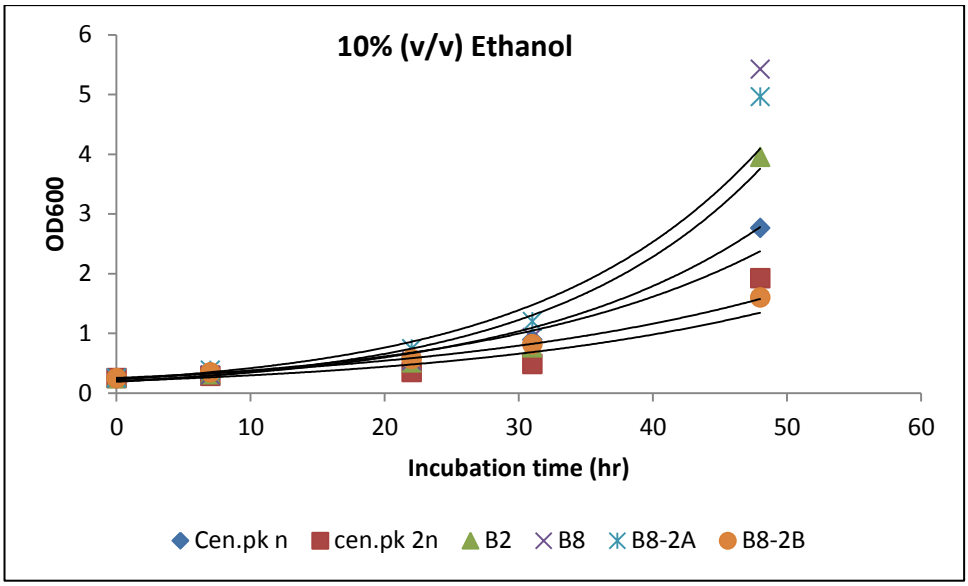

Figure 3.30: Growth curves of haploid and diploid cultures under 10% (v/v) ethanol stress conditions.

The maximum specific growth rates (μ_{max}) of the cultures grown under 0% and 10% ethanol stress conditions are given in Table 3.25.

Table 3.25: Maximum specific growth rates of haploid and diploid cultures.

	CEN.PK (n)	CEN.PK (2n)	B2	B8	B8-2A	B8-2B
μ_{max} *(0%)*	0.37	0.53	0.40	0.39	0.44	0.41
μ_{max} *(10%)*	0.05	0.04	0.05	0.06	0.06	0.04
μ_{max} *(10%)* / μ_{max} *(0%)*	0.13	0.07	0.14	0.16	0.14	0.09

Maximum specific growth rates of the cultures were not significantly different (Table 3.25). However, when the μ_{max} ratio for each culture under 0% and 10% (v/v) ethanol stress was considered, the mutants B2 and B8 seemed to have a higher ethanol resistance than both haploid and diploid wild type cultures. The haploid segregants selected from diploid B8 mutant had a higher ratio than the diploid culture. However, the resistance levels of these two segregants were not similar. One of the segregants was more resistant than the other one. These two segregants were the ones that could survive after dissection. The other two segregants were dead. Moreover, the diploid wild type culture had lower resistance to ethanol than the haploid wild type culture.

3.6.15 Sequencing of homothallic switching endonuclease (HO) gene

HO genes of wild type and mutants B2 and B8 were amplified with -1000 bp and +200 bp flanking regions. The PCR products for each culture were visualized in agarose gel (Figure 3.31). 1 kb DNA ladder #3232L (BioLabs) was used as the size

marker on the gel. Ligation of the PCR products was performed by using pGEM-T Easy vector system. After transformation, the colonies with the inserts were selected by blue-white screening.

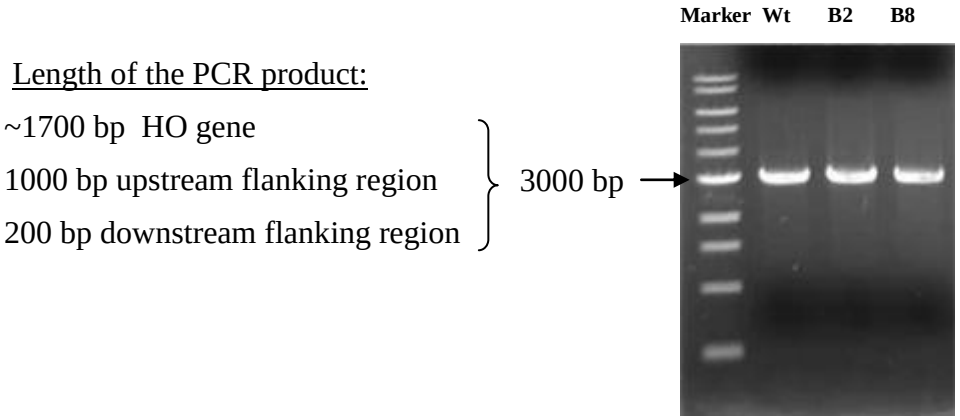

Figure 3.31: PCR products of the mutant B2, B8 and wild type cultures after amplification with HO primers.

Sequencing results showed that there was no mutation in the HO gene of the mutants. The HO sequences in wild type and the mutants "B2 and B8" were exactly the same as the HO sequence reported in *Saccharomyces* Genome Database (Url-1).

3.6.16 Determination of expression levels of HO and MKT1 by qRT-PCR

qRT-PCR analysis was performed according to protocol of DNA SYBR Green I Master (Roche) kit by using Roche-LightCycler® 480. The gene expression levels calculated according to the $2^{(-(\Delta Ct))}$ method are shown in Table 3.26.

