ISTANBUL TECHNICAL UNIVERSITY ★ INSTITUTE OF SCIENCE AND TECHNOLOGY

MOLECULAR CHARACTERIZATON OF ETHANOL RESISTANCE IN *Saccharomyces cerevisiae*

M.Sc. THESIS

Arman AKŞİT

Department of Advanced Technologies

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İSTANBUL TEKNİK ÜNİVERSİTESİ ★ FEN BİLİMLERİ ENSTİTÜSÜ

Saccharomyces cerevisiae'de Etanol Direncinin Moleküler Karakterizasyonu

YÜKSEK LİSANS TEZİ

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Moleküler Biyoloji-Genetik & Biyoteknoloji Programı

Tez Danışmanı: Prof. Dr. Zeynep Petek ÇAKAR

HAZİRAN 2012

Arman Akşit, a **M.Sc.** student of ITU **Institute of Science and Technology,** 521101100, successfully defended the **thesis/dissertation** entitled "**Molecular Characterization of Ethanol Resistance in *Saccharomyces cerevisiae***", which he prepared after fulfilling the requirements specified in the associated legislations, before the jury whose signatures are below.

Thesis Advisor :	**Prof. Dr. Zeynep Petek ÇAKAR** Istanbul Technical University	.....
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“Life is like riding a bicycle. To keep your balance you must keep moving.”

Albert Einstein

FOREWORD

I owe a great debt of gratitude to my supervisor Prof. Dr. Zeynep Petek ÇAKAR for not only welcoming me to this program but also for her guidance throughout my studies. She offered invaluable help in all possible ways. I am thankful to her for her encouragements and helpful critics during this research project.

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ABBREVIATIONS

CDW	**:** Cell dry weight
EMS	**:** Ethyl methane sulfonate
FDA	**:** Food and Drug Administration
GRAS	**:** Generally Regarded As Safe
HPLC	**:** High performance liquid chromatography
HSP	**:** Heat shock protein
MAT	**:** Mating type
MPN	**:** Most probable number
OD	**:** Optical density
PBS	**:** Phosphate Buffered Saline
RI	**:** Refractive Index
ROS	**:** Reactive oxygen species
RT	**:** Retention Time
SEM	**:** Scanning Electron Microscope
YEPDG	**:** Yeast extract - peptone - dextrose - glycerol
YMM	**:** Yeast minimal medium
YPD	**:** Yeast extract - peptone - dextrose (Yeast complex medium)

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MOLECULAR CHARACTERIZATION OF ETHANOL RESISTANCE IN *Saccharomyces cerevisiae*

SUMMARY

Yeast *Saccharomyces cerevisiae* has been utilized by humankind for thousand of years for several purposes such as baking, brewing and wine production. Because of being a model organism and the first eukaryote to have its genome completely sequenced, *Saccharomyces cerevisiae* is of great importance for eukaryotic cellular and molecular biology. Because of its very high capability for rapid and efficient conversion of sugars into ethanol, and tolerance to high ethanol concentrations (8%, v/v). *S. cerevisiae* represents the primary yeast 'cell factory' in biotechnology and is the most exploited microorganism known, producing potable and industrial ethanol, which is the world's premier biotechnological commodity. Due to increasing price and accelerating depletion of fossil fuels attracted widespread attention on bioethanol production because of its better properties such as being renewable and sustainable product, reducing air pollution, greenhouse gas, CO_2 for global warming. In high or very high gravity fermentations, it is possible to produce high-titers of ethanol. However, *S. cerevisiae* is sensitive to very high concentrations of ethanol (14%, v/v). Accumulation of ethanol incide the cell has remarkable adverse effects on cellular growth and viability. So, obtaining high ethanol-tolerant strain is desirable for bioethanol production from biomass. For this reason, understanding the ethanol stress tolerance mechanism in *Saccharomyces cerevisiae* is crucial, whereby it can be studied on tailored and improved yeasts obtained by numerous different strategies such as evolutionary engineering approach. Evolutionary engineering is a powerful strategy, which has successfully been used for strain improvement. This strategy is based on the selection of the mutants with a desirable phenotype among a genetically diverse population. The aim of this study was to gain more insight into the mechanisms responsible for ethanol stress tolerance by investigating genetic, physiological and phenotypic components and indicators. In a previous study, ethanol resistant mutants were obtained via constant (5%, v/v ethanol) and increasing (5% to 11,4% ,v/v ethanol) stress application strategies of evolutionary engineering method. After that, individual mutants of increasing stress application strategy were characterized phenotypically in terms of their cross-resistance for other industrial stresses. In this study, these mutants were analyzed transcriptomically by microarray analysis. In parallel with transcriptomic analysis, growth curves were graphed and co-evaluated with extracellular metabolite levels regarding glucose, acetate, ethanol, glycerol and intracellular metabolite levels including glycogen and trehalose. Production and consumption of metabolites were estimated by using data obtained from HPLC analysis. These results were consistent with the phenotypical characterization results from solid and liquid media. In this study, compared to wild type, glycogen and trehalose production and consumption were so high in ethanol-resistant mutants under control conditions.

*Saccharomyces cerevisiae'*de ETANOL DİRENCİNİN MOLEKÜLER KARAKTERİZASYONU

ÖZET

Saccharomyces cerevisiae mayası binlerce yıldan beri insanoğlu tarafından ekmek yapımı, bira yapımı ve şarap yapımı gibi çeşitli amaçlarla kullanılmaktadır. Bir model organizma ve genom dizilenmesi tamamlanan ilk ökaryot olması sebebiyle *S. cerevisiae* ökaryotik hücre ve moleküler biyolojisi için büyük öneme sahiptir. Şekerleri etanole hızlı ve etkili bir şekilde dönüştürme yeteneği, ve yüksek etanol konsantrasyonlarına (8%, v/v) dayanıklı olması sebebiyle, *S. cerevisiae* biyoteknolojideki öncü maya "hücre fabrikası"nı temsil eder ve dünyanın başlıca biyoteknolojik ürünü olan içilebilir ve endüstriyel etanol üretiminde en fazla kullanılan mikroorganizmadır. *S. cerevisiae* mayası bira, şarap, ekmek üretim endüstrilerindeki kullanımı dışında, son yıllarda biyo-yakıt olarak kullanılan biyo-etanol üretim proseslerindeki kullanımı ile de çok büyük önem arz etmektedir. Fosil yakıtların fiyat artışı ve hızlı tükenmesi biyoetanol üretimi ve biyoetanol üretim mekanizmalarının bu mayada araştırılmasını sağlamıştır. Ayrıca biyo-etanol, fosil yakıtlarla kıyaslandığında, yenilenebilir ve sürdürülebilir ürün olma, hava kirliliğini, sera gazlarını ve CO_2'i azaltma gibi daha iyi özelliklerinden dolayı her geçen gün daha fazla önem kazanmaktadır. Yüksek miktarlarda etanol üretmek için yüksek veya çok yüksek yoğunluklu fermentasyon koşulları kullanılmaktadır. Fakat, *S. cerevisiae* mayası çok yüksek konsantrasyonlarda (14%, v/v) etanole karşı hassastır. Etanol, biyolojik membranlardan difüzyonla serbestçe geçerek hücre büyümesinin inhibisyonuna ve hücre canlılığının azalmasına, ayrıca etanol fermentasyon hızının ve son verimin düşmesine neden olmaktadır. Yüksek konsantrasyonlarda etanolün protein konformasyonunu bozduğu, glukoz, amonyum, maltoz ve amino asitlerin alınımını etkilediği bilinmektedir. Bu nedenlerden dolayı, biyokütleden biyoetanol üretimi için, yüksek konsantrasyonda etanole toleranslı suş eldesi arzu edilmektedir. Bunun için, *S. cerevisiae'*deki etanol stres tolerans mekanizmalarının anlaşılması oldukça önemlidir ki bu, evrimsel mühendislik gibi çeşitli farklı stratejilerle elde edilmiş özellikleri geliştirilmiş mayalar sayesinde çalışılabilir. Evrimsel mühendislik, tersine metabolizma mühendisliği stratejisinin bir alt dalıdır ve suş geliştirmesinde başarıyla kullanılan, çok güçlü bir yöntemdir. Bu yöntem, genetik özellikler bakımından karışık bir popülasyondaki istenilen fenotipe sahip olan mutantların seçilimine dayanmaktadır.

Bu çalışmanın amacı, etanol stres direncinden sorumlu mekanizmalar hakkında; genetik, fizyolojik ve fenotipik unsur ve göstergelerin araştırılması ile daha geniş bilgiler elde etmektir. Önceki bir çalışmada etanole dirençli mutantlar, evrimsel mühendislik yönteminin sabit (%5 etanol, v/v) ve artan (% 5'den %11,4'e) stres uygulamaları kullanılarak elde edilmiştir. Daha sonra, artan stres uygulaması stratejisi ile elde edilen mutant bireyler başka endüstriyel streslere çapraz dirençleri açısından fenotipik olarak karakterize edilmişlerdir. Bu çalışmada, bu mutantlar mikrodizileme analizi ile transkriptomik olarak analiz edilmiştir. Transkriptomik

analizlere paralel olarak büyüme eğrileri elde edilmiş; ayrıca glukoz, asetat, etanol ve gliserol gibi hücre dışı metabolitler ile glikojen, trehaloz gibi hücre içi metabolitlerin üretimi araştırılmıştır. Hücre dışı metabolit miktarları, yüksek basınçlı sıvı kromatografisi (HPLC) analizlerinden elde edilmiş veriler sayesinde değerlendirilmiştir. Stres dirençleri, en muhtemel sayı (MPN) ve damlatma deneyleri ile belirlenmiştir. Bunun haricinde, etanole dirençli mutant kültürlerde mitokondriyal kusur görülme sıklığı araştırılmış ve bu mutant kültürlerin genetik olarak stabil olup olmadıkları incelenmiştir. Evrimsel mühendislik yöntemi ile elde edilen etanol stresine dirençli mutantlardan B2'nin en dirençli mutant olduğu, B8'in ise birden fazla stres etmenine çapraz direnç gösterdiği önceki çalışmalardan bilinmektedir. Hayatta kalma (*viability*) testinde, sürekli strese maruz kalan hücrelerin stres sonrasında vejatatif olarak büyüyebilme kabiliyetleri incelenmiştir. Bu deney sonucunda beklendiği gibi mutant B2'nin hayatta kalma seviyesi mutant B8 ve yabani tip kültüre göre daha yüksek bulunmuştur. Bu sonuç, önceden elde dilmiş sonuçları doğrulaması açısından oldukça önemlidir. 5-tüp MPN metodu ile, mutant B2 ve B8'in genetik kararlılıkları test edilmiştir. Her bir pasajlama sonrasında stres direnç seviyeleri açısından düzenli bir azalma görülmediği için etanole dirençli mutant B2 ve B8'in genetik olarak kararlı olduğu belirlenmiştir. Petite sıklığı (*petite frequency*) deneyinde yabani tip, ara popülasyonlar A2 ve A23, son popülasyon A98 ve mutant bireyler B2 ve B8 kullanılmıştır. Solunum yapamayan, büyümek için yalnızca fermentasyonu kullanan hücreler, eser miktarda fermente edilebilir karbon kaynağı varlığında çok az da olsa büyüyebilirler. Bu nedenle küçük (petite) koloniler oluştururlar. Petite sıklığı, petite kolonilerin sayısının toplam kolonilere oranı olarak ifade edilmektedir. Bu deney sonucunda, etanol stresine dirençli mutantlarda neredeyse hiç petite görülmediği; oysaki yabani tipte petite sıklığının çok yüksek miktarda olduğu tespit edilmiştir. Bu sonuç, etanol stres direnci için sağlam ve fonksiyonel mitokondriye gereksinim olduğunu göstermesi açısından oldukça önemlidir. Herhangi bir stres etmeninin olmadığı kontrol koşulunda elde edilmiş büyüme eğrilerine göre, yabani tip kültür ile mutant kültürler B2 ve B8'in spesifik büyüme hızlarının aynı olduğu görülmüştür. Bu sonuç; başlangıçta rasgele mutasyon geçirmiş olan yabani tip kültürden evrimsel mühendislik stratejisi ile elde edilmiş olan etanole dirençli mutant bireyler B2 ve B8'de, stressiz koşulda büyüme yeteneğinde bir azalma olmadığını göstermesi açısından oldukça önemlidir. Yine stres içermeyen koşulda, trehaloz ve glikojen üretim miktarları incelenmiştir. Etanole dirençli mutantlar B2 ve B8'de yabani tip kültüre kıyasla dikkate değer farklılıklar saptanmıştır. Stressiz koşulda, yabani tip ne trehaloz ne de glikojen üretememiştir. Fakat mutant kültürler, bu karbohidratlardan anlamlı miktarlarda üretmişlerdir. Trehalozun hücre için önemi özellikle stres koruyucu fonksiyonundan ileri gelmektedir. Stres uygulaması olmamasına rağmen mutant kültürlerde yabani tip kültüre kıyasla bu karbohidratlardan önemli miktarlarda üretilmesi, etanole dirençli mutant kültürlerde stressiz koşulda dahi stres koruyucu fonksiyonların oldukça etkin olduğunu göstermektedir. Hücre içi metabolit miktarlarının tayini için öncelikle yabani tip kültür ve mutant B2, hem stres içermeyen hem de %8 (v/v) oranında etanol içeren sıvı besiyerinde eş zamanlı olarak inkübe edilmiştir. Belirli zaman aralıklarında toplanmış örneklerdeki hücre dışı metabolit miktarları "Yüksek Basınçlı Sıvı Kromatografisi" (HPLC) yöntemi kullanılarak analiz edilmiş ve büyüme eğrileriyle birlikte değerlendirilmiştir. Beklendiği üzere stres varlığında hem B2'nin hem de yabani tip kültürün spesifik büyüme hızı azalmıştır. Ancak, spesifik büyüme oranındaki azalma yabani tip kültürde çok daha fazla bulunmuştur. Bu nedenle 8% (v/v) etanol stres varlığında etanole dirençli mutant B2'nin daha iyi büyüdüğü

anlaşılmaktadır. Bu sonuç, önceden yapılmış olan deneyleri doğrulaması bakımından dikkat çekicidir. Beklendiği gibi hem yabani tip kültürde hem de B2 kültüründe, etanol stresi varlığında glukoz tüketim hızı yavaşlamış ve 48. saat itibariyle glukoz tam olarak tüketilememiştir. Bu sonuca paralel olarak etanol üretim hız ve miktarında da düşüş gözlenmiştir. Stres varlığında da yokluğunda da mutant B2'nin yabani tip kültüre göre daha fazla gliserol ürettiği tespit edilmiştir. Ayrıca etanol stresi varlığında yabani tip kültürde asetat üretiminin düştüğü; halbuki B2'de anlamlı bir artış olduğu belirlenmiştir.

Mikrodizileme analizine göre, yabani tipe kıyaslandığında B2 ve B8'de anlatım seviyesi en az iki kat değişen genler sırasıyla 228 ve 396 adettir. Mikrodizileme analizi için kullanılan kültürler stressiz koşulda inkübe edildikleri halde, yabani tip kültüre kıyasla B2 ve B8'in gen anlatımında çok anlamlı bir fark belirlenmiştir. Bu sonuç, stres içermeyen ortamda B2 ve B8'in fizyolojik olarak yabani tip kültürden farklı oluşunu açıklaması açısından oldukça önemlidir. Her iki mutantta da expresyonu en fazla azalan gen, homotalik dönüşüm endonükleazını kodlayan HO geni olarak bulunmuştur. HO geni, mayada eşey tipinin değişim mekanizmasını başlatmaktan sorumludur, bu nedenle haploid hücrelerde anlatımı yapılan bir gendir. Önceden yapılmış olan çalışmalarda mutant B2 ve B8'in diploid hale geçmiş oldukları tespit edilmiştir. Mikrodizileme analizi bu tespiti doğrulamıştır. Ayrıca, her iki mutantta da expresyonu en fazla artan gen, mayoz bölünmeyi uyaran IME4 geni olarak bulunmuştur. Bu genin anlatımı diploid hücrelere özgü olduğundan, mutant kültürlerde bu genin ifadesinin seviyesindeki artış aynı şekilde mutant kültürlerin diploit oluşunu kanıtlamaktadır.

Elde edilen bu sonuçlar, evrimsel mühendislik yöntemi ile elde edilmiş mutant kültürlerin, yabani tip kültüre kıyasla fenotipik ve fizyolojik açıdan farklı olduğunu göstermiş, transkriptomik analiz sonucu da bu farklılığı doğrulamıştır.

1. INTRODUCTION

1.1 General Information About *Saccharomyces cerevisiae*

Saccharomyces cerevisiae, is a yeast which has been utilized by mankind for several purposes, is commonly known as brewer's yeast, baker's yeast or budding yeast (Madigan TM. *et al.*, 2009). *S. cerevisiae* which was firstly introduced by Meyen in 1837 (Feldman, 2010) is a small, single-celled member of the kingdom of fungi (Alberts, 2008) and belongs to phylum Ascomycetes and genus Saccharomyces. The phylum ascomycetes takes its name from the production of asci (singular, ascus) in which mating of two haploid nuclei from different mating types occurs and results in a diploid nucleus that then undergoes meiosis to give haploid ascospores. The systematic classification of *S. cerevisiae* is shown in Table 1.1.

Table 1.1 : Systematic classification of *S. cerevisiae.*

Kingdom	Fungi
Phylum	Ascomycota
Class	Hemiascomycetales
Order	Saccharomycetales
Family	Saccharomycetacae
Genus	Saccharomyces
Species	*S. cerevisiae*

The cells of *Saccharomyces cerevisiae* are characteristically spherical, oval, or cylindrical in shape (Madigan TM. *et al.*, 2009). The sizes of haploid and diploid cells show variations depending on the phase of growth and type of the strain (Esslinger, 2009). Generally haploid cells are 4 µm diameter spheroids and diploid cells are 5 x 6 µm ellipsoids. Also, for a haploid and diploid cell, mean cell volumes are 29 and 55 µm3, respectively (Walker, 1998). Furthermore, mean cell size of *S. cerevisiae* increases with cell age (Walker, 1998; Feldmann, 2010). A scanning

electron micrograph of a cluster of *S.cerevisiae* cells is shown in Figure 1.1 (Alberts, 2008).

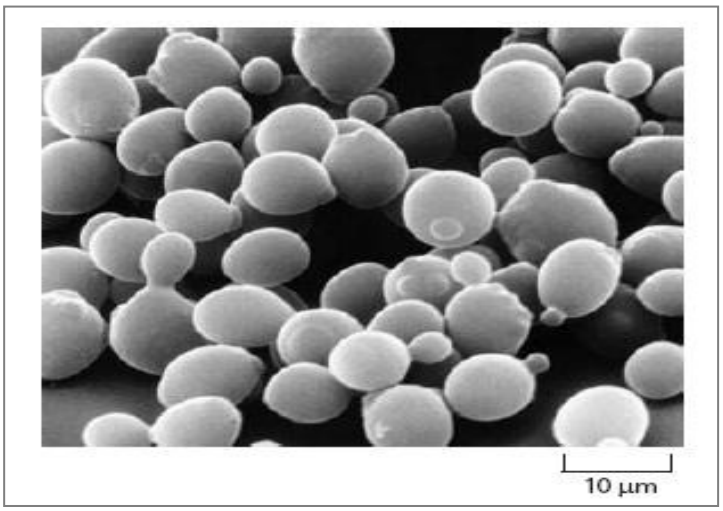

Figure 1.1 : A typical image of *Saccharomyces cerevisiae* gained by scanning electron microscope (SEM) (Alberts, 2008).

Saccharomyces cerevisiae has structural compartments such as nucleus, mitochondria, golgi apparatus, secretory vesicles, endoplasmic reticulum, vacuoles and micro bodies in the cell structure like in the other higher eukaryotes. Besides these features, in common with other fungi, they have cell wall which is thick and tough. The presence of a cell wall differentiates a yeast cell from an animal cell. Due to not having a chloroplast compartment in the cell they are not plants, too. So, yeast cells are considered as being close to both animal and plant cells (Walker, 1998; Alberts, 2008). Figure 1.2 that was obtained with transmission electron microscope (TEM) shows the nucleus, mitochondrion and the cell wall of a yeast cell (Alberts, 2008).