Table 3.26: The expression levels of MKT1 and HO genes, as determined by qRT-PCR.

Sample name	HO-ACT1 ΔCt	$2^{(-(\Delta Ct))}$	Normalized to wild type	MKT1-ACT1 ΔCt	$2^{(-(\Delta Ct))}$	Normalized to wild type
Wt	8.52	0.002724	1.00	7.08	0.007391	1.00
Wt_{EMS}	8.74	0.002339	0.86	6.13	0.014279	1.93
A23	9.38	0.001501	0.55	6.09	0.014680	1.99
A24	10.62	0.000635	0.23	5.44	0.023035	3.12
A32	12.35	0.000192	0.07	5.60	0.020617	2.79
A43	13.33	0.000097	0.04	6.82	0.008851	1.20
A53	12.87	0.000134	0.05	5.60	0.020617	2.79
A66	13.05	0.000118	0.04	5.89	0.016863	2.28
A73	12.87	0.000134	0.05	6.36	0.012174	1.65
A86	16.64	0.000010	0.00	5.87	0.017098	2.31
A98	12.85	0.000135	0.05	5.93	0.016402	2.22
B2	11.90	0.000262	0.10	5.87	0.017098	2.31
B8	13.34	0.000096	0.04	6.51	0.010972	1.48

The relevance of error rate and efficiency are essential for precise and reliable real time PCR of each gene. The error and efficiency values are given in Table 3.27.

Table 3.27: The error and efficiency values of ACT1, HO and MKT1 genes.

	ACT1	**HO**	**MKT1**
Error	0.0108	0.0197	0.127
Efficiency	1.927	1.918	1.820

The relative expression levels normalized to wild type are shown in Figure 3.32.

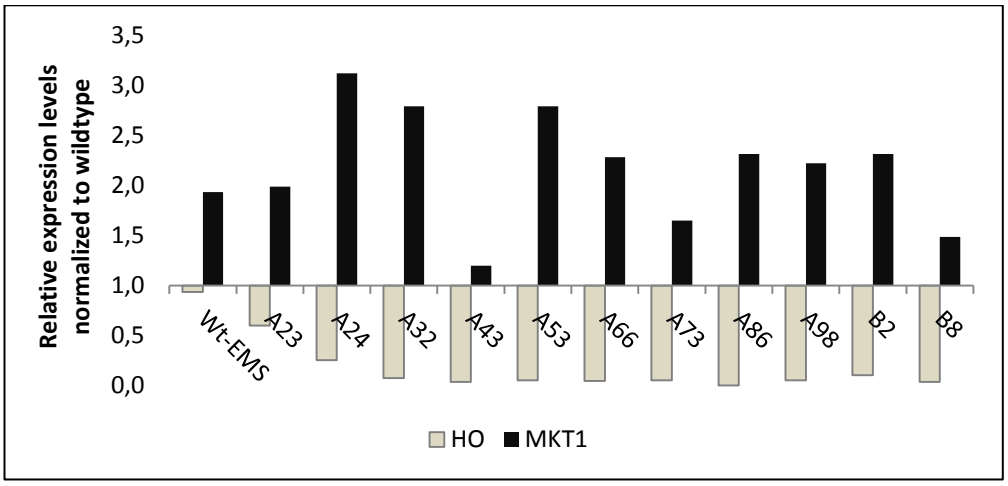

Figure 3.32: Relative expression levels of HO and MKT1 genes normalized to wild type values.

According to normalized values, all cultures had lower HO expression levels than the wild type. HO expression levels of intermediate populations A23 and A24 were affected less than the other cultures. Additionally, it was observed that the HO expression levels of the cultures other than A23 and A24 were similar to each other.

In contrast to HO expression levels, the expression levels of MKT1 were higher than those of the wild type for all cultures tested. However, there was no correlation between the passage numbers and the expression levels.

3.6.17 Analysis of mitochondrial defect by petite frequency assay

Mitochondrial defects of the cultures were analyzed by petite frequency assay as described previously (Dimitrov et al., 2009). After 5 days of incubation at 30°C, the *petite* and *grande* colony formation was observed (Figure 3.33).

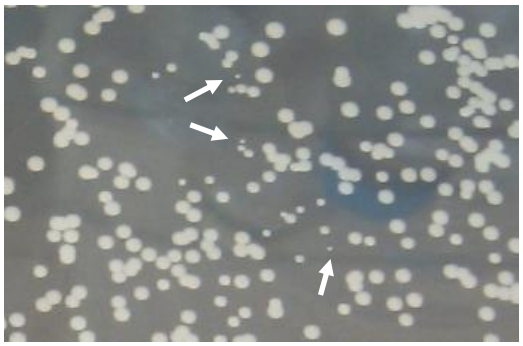

Figure 3.33: *Petite* and *grande* phenotypes on YEPDG plates. Some of the petite colonies are indicated with arrows.

At least four independent colonies from each sample were analyzed on YEPDG. The petite frequency was calculated by the ratio of the number of petite colonies to the number of total colonies for each YEPDG plate. The average petite frequency values for each sample are shown in Figure 3.34.