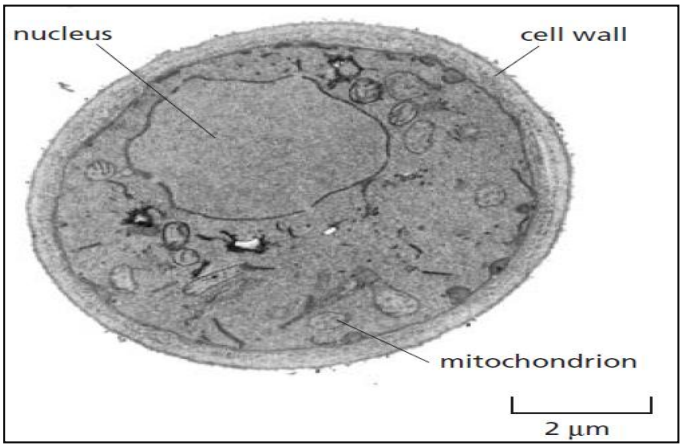

Figure 1.2 : A transmission electron micrograph of a cross section of a yeast cell, showing its nucleus, mitochondrion, and thick cell wall (Alberts, 2008).

Cell wall of *S. cerevisiae* contains structural components such as proteins, lipids, pigments carboxylate, phosphate, sulphydryl, amine groups and distinct, potential metal-complexing sites. Mannan-β-glucan stands out as the main structural polymer of the cell wall (Zetic VG. *et al.*, 2001).

Vacuole is the other cellular component, which is the largest compartment of the cell. It involves in numerous functional processes, including the homeostasis of cell pH and the concentration of ions, storage of polyphosphate and amino acids, osmoregulation and degradation processes (Kubota *et al.* 2004; Fujita *et al.* 2006).

Saccharomyces cerevisiae is a facultative anaerobe organism which is able to growth on various fermentable and non-fermentable carbon sources. If yeast is grown on fermentable carbon sources such as glucose, it produces essentially its metabolic energy from glycolysis. The Pasteur Effect, an inhibiting effect of oxygen on the fermentation process, takes place in case there is high concentration of oxygen. If the glucose concentration is high, the Pasteur Effect is no longer usable. In this situation, "Crabtree effect", in which cells proceed to ferment, takes place (Francesca G. et al, 2010).

Due to its ability to ferment sugars to ethanol and carbon-dioxide, it has long been utilized for production of food and alcoholic beverages for ages. It is remarkably important for the baking industry for raising dough. Carbon dioxide is trapped within tiny bubbles and causes dough rising. Also these organisms can be taken as a vitamin supplement due to their high content of B vitamins, niacin and folic acid (URL-1).

Saccharomyces cerevisiae, a model organism and the first eukaryote to have its genome completely sequenced, is of great importance for eukaryotic cellular and molecular biology for more than 50 years (Xiao W., 2006). Besides its usage in bakery and brewery, together with its well-defined genetic system, rapid growth, cell division, cellular mechanisms of replication, recombination, biochemistry, autonomously replicating plasmid, the ability readily to form distinct colonies on simple defined medium, the ease of replica plating and mutant isolation, a highly versatile DNA transformation system, it has been employed during development of recombinant DNA technology (URL-1). The complete sequence of its genome functions as a reference towards the sequences of other higher eukaryotic genes including those of human (Xiao W., 2006). Another reason for its popularity in basic

and applied research is resulted from its classification as GRAS (generally regarded as safe) by the U.S. Food and Drug Administration (FDA) (Nevoigt, 2008).

Unlike most other microorganisms, strains of *Saccharomyces cerevisiae* can exist both as haploid and diploid form. Both vegetative and sexually reproduction can be seen in *S. cerevisiae* (Figure 1.3). If adequate amount of nutrients is present, both haploids and diploids can achieve vegetative growth and undergo mitosis (Dickinson J. R., 2004).

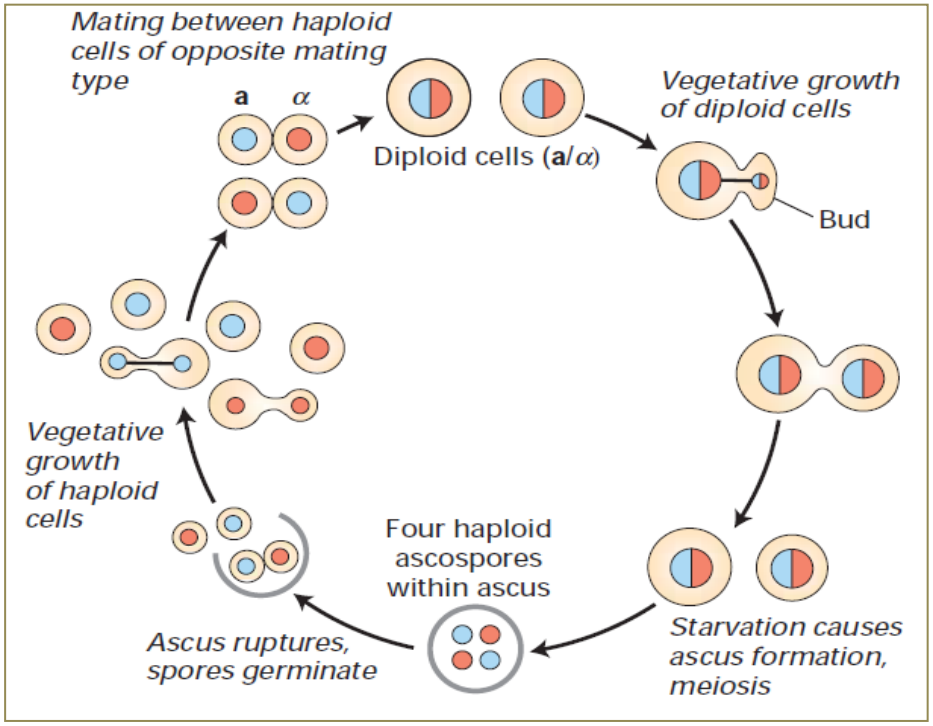

Figure 1.3 : Sexual and vegetative reproduction of the yeast *Saccharomyces cerevisiae.*

Budding is the form of vegetative reproduction that occurs in *S. cerevisiae*. The division of budding yeast is asymmetrical; the daughter cell is smaller than the mother. Buds may arise from any point on the cell wall; however, they do not arise from the same point more than once (Briggs, 2004). In the budding process, a new cell forms as a small outgrowth of the mother cell in case mother cells attain a critical cell size. After enlargement of the bud, nuclear division, cell wall formation and finally, separation of daughter cell from the parent cell take place (Walker, 1998; Briggs, 2004). Scanning electron micrograph of the budding yeast is shown in Figure 1.4.

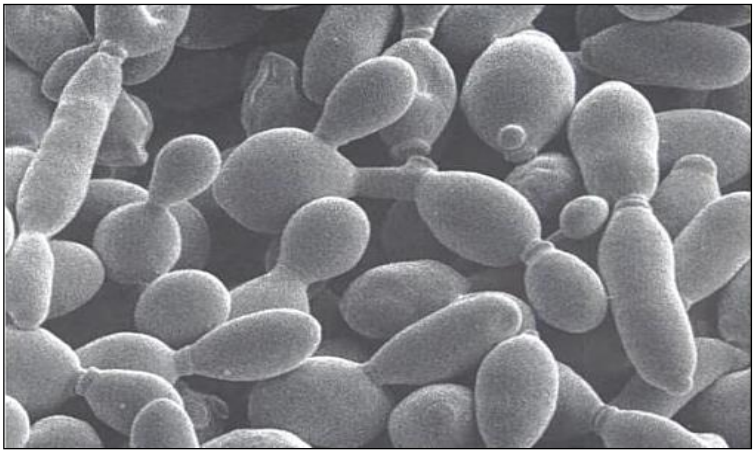

Figure 1.4 : Typical image of *Saccharomyces cerevisiae* cells during budding (Chapman and Roberts, 1997).

Haploids and diploids have different budding pattern. In rich media, haploids and homozygous diploids (*MAT***a**/*MAT***a** and *MAT*α/*MAT*α) bud in an axial pattern. However, heterozygous (*MAT***a**/*MAT*α) diploid cells display polar budding (Dickinson J. R., 2004).

Even if nutrients are supplied unlimitedly, an individual yeast cell could not continue to produce new daughter cells forever. Every yeast has a characteristic finite lifetime (Dickinson J. R., 2004). Yeast cells bud nearly 20 times in a life period (Burke *et al.*, 2000). Age of a cell can be determined by the number of bud scars present on the cell wall. Figure 1.5 shows bud scars present on the cell surface of a single *S. cerevisiae* cell.

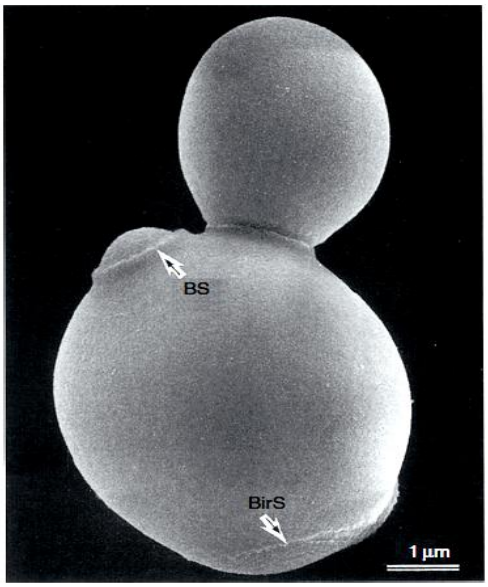

Figure 1.5 : Bud scar formation of an individual cell shown by SEM. BS, bud scar; and BirS, birth scar (Schaechter, 2009).

Some cells show sexual reproduction via mating process, in which two yeast cells come together and fuse. Figure 1.6 shows sexual life cycle of *Saccharomyces cerevisiae*. There are two different mating types of haploid *Saccharomyces cerevisiae* designated as **a** and α. Mating types can be considered analogous to male and female gametes. Haploids of mating type **a** produce "**a** factor". Also, haploids of mating type α produce "α factor". Both of these factors are peptide hormones (pheromones) that attract cells of the opposite mating type. That is, cells of type a mate only with cells of type α, or vice versa. Due to this feature, mating type of a cell can be determined genetically by mating this cell with a cell whose mating type is known (Madigan TM. *et al.*, 2009).

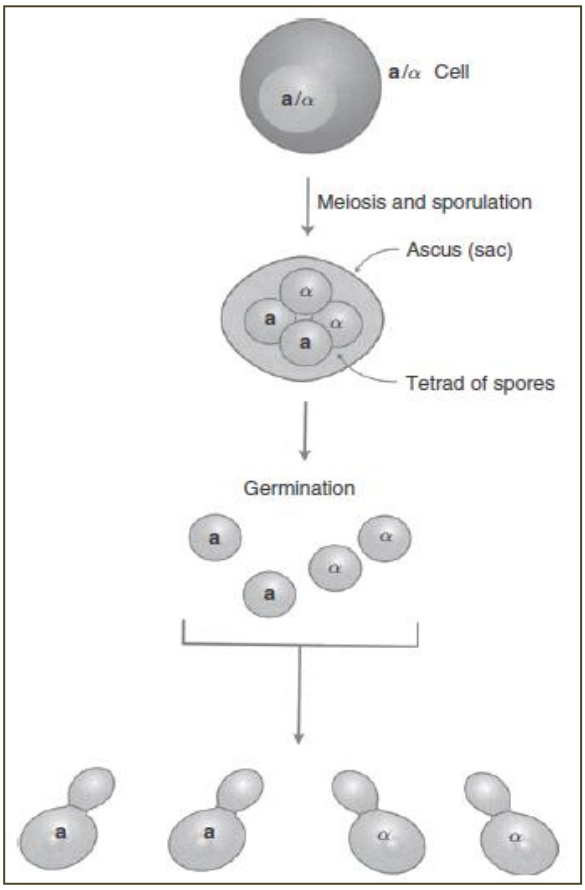

Figure 1.6 : Sexual life cycle of *Saccharomyces cerevisiae* (Schaechter, 2009).

S. cerevisiae cells are classified as heterothallic or homothallic depending on the capability for mating type switching (Figure 1.7). Homothallic strains are only maintained in diploid form throughout life cycle because of their competence in mating type switching which results from presence of a dominant allele termed HO (HOmothallic switching endonuclease).

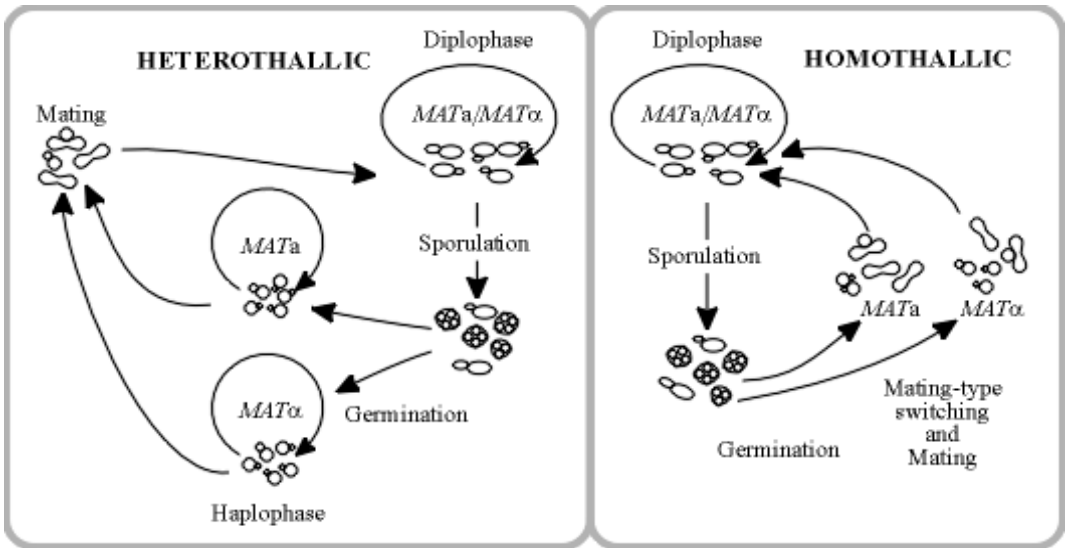

Figure 1.7 : Reproduction cycles of heterothallic and homothallic strains of *S. cerevisiae* (Sherman F., 2002).

During vegetative growth, the HO gene present in such yeast strains brings about a high frequency of switching between two different mating types. Under the influence of HO gene, the mating type locus, MAT, of such strains changes from *MATα* to *MATa* or vice versa (Priest and Campbell, 1996). That is, *MATa* cells produce *MATα* buds and *MATα* cells produce *MATa* buds. This results in self-diploidization due to mating with the cell of opposite mating type. As a result, there can be seen only a transient haplophase in homothallic strains. However, heterothallic strains are HO^{-}, carry non-functioning form of the HO and show both diploid and haploid forms (Haber, 1998; Madigan, 2006; Briggs, 2004).

This switch of mating type takes place when the active mating gene is replaced with one of two silent genes (Figure 1.8). Mating type is determined by expression of genes at the MAT (for mating type) locus on chromosome III, at which either gene a or gene α can be inserted. At this locus MAT promoter controls transcription of whichever gene is present. If gene a is at that locus, then the cell mating type is a, whereas if gene α is at that locus, the cell is mating type α. Copies of genes a and α are not expressed elsewhere in the genome. These silent copies are the source of the inserted gene. In this switch mechanism, firstly the gene, which will be inserted, is copied from its silent site and inserted into MAT region, replacing the gene already present. The old mating type gene is excised and discarded, and the new gene is inserted. Whichever gene is inserted in the MAT locus is the one that will be transcribed (Madigan *et al.*, 2009).

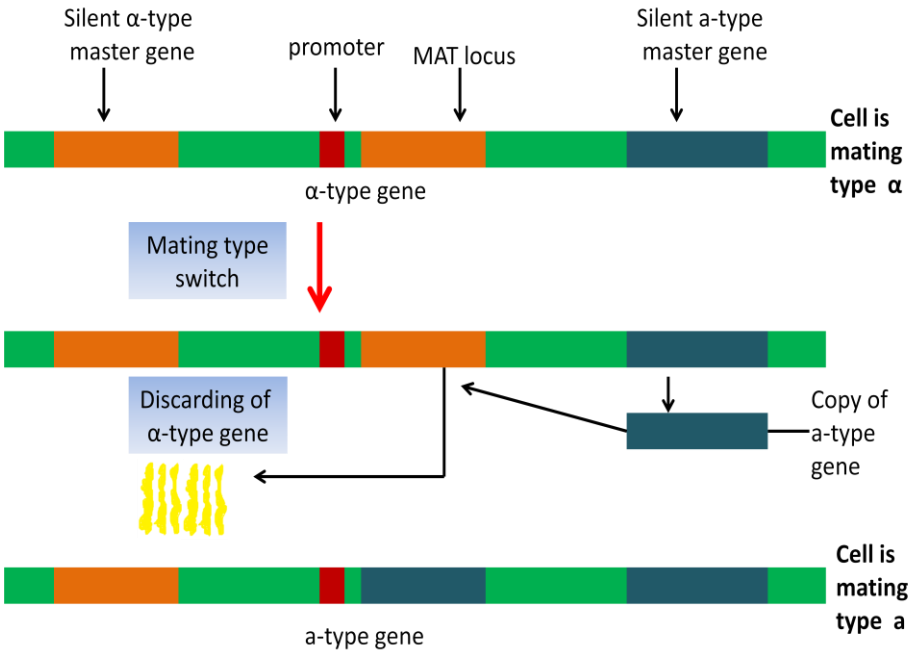

Figure 1.8 : The cassette mechanism that switchs yeast *S. cerevisiae* from mating type α to a (Madigan TM. *et al.*, 2009).

Pheromone produced by cells of one mating type binds to cognate G-coupled receptor on the cell surface of cells of the opposite mating type. When the opposite mating factor binds to cell surface receptor, it causes haploid cells to arrest in G1 phase of the cell cycle. Under this condition proliferation ceases and "shmoo" structure, which is characterized by formation of protuberance towards each other, is formed (Dickinson J. R., 2004) (Figure 1.9). Ultimately, via cell contact and fusion, diploid *MAT**a***/*α* cells are generated. Even though diploid *MAT**a***/*α* cells have not the ability of mating, due to nutrient depletion they can undergo meiotic division to form haploid cells (Feldman,2005; Zetic *et al*, 2001).

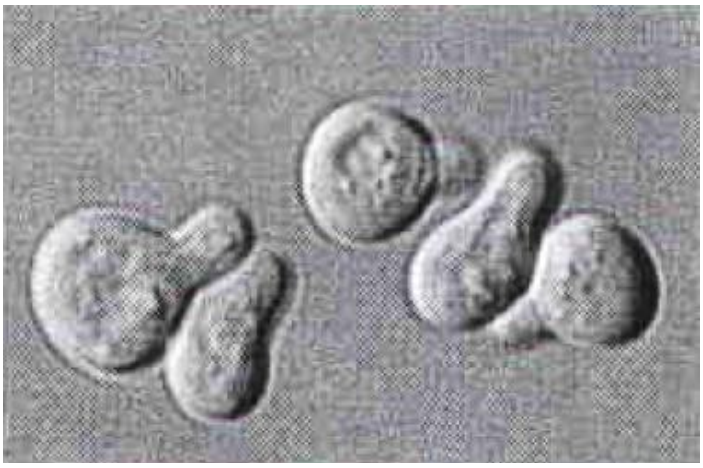

Figure 1.9 : Shmoo formations in response to mating factor of the opposite mating type of cell.

When there is no available nutrient, both haploids and diploids arrest as stationary phase cells. They can be distinguished from proliferating yeast cells because of their different morphology and biochemistry. They are round, phase-bright, unbudded and stores higher levels of trehalose and glycogen compared to proliferating cells (Werner-Washburne *et al.* 1993).

Sporulation occurs only if nitrogen source is absent and there is a poor carbon source like acetate. Fermentable carbon sources, for instance glucose, even at low concentrations repress this event. Sporulation takes place in normal heterozygous diploids *MAT**a**/α* but not in haploids, homozygous diploids and respiratory deficient "petite" cells. In such condition subsequent to meiosis, spores are formed within an ascus. If the spores are returned to rich nutrient conditions they will germinate and start to growth as haploids (Dickinson J. R., 2004).

Doubling time of laboratory haploid strains is approximately 90 minutes in yeast complex rich medium "YPD" (1% yeast extract, 2% peptone and 2% dextrose), and is about 140 min. in synthetic media. As a general rule, *S. cerevisiae* cells are cultivated at 30 ºC as optimum temperature for growth. But, some strains show reduced growth rates in synthetic media. To prevent such a condition and to obtain high amounts of cells can be accomplished with special conditions by adjusting pH value, adding nutrients and filtered-sterilized media and extreme aerating (Sherman, 2002).

Large-scale molecular analysis of *S.cerevisiae* started just after obtaining DNA sequence of whole genome sequence which was completed in 1997. The importance of this genome sequencing is that it is the first eukaryotic genome that was completely sequenced. It contains about 13,117,000 nucleotide pairs, including the small contribution (78,520 nucleotide pairs) of the mitochondrial DNA and approximately 6300 genes, compactly organized on 16 chromosomes. *S. cerevisiae* genome size is approximately 2.5 fold the genome of the prokaryotic model organism, *E. coli*. It is estimated that yeast shares about 23% of its genome with that of humans. Therefore, many important human proteins such as signaling proteins, cycle proteins were firstly discovered by studying their homologs in *S. cerevisiae* (URL-2; Alberts, 2008).

1.2 Industrial and Biotechnological Importance of the Yeast *Saccharomyces cerevisiae*

Saccharomyces cerevisiae is one of the earliest domesticated organisms in human civilization history, and it has been exploited for thousands of years in traditional fermentation processes to produce beer, wine, and bread (Schaechter, 2009). Because of its very high capability for rapid and efficient conversion of sugars into ethanol, and tolerance to high ethanol concentrations, *S. cerevisiae* has been a popular microorganism for both traditional and industrial production processes (Piskur *et al.*, 2006).

The products of modern yeast biotechnologies affect many commercially important sectors such as food, beverages, chemicals, industrial enzymes, pharmaceuticals, agriculture, and the environment (Table 1.2). *S. cerevisiae* represents the primary yeast 'cell factory' in biotechnology and it is the most exploited microorganism known, producing potable and industrial ethanol, which is the world's premier biotechnological commodity (Schaechter, 2009).

Table 1.2 : Industrial commodities produced by *Saccharomyces cerevisiae*.

Commodity	Examples
Beverages	Potable alcoholic beverages such as beer and wine
Food and animal feed	Baker's yeast, yeast extracts, fodder yeast, livestock growth factor, and feed pigments
Chemicals	Fuel ethanol (bioethanol), carbon dioxide, glycerol, and citric acid vitamins
Enzymes	Invertase, inulinase, pectinase, lactase and lipase
Recombinant proteins	Hormones (e.g. insulin), viral vaccines (e.g. hepatitis B vaccine), antibodies, growth factors, interferons, blood proteins, and enzymes

In addition to their traditional roles in food and fermentation industries, yeasts play important roles in the environment and in the health care sector of biotechnology. Also, they are so important in fundamental biological and biomedical research as model eukaryotic cells (Schaechter, 2009) (Figure 1.10).

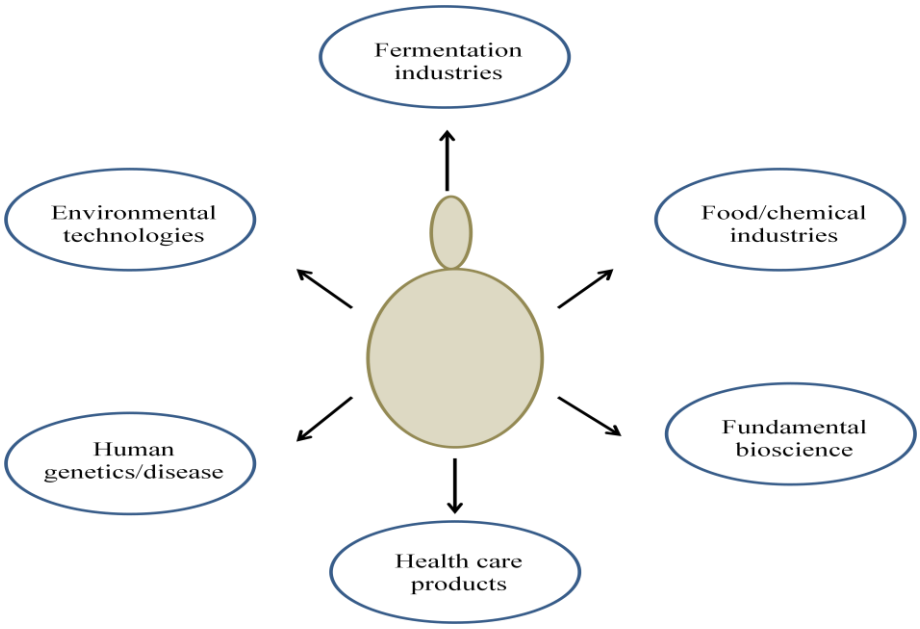

Figure 1.10 : Uses of yeast in biotechnology.

In agriculture, it has been shown that live cultures of *S. cerevisiae* stabilize the rumen environment of ruminant animals (e.g., cattle) and improve the nutrient availability to increase animal growth or milk yields. Maybe, it can be caused by preventing oxidative stress to rumen bacteria, or stimulating rumen bacterial growth via supplying malic and other dicarboxylic acids. *S. cerevisiae* is determined as beneficial to plants in preventing fungal disease. For instance, it has potential as a phytoalexin elicitor in stimulating cereal plant defenses against fungal pathogens. Also, in medicine yeasts are employed for production of novel human therapeutic agents by using yeast recombinant DNA technology. Yeasts are also very important as experimental models in biomedical research, especially in the fields of oncology, pharmacology, toxicology, virology, and human genetics (Schaechter, 2009) (Table 1.3).

Table 1.3 : Value of yeasts in biomedical research.

Biomedical field	Examples
Aging	Mechanisms of cell aging and apoptosis
Pharmacology	Drug metabolism, multidrug resistance
Oncology	Basis of cell cycle control, human oncogene regulation, anti-cancer drugs
Human Genetics	Basis of human diseases and genome/proteome projects
Virology	Viral gene expression, antiviral vaccines

There are several reasons about why *Saccharomyces cerevisiae* is favored as an experimental model. Firstly, it is a unicellular eukaryotic organism with a relatively uncomplicated and short life cycle. Secondly, it has a small genome comprising about 6000 genes, which has been completely sequenced (Goffeau *et al.*, 1996) and extensively mapped. Thirdly, major processes in cell biology such as signal transduction (e.g. Whitmarsh and Davis, 1998), control of cell cycle progression (e.g. Nasmyth, 1996), the basis of the switch from mitosis to meiosis (Nasmyth, 1996), genetic recombination (Stahl, 1996), intracellular trafficking of proteins (Kim *et al.*, 1998), response to stress (e.g. heat shock genes; Glover and Lindquist, 1998), and protein degradation (e.g. Xie and Varshavsky, 1999) has been understood beacuse of the special characteristics of this organism. Fourthly, since it has been revealed that many yeast genes have orthologs in the human genome, including some disease-causing genes, this observation makes the budding yeast an appealing model for studies of medicine and processes encountered in multi-cellular organisms. Lastly, yeast *Saccharomyces cerevisiae* is a desirable organism, because it is relatively cheap to grow in large quantities on simple medium (Gershon and Gershon, 2000).

1.3 Stress Exposure and Responses in *Saccharomyces cerevisiae*

All cell types, even individual cells in multi-cellular organisms have the ability to respond to changes in environmental conditions. To give a response, complex pathways of sensing and signal transduction leading to adaptation of cell growth and proliferation are operated. Also, gene expression programme, metabolic activities and other features of the cell are adjusted. Environmental conditions that threaten the survival of a cell, or prevent it from performing optimally are referred to as cell stress. Unicellular organisms like *Saccharomyces cerevisiae* must maintain their capacity to proliferate when sudden environmental changes happen. For this reason, the cellular response to stress is apparently aimed at protecting cells from the harmful effects of stress and at repairing damage. Stress factors can be temperature, pressure, radiation, toxic chemical agents, concentration of solutes and water, presence of certain ions, pH and nutrient availability. Generally, stress responses are investigated by rapidly shifting yeast cells from one condition to another, for example, from optimum growth temperature to very high temperature or from a medium without ethanol to high concentrations of ethanol. After this shift, cellular behaviour can be

examined by gene or protein expression analysis. By means of such analysis, candidate genes and proteins for stress response are determined (Hohmann S., 2003).

Cell proliferation is directly affected by cellular stress responses. Generally, stress treatments result in a transient arrest of the cell cycle, generally in G1. This arrest is crucial for the cell to prevent damage caused by environmental change. Nutrient starvation is a well studied stress factor which causes cell cycle arrest in G1. Glucose is the preffered carbon source of *Saccharomyces cerevisiae* and depletion of glucose stimulates cellular stress response. An important feature for cell to adapt this nutrient depletion is that cell can acquire tolerance to different stress conditions (Hohmann S., 2003).

Protective responses of living cells were fistly determined when heat shock response was investigated. At higher temperatures, cells increase production of heat shock proteins (Hsp). Many of Hsp serve as molecular chaperones and control the conformation of proteins to keep them functional. This investigation revealed two important features of stress response. First, the response causes acquisition of stress tolerance. That is, once cells are treated with a mild stress, they become more resistant to extreme stress conditions. Second, molecular mechanisms, which is the reason of stress response, also play a significant role in normal unstressed cells (Hohmann S., 2003).

Also, in several cases it has been observed that treatment of one type of stress caused tolerance to other types of stress. This phenomenon is defined as “cross-resistance”, and it implies that different stress factors require common cellular responses such as production of heat shock proteins or glycerol and trehalose. This important part of stress response is distinctly called as “general stress response” or “environmental stress response” (Hohmann S., 2003).

1.3.1 Ethanol stress and tolerance

Ethanol has been produced for thousand of years by employing *Saccharomyces cerevisiae* which is a superb ethanol producer. However, it is sensitive to ethanol at higher concentrations, especially during industrial bioethanol production which is carried out under (very) high gravity fermentation conditions (Legras *et al.* 2007). And ethanol accumulation has remarkable adverse effects on cellular growth and viability (Stanley *et al.*, 2010). By means of the developments in omic technologies

and systems biology, it has been possible to elucidate when and why *Saccharomyces cerevisiae* produce ethanol at high concentrations (Patrascu *et al.*, 2009). Also, these technologies have been utilized to study molecular mechanisms of ethanol tolerance. However, the whole mechanism of ethanol tolerance is indefinite so far.

Fossil oil is used primarily as transportation energy source. However, its increasing price and accelerating depletion attracted widespread attention on bioethanol production because of the better properties of bioethanol such as being renewable and sustainable product, reducing air pollution, greenhouse gas, CO_2 for global warming (Outlaw *et al.* 2005; Liu *et al.* 2008; Vertes *et al.* 2009).

In high or very high gravity fermentations, it is possible to produce high-titers of ethanol. However, it is sensitive to high concentrations of ethanol. Ethanol diffuses freely across biological membranes. So, this results in inhibition of cell growth and decrease in cell viability. Also, it causes reduced ethanol fermentation rate and final yield (D'Amore *et al.* 1990; Bai *et al.* 2004; Ding *et al.* 2009). It has been showed that high concentration of ethanol perturb protein conformation, affect uptake of glucose, ammonium, maltose, and amino acids (Piper, 1995).

In addition to ethanol stress, the yeast *Saccharomyces cerevisiae* are affected negatively from various other stress factors such as nutrient deficiency, high temperature, osmotic pressure. Figure 1.11 shows these stress factors.

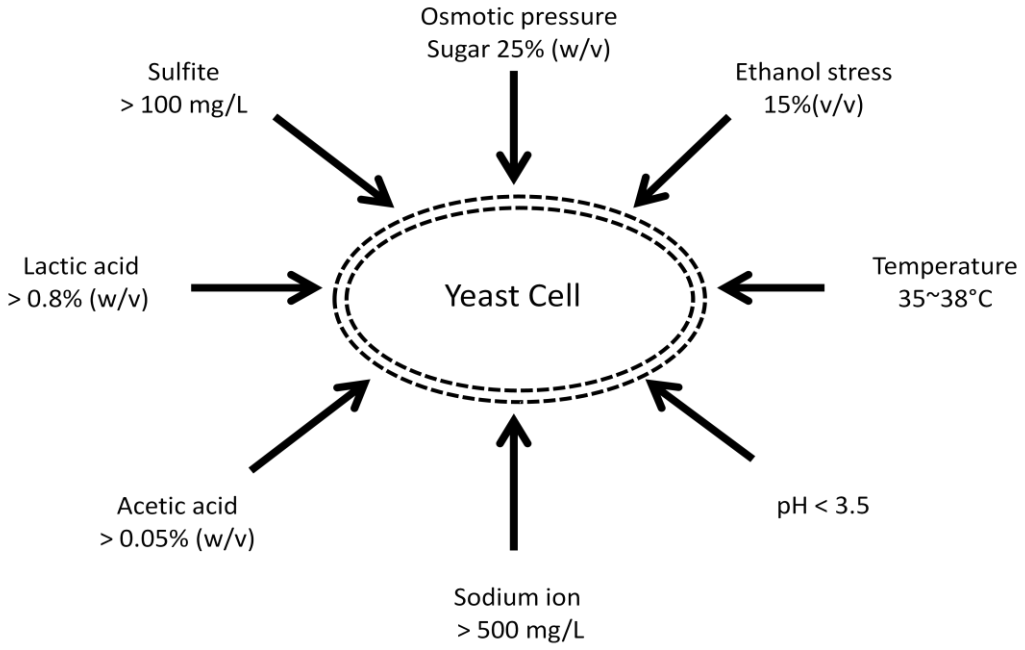

Figure 1.11 : Potential environmental stresses on *Saccharomyces cerevisiae* during alcoholic fermentation.

Obtaining high ethanol-tolerant strain is desirable for bioethanol production from biomass. To date, several hundred genes have been determined to be associated with tolerance to ethanol. These genes involves a broad range of functional categories including heat shock proteins, membrane and cell wall organization, transport, amino acid metabolism, cell cycle and growth, nucleotide metabolism, lipid, fatty acid and ergosterol metabolism (Liu ZL, 2012).

Main target sites of ethanol is thought as cell membranes, especially plasma membrane (D'Amore and Stewart 1987) (Figure 1.12). Many genes relevant with membrane composition were determined to be responsible for ethanol tolerance. Increasing fluidity of the plasma membrane caused by ethanol stress is compensated by monounsaturated fatty acids such as palmitoleic and oleic acid in the *Saccharomyces cerevisiae*. It was shown that higher ergosterol content in cellular membrane is associated with higher ethanol tolerance in yeast (del Castillo Agudo, 1992).

Recently, some genes in PDR family were determined as candidate genes for ethanol tolerance (Ma and Liu, 2010). Many PDR genes operate as transporters of ATP-binding cassette proteins and mediate translocation of ions and a wide range of substrates. Because of their functions on cell wall and plasma membrane, these PDR genes are considered to be involved in remodelling of cell membrane in response to ethanol stress (Ma and Liu 2010).

The vacuole is another compartment which is related with ethanol tolerance in the *Saccharomyces cerevisiae*. It functions in homeostasis of cell pH and the concentration of ions, osmoregulation, storage of amino acids and degradation processes. It was observed that deletion mutants of many genes associated with vacuolar membrane structure and protein sorting machinery, were affected badly by ethanol stress (Kubota *et al.* 2004; Teixera *et al.* 2009). Effect of ethanol for increased cell permeability results in increased proton influx and acidification inside the cell (Rosa and Sa'-Correia, 1996). To resist ethanol stress, intracellular H^+ must be transported to vacuoles by courtesy of H^+ V-ATPase to maintain a convenient pH homeostasis inside the cell (Forgac 1998; Inoue *et al.* 2005).

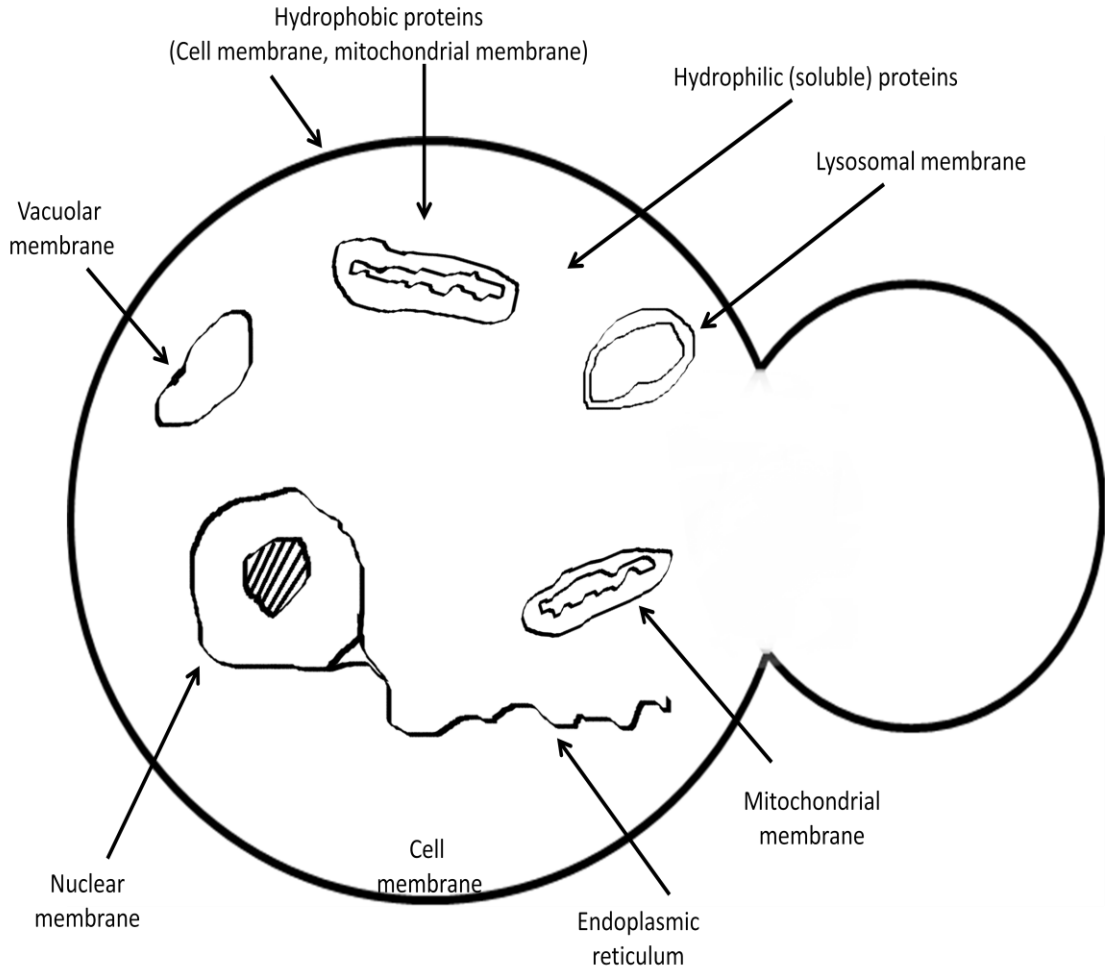

Figure 1.12 : Possible target sites for ethanol inhibition in yeast cells (D'Amore and Stewart, 1987 cited by Zhao and Bai, 2008).

The mitochondrion is important for ATP generation and take part in the biosynthesis of phospholipids, degradation of amino acids and fatty acids, and also the storage of metal ions (Scheffler 1999). To date, many genes were identified to be related with ethanol tolerance. The ethanol-resistant strains showed a lower frequency of ethanol-induced respiratory deficient (petite mutant) than ethanol-sensitive strains (Chi and Arneborg 1999). This designates that under stress conditions *Saccharomyces cerevisiae* cell must maintain its functional mitochondria to be able to tolerate ethanol.

Peroxisome is also a compartment in the cell. In the peroxisome compartment some oxidative reactions such as betaoxidation of very-long chain fatty acids occur. And peroxisome was found to be relevant with ethanol tolerance because of its function in synthesis or degradation of membrane phospholipids in cell membrane remodelling

(Lockshon *et al.* 2007). Also it metabolizes peroxides and other reactive oxygen species (ROS) caused by ethanol stress (Schrader and Fahimi 2004).