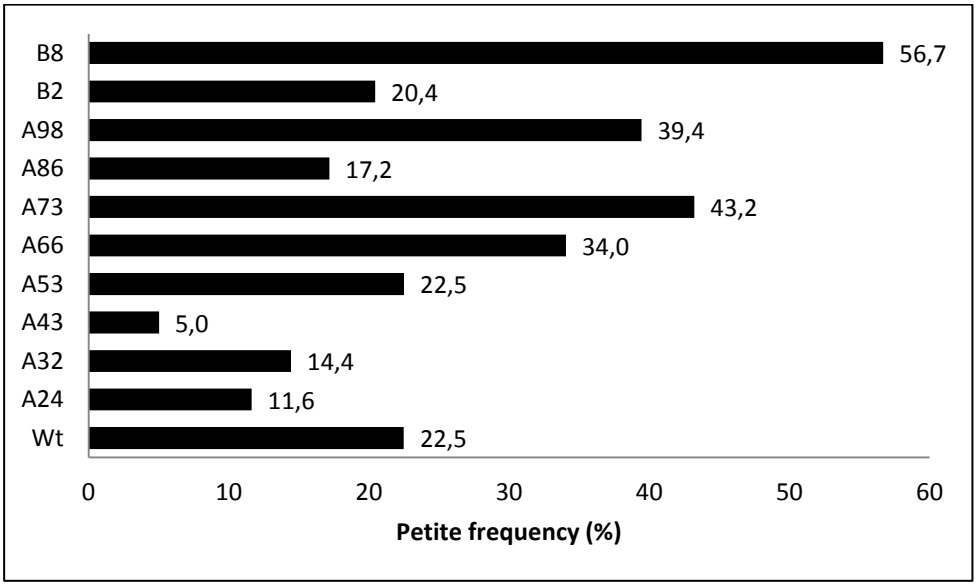

Figure 3.34: Petite frequency values.

The results demonstrated that mutant B8 had the highest petite frequency when compared to the other cultures. However, the petite frequency value of B2 was close to that of the wild type. No relationship between the petite frequency values and the passage numbers could be found.

3.6.18 Gradually increasing stress selection studies using different starting cultures

The evolutionary stress selection strategy under increasing ethanol levels (as explained in Section 2.2.2) was repeated by using the *EMS-mutagenized ΔHO* and

ΔMKT1 deletion strains of *CEN.PK 113-7D*, *EMS-mutagenized X2180-2A* strain and *non-mutagenized CEN.PK 113-7D* cultures as the starting population. The diploidization of the cultures was checked under the light microscope after incubation in sporulation media (Section 2.2.11) after each step of stress selection. The results are given in Table 3.28.

Table 3.28: Diploidization analysis of the EMS-mutagenized CEN.PK ΔHO, EMS-mutagenized CEN.PK ΔMKT1, EMS-mutagenized X2180-2A and non-mutagenized CEN.PK cultures.

Culture name	The final stress level	Diploidization state
EMS-mutagenized CEN.PK ΔHO	7.4% (v/v) ethanol	Diploidization observed
EMS-mutagenized CEN.PK ΔMKT1	8.4% (v/v) ethanol	No diploidization
EMS-mutagenized X2180-2A	9.2% (v/v) ethanol	No diploidization
Non-mutagenized CEN.PK	8.9% (v/v) ethanol	No diploidization

Only the stress selection with the *EMS-mutagenized CEN.PK ΔHO* starting culture resulted with diploidization. *EMS-mutagenized CEN.PK ΔMKT1*, *EMS-mutagenized X2180-2A* and *non-mutagenized CEN.PK* cultures remained as haploid cultures. These cultures could not tolerate higher ethanol levels than those indicated in Table 3.28. Thus, the stress selection process for each culture was terminated at those final stress levels.

3.6.19 Comparison of protein expression levels

Protein expression levels of ethanol-resistant mutant B8 were analyzed under non-stress conditions and compared to that of the wild type. Table 3.29 shows a list of proteins with decreased expression levels in B8.

Table 3.29: Down-regulated proteins in mutant B8, compared to the wild type.

Accession	Description	Fold change
P12709	G6PI Glucose 6 phosphate isomerase	0.62
P07274	PFY1 Profilin	0.58
P31787	ACB1 Acyl CoA binding protein	0.57
P01095	PBI2 Protease B inhibitors 2 and 1	0.48
P14832	CPR1 Peptidyl prolyl cis trans isomerase	0.44
P09435	HSP73 YEAST Heat shock protein SSA3	0.43
Q03048	COFI Cofilin	0.33

Expression levels of 7 proteins decreased more than 1.5 times in B8 when compared to wild type. These proteins were glucose 6 phosphate isomerase, profilin, acyl-coA binding protein, protease B inhibitors 2 and 1, peptidyl prolyl cis trans isomerase, heat shock protein SSA3 and cofilin. Glucose 6 phosphate isomerase is involved in

glycolysis. Profilin and cofilin have roles in actin filament polymerization. Acyl-CoA binding protein functions as an intracellular carrier of acyl-CoA esters in fatty acid metabolic process. Protease B inhibitors 2 and 1 are involved in facilitation of vesicle fusion such as homotypic vacuole and ER-derived COPII vesicle fusion with the Golgi. Peptidyl prolyl cis trans isomerase catalyzes the cis-trans isomerization of proline imidic peptide bonds in oligopeptides and is involved in histone deacetylase complexes (Url-1).

A total of 41 proteins were determined to be up-regulated in mutant B8 when compared to wild type. The list of up-regulated proteins is given in Table 3.30.

Figure 3.35 classifies the up-regulated proteins according to their function.