Another finding is that some genes related with amino acid biosynthesis were shown as important players for ethanol stress. Especially, the roles of proline and tryptophan in ethanol tolerance were investigated broadly. In a highly ethanol-tolerant strain, expression levels of tyrptophan biosynthesis genes were higher compared to ethanol-sensitive strain (Hirasawa *et al.* 2007). Amino acid proline functions in increasing stability of membranes and proteins, and preventing protein aggregation during protein refolding (Rudolph and Crowe, 1985; Samuel et al, 2000). Importance of proline in ethanol tolerance was demonstrated by the help of deletion and overexpression studies (Kubota *et al.* 2004).

Ethanol stress deteriorates conformation of proteins and results in aggregation of denatured proteins. Under this stress condition, heat shock proteins which primarily acts as chaperones, are stimulated for protecting proteins, and also cell structure and organelles. Besides HSP genes, some other genes encoding chaperones, such as *SSA1*, *SSA3*, *SSA4*, which involved in protein folding are also up-regulated upon stress exposure (Alexandre *et al.* 2001; Marks *et al.* 2008).

Trehalose and glycogen are two important storage carbohydrates of the yeast *Saccharomyces cerevisiae*. The amount of these two storage carbohydrates varies strongly and rapidly in response to fluctuations in environmental conditions. According to growth, nutrient availability and stress conditions, levels of glycogen and trehalose vary remarkably (Francois and Parrou, 2001). Trehalose has been shown to function by reducing membrane permeability and by ensuring proper folding of proteins (Mansure *et al.* 1994; Singer and Lindquist 1998).

Pathway of glycolysis plays an important role in cell growth and ethanol fermentation. It is very close to trehalose pathway, and expression pattern of glycogen is similar to that of trehalose. It supplies both ATP as an energy source and a variety of carbon intermediate metabolites for biosynthesis of aminoacids, lipids and nucleotides. It is found that genes *HXK1* and *GLK1* whose products catalyze important steps in glycolysis were up-regulated under ethanol stress. Also, expression level of *TDH1* was determined as 20-fold up-regulated under ethanol stress compared to normal condition (Ma and Liu, 2010).

Yeast response to ethanol stress is triggered via a complex signal transduction pathway. In this pathway, transcription factors Msn2p/Msn4p, Yap1p, and Hsf1p appear as key regulators for ethanol tolerance. Ethanol stress, as a general stress, stimulates Msn2p/Msn4p to trigger environmental stress response. Msn2p/Msn4p induces gene expression by binding to stress response element (STRE) (Marchler *et al.*, 1993). Under oxidative stress, Yap1p is activated by conformational change and transit from cytoplasm to nucleus (Rodrigues-Pousada *et al.* 2010). Hsf1p regulates transcription of many genes upon stress exposure such as heat shock, ethanol or other stresses (Hahn *et al.*, 2004).

In conclusion, ethanol tolerance of yeast involves lots of genes at multiple quantitative trait loci and interactions of complex networks at genome level (Ogawa *et al.*, 2000; Alexandre *et al.*, 2001; Ma and Liu, 2010). Many genes activated under ethanol stress are also activated under other stresses such as oxidative, osmotic, heat shock and toxic chemical. Yeast ethanol tolerance can be gained by evolutionary methods, such as evolutionary engineering (Çakar *et al.*, 2005; Dinh *et al.*, 2008), genome shuffling (Shi *et al.* 2009), and global transcription machinery engineering (gTME) (Alper *et al.*, 2006).

1.4 Metabolic Engineering

Metabolic engineering was introduced by Jay Bailey in 1991 as a subdiscipline of engineering and defined as "the improvement of cellular activities by manipulation of enzymatic, transport, and regulatory functions of the cell with the use of recombinant DNA technology" (Bailey, 1991). The goal of metabolic engineering is the directed modification of metabolic fluxes. This approach has played an important role in improving yeast strains for all industrial applications. To do that, genetic engineering which is the targeted manipulation of a cell's genetic information was used. Taken definition made by Bailey into consideration, it was observed that there was some cases which can not be explained by this definition. For example; since stress tolerance is not a cellular activity, this definition could not explain stress tolerance improvement. For this reason, the term "cellular activities" was replaced by "product formation or cellular properties" (Nevoigt, 2008).

Table 1.4 : Engineering approaches for microbial strain improvement (Nevoigt, 2008).

Strategy	Description	Advantage(s)
Rational Metabolic Engineering	Engineering metabolic pathways (mainly enzymes, transporters or regulatory proteins) based on available information	Strain improvement caused by known genetic modifications can be transferred to other strain
Inverse Metabolic Engineering	Starts from the phenotype; analysis of the genetic basis responsible from the phenotype; transfer of the genetic basis to the production strain	Independent of preliminary knowledge about pathways, enzymes, and their kinetics; can reveal novel, unknown target genes for improvement
Evolutionary Engineering	Includes all random genetic modifications and perturbation; relies primarily on random mutagenesis of the entire genome or its parts; identification of genetic modifications is difficult	Independent of preliminary knowledge about pathways, enzymes, and their kinetics; multigenic traits can be addressed

Metabolic engineering is seen as an iterative process including several rounds of engineering, analysis, and modeling of metabolic fluxes. Rational metabolic engineering was facilitated by approaches such as inverse metabolic engineering and evolutionary engineering. Table 1.4 shows the engineering approaches for microbial strain improvement (Nevoigt, 2008).

1.4.1 Rational metabolic engineering

Traditional metabolic engineering is referred to as "rational, constructive". Rational metabolic engineering is an approach for the engineering of enzymes, transporters, or regulatory proteins based on available information about the pathways, enzymes, and their regulation. Even though rational metabolic engineering has been very successful in several applications, it caused to some unwanted side effects or failed in some cases (Nevoigt, 2008). Because trying to reengineer a complex cellular machine without a detailed plan and extensive knowledge of how it really works is the major problem in rational metabolic engineering. Also, limitations such as requirement of comprehensive genetic and biochemical information on the organism of interest, complexity of cellular physiological responses, and difficulties of cloning in industrial strains are responsible for such failures (Çakar *et al.*, 2012). To be able to estimate and understand all the secondary responses of a certain metabolic engineering approach, complex global metabolic network and its responses to changing environmental conditions must be understood. Systems biology is a new

discipline to be able to understand and model the cell as a whole. And it is very helpful to evaluate many information obtained by metabolic engineering approaches (Nevoigt, 2008).

1.4.2 Inverse metabolic engineering

Bailey *et al.* introduced an alternative strategy called "inverse metabolic engineering" as a subdiscipline of metabolic engineering. They defined this approach as "the elucidation of a metabolic engineering strategy by: first, identifying, constructing, or calculating a desired phenotype; second, determining the genetic or the particular environmental factors conferring that phenotype; and third, endowing that phenotype on another strain or organism by directed genetic or environmental manipulation" (Bailey *et al.*, 1996). It has some advantages over rational metabolic engineering in terms of phenotypic differences. Starting point in this approach is a known and desired phenotype. Since, inverse metabolic engineering starts with the last step of the rational approach as its first step, it is referred as the bottom-up approach (Bailey *et al*., 1996). The second step, which is the identification of the genetic basis for the different trait values, is critical and the biggest challenge in inverse metabolic engineering (Çakar *et al*, 2009). However, omic technologies such as transcriptomics, metabolomics, proteomics and fluxomics have simplified the clarification of phenotype-genotype relationships. The last and the most important step in inverse metabolic engineering is to verify potential target genes determined by "omics" technology (Nevoigt, 2008).

Inverse metabolic engineering has several advantages over rational approaches. Firstly, there is no need for prior knowledge with respect to the proteins/enzymes of a pathway and their regulation. Secondly, industrial strains can be employed to determine important genetic players. Thirdly, modified strain can be considered "self cloned", which is an important subject for public acceptance especially in the food field. Lastly, novel genetic targets for strain improvement, which couldn't be found by rational approaches, can be explored (Çakar *et al.*, 2012; Nevoigt, 2008).

After all, inverse metabolic engineering for strain improvement is a very useful and successful approach. Nevertheless, the combination of rational approaches and inverse metabolic engineering is likely to be the most successful way for strain improvement (Nevoigt, 2008).

1.4.3 Evolutionary engineering

The process of repeated cycles of evolution, followed by selection of cells with the desired phenotype is generally called 'evolutionary engineering' (Sauer, 2001; Nevoigt, 2008). According to this definition, it encompasses several methods such as random mutation and selection, genome shuffling, global transcription machinery engineering, random knockout and overexpression libraries, ribosome engineering (Çakar ZP., 2009). Evolutionary engineering is one of the approaches that can be applied to generate various genetic alterations and diversity in performance, which is the first step in inverse metabolic engineering studies (Çakar *et al.*, 2012).

Strain improvement is started with random mutagenesis by using chemicals or UV light, and followed by selection of desired variants from the mutant population on solid media, or in liquid cultures, as batch or chemostat selection (Çakar ZP., 2009). By this way, baker's yeast *Saccharomyces cerevisiae* has been improved in several studies in terms of physiology and production feature. Multiple stress resistant *Saccharomyces cerevisiae* strains were obtained in this manner (Çakar *et al.*, 2005).

As distinct from metabolic engineering, inverse metabolic engineering relies on random methods in which genetic modifications are not directed towards specific sites. However, it is not easy to determine all genetic modifications responsible for the desired phenotype. Because, random mutagenesis results in random mutations spread over the entire genome. Also, it is impossible to transfer the genetic information to other strains without any knowledge about which genetic alterations caused formation of that phenotype. This challenge can be handled by using comparative transcriptome analysis such as DNA microarray analysis (Çakar ZP., 2009).

To sum up, evolutionary engineering is an effective method for microbial strain improvement in terms of phenotypic traits. And it will become a more powerful strategy with rational approaches and omic technologies.

1.5 The Aim of This Study

In a previous study, by utilizing evolutionary engineering strategies ethanol-resistant mutant individuals were obtained from EMS (Ethyl-Methane-Sulfonate) mutagenized initial population of *S. cerevisiae* cells (Turanlı-Yıldız B., 2012). The

aim of this study was to perform molecular, metabolic and phenotypical characterization of ethanol-resistant mutants of the yeast *S. cerevisiae*. Individuals, which were exploited in this study, were selected as a result of two different selection strategies (constant and increasing) and under continuous stress application. Individuals obtained via increasing stress application strategy, were more ethanol-resistant compared to individuals of constant stress application. B2 was the most ethanol-resistant individual mutant, whereas mutant individual B8 was the most multi-resistant to various industrially important stress factors. Because of the improved properties of these individuals, almost all of the experiments were performed by using these two individuals. In addition to molecular and phenotypical characterization, metabolic characterization of the mutants was performed via HPLC analysis. Glucose consumption and growth performances of the mutants were compared with trehalose, glycogen, glycerol, ethanol and acetate productions and consumptions. Finally, whole genome microarray analysis was performed to determine whether there is difference between wild type and mutants in terms of gene expression levels.

2. MATERIALS AND METHODS

2.1 Materials and Laboratory Equipments

2.1.1 Yeast strain

Saccharomyces cerevisiae CEN.PK113.7D (*MATa*, *MAL2-8*^{c}, *SUC2*) was kindly provided by Dr. Peter Kötter (Johann Wolfgang Goethe-University, Germany). Beforehand, *Saccharomyces cerevisiae* CEN.PK113.7D was named as 905 (wild type). Ethyl methane sulphonate (EMS), a chemical mutagen, was utilized to obtain mutagenized *Saccharomyces cerevisiae* CEN.PK 113-7D cells by applying this mutagen to the wild type population. The mutant cell population, 906, had been previously exposed to different stress conditions throughout successive batch cultivations. As a result of this evolutionary engineering strategy, some individuals selected after continuous and increasing ethanol stress application were found highly ethanol-resistant and employed for next experiments. The mutant individuals selected from the final population corresponding to 11.4%, v/v ethanol stress condition were named as B2 and B8, respectively (Turanlı-Yıldız B., 2012). In this study, 905 was used as the wild type. "B2" and "B8" were utilized as ethanol-resistant mutants.

2.1.2 Yeast culture media compositions

2.1.2.1 Yeast minimal medium (YMM)

Table 2.1 : Yeast minimal medium (YMM) ingredients and amounts.

Yeast Minimal Medium (YMM)	
Component	**Amount**
Yeast Nitrogen Base without amino acids	6.7 g
Dextrose	20 g
Agar (only for solid media)	20 g
in 1 liter of distilled water (ddH_2O)	

2.1.2.2. Yeast extract peptone dextrose (YPD) medium

Table 2.2 : Yeast complex medium (YPD) ingredients and amounts.

Yeast Extract Peptone Dextrose (YPD)	
Component	**Amount**
Yeast Extract	10 g
Dextrose	20 g
Peptone	10 g
Agar (only for solid media)	20 g
in 1 liter of distilled water (ddH_2O)	

2.1.2.3 Yeast minimal medium (YMM) without dextrose

Table 2.3 : Yeast minimal medium (YMM) without dextrose ingredients and amounts.

Yeast Minimal Medium without Dextrose	
Component	**Amount**
Yeast Nitrogen Base without amino acids	6.7 g
Agar (only for solid media)	20 g
in 1 liter of distilled water (ddH_2O)	

2.1.2.4 Xylose medium

Table 2.4 : Xylose medium ingredients and amounts.

Xylose Medium	
Component	**Amount**
Yeast Nitrogen Base without amino acids	6.7 g
Xylose	20 g
Agar (only for solid media)	20 g
in 1 liter of distilled water (ddH_2O)	

2.1.2.5 Yeast extract peptone dextrose glycerol (YEPDG) medium

Table 2.5 : Yeast extract peptone dextrose glycerol (YEPDG) medium (Solid) ingredients and amounts.

Yeast Extract Peptone Dextrose Glycerol (YEPDG) Medium	
Component	**Amount**
Yeast Extract	10g
Peptone	20 g
Glucose	1 g
Glycerol	30 ml
Agar	20 g
in 1 liter of distilled water (ddH_2O)	

2.1.3 Laboratory equipment

The general laboratory equipments used in this study are given in Table 2.6.

Table 2.6 : The general laboratory equipments used in this study.

Laboratory Equipment	Supplier
Micropipettes	Eppendorf (Germany)
Microcentrifuge	Eppendorf Microcentrifuge 5424 (Germany)
Benchtop Centrifuge	Beckman Coulter Allegra 25R Benchtop Centrifuge (USA)
Magnetic Stirrer	Labworld (Germany)
Autoclaves	Tomy SX 700E (China)
Laminar Flow	Biolab Faster BH-EN 2003 (Italy)
UV-Visible Spectrophotometer	Shimadzu UV-1700 (Japan)
Light Microscope	Olympus CH30 (USA)
Thermomixer Compact	Eppendorf (Germany)
Multiplate Spectrophotometer	BioRad Benchmark Plus (UK)
Nano Drop 2000 Spectrophotometer	Thermo Fischer Scientific
Desiccator	Finemech (USA) Bola-Star Vitrium Desiccator
Deep Freezes and Refrigerators	- 80˚C Heto Ultrafreeze 4410 (Denmark) - 20˚C Arçelik (Turkey) + 4˚C Arçelik (Turkey)
Ultrasonicator	Transsonic TP690
Vortex mixer	Heidolph (Germany)
Orbital Shaker	Certomat S-2 Sartorius (Germany)
Water Bath	Julabo SW22 (Germany)
Transilluminator	Vilber Lourmat
pH meter	Mettler Toledo MP220 (Switzerland)
HPLC System - System Controller - Liquid Chromatography - Degasser - Refractive Index Detector - Auto Injector - Column Oven - Column	 - Shimadzu SCL - 10A (Japan) - Shimadzu LC-10AD (Japan) - Shimadzu DGU-14A (Japan) - Shimadzu RID-10A (Japan) - Shimadzu SIL-10AD (Japan) - Shimadzu CTO-10AC (Japan) - Aminex HPX-87H (300 x 7.8mm) Bio-Rad (USA)

2.1.4 Chemicals, buffers, solutions, kits and enzymes

The chemicals, solutions, kits and enzymes used in the study are given in Tables 2.7 to 2.10.

Table 2.7 : The chemicals used in this study.

Chemical	Supplier
Glycerol	Duchefa Biochemie (Holland)
Ethanol (absolute)	J.T.Baker (Holland)
Sodium Acetate	J.T.Baker (Holland)
Potassium Acetate	Carlo Erba Reagents (Italy)
Sodium carbonate	Riedel – de Haen (Germany)
Acetic acid	Riedel – de Haen (Germany)
Sulphuric acid	Riedel – de Haen (Germany)

Table 2.8 : List of solutions/buffers used in this study.

Buffer/Solution	Stock Concentration
Glycerol	%30 v/v
Acetate	%20 v/v
Ethanol	%20 v/v
Phosphate buffered saline (pH = 7.4)	50 mM
Na_2CO_3	0.25 M
CH_3COOH	1 M
CH_3COONa (pH = 5.2)	0.2 M
H_2SO_4	5 mM

Table 2.9 : List of kits used for specific purposes in this study.

Kit	Supplier
RNeasy Mini Kit	Qiagen (Germany)
RNA 6000 Nano Assay Kit	Agilent (USA)
One-Color RNA Spike-In Kit	Agilent (USA)
Absolutely RNA NanoPrep Kit	Agilent (USA)

Table 2.10 : Enzymes and special chemicals used for quantitative assessment of glycogen and trehalose content.

Product	Supplier
Glucose oxidase/peroxidase reagent	Sigma
o-dianisidine dihydrochloride tablet	Sigma
Trehalase (from porcine kidney)	Sigma
α-Amyloglycosidase	Roche

2.1.5 HPLC stock solutions and HPLC standards

For the HPLC experiments, a standard curve was created in order to calculate the concentration of particular metabolite in samples.

Stock solutions components and amounts are listed in Table 2.11. 5 mM sulfuric acid (H_2SO_4) is used as eluent.

Table 2.11 : Stock Solutions of HPLC Standards.

SOLUTION A	
Component	**Amount**
Glucose	120 g
in 1 liter of distilled water (ddH_2O)	

SOLUTION B	
Component	**Amount**
Acetate	4 g
Glycerol	2 g
Ethanol	30 g
in 1 liter of distilled water (ddH_2O)	

SOLUTION C	
Component	**Amount**
Maltose	20 g
Citric Acid	20 g
Succinic Acid	20 g
in 1 liter of distilled water (ddH_2O)	

Standard solutions used to create HPLC standard curve were prepared according to the following dilutions given in Table 2.12 and Table 2.13. In these two tables there is difference in terms of solutions (Solution A&B or solution C).

Table 2.12 : HPLC Standard Solution Dilutions (Std: Standard Solution).

Standard Solutions	**Mixing Volumes**		**Final Volume**
Standard 1	1 ml Solution A 3 ml Solution B	2 ml Eluent	6 ml
Standard 2	0.75 ml Std 1	0.25 ml Eluent	1 ml
Standard 3	0.50 ml Std 1	0.50 ml Eluent	1 ml
Standard 4	0.25 ml Std 1	0.75 ml Eluent	1 ml
Standard 5	0.125 ml Std 1	0.875 ml Eluent	1 ml
Standard 6	0.063 ml Std 1	0.937 ml Eluent	1 ml

Table 2.13 : HPLC Standard Solution Dilutions (Std: Standard Solution).

Standard Solutions	Mixing Volumes		Final Volume
Standard 1	1 ml Solution C	-	1 ml
Standard 2	0.75 ml Std 1	0.25 ml Eluent	1 ml
Standard 3	0.50 ml Std 1	0.50 ml Eluent	1 ml
Standard 4	0.25 ml Std 1	0.75 ml Eluent	1 ml
Standard 5	0.125 ml Std 1	0.875 ml Eluent	1 ml
Standard 6	0.063 ml Std 1	0.937 ml Eluent	1 ml

Concentrations of metabolites in the prepared standard solutions are shown in Table 2.14.

Table 2.14 : Metabolite Concentrations in HPLC Standard Solutions (Std: Standard Solution, RT: Retention Time, RI: Refractive Index).

Metabolite (g/L)	Std1	Std2	Std3	Std4	Std5	Std6	RT (min)	Detector
Glucose	20	15	10	5	2.5	1.25	9.43	RI
Glycerol	1	0.75	0.5	0.25	0.125	0.0625	13.52	RI
Acetate	2	1.5	1	0.5	0.25	0.125	14.83	RI
Ethanol	15	11.25	7.5	3.75	1.875	0.9375	19.65	RI
Maltose	20	15	10	5	2.5	1.25	6,95	RI
Citric Acid	20	15	10	5	2.5	1.25	7,54	RI

2.2 Methods

In a previous study, by applying evolutionary engineering strategy, ethanol-resistant mutant individuals were successfully obtained from EMS (Ethyl-Methane-Sulfonate) mutagenized initial population of *S. cerevisiae* cells (906). Mutant cells were selected at continuous ethanol stress and they showed cross-resistances towards other stress conditions including oxidative, heat, sorbitol, freeze-thaw, cold, methanol, phenyl-ethanol stresses.

2.2.1 Preparation of growth media

For liquid media preparation, firstly constituents, given in the section 2.1.2, are mixed with distilled water in a flask. For solid (agar) plates, 2 % agar is added to the liquid in the flask and shaken to disperse before autoclaving. The flask is plugged with a nonabsorbent cotton wool which is covered with tin foil to keep it dry and to

prevent contamination risk. Autoclave is done at 121 ºC at 1 atmosphere for 15 minutes. Autoclave is opened when it has cooled adequately and pressure of it reachs to zero. By using gloves, flasks are taken and allowed to cool. Medium including agar can be poured to plates when its temperature reachs approximately 50 ºC. For this, agar containing medium is gently shaken for agar dispersal and is poured 20-25 mL into each sterile Petri dish. After drying of plates, they are stored at 4 ºC.

2.2.2 Preparation of stock cultures

Frozen stock cultures were prepared following the obtainment of ethanol-tolerant mutant individuals. For this purpose, firstly, mutant cultures were vortexed to obtain cells highly as possible as. Then, 1000 µl of cultures were washed twice with YMM by centrifuging at 14000 rpm for 5 minutes in sterile 1.5 ml microfuge tubes. Supernatants were discarded and cell pellets were resuspended in fresh, sterile 1000 µl 30% Glycerol (v/v). Finally, the suspensions were vortexed and transferred to -80 ºC for long-term storage to be used for further analysis.

2.2.3 Cultivation of yeast cells

In this study, wild type strain 905 (n), wild type diploid (2n), mutant individuals B2, B8, and segragants of B2 were utilized. Frozen stock cultures from -80 ºC were inoculated in 10 ml YMM in 50 ml test tubes. Those cell solutions were incubated overnight at 150 rpm, a temperature of 30 ºC. After overnight growth, cells were adjusted to 0.2 initial optical density at 600 nm and pre-cultured unless otherwise stated.

2.2.4 Contamination tests

Wild type and ethanol resistant mutants were checked for contamination. Contamination tests were done on Xylose-agar plates. This test is based on the inability of *Saccharomyces cerevisiae* to use xylose as a carbon source.

For a contamination test, firstly, cells were precultured on YMM-agar plates. Samples were taken from these plates with replica plate method onto the Xylose-agar plate. Same were done on YMM-agar plate and this plate was used as the control. The plates were then incubated at 30 ºC. Growth on Xylose-agar plate was monitored visually over 48 hours of incubation.

2.2.5 Sporulation of the yeast diploid cells

Diploid cells can be distinguished from hapliod cells under the light microscope since they are generally larger and show a distinct budding pattern, and can be sporulated by using "Yeast Sporulation Medium" given in the section 2.1.2 to obtain haploid spores. Also, to test whether haploid strains became diploid or not, cells can be sporulated by using this sporulation medium which contains potassium acetate. If there is a diploidization present, sporulation causes tetrad formation.

2.2.6 Determination of stress resistances

2.2.6.1 Most Probable Number (MPN) method

Most Probable Number (MPN) is a high-throughput and statistical technique with 95% confidence to estimate the population density of viable microorganisms by using serial dilutions in 96-well plates which contained 180 µl YMM in each well (Russek and Colwell, 1983; Cakar *et al.*, 2005). It is based upon the probability of numbers of observed positive growth in answer to a standard dilution series of sample inoculum. Due to the ability of aliquots to grow at higher dilutions, the most-probable number of survivors was predicted by using tables designed for the case of 'five-tube MPN'. With respect to this protocol, cells were inoculated into YMM with and without stress conditions in 96-well plates with five replicates and serially diluted up to 10^8 fold dilution. After 72 hours of incubation, the growth in the wells is jotted down and the MPN score is converted to cell numbers by using MPN table (Appendix A) based on Poisson regression analysis.

2.2.6.2 Spotting assay

Spotting assay is a powerful experiment to determine ethanol resistance levels of the cultures. In this study wild type (n), wild type (2n), mutant individual B2 and segragants of B2 were used. These cultures were precultured as mentioned before. After preculturing step, cultures that grow readily were chosen for the main experiment and were cultivated in 50 ml test tubes as initial OD_{600} value is 0.2. This cultures were used when their OD_{600} values reached to a number between 2-3. And, cells were collected as OD_{600} is 5 and transferred into microfuge tubes. After obtaining proper amount from each sample, solid YPD plates were prepared which include proper concentration of ethanol. Table 2.15 shows the appropriate levels of solutions used to prepare solid YPD plates.

Table 2.15 : Contents of solid ethanol plate

Stress Level	*2X YPD (solid)*	*EtOH*	*ddH_2O*	*Total*
0% (v/v) Ethanol	25 ml	-	25 ml	50 ml
10 % (v/v) Ethanol	25 ml	5 ml	20 ml	50 ml
12 % (v/v) Ethanol	25 ml	6 ml	19 ml	50 ml
14 % (v/v) Ethanol	25 ml	7 ml	18 ml	50 ml

Also, control plate (0% v/v ethanol) was prepared to determine the difference between stress and control conditions in terms of ability to grow. Mixture of YPD and ethanol was made by using 50 ml test tubes. Before spotting of samples onto YPD plates, dilutions were done by using 96-well plate as 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}. After that, 5 µl from each culture were spotted onto solid plates.

2.2.7 Analysis of viability

Viability of ethanol-resistant mutants were determined with respect to wild-type (Hu *et al.*, 2007). To do that, firstly cells were incubated in YPD medium until they reached stationary phase (OD_{600}~6). After centrifugation at 14000 rpm for 5 min, the supernatant was removed. Cell pellets were then washed in 2 ml sterile water and resuspended in water with the final cell concentration of 10^5-10^6 cell/ml. Then, 10 µl from the cell suspension was added into 5 ml ethanol stress medium containing ethanol at varying concentrations and 0.1 M acetate buffer (pH 4.2) including 1% glucose (g/L). Table 2.16 shows the acetate buffer solutions containing appropriate amounts of ethanol. The medium was incubated at 30 ºC, 150 rpm for 72 h. A cell suspension of 5 µl was transferred onto a YPD plate and incubated at 30ºC for 48 h. The phenotype of ethanol tolerance was scored as the ethanol concentration at which the cells were visually the same as that of the cells with 10^{-2} dilution factor at 0% (v/v) ethanol concentration. Then, cells were incubated for 72 h at 30ºC after which the viabilities were recorded.

Table 2.16 : Acetate buffer containing proper concentrations of ethanol

Ethanol (%)	Amount of Ethanol (µl)	Acetate Buffer (inc. 1% glucose) (µl)
0	-	5000
10	500	4500
11	550	4450
12	600	4400
13	650	4350
14	700	4300

2.2.8 Stability test

The aim of this experiment is to determine whether there is a difference in terms of stress resistances of mutant strains after each passage. In this test, inoculation from previous day's inoculated culture was repeated for 5 days, successively. Firstly, wild type and mutant strains B2 and B8, which were taken from -80 ºC stock, were pre-cultured into fresh YMM by using 100 µl from each stock. Then cultures were incubated at 30ºC and 150 rpm, as always. After overnight incubation, stocks of these cultures were prepared. Then, these cultures were inoculated again into fresh YMM. For 5 successive days, this procedure was employed. Five days later, -80 ºC stocks of cultures of B2, B8 and wild type strains were inoculated into fresh YMM. When OD_{600} values of cultures were approximately 2 or 3, MPN method was performed. Stress level was chosen as 10% (v/v) ethanol. 10^8 fold dilution of the cultures at control condition was not sufficient to show negative growth on 96-well plate. So, cultures were inoculated as 100 fold diluted at this condition.

2.2.9 Petite frequency assay

Cell cultures taken from the glycerol freezer stock were streaked onto YPD plates to enable growing as single colonies for 2 days. Before spreading on YEPDG, single colonies of the chosen mutant were suspended in PBS with different dilution factors; 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5} for optimizing the suitable dilution ratio. Four independent colonies for each streaking plate were diluted in sterile 1 ml of PBS as 10^{-4} to spread onto 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.

2.2.10 Obtaining growth curve of wild type and mutant individuals

Firstly, -80 ºC stock cultures of wild type and mutant B2 were inoculated into 50 ml test tubes in which 10 ml of YMM is present. After overnight incubation, these cultures were inoculated into 500 ml flasks including 100 ml YMM as initial OD_{600} value is 0.2. Again, subsequent to overnight incubation, cultures were inoculated in 400 ml YMM in 2000 ml flasks as initial OD_{600} value is 0.3 or 0.4. Incubation of precultures was carried out at 30 °C, 150 rpm. OD_{600} values were measured by using Shimadzu UV-1700 Spectrophotometer. As well for the incubation of precultures, wild type and mutant B2 in 2 lt flasks were incubated at 30 ºC and 150 rpm for 48 hours. Every 2 hours, OD_{600} values were measured and samples for later analysis were taken if necessary. Growth curves of mutants were obtained in this manner.

2.2.11 Measurement of cell dry weight (CDW)

In this study, cellular dry weight analysis was performed for each sample. Firstly, 1.5 ml microfuge tubes were dried in an oven at 80 ºC for 48 hours. After two days of drying, they were placed in desiccators for 30 minutes to cool down to room temperature, and then weighed. Throughout the growth curve experiment, 1 ml of samples were taken into dried microfuge tubes via pipetting at particular time intervals. Cells were centrifuged at 14000 rpm for 5 minutes, supernatants were discarded. The tubes were dried in an oven at 80 ºC to evaporate the remaining liquid on the cell pellet. After 48 hours of drying, the tubes with dried cell extracts were again placed in a desiccator for 30 minutes and then weighed. The cellular dry weight (CDW) was calculated by subtracting the first weight from the second weight.

2.2.12 Measurement of glycogen and trehalose content

The aim of this analysis is to compare the glycogen and trehalose amounts of the wild-type and mutants. During growth curve experiment, samples are taken for the measurement of glycogen and trehalose amount. When the samples will taken is determined according to the growth phase of the cultures. Firstly, culture amount corresponding to 25 optical density unit of cells are taken. Then, they were transferred to 1.5 ml microcentrifuge tubes. After that, tubes were centrifuged at 14000 rpm for 5 minutes and supernatant was discarded. Finally, cell pellets were stored at -20 °C.

Measurement of the glycogen and trehalose amounts in all samples, which were previously obtained, was carried out as described previously (Parrou and Francois, 1997). Firstly, 250 µl 0.25 M sodium carbonate was added onto the pellets of the cells and then they were vortexed. After that, microfuge tubes containing cells cultures were placed to 95 °C for 4 hours. At the end of 4-hour incubation, cell suspensions were adjusted to pH 5.2 with 1M 150 µl acetic acid and 600 µl 0.2 M sodium acetate buffer (pH 5.2). Cell suspensions were mixed properly. Subsequently, 1000 µl of the samples were divided equally into new test tubes to utilize one tube for trehalose determination and the other for glycogen analysis. 10µl trehalase (trehalase was already liquid) was pipetted into 500 µl cell suspensions and the tubes were placed to 37 °C for overnight incubation. Moreover, 20 µl alpha-amyloglycosidase (it is solubilized in 0.2 M sodium acetate 'pH 5.2') was pipetted into other half of cell suspensions and the tubes were placed into a rotary shaker whose temperature is 57 °C for overnight incubation.

The next day, glucose standards which is used for the determination of both glycogen and trehalose concentration, were prepared. 20 µl of standards and all the samples were added to different wells of 96-well plate. Then, 200 µl of glucose oxidase/peroxidase reagent was added into each well of 96-well plate. After 30 minutes incubation at 37 °C, the absorbance of the samples was measured at 490 nm.

2.2.13 Determination of metabolite contents by HPLC

The scope of HPLC analysis for glucose, acetate, ethanol and glycerol is to figure out the relationship between the growth and metabolite production/consumption. Wild type (905) and B2 cultures that utilized in this study were firstly inoculated into Yeast Minimal Medium (YMM) in 50 ml test tubes by setting initial OD_{600} as 0.05. After overnight incubation at 30 °C and 150 rpm shaking speed, OD_{600} values were measured by spectrophotometer (Shimadzu). These cultures were inoculated into 100 ml fresh YMM in 500 ml flasks with an initial OD_{600} 0.2. After overnight incubation to these pre-cultures were inoculated into 400 ml fresh YMM with or without stress factor in 2 L flasks with an initial OD_{600} 0.4. Stress level was chosen as 8 % (v/v) ethanol. Both of these cultures were incubated at 30 °C and 150 rpm shaking speed.

The growth was monitored by measuring OD_{600} in triplicates. OD_{600} values were measured at every sampling time. While acquiring the growth curve of the wild type

and mutant, 1 ml from all of the cultures were taken into microfuge tubes with known-weight (for cell dry weight experiment) with 1.5 hours intervals and the flasks were replaced to 30 °C immediately. After centrifugation (14000 rpm, 5 min), supernatant was taken with syringe and transferred to new microfuge tubes upon filtration (0.22 μm pore size). Those supernatants were kept at -20 °C for further High Performance Liquid Chromatography 'HPLC' analysis. Each of microfuge tubes which contained the cultures' pellets were kept to the -20 °C.

Metabolite analysis via HPLC was performed by using the Aminex© HPX-87H column which was eluted with H_2SO_4 (5 mM) with a flow rate of 0.6 ml/min and at 60 ºC. Injection volume per sample was adjusted as 20 μl. Metabolite compositions were determined by courtesy of the comparison of 6 known standards (see section 2.1.5). The standard solutions were analyzed to obtain a calibration curve. By using that calibration curve, the concentrations of metabolites in each sample were determined.

2.2.14 Microarray analysis

2.2.14.1 Culturing and RNA purification of wild type and mutants

Wild type, B2 and B8 pre-cultures were inoculated to 100 ml YMM in 500-ml flasks with an initial OD_{600} of 0.1. They were incubated at 30 °C and 150 rpm shaking speed until they reached an OD_{600} of ~1 (10^7 cell/ml). Total RNA was extracted by using RNeasy Mini Kit (QIAGEN). Sample preparation of wild type, B2 and B8 were performed for four and three times respectively.

2.2.14.2 RIN and RNA content determination before microarray analysis

Quality of all RNA samples was determined with BioAnalyzer 2100 (Agilent) by using RNA 6000 Nano Assay Kit (Agilent) following the description of the corresponding manual. Before the experiment, RNA contents of all of the samples were measured with NanoDrop 2000 (ThermoScientific) and they were set to 100 μg/μl concentration by making proper dilutions.

2.2.14.3 Sample preparation for microarray analysis

Before labeling reaction, spike mix preparation was performed with One-Color RNA Spike-In Kit (Agilent). T7 promoter Primer mix, cDNA master mix, transcription

master mix were prepared as written in the One-Colour Microarray-based Gene Expression Analysis Protocol (Agilent). At the end, labeled RNA samples were purified with Absolutely RNA NanoPrep Kit (Agilent Technologies).

2.2.14.4 Hybridization and microarray wash

Labelled cRNAs were hybridized to Agilent yeast microarrays. The microarrays were incubated in a special hybridization chamber for ~18-20 h at 65 °C. At the end of hybridization process, wash step was carried out with gene expression wash buffers (Agilent).

2.2.14.5 Scanning and feature extraction

Agilent Laser Scanner was used for microarray samples and feature extraction was performed with Agilent Feature Extraction software. Also, GeneSpring GX 12.00 software was utilized for interpretation of data obtained from microarray analysis.

3. RESULTS AND DISCUSSIONS

3.1 Stress Resistances of Wild Type and Mutants

3.1.1 Analysis of stress resistances via MPN method

The number of cells of individual mutants obtained by means of continuous stress strategy (via constant stress application) were investigated by using the statistical high-throughput most probable number (MPN) assay. For this purpose, individuals obtained by using constant stress selection strategy were cultivated in the presence of 5% (v/v) ethanol in 96-well plates by MPN method in order to determine the ethanol resistance levels. The number of cells was calculated by using MPN tables with 95% confidence limits. Also, the survival rate values were gained by dividing number of cells after stress exposure by that of non-treated cells.

The results, given in Figure 3.1, showed that there were no significant improvement in ethanol resistance of individual mutants when compared to the wild type culture.

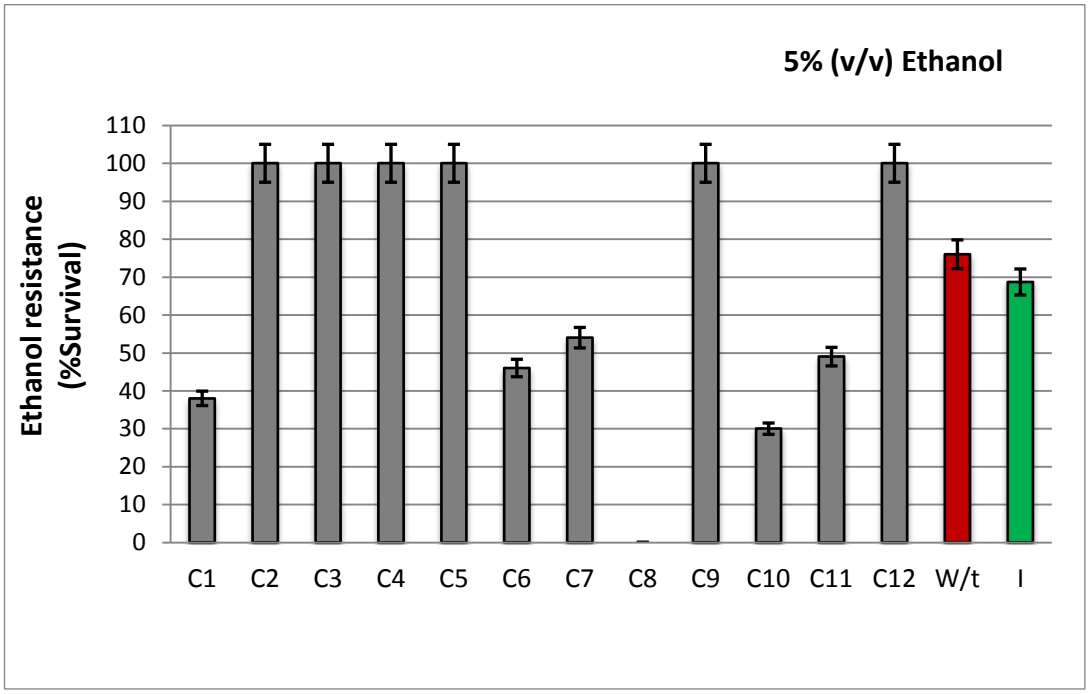

Figure 3.1 : Ethanol resistance levels (% survival) of mutant individuals isolated from continuously applied, constant ethanol stress selection (CONST) strategy after 72nd hour of incubation.

These constant mutants were obtained at low concentration of ethanol (5%, v/v), which can be tolerated by even wild type strain. This stress level is mild for the wild type culture. Thus, the mutants seem not significantly resistant to ethanol stress under this condition when compared to wild type.

3.1.2 Analysis of ethanol stress resistances via spotting assay

The level of stress resistances were determined by spotting of cultures, which were serially diluted, onto solid YPD plate, including appropriate concentration of ethanol. Initially, segregants of both B2 and B8 were inoculated from -80 ˚C stock. But, only segragants of B2 were utilized in this study because of their ability to grow in pre-culturing step. As expected, for almost every case, half of the segragants showed better growth compared to other half. In this study, numbers and letters represent different asci and segregants obtained from each ascus, respectively.

Figure 3.2 shows the growth properties of these cultures under control and ethanol stress conditions at 72nd hour of incubation.