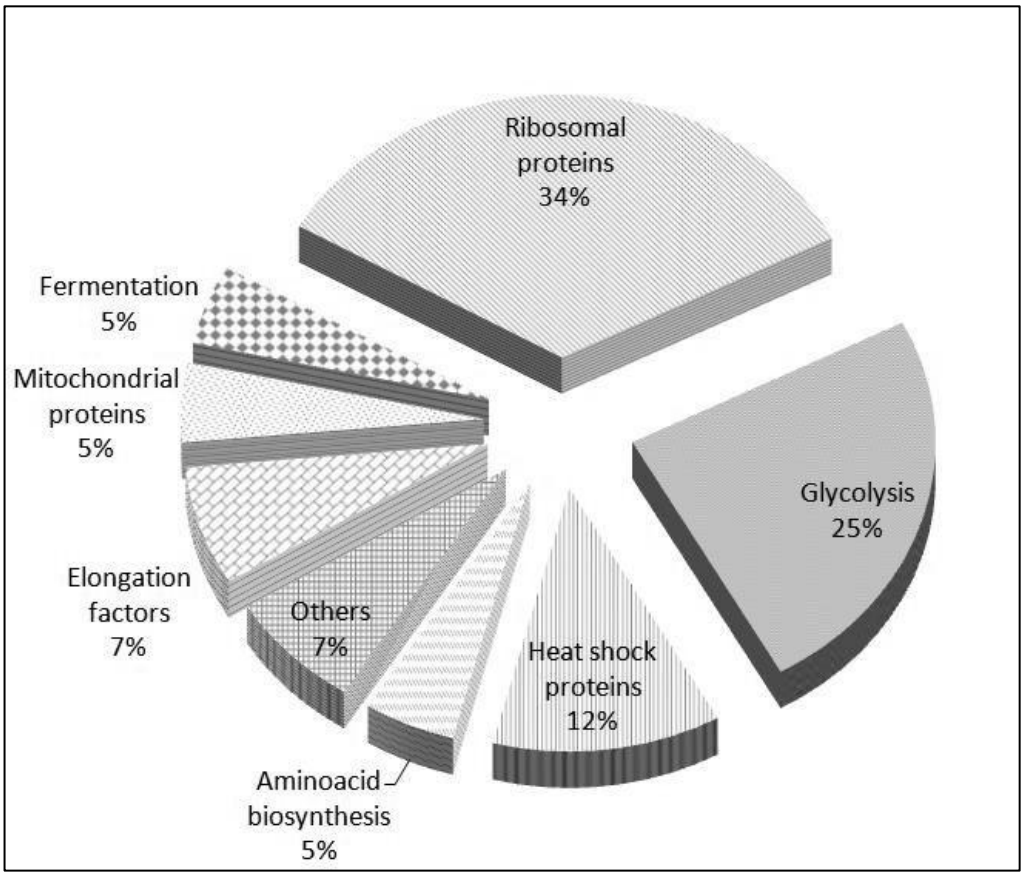

Figure 3.35: Functional classification of up-regulated proteins in mutant B8, compared to the wild type.

Table 3.30: Up-regulated proteins in mutant B8, compared to the wild type.

Accession	Description	Fold change
Q6FKY1	Phosphoglycerate kinase	6.36
O74261	Heat shock protein 60 mitochondrial	3.86
O59841	Glyceraldehyde 3 phosphate dehydrogenase	2.41
P26781	40S ribosomal protein S11	2.18
P39938	40S ribosomal protein S26 A	2.00
Q9Y7B5	Acetyl coenzyme A synthetase 2	2.00
P02994	Elongation factor 1 alpha	1.97
P0C2I0	60S ribosomal protein L20	1.95
P0C0W1	40S ribosomal protein S22 A	1.93
Q756H2	Enolase	1.93
P16474	78 kDa glucose regulated protein homolog	1.92
P07279	60S ribosomal protein L18	1.86
P00360	Glyceraldehyde 3 phosphate dehydrogenase 1	1.82
P07262	NADP specific glutamate dehydrogenase 1	1.80
P10664	60S ribosomal protein L4 A	1.79
P02407	40S ribosomal protein S17 A	1.79
P04451	60S ribosomal protein L23	1.79
P40054	D 3 phosphoglycerate dehydrogenase 1	1.79
Q12690	60S ribosomal protein L13 A	1.77
P05694	5 methyltetrahydropteroyltriglutamate homocysteine methyltransferase	1.75
P12398	Heat shock protein SSC1 mitochondrial	1.75
P05735	60S ribosomal protein L19	1.73
P17079	60S ribosomal protein L12	1.72
P00445	Superoxide dismutase Cu Zn	1.70
P04840	Mitochondrial outer membrane protein porin 1	1.67
P11986	Inositol 3 phosphate synthase	1.67
Q6FQY4	Enolase 2	1.67
P19882	Heat shock protein 60 mitochondrial	1.65
P26321	60S ribosomal protein L5	1.65
P02365	40S ribosomal protein S6	1.65
P00924	Enolase 1	1.63
A6ZQJ1	ATP dependent RNA helicase eIF4A	1.62
P40150	Heat shock protein SSB2	1.62
Q6FV12	Pyruvate kinase 2	1.62
P20369	Alcohol dehydrogenase 1	1.60
P00549	Pyruvate kinase 1	1.57
P06169	Pyruvate decarboxylase isozyme 1	1.57
P35271	40S ribosomal protein S18	1.52
P00359	Glyceraldehyde 3 phosphate dehydrogenase 3	1.52
P00942	Triosephosphate isomerase	1.52
P32324	Elongation factor 2	1.51

Among 41 proteins expressed more than 1.5 fold in mutant B8 compared to wild type, 34% belonged to ribosomal proteins and 12% belonged to heat shock proteins that are components of general stress response mechanism. Another important group of up-regulated proteins were glycolytic proteins (25%). Other up-regulated proteins were grouped as elongation factors, mitochondrial proteins and those involved in amino acid biosynthesis, and fermentation processes.