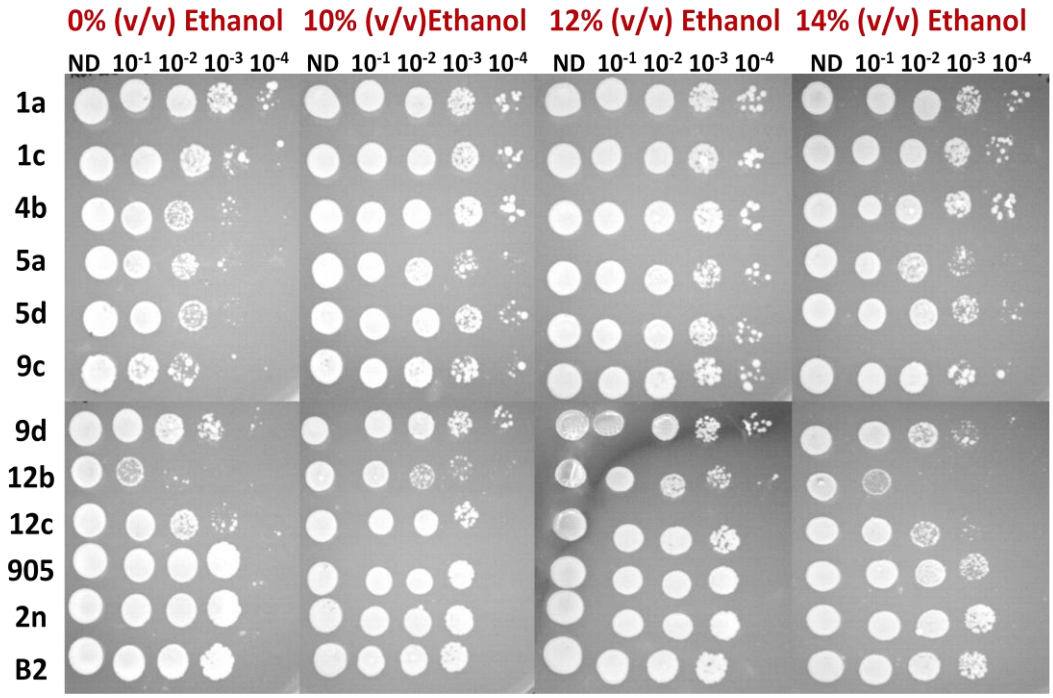

Figure 3.2 : Spotting results of cultures at 72nd hour of incubation.

It can be seen that all of the cultures showed resistance at high concentration of ethanol, even at 14% (v/v) ethanol stress. The resistances of cultures, especially those of wild type (905) and 2n at these high levels of ethanol, were not expected. Since ethanol is a volatile compound, it is possible to volatilize readily at high temperatures

as well at our laboratory. Nevertheless, it can be inferred that segregants showed higher resistance than n, 2n and B2. Only one of them, 12b, was very sensitive to 14% (v/v) ethanol stress. Also, these segragants showed decreased growth under control condition, but not under ethanol stress up to 12% (v/v). This implicates that there might have been a metabolic change for segregants to utilize ethanol as a carbon source.

3.1.3 Analysis of viability

In this experiment wild type, B2 and B8 were used to determine their ability for vegetative growth after continuous stress exposure. Cultures obtained under control condition were diluted 10^2 fold, then were spreaded onto solid YPD plate. Cultures of stress treatment were also spreaded onto solid YPD plates. At 48 hour of incubation the phenotype of ethanol tolerance was scored as the ethanol concentration at which the cells were visually the same as that of the cells with 10^{-2} dilution factor at control condition. Figure 3.3 shows the viability under control, 10% (v/v) and 11% (v/v) ethanol stress conditions at 48 hour of incubation. Since, there was no growth under 12% (v/v), 13% (v/v) and 14% (v/v) ethanol conditions, these results are not shown here.

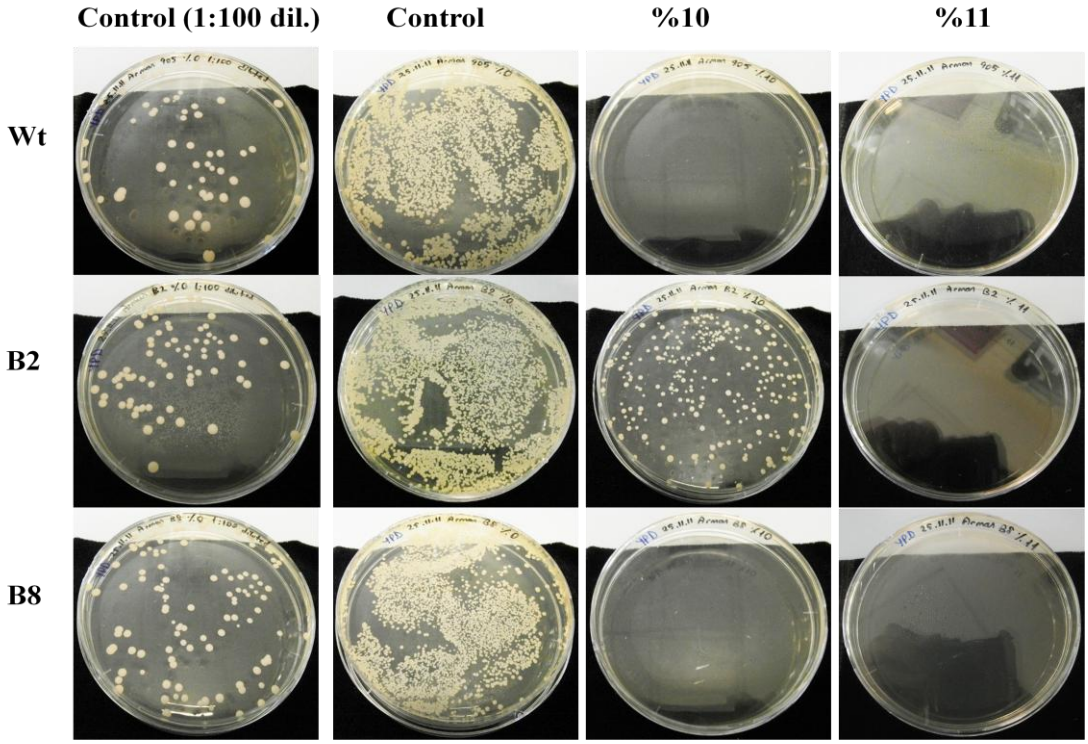

Figure 3.3 : The growth of the viable colonies following 10% (v/v) and 11% (v/v) ethanol stress exposure.

According to the results, it is obvious that B2 has higher score than both B8 and wild type. Because there is no growth for B8 and wild type under 10% (v/v) ethanol stress, it can be estimated from these results that score of both wild type and B8 is lower than 10% ethanol (v/v). B2, which is able to grow at 10% (v/v) ethanol stress, were shown to be more viable compared to those of control condition which was diluted 100 fold. For that reason, we can interpret that score of B2 is between 10% (v/v) and 11% (v/v) ethanol.

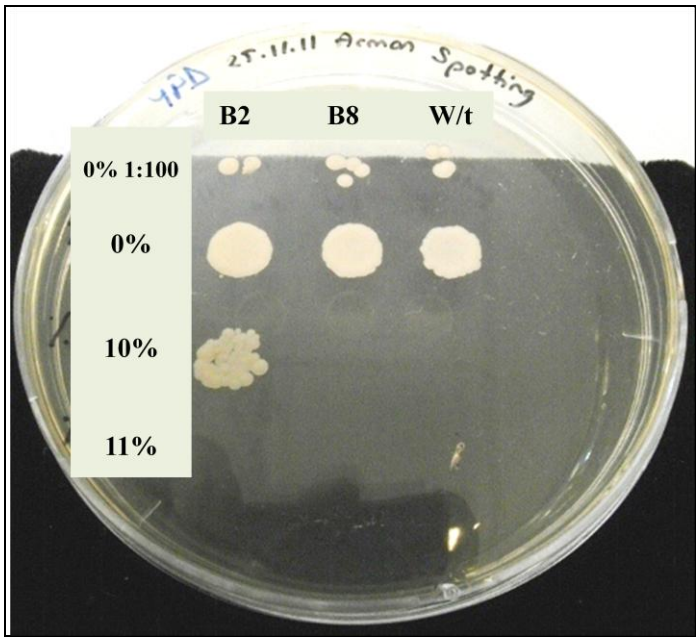

Figure 3.4 : Spotting results of the same cultures which were previously exposed to ethanol stress and utilized for analysis of viability.

The cultures used for this experiment, were also inoculated by spotting onto solid YPD plates. Figure confirms the results of viability analysis which were previously tested by spreading onto YPD plates. Likewise, B2 culture was the sole in terms of the ability to grow vegatatively after exposure to 10% (v/v) ethanol stress.

3.2 Analysis of petite frequency

Whether respiratory-deficient (petite) mutants are present or not was examined by using intermediate populations A2, A23; final population A98; individual mutants B2, B8; and wild type strain. A2 and A23 were chosen due to being haploid cultures as well wild type. A98, B2 and B8 were both diploids and more resistant to ethanol compared to wild type and intermediate populations. By this means, the relationship between ethanol resistance, diploidisation and respiratory deficiency were examined.

Figure 3.5 shows the respiratory-deficient mutant colonies of wild type strain after 120 hour of incubation at YPEDG medium.

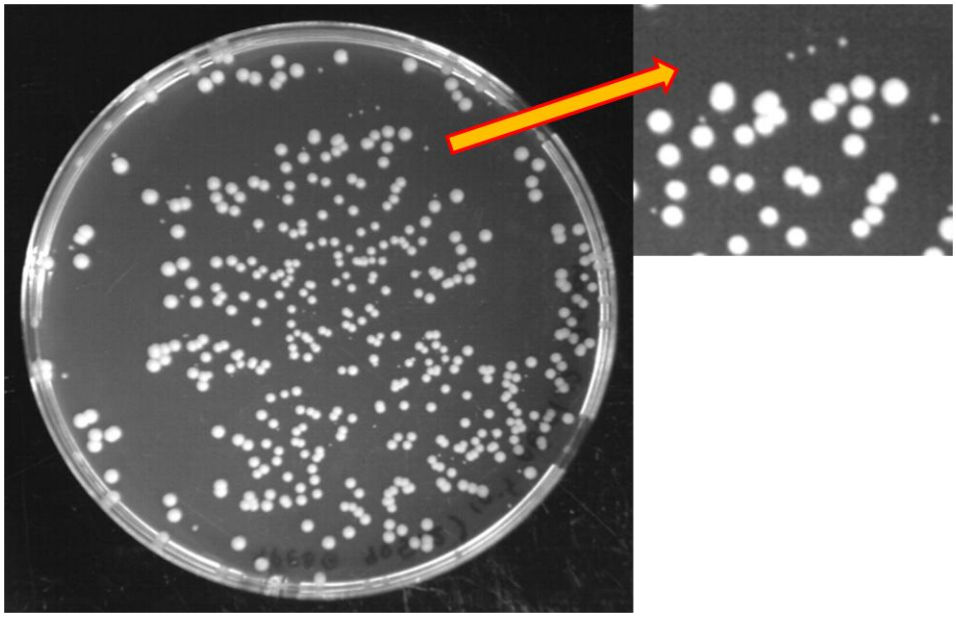

Figure 3.5 : *Petite* and *grande* colonies of wild type strain on YEPDG plates.

In Figure 3.6, the average petite frequency of four different colonies selected from each culture was depicted.

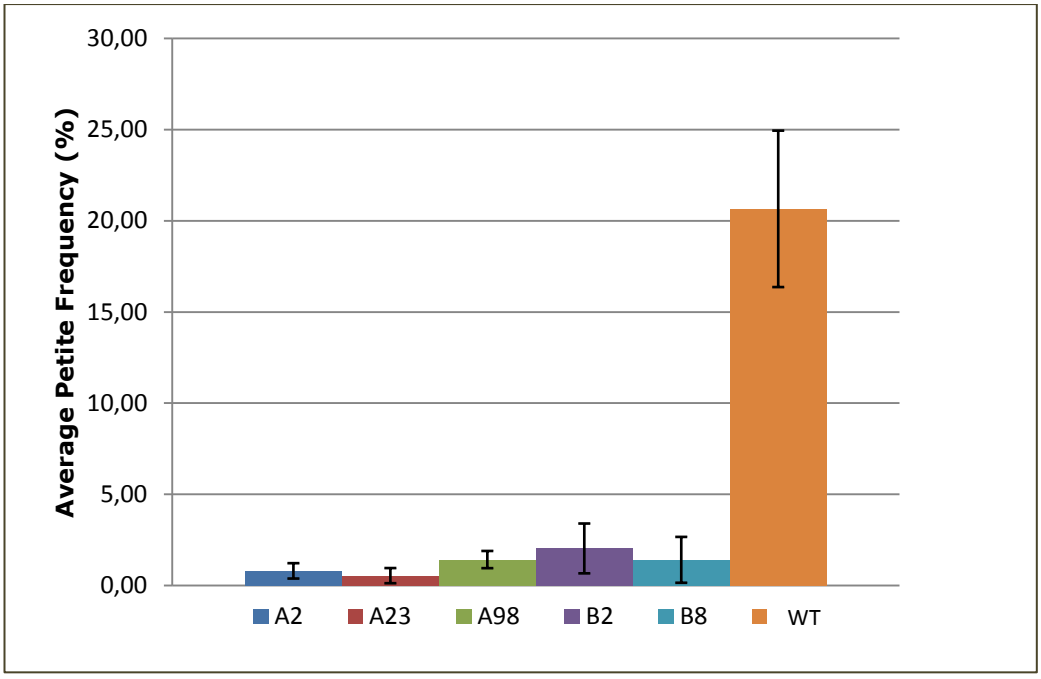

Figure 3.6 : Average petite frequency values of wild type and mutant strains.

With regard to results, wild type (905) showed the highest frequency of petite colonies compared to mutants. Average petite frequency of wild type was at least 8 fold higher than the other ones. This is an expected result because in another study it

was shown that the ethanol-tolerant strains exhibited a lower frequency of ethanol-induced respiratory deficient than ethanol-sensitive strains (Chi and Arneborg, 1999). This demonstrates that the ethanol tolerance of *S. cerevisiae* is dependent on the maintenance of functional mitochondria under the stress.

3.3 Genetic stability analysis of individual mutants

The number of cells/ml and percent survival values of overnight cultures of mutant individuals *B2, B8* and wild type cultures under continuous 10% (v/v) ethanol stress were determined by 5 tube-MPN method. The cultures without ethanol stress were used as control groups. The results obtained at 72nd hours of incubation were given in Table 3.1 and Figure 3.7.

Table 3.1 : Number of cells / ml and percent survival values under ethanol stress at 72nd hour of incubation.

	Cells/ml (at control condition)	Cells/ml (under % 10 v/v ethanol stress)	Percent survival	Survival as fold of wild type
B2(1)	3500000	1700000	48.6	12,7
B2(2)	2400000	920000	38.3	10,1
B2(3)	1600000	540000	33.8	8,8
B2(4)	4900000	1600000	32.6	8,6
B2(5)	1600000	2400000	150	39,4
B8(1)	1100000	540000	49.1	12,9
B8(2)	920000	240000	26.1	6,8
B8(3)	790000	700000	88.6	23,3
B8(4)	920000	350000	38	10,0
B8(5)	1100000	540000	49.1	12,9
WT	9200000	350000	3.8	-

All of the mutants analyzed in this experiment showed higher resistance values under 10% (v/v) ethanol stress in terms of survival rate normalized to wild type. Although there were differences in resistance values, there was no decrease regularly in terms of resistance after each passage. For this reason, it can be inferred that all of the mutants showed stability for five successive passages.

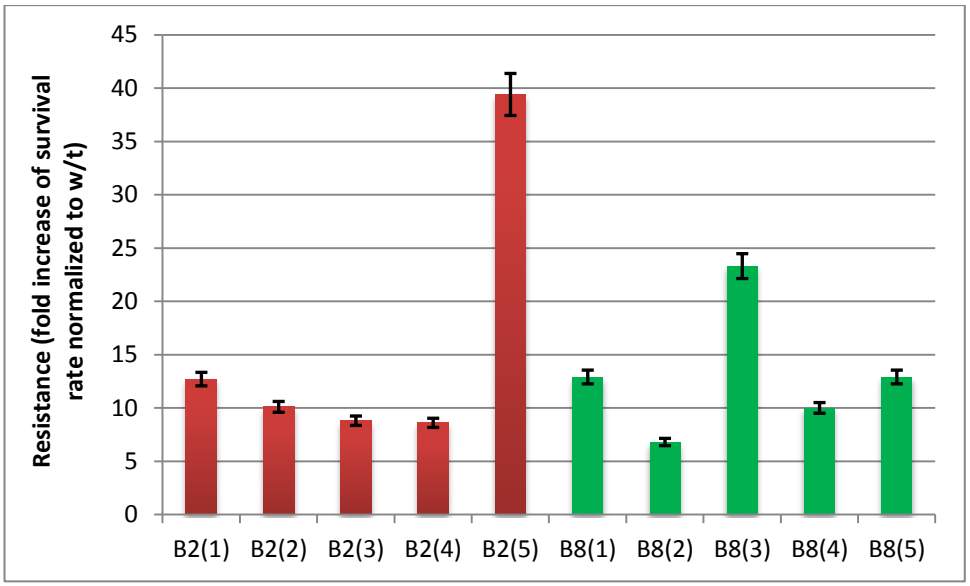

Figure 3.7 : Ethanol resistance values of mutant individuals after five successive passages.

It can be seen in Figure 3.7 that the minimum value for survival ratio as fold of wild type is 8.6 and 6.8 for B2 and B8, respectively. Also, B2(5), which was obtained at 5^{th} day, was the most resistant of the all passages of B2. B8(3), which was obtained at 3^{rd} day, was the most resistant of the all passages of B8.

3.4 Growth Curves of Wild Type and Mutant Strains

Growth curve of wild type and mutant individuals were obtained by measuring OD_{600} values at certain time of incubation. Also, samplings of cultures for trehalose-glycogen content determination and cell dry weight was made at appropriate OD_{600} values.

Table 3.2 : OD_{600} values of B2, B8 and wild type at appropriate times.

Time(hour)	OD_{600} of B2	OD_{600} of B8	OD_{600} of WT
0	0.3	0.3	0.3
2	0.615	0.555	0.51
4	1.24	1.31	1.22
6	2.46	2.64	2.49
7	3.42	3.56	3.64
8.25	4.45	4.25	4.55
9.75	4.64	4.58	5.23
10.5	5.03	4.61	5.51
24.5	5.49	4.60	5.31
30	6.38	5.66	6.6

Table 3.2 shows the growth characteristics of cultures. Rows that shaded with color indicates the time at which samples are gathered for trehalose and glycogen content determination.

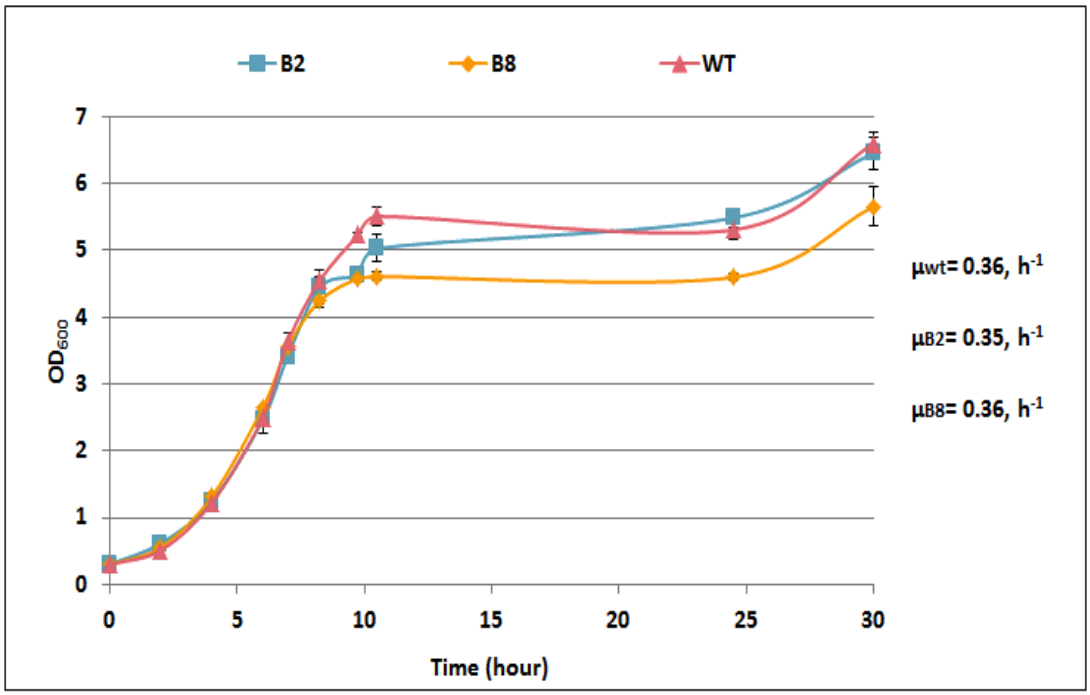

Figure 3.8 : Growth curves and specific growth rate values of wild type, B2 and B8.

As shown in Figure 3.8, all of the cultures growed fast until 8 hour of incubation. After that time, their growth rate decreased. Growth characteristics of all these cultures were very similar, and their final OD_{600} values were between 5.5 and 6.5. An important result was that specific growth rates of mutants were nearly the same to that of wild type. It indicated that there was no decrease in terms of growth ability for these mutants, which had been mutagenized at the initial step of evolutionary engineering.

3.5 Determination of CDWs

CDWs of wild type obtained at particular time intervals were compared to those of mutant individuals. CDW analysis was performed in triplicate according to the procedure that was explained in section 2.2.11. Average cell dry weights of all individuals were shown in Figure 3.9. According to these results, all of the cultures appeared to be similar in terms of cdw values. And these values were coherent with growth curve data.

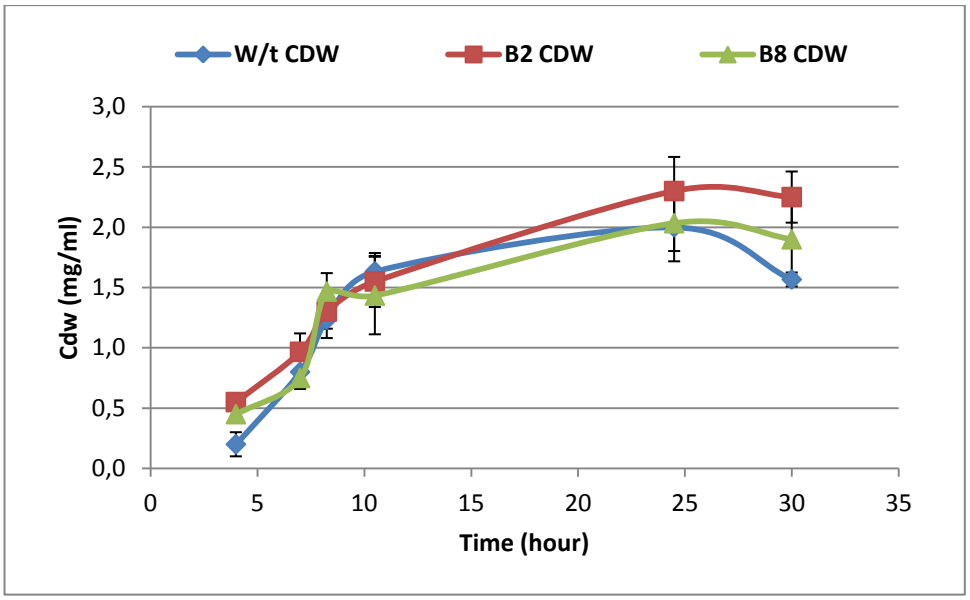

Figure 3.9 : Average CDW values of wild type, B2 and B8.

As shown in this figure, at 30 hour of incubation average cdw values for wild type, B2 and B8 were 1.5, 2.3 and 1.9 (mg/ml), respectively.

3.6 Determination of Trehalose and Glycogen Content

Trehalose and glycogen amounts of cultures were measured at 490 nm according to the glucose standards shown in Figure 3.10. The content of both of these sugars were determined in terms of cdw (mg/ml) values. The results together with the OD_{600} values were given in the Figures from 3.11 to 3.13, respectively.

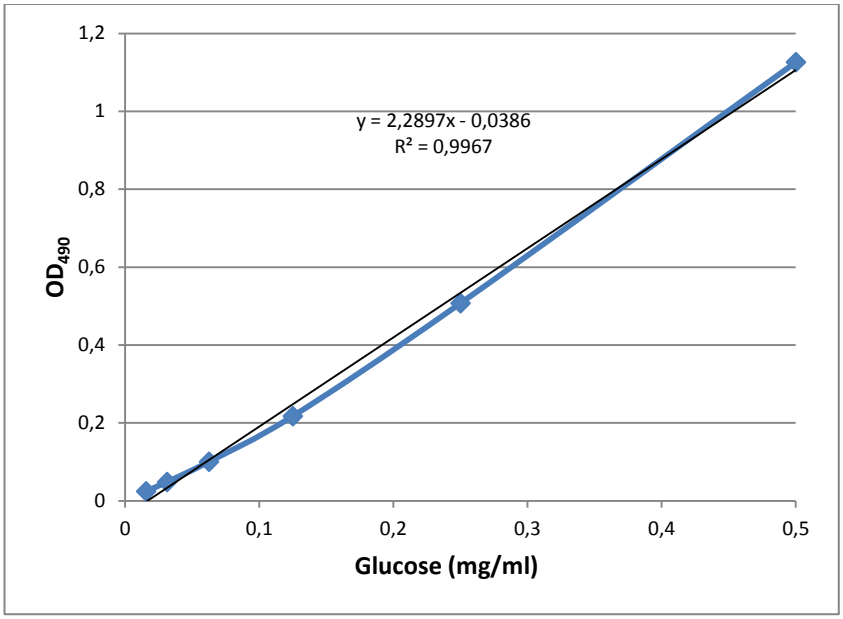

Figure 3.10 : Glucose standards prepared to determine amounts of trehalose and glycogen.

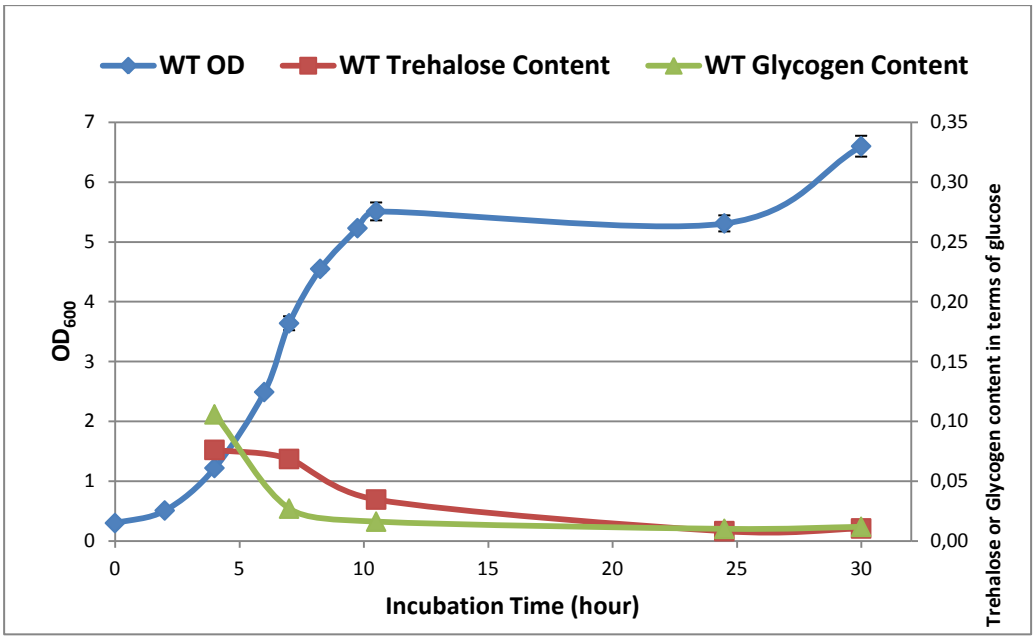

Figure 3.11 : OD_{600} versus trehalose and glycogen content of wild type.

Figure 3.11 shows that until 7 hour of incubation, glycogen content and until 10 hour of incubation, trehalose content of wild type decreased harshly despite it growed easily. Also, the most striking result was that wild type could not produce neither glycogen nor trehalose.

As shown in the Figures 3.11 to 3.13, all of the cultures reached an OD_{600} value between 4.5 and 5.5 after 10 hour incubation. After 30 hour of incubation wild type and B2 cultures reached to approximately OD_{600} 6, and B8 reached to OD_{600} 5.66.

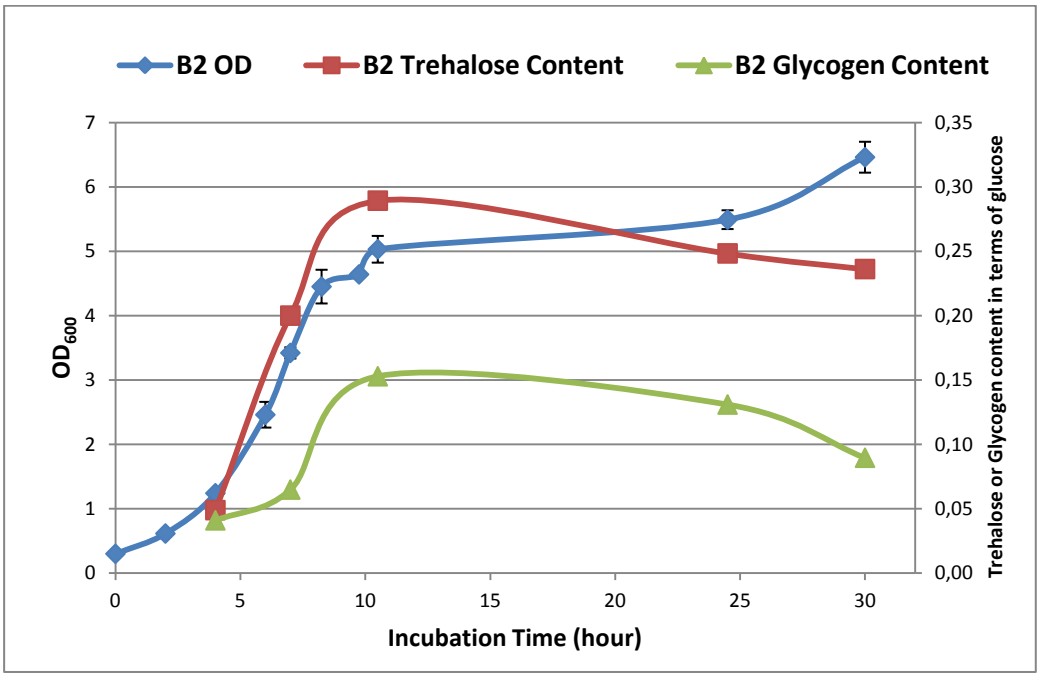

Figure 3.12 : OD_{600} versus Trehalose and Glycogen content of B2.

Figure 3.12 shows that, growth characteristics of B2 was similar to those of wild type. However, B2 produced significant amounts of trehalose and glycogen until 10 hour of incubation. After 10th hour, it started to utilize both of these sugars.

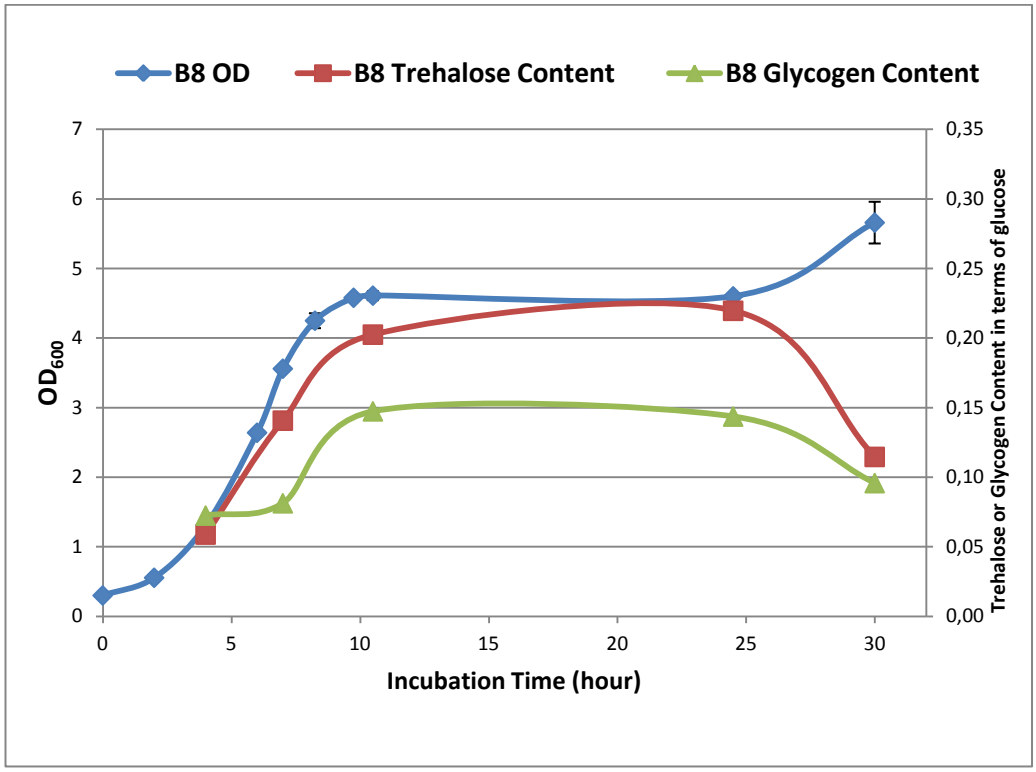

Figure 3.13 : OD_{600} versus Trehalose and Glycogen content of B8.

Figure 3.13 shows that B8 reached to ~5 OD_{600} at 10 hour of incubation, also it produced significant amounts of trehalose and glycogen similarly to B2. However, trehalose content (mg/ml CDW) produced by B8 was less compared to B2.

This results show that at control condition all cultures showed different growth characteristics. Wild type strain could not produce trehalose and glycogen under control condition. Even mutant individuals, which were obtained from the same final population of increasing selection strategy, produced different amounts of trehalose which is a storage carbohydrate and very important for stress resistance.

3.7 Measurement of Metabolite Contents by HPLC

The growth of wild type (905) and B2 cultures that utilized in this study were monitored by measuring OD_{600} in triplicates. OD_{600} measurements were performed at every sampling time. Table 3.3 and 3.4 shows OD_{600} values for wild type and B2, respectively.

Table 3.3 : OD_{600} values of WT strain.

t	WT OD_{600}			WT %8 EtOH OD_{600}		
0	0.41	0.40	0.42	0.43	0.43	0.42
1.5	0.708	0.676	0.704	0.472	0.468	0.492
3.5	1.63	1.58	1.64	0.55	0.55	0.6
4.83	2.62	2.32	2.57	0.61	0.665	0.66
6	3.09	3.12	3.135	0.75	0.63	0.665
7.5	3.785	3.715	3.82	0.94	0.85	0.805
10	4.355	4.81	4.38	1.035	1.115	1.095
12	5.62	5.39	5.5	1.25	1.27	1.32
24	5.27	5.67	5.22	2.73	2.74	2.65
30	5.14	5.35	5.32	3.41	3.4	3.34
48	6.82	6.34	6.66	4.08	4.22	4.12

As shown in Table 3.3 and 3.4, OD_{600} measurements were performed at 11 different time points. Initial OD_{600} values were measured as 0.41 and 0.43 at control condition and stress condition, respectively.

Table 3.4 : OD_{600} values of B2 strain.

t	B2 OD_{600}			B2 %8 EtOH OD_{600}		
0	0.42	0.41	0.41	0.44	0.43	0.44
1.5	0.684	0.76	0.692	0.428	0.428	0.42
3.5	1.62	1.625	1.825	0.605	0.57	0.67
4.83	2.475	2.515	2.49	0.775	0.77	0.705
6	3.075	3.085	3.045	0.795	0.835	0.79
7.5	3.43	3.46	3.47	0.96	0.93	1.02
10	4.205	4.01	4.095	1.535	1.425	1.44
12	5.09	4.99	4.92	1.53	1.61	1.8
24	5.31	5.49	5.53	3.35	3.38	3.4
30	5.27	5.36	5.21	3.5	3.53	3.54
48	5.72	6.48	6.06	4.22	4.52	4.4

Table 3.5 shows average OD_{600} values and standard deviation values corresponding to each sampling time.

Table 3.5 : Average OD_{600} Values and Standard Deviations of Cultures.

t	WT OD_{600} AVG	WT OD_{600} STDEV	WT 8% EtOH OD_{600} AVG	WT 8% EtOH OD_{600} Stdev	B2 OD_{600} Avg	B2 OD_{600} StDev	B2 8% EtOH. OD_{600} AVG	B2 8% EtOH OD_{600} StDev
0	0.41	0.00	0.43	0.00	0.41	0.02	0.43	0.02
1.5	0.70	0.02	0.48	0.01	0.71	0.04	0.43	0.00
3.5	1.62	0.03	0.57	0.03	1.69	0.12	0.62	0.05
4.83	2.50	0.16	0.65	0.03	2.49	0.02	0.75	0.04
6	3.12	0.02	0.68	0.06	3.07	0.02	0.81	0.02
7.5	3.77	0.05	0.87	0.07	3.45	0.02	0.97	0.05
10	4.52	0.26	1.08	0.04	4.10	0.10	1.47	0.06
12	5.50	0.12	1.28	0.04	5.00	0.09	1.65	0.14
24	5.39	0.25	2.71	0.05	5.44	0.12	3.38	0.03
30	5.27	0.11	3.38	0.04	5.28	0.08	3.52	0.02
48	6.61	0.24	4.14	0.07	6.09	0.38	4.38	0.15

According to the Table 3.5, both OD and standard deviation values were coherent. Depending on these values growth properties of these cultures at control and stress condition were shown at Figure 3.14.

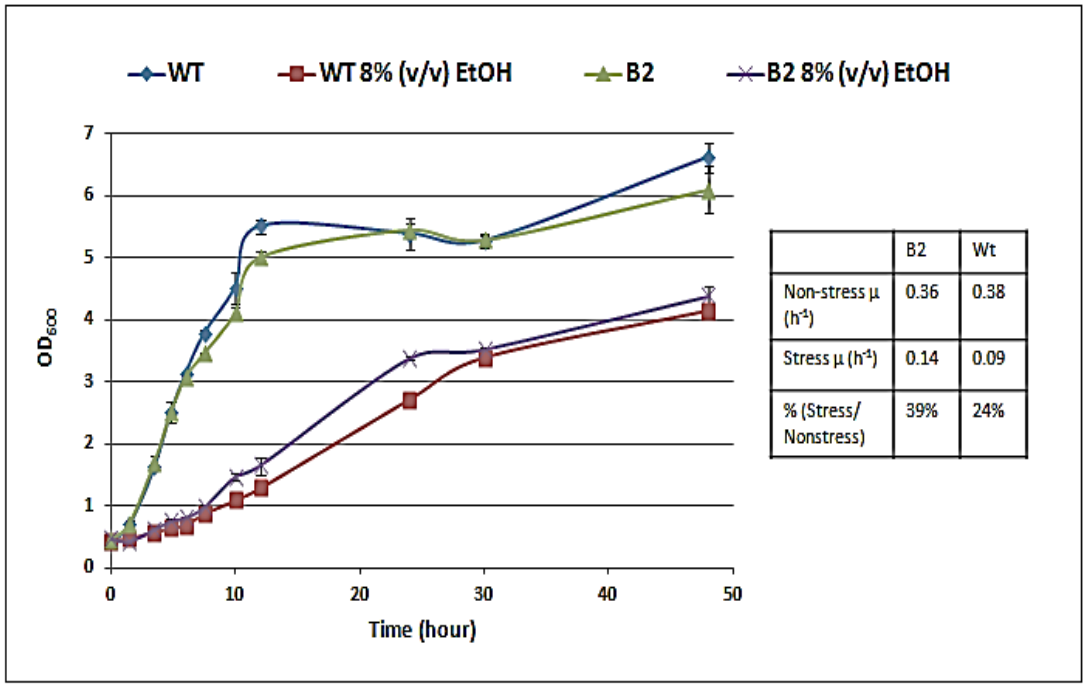

Figure 3.14 : Growth curves and specific growth rate values of wild type and B2 strain in the presence and absence of ethanol.