4. DISCUSSION AND CONCLUSIONS

The aim of this study was to obtain ethanol-resistant *Saccharomyces cerevisiae* cells and to investigate the molecular basis of ethanol resistance in *S. cerevisiae*. For obtaining ethanol-resistant *S. cerevisiae*, evolutionary engineering, which is an inverse metabolic engineering strategy was adopted. Using this strategy, first the ethanol-resistant mutant strains were obtained and then the genetic basis of their phenotype character was investigated. In contrast to the rational metabolic engineering approaches, obtaining the desired phenotype at the beginning of the study ensures the presence of all molecular components of the related resistance mechanism in the mutants to be investigated.

Prior to evolutionary selection strategy, the initial wild type culture was exposed to a chemical mutagenesis step for increasing the genetic diversity of the starting population for selection. Lethality rate and frequency of base pairing substitutions have been reported as critical for an effective mutagenesis reaction (Lawrence, 1991). After exposure to the chemical mutagen EMS, the death ratio of the culture was determined to be 90%. Thus, the starting population was accepted to be diverse enough for selecting the mutants of interest.

EMS-mutagenized and non-mutagenized wild type cultures were screened under different continuous ethanol stress conditions. The growth pattern for both cultures was similar at various ethanol stress levels (Figure 3.1). However, it was observed that under non-stress conditions, the mutagenized culture reached to a higher OD_{600} value of approximately 7.5, while the wild type culture had an OD_{600} value lower than 6.0 after 48 hours of incubation in YMM. This result demonstrated that mutagenesis did not result with a pool of 'crippled' cells with growth deficiency, but a heterogeneous population with a higher potential of growth in minimal medium was obtained.

It was observed that about 7% (v/v) ethanol stress resulted with 50% reduction in growth in wild type and EMS-mutagenized wild type cultures (Figure 3.1). Thus, for

evolutionary selection strategy, the initial ethanol stress level was assigned as a lower ethanol concentration (5%, v/v). This mild stress condition was chosen in order to allow growth of most of the cells in the culture and to not to decrease the number of survivors rapidly within the first few cultivations under stress conditions.

By transferring the survivors of a stress level to the next stress level, 98 passages were obtained through two different selection strategies: "constant stress selection" and "gradually increasing stress selection" (Figure 3.2). Survival ratio values obtained after constant stress application at 5% (v/v) ethanol stress for 98 passaging steps were generally between 0.8-1.0 (Figure 3.3). However, the survival ratio values of the passages obtained by increasing stress application strategy were shown to be oscillating within a wide range (Figure 3.4). This might be considered as an expected result due to the severe effects of the increasing ethanol levels and repeating steps of the same stress levels in-between those sharp declines in survival rates for providing the cell culture a mild phase to tolerate severe conditions. An example to the repeating steps of the same stress level is the 10 times of repetition at 8.4% (v/v) ethanol stress corresponding to 23^{rd} -32^{nd} passaging steps at INCR strategy (Table 3.2).

The intermediate populations were screened at 7% (v/v) ethanol stress in order to observe the evolutionary improvement in terms of stress resistance. The survival values of the intermediate populations obtained throughout the stress selection studies did not display a gradually increasing pattern as reported in a previous work (Çakar et al., 2005). This result might have occurred as an extension of the initially applied chemical mutagenesis step and the genetic diversity of the population. Even though the increasing levels of stress conditions were capable of enriching the ethanol resistant mutants in the population and leading to increased homogeneity of the mutagenized populations, it was not possible to precisely quantify the stress resistance level of even the final mutant population. The experimental results gave a rough estimation of the resistance level of the population, corresponding to the mean value of a group of cells with distinct features. This was the reason for continuing further investigations with individual mutants, rather than the heterogenous populations.

At the 98^{th} steps of both CONST and INCR selection strategies, the stress selection protocols were terminated and 98^{th} passages were accepted as the final populations.

Individual mutants from these final populations were randomly selected on solid media. The mutants selected from the final population of constant stress selection strategy were tested under 5% (v/v) ethanol stress condition. The results revealed that resistance levels of all of the selected mutants at 5% (v/v) ethanol stress were approximately the same. In addition, their stress resistances were not significantly higher than that of the wild type (Figure 3.6). This result might be explained by the mildness of the applied stress level. When growth of the mutagenized and wild type cultures was investigated under 0-15% continuous ethanol (v/v) stress in the beginning of the study, it was observed that 5% ethanol (v/v) was a tolerable stress condition for both cultures (Figure 3.1). Thus, this mild stress might not be sufficient to distinguish the mutants from the wild type culture.

The individual mutants obtained by the increasing stress selection strategy were screened under 10-12% (v/v) continuous ethanol stress conditions and the number of cells were determined by MPN method under each stress condition. The mutants B2 and B8 were found to be the highest ethanol-resistant mutants when compared to the wild type and the other mutants. However, it is remarkable that all of the selected mutant individuals had significantly higher ethanol resistance levels when compared to wild type. At 10%, 11% and 12% (v/v) ethanol stress where the mutagenized starting population could grow as poorly as the wild type (Figure 3.1), the mutant individuals obtained from the selection, however, had survival rates up to 4558-fold of the wild type (Figure 3.7). The resistance levels of the mutants as fold of wild type were promising when compared with the commercial wine yeast strains analyzed by Carrasco et al (2001). In that study, it was shown that 14 wine yeast strains analyzed were tolerant up to 10% (v/v) ethanol (Carrasco et al., 2001). These results altogether revealed that selection under gradually increasing ethanol stress seemed to be more effective for enrichment of the ethanol resistant strains within a heterogeneous population. For this purpose, the mutants selected by gradually increasing ethanol stress application were chosen for further characterization, rather than the mutants selected by constant stress application.

Since complex culture media are generally preferred in industrial applications because of economic concerns, it was necessary to investigate the stress resistance levels of the selected mutants in complex media. The evolved mutants indeed retained their resistance characteristics when grown both in minimal and complex

media (Figure 3.8). This result is desirable for the selection strategy regarding potential industrial applications.

Moreover, it was previously stated that the capability of the cells to carry out fermentation process is mainly influenced by the produced ethanol concentrations (Carrasco et al., 2001; Ma and Liu, 2010). Significantly higher resistance levels of the mutants when compared to wild type provide the evolved mutants a crucial advantage for production of higher levels of ethanol.

Another approach for testing the ethanol resistance levels of the individual mutants was exposure to 20% (v/v) shock (pulse) ethanol stress for 90 minutes. Interestingly, the mutants with the highest survival rate values under continuous ethanol stress had the lowest survival rates under 20% (v/v) pulse ethanol stress condition (Table 3.5). Interestingly, the most resistant mutant under continuous ethanol stress (B2), had a lower survival rate than wild type when exposed to pulse ethanol stress. These results suggested that the cellular responses that are triggered upon continuous and pulse ethanol stress conditions might not be overlapping.

As an alternative methodology to the analysis of cell growth upon stress by MPN method, a viability assay was used to test if the cells could carry out vegetative growth after stress exposure. The number of colonies on solid media representing the viable cells were higher in all of the mutant cultures when compared to the wild type (Figure 3.9). The evolved mutants B2 and B8 were significantly viable following 11% and 12% (v/v) ethanol stress exposure, whereas the wild type cells could not tolerate these stress conditions. Viability of the cells was also tested later by methylene blue staining during aerated fed-batch fermentation experiments.

From an industrial point of view, the resistance of the strains against stress conditions to which the cells are generally exposed to during bioprocesses, is considered as a crucial parameter for efficient productions. Thus, the survival rates of the evolved mutants were determined under oxidative (0.3M H_2O_2), osmotic (2M sorbitol), freeze-thaw (-20°C and liquid nitrogen), and high temperature (60°C) stresses, to determine any potential cross-resistance. Results showed substantial differences among the mutant cultures. The mutant B8 with cross-resistance against many stress types was shown to be the most multi-stress resistant strain; while the mutant B2 with the highest ethanol resistance did not show a significant cross-

resistance to the other stresses tested (Figures 3.10-3.14). Another interesting result was that the highest resistance levels among the stresses tested were obtained after freeze-thaw stress exposure (Figure 3.13). In line with the previous work of Çakar et al (Çakar et al., 2005), these results confirmed the connection of the freeze-thaw stress resistance with the multi-stress resistance characteristics.

In addition to the industrial stress conditions mentioned above, the mutant strains were exposed to phenyl-ethanol and methanol stresses as other potential industrial stress conditions. The results revealed that the the mutants B2 and B8 could tolerate higher levels of phenyl-ethanol when compared to wild type. It was also observed that the mutant B8 was more tolerant at 3 g/L - 5 g/L phenyl-ethanol stress than the mutant B2 (Figure 3.15). In addition, mutant B8 was the most resistant mutant individual among the other mutants and the wild type under methanol stress. This was an expected result due to the seemingly multi-resistant character of the mutant B8 which was observed during previously mentioned cross-resistance studies.

In addition to stress resistance tests, the mutants were also investigated for determination of their ethanol production. The percent ethanol production per mg cell dry weight was determined after 48 hours of batch cultivation for the mutants in comparison with the wild type. The mutants B2 and B8, with the highest ethanol resistance values, were found to have the highest ethanol production as well, when compared to the other mutants and the wild type. On the basis of these results, it might be proposed that the high ethanol resistance of the selected mutants might well be associated with their higher ethanol production.

In addition to ethanol production capacity, the maximum specific growth rate of the production strain is also important for industrial applications. Strains with high ethanol resistance and high ethanol production capacities are desired, along with rapid growth during production processes. The growth curves of the evolved mutants B2 and B8 showed that these mutants had similar growth rates with the wild type under non-stress conditions, and higher growth rates than the wild type under 10% (v/v) continuous ethanol stress condition in batch cultivations Figures (3.19-3.20). This result supported the potential of the evolved mutants in terms of productivity.

For a better understanding of this phenomenon, a growth physiology experiment was designed in an aerated fed-batch cultivation system, where glucose, ethanol,

glycogen, trehalose levels were determined in addition to biomass concentration and cell viability. The optimal aeration parameters of the present cultivation had been determined previously for high ethanol production levels (Alfenore et al., 2004). In the mutants B2 and B8, both the maximum specific growth rates, ethanol production levels were higher than the wild type (Table 3.19). This result confirmed the previous data obtained in batch cultivations. Interestingly, it was observed that the decrease in the viability levels of the mutants were significantly lower than that of the wild type during cultivation. Another significant result was that the two evolved strains B2 and B8 continued to produce ethanol when growth of the culture had stopped (Figure 3.21). This phase was previously defined as the uncoupling phase and the better capacity of ethanol production was linked to the length of this phase (Cot et al., 2007; Benbadis et al., 2009). The ethanol production results together with the higher viability levels implied that this higher performance of the mutants may be explained by the longer uncoupling phase.

The glycogen levels of the mutant B2 were found to be significantly higher than the mutant B8 and the wild type (Figure 3.21). This result might be accepted as another example of the differences in the physiological behaviour of these two mutants. In contrast to the glycogen contents, trehalose levels during cultivation were similar in the mutants and the wild type strain (Figure 3.21). A similar result was observed in a previous study under non-stress conditions (Mahmud et al., 2009). Analysis of the trehalose levels of the mutants under stress conditions might give differerent results in mutants and the wild type.

In addition to physiological analyes of the mutants and the wild type, genetic analyses were also perfromed for the investigation of the molecular basis of ethanol resistance. For this purpose, first, HSP104 expression levels were determined by RT-PCR. HSP104 encodes a heat shock protein that is responsible in refolding and reactivation of previously denatured and aggregated proteins. It is known to be responsive to ethanol stress (Sanchez et al., 1992). However, RT-PCR results showed that HSP104 expression levels were not significantly different between the mutants and the wild type. However, the ratio of the expression levels of HSP104 gene in the mutant B2 was shown to be lower than that of mutant B8 (Table 3.20). This result might be correlated with the higher ethanol tolerance of the mutant B2. Due to its higher ethanol tolerance, B2 might have possibly generated a weaker response

againts 7% (v/v) ethanol concentration when compared with the mutants B8 and the wild type, resulting with a lower ratio of HSP104 expression at stress/non-stress conditions. However, it is important to note once more that the differences were not major.

For further genetic characterization of the mutants, backcrossing of the mutants with a wild type strain of opposite mating type was planned to follow the genetic segregation of the resistance phenotype. However, backcrossing could not be achieved. Microscopic observations after incubation on sporulation media (Figure 3.24) and flow cytometry results (Figure 3.25) verified that the haploid starting population was no longer a haploid culture. It was determined that the diploidy emerged at around 7-8% (v/v) ethanol stress level during the stress selection protocol (Figure 3.26). Interestingly, it was determined that the diploidization was specific to increasing ethanol stress selection, since no other stress selection strategy tested, including the constant ethanol stress selection strategy, resulted with diploidization (Table 3.22). This is a valuable information, because there are no other studies in literature so far, pointing the effect of ethanol stress exposure on diploidization in yeast.

It is known that *S. cerevisiae* cells are capable of mating type switching and concomitantly forming a self diploidized culture (Haber, 1998). By analyzing the fingerprinting patterns of the diploidized cultures, the self diploidization of the mutant cultures were verified (Figure 3.27).

Diploid mutant cultures were sporulated and the ascus structures were dissected to yield four haploid segregants. The growth of the segregants of mutants B2 and B8 were analyzed on solid media (Figure 3.28). Two of the segregants of mutant B2 resulted with slow growth and two of the segregants of mutant B8 could not grow on YPD medium after dissection. This might be due to an improper segregation during division.

Moreover, the specific growth rates of the two segregants of B8 that could grow on solid plates were obtained by growth physiological analysis. It was shown that both segregants had similar maximum specific growth rates to the mutant B8 under non-stress conditions, whereas one of these segregants had a slightly lower growth rate than B8 under stress conditions, although not significant.

The mating type switching mechanism is known to be initiated by the site-specific HO endonuclease (Haber, 1998). However, HO gene is either deleted or silenced in the laboratory strains in order to avoid undesired diploidization. By sequencing the HO gene with its -1000 bp and +200 bp flanking regions in the mutants B2 and B8, the presence of HO in the genome was verified and it was observed that no mutation was present neither in HO gene nor in the possible transcription factors located in the flanking regions.

MKT1 encodes a protein that forms a complex with Pbp1p and mediates posttranscriptional regulation of HO (Tadauchi et al., 2004). The expression levels of HO and MKT1 in the mutants B2 and B8 were thus determined, together with the intermediate populations obtained during increasing ethanol stress selection. The expression levels of these genes were expected to provide a potential link with diploidization. It was clearly observed that the HO expression levels of all cultures were lower than that of the wild type. Inversely, the MKT1 expression levels of all cultures tested were higher than that of the wild type. However, no correlation could be made between the gene expression levels and the diploidization states of the intermediate populations. In addition, the analysis of petite colony frequency, as it has previously been correlated with MKT1 expression (Dimitrov et al., 2009), was performed (Figure 3.34). However, petite colony frequency results could not be correlated with MKT1 expression and the diploidization process in this study. By employing evolutionary engineering strategy on ΔHO and ΔMKT1 cultures, the occurrence of diploidization was investigated, along with the potential effects of HO and MKT1 genes on diploidization. ΔHO culture diploidized. On the other hand, ΔMKT1 culture did not form diploids even at high ethanol stress levels (Table 3.28). These results may imply that the absence of HO seems to be tolerated by another factor, but absence of MKT1 directly affects mating type switching. However, this effect could not be observed in the expression levels of these genes. It was reported that the alterations in the expression levels of MKT1 had slight effects on HO mRNA level, while it resulted with stronger effects on protein level (Tadauchi et al., 2004). This might be the reason for not observing a direct correlation between these two data sets.

Protein expression levels of the ethanol-resistant mutant B8 were also determined by global proteomic analysis and compared to that of the wild type under non-stress

conditions. The expression levels of a total of 48 proteins were shown to be significantly altered in the mutant strain when compared to wild type. Expression levels of 7 proteins decreased more than 1.5 times when compared to wild type (Table 3.29). Expression levels of glucose 6 phosphate isomerase (Pgi1p), acyl CoA binding protein (Acb1p), protease B inhibitors 2 and 1 (Pbi2p), peptidyl prolyl cis trans isomerase (Cpr1p), heat shock protein SSA3 (Ssa3p) and actin filament polymerization proteins cofilin (Cof1p) and profilin (Pfy1p) decreased in the mutant strain under non-stress conditions. Down-regulation of cofilin and profilin in mutant B8 might be related to growth deficiencies observed after meiotic segregation (Figure 3.28).

In contrast to the down-regulation of Ssa3p, SSA3 gene was previously shown to be overexpressed by 3.8 fold after 30 min of 7% (v/v) pulse ethanol stress (Alexandre et al., 2001). This result might be considered as an indicator of the contrast between the resistance mechanisms against continuous and pulse ethanol stress conditions.

Out of 41 proteins expressed more than 1.5 folds in mutant B8 compared to wild type, 34% belonged to ribosomal proteins, most of which have not been previously associated with ethanol tolerance. Most of the ribosomal proteins up-regulated in mutant B8 were determined to be involved in haploinsufficiency group. Haploinsufficiency is defined to be a dominant behaviour in diploids which are heterozygous for a loss-of-function allele (Deutschbauer et al., 2005). This result might be the reason for growth deficiency of segregants of B8 (Figure 3.28) where gene dosage effect might have led to incomplete function.

Another group of proteins that are shown to be affected the most belonged to heat shock protein family which are components of general stress response mechanism. These data indicate that the stress response mechanism is not turned off in mutant B8 even under non-stress condition.

Twenty five percent of up-regulated proteins that are shown to be affected the most are responsible in glycolysis/gluconeogenesis pathways. Other upregulated proteins can be grouped as elongation factors and proteins involved in amino acid biosynthesis and fermentation. Superoxide dismutase and mitochondrial outer

membrane protein porin 1 were the two mitochondrial proteins with increased expression levels (Table 3.30).

These results altogether may be explained by the fact that ethanol stress response is shown to be in accordance with energy limitations and it leads to increased expression of genes responsible in energy producing pathways such as glycolysis and mitochondrial action as pointed out by Stanley and his coworkers (Stanley et al., 2010b). Sod1, Tdh1 and Eno1 proteins, which were shown to be induced under various other stress conditions (Trabalzini et al., 2003), were also up-regulated in the mutant B8 of the present study.

To summarize, evolutionary engineering, which is an inverse metabolic engineering strategy, is obviously an efficient tool for obtaining highly ethanol resistant *S. cerevisiae* and understanding the molecular mechanisms of high ethanol resistance in *S. cerevisiae*. The mutants obtained in this study had high ethanol resistance in both minimal and complex media; in addition to high ethanol production, rapid growth under both non-stress and ethanol stress conditions, and cross-resistance against several industrial stress conditons. Thus, the mutants obtained and analyzed in this study could be promising laboratory models for efficient industrial ethanol production bioprocesses. Moreover, based on the emergence of diploidy in response to ethanol stress conditions, the current study emphasized the importance of polyploidy for ethanol-resistant phenotypes. Further studies are still necessary to clarify the whole molecular basis of ethanol resistance in yeast.

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APPENDICES

Appendix A1 : MPN Score Table for Five Tubes

Appendix A1

Table A1: MPN Score Table for Five Tubes (Lindquist, 2012)

Number of Positive Tubes in			MPN in the inoculum of the
First set	Middle set	Last set	middle set of tubes
0	0	0	‹0.01
0	0	1	0.02
0	1	0	0.02
0	2	0	0.04
1	0	0	0.02
1	0	1	0.04
1	1	0	0.04
1	1	1	0.06
1	2	0	0.06
2	0	0	0.05
2	0	1	0.07
2	1	0	0.07
2	1	1	0.09
2	2	0	0.09
2	3	0	0.12
3	0	0	0.08
3	0	1	0.11
3	1	0	0.11
3	1	1	0.14
3	2	0	0.14
3	2	1	0.17
4	0	0	0.13
4	0	1	0.17
4	1	0	0.17
4	1	1	0.21
4	1	2	0.26
4	2	0	0.22
4	2	1	0.26
4	3	0	0.27
4	3	1	0.33
4	4	0	0.34
5	0	0	0.23
5	0	1	0.31
5	0	2	0.43
5	1	0	0.33
5	1	1	0.46
5	1	2	0.63
5	2	0	0.49
5	2	1	0.7
5	2	2	0.94
5	3	0	0.79
5	3	1	1.1
5	3	2	1.4
5	3	3	1.8
5	4	0	1.3
5	4	1	1.7
5	4	2	2.2
5	4	3	2.8
5	4	4	3.5
5	5	0	2.4
5	5	1	3.5
5	5	2	5.4
5	5	3	9.2
5	5	4	16
5	5	5	›24

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BOOK CHAPTERS

- **B. Turanli-Yildiz**, Z. P. Çakar, 2011. Tersine Metabolik Mühendislik ve Uygulamaları. *Metabolizma Mühendisliği.* Birleşik Matbaacılık. ISBN: 978-975-483-911-1.
- **B. Turanli-Yildiz**, C.Alkim Z. P. Çakar, 2012. Protein Engineering Methods and Applications, *Protein Engineering*, Pravin Kaumaya (Ed.), ISBN: 978-953-51-0037-9, InTech.

SELECTED PRESENTATIONS

- **B. Turanli Yildiz,** T. Sezgin, Z.P. Çakar, L. Benbadis, J. François. Characterization of two ethanol-resistant mutants of *Saccharomyces cerevisiae* obtained by evolutionary engineering. Poster presentation. 4th Conference on Physiology of Yeast and Filamentous Fungi (PYFF4), 1-4 June 2010, Rotterdam, The Netherlands.
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GRANTS, AWARDS

- Research Fellowship Grant from Federation of European Microbiological Societies (FEMS), 2009.
- ITU Department of Molecular Biology and Genetics, BSc Graduation award, ranking the 3rd, 2003.