Figure 3.14 shows that, wild type and B2 strain showed similar growth in terms of optical density values. Because 8% (v/v) ethanol is not a very high stress condition, both of these cultures was able to grow at this stress condition. This was an expected

result. Also, under stress condition, growth rates of both cultures decreased compared to those of under control condition. However, it was clear that specific growth rate of B2 was significantly higher than the wild type at stress condition. It indicated that mutant B2 growed better compared to wild type in the presence of ethanol stress condition.

After obtaining growth curve of these cultures, HPLC analysis were performed as described in 2.2.13. To be able to interpret the results obtained, standards of metabolites were prepared and measured by HPLC tool. Figure 3.15 shows Glucose standards.

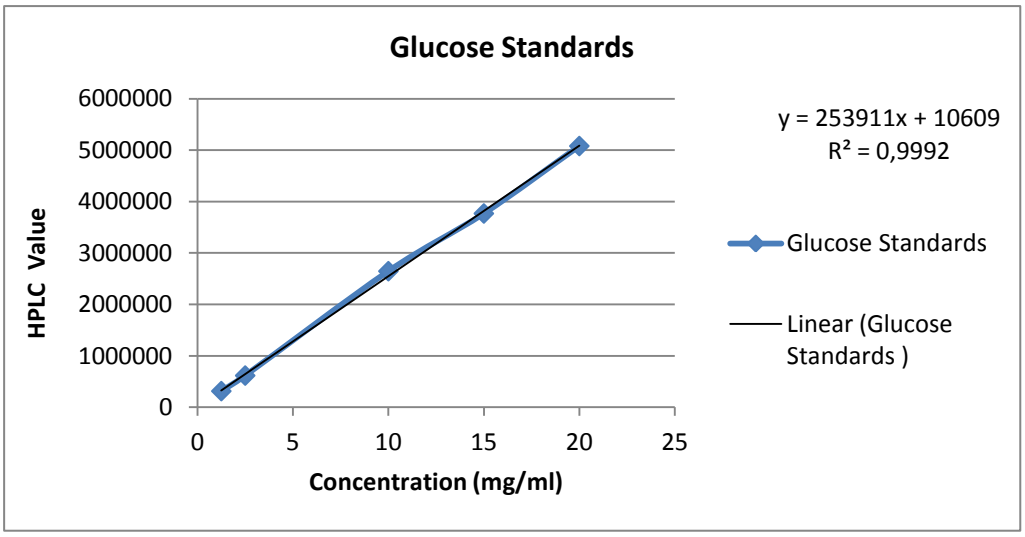

Figure 3.15 : Calibration curve for glucose standards.

Similarly; standards for acetate, glycerol, ethanol, maltose, citric acid, succinic acid were shown at Figures from 3.16 to 3.21 respectively.

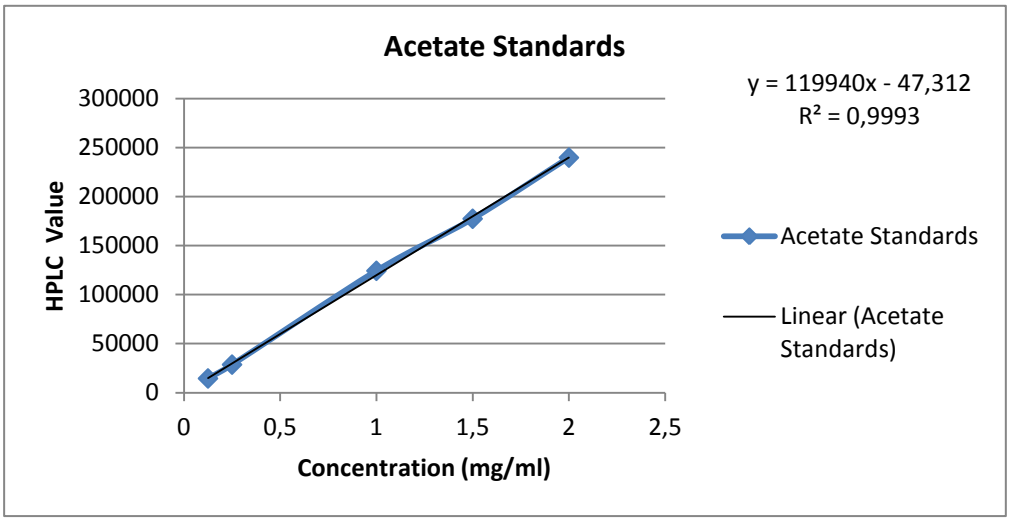

Figure 3.16 : Calibration curve for acetate standards.

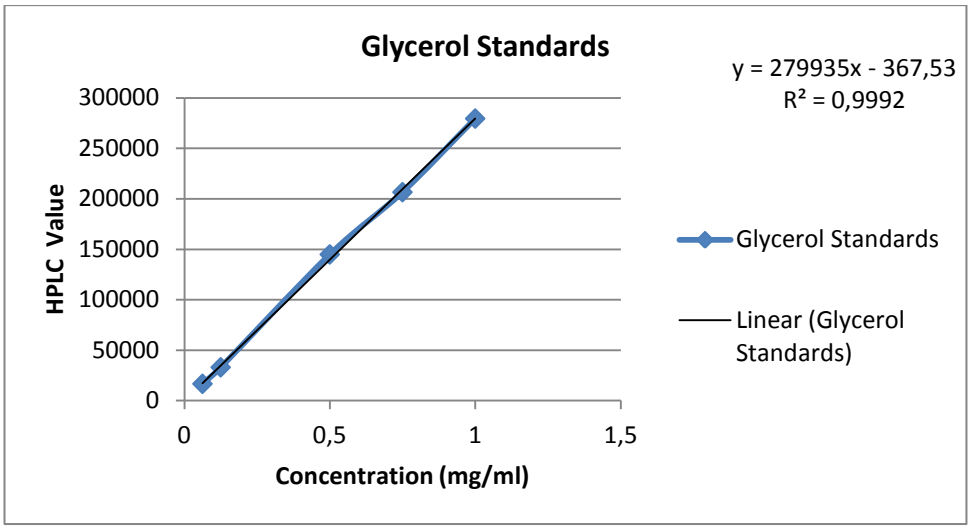

Figure 3.17 : Calibration curve for glycerol standards.

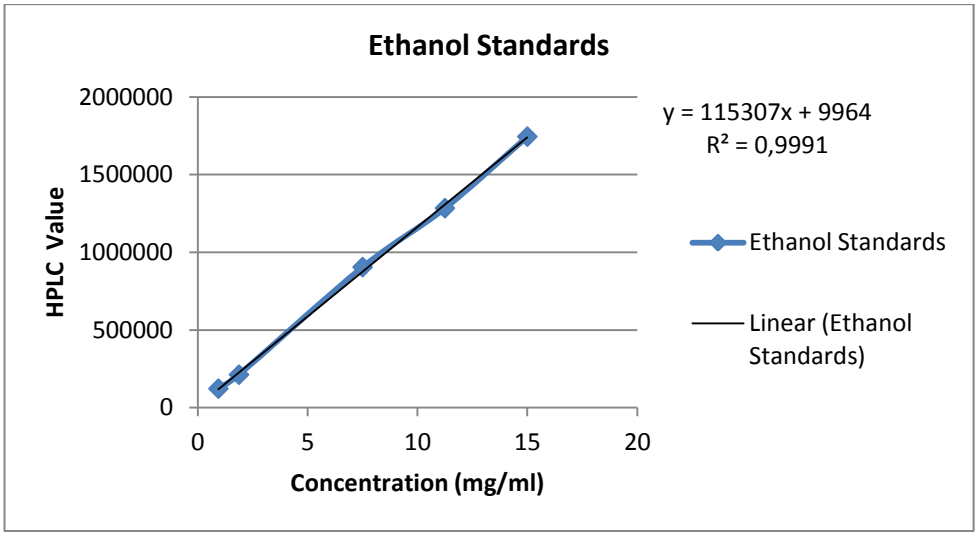

Figure 3.18 : Calibration curve for ethanol standards.

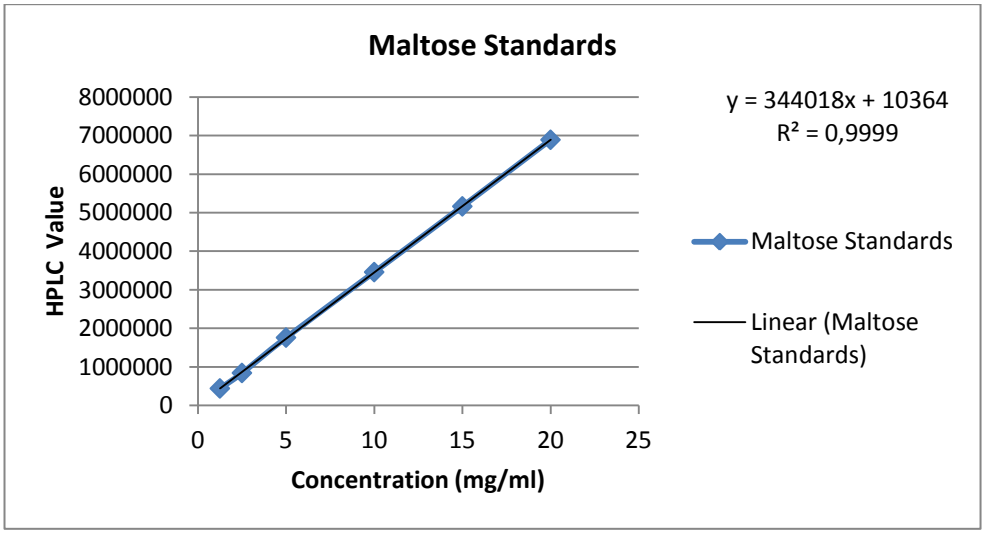

Figure 3.19 : Calibration curve for maltose standards.

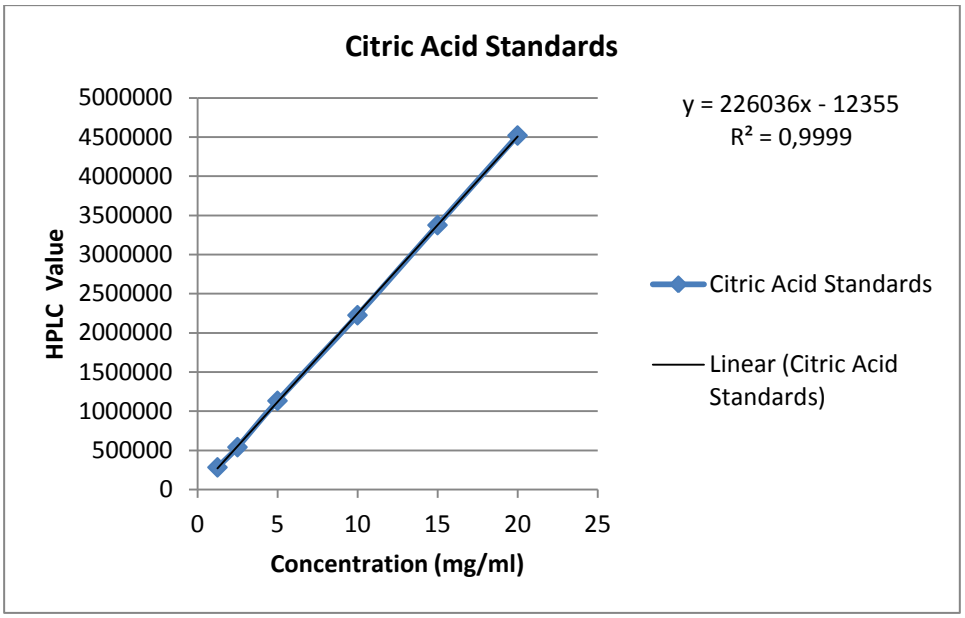

Figure 3.20 : Calibration curve for citric acid standards.

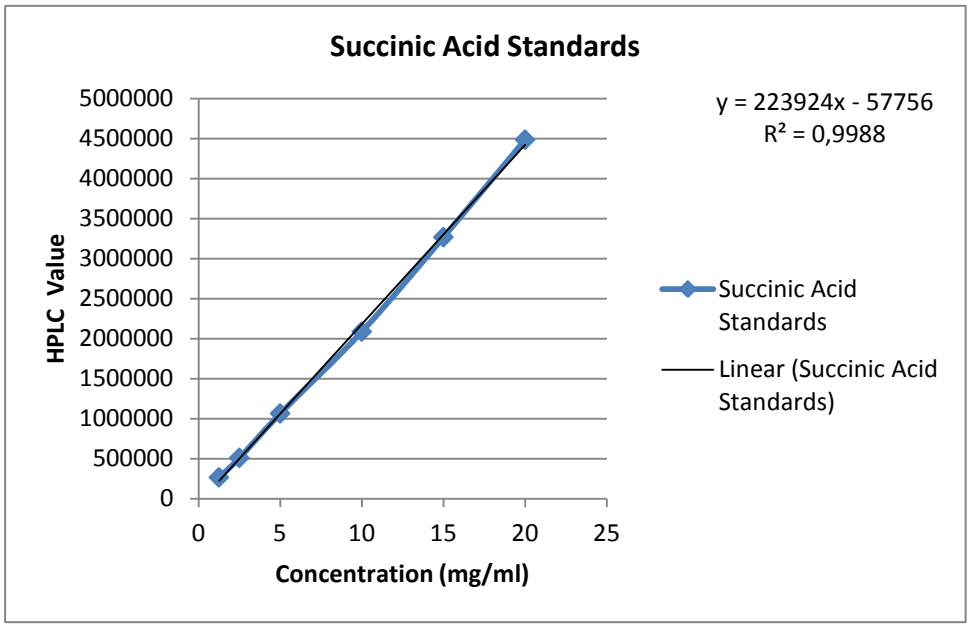

Figure 3.21 : Calibration curve for succinic acid standards.

Glucose consumption and glycerol/acetate/ethanol/citric acid production of wild type culture were shown on the Table 3.6. Average and standard deviation values were obtained from three different sampling.

Table 3.6 : Average amounts of metabolites (g/L) obtained from HPLC analysis for wild type and corresponding standard deviation values.

WT										
t	**Glucose AVG**	**Glucose StDev**	**Glycerol AVG**	**Glycerol StDev**	**Acetate AVG**	**Acetate StDev**	**Ethanol AVG**	**Ethanol StDev**	**Citric Acid AVG**	**Citric Acid StDev**
0	20.63	0.13	0.10	0.00	0.03	0.00	0.46	0.00	1.22	0.01
1.5	20.05	0.14	0.14	0.00	0.03	0.00	0.61	0.02	1.21	0.01
3.5	18.51	0.03	0.20	0.00	0.05	0.00	1.14	0.04	1.17	0.00
4.83	17.10	0.01	0.28	0.00	0.06	0.00	1.92	0.03	0.82	0.00
6	14.36	0.05	0.38	0.02	0.09	0.04	2.87	0.06	0.81	0.00
7.5	10.79	0.01	0.51	0.00	0.10	0.00	4.20	0.01	0.80	0.00
10	5.14	0.01	0.81	0.00	0.13	0.00	6.19	0.03	0.74	0.08
12	1.91	0.01	0.99	0.00	0.18	0.01	7.56	0.03	0.80	0.00
24	0.00	0.00	1.04	0.00	0.28	0.00	7.94	0.01	0.79	0.01
30	0.00	0.00	1.05	0.00	0.32	0.00	7.76	0.04	0.78	0.02
48	0.00	0.00	1.07	0.04	0.40	0.00	7.53	0.08	0.81	0.04

Glucose consumption and glycerol/acetate/ethanol/citric acid production of wild type culture that was grown at 8% (v/v) ethanol concentration were shown on the Table 3.7. Average and Standard deviation values were obtained from three different sampling.

Table 3.7 : Average amounts obtained from HPLC analysis for WT under 8% (v/v) Ethanol stress and corresponding Standard Deviation Values.

WT Stress										
t	**Glucose AVG**	**Glucose StDev**	**Glycerol AVG**	**Glycerol StDev**	**Acetate AVG**	**Acetate StDev**	**Ethanol AVG**	**Ethanol StDev**	**Citric Acid AVG**	**Citric Acid StDev**
0	18.89	0.02	0.09	0.00	0.02	0.00	2.79	0.02	1.13	0.00
1.5	18.75	0.03	0.18	0.00	0.03	0.00	2.26	0.08	1.13	0.00
3.5	18.41	0.05	0.13	0.00	0.03	0.00	1.70	0.02	1.12	0.00
4.83	18.88	0.03	0.13	0.00	-	-	2.30	0.06	0.78	0.00
6	18.67	0.02	0.14	0.00	0.04	0.00	2.19	0.03	0.78	0.00
7.5	18.41	0.07	0.14	0.00	0.04	0.00	2.31	0.04	0.78	0.00
10	17.81	0.03	0.15	0.00	0.05	0.00	1.35	0.07	0.77	0.00
12	17.54	0.05	0.16	0.00	0.06	0.00	2.45	0.06	0.77	0.00
24	12.86	0.03	0.26	0.00	0.10	0.00	3.44	0.04	0.75	0.00
30	10.26	0.07	0.33	0.00	0.13	0.00	4.14	0.11	0.73	0.00
48	4.08	0.01	0.52	0.00	0.23	0.01	6.56	0.10	0.73	0.00

Table 3.8 : Average amounts obtained from HPLC analysis for B2 and corresponding Standard Deviation Values.

B2										
t	Glucose AVG	Glucose StDev	Glycerol AVG	Glycerol StDev	Acetate AVG	Acetate StDev	Ethanol AVG	Ethanol StDev	Citric Acid AVG	Citric Acid StDev
0	20.60	0.03	0.15	0.00	0.02	0.00	0.44	0.00	1.23	0.00
1.5	20.00	0.10	0.20	0.00	0.03	0.00	0.60	0.00	1.21	0.01
3.5	18.53	0.00	0.27	0.00	0.04	0.00	1.25	0.03	0.82	0.00
4.83	16.66	0.02	0.37	0.00	0.05	0.00	1.98	0.03	0.81	0.00
6	13.55	0.02	0.50	0.00	0.06	0.00	3.06	0.01	0.80	0.00
7.5	10.14	0.04	0.75	0.00	0.08	0.00	4.28	0.01	0.78	0.00
10	5.77	0.01	1.08	0.00	0.12	0.00	5.87	0.01	0.79	0.00
12	3.30	0.01	1.24	0.00	0.13	0.00	6.78	0.03	0.79	0.00
24	0.00	0.00	1.36	0.01	0.10	0.00	7.64	0.01	0.77	0.00
30	0.00	0.00	1.36	0.00	0.10	0.00	7.47	0.01	0.76	0.00
48	0.00	0.00	1.36	0.00	0.10	0.00	7.56	0.03	0.78	0.01

Glucose consumption and glycerol/acetate/ethanol/citric acid production of B2 were shown on the Table 3.8. Average and Standard deviation values were obtained from three different sampling.

Table 3.9 : Average amounts obtained from HPLC analysis for B2 under 8% (v/v) Ethanol stress and corresponding Standard Deviation Values.

B2 Stress										
t	Glucose AVG	Glucose StDev	Glycerol AVG	Glycerol StDev	Acetate AVG	Acetate StDev	Ethanol AVG	Ethanol StDev	Citric Acid AVG	Citric Acid StDev
0	18.84	0.01	0.14	0.00	0.02	0.00	2.28	0.04	1.13	0.00
1.5	18.70	0.02	0.17	0.00	0.02	0.00	2.18	0.07	1.13	0.00
3.5	18.82	0.02	0.25	0.00	0.00	0.00	1.90	0.02	0.78	0.00
4.83	18.71	0.02	0.20	0.00	0.00	0.00	2.05	0.05	0.78	0.00
6	18.34	0.01	0.20	0.00	0.04	0.00	1.66	0.05	0.78	0.00
10	17.36	0.02	0.23	0.00	0.05	0.00	2.31	0.33	0.77	0.00
12	16.61	0.03	0.25	0.00	0.06	0.00	2.45	0.06	0.76	0.00
24	11.12	0.03	0.48	0.00	0.14	0.00	4.16	0.12	0.73	0.00
30	9.33	0.00	0.57	0.01	0.18	0.00	3.62	0.31	0.74	0.00
48	5.09	0.01	0.80	0.00	0.28	0.00	4.45	0.04	0.74	0.00

Glucose consumption and glycerol/acetate/ethanol/citric acid production of B2 that was grown at 8% (v/v) ethanol concentration were shown on the Table 3.9. Average and Standard deviation values were obtained from three different sampling.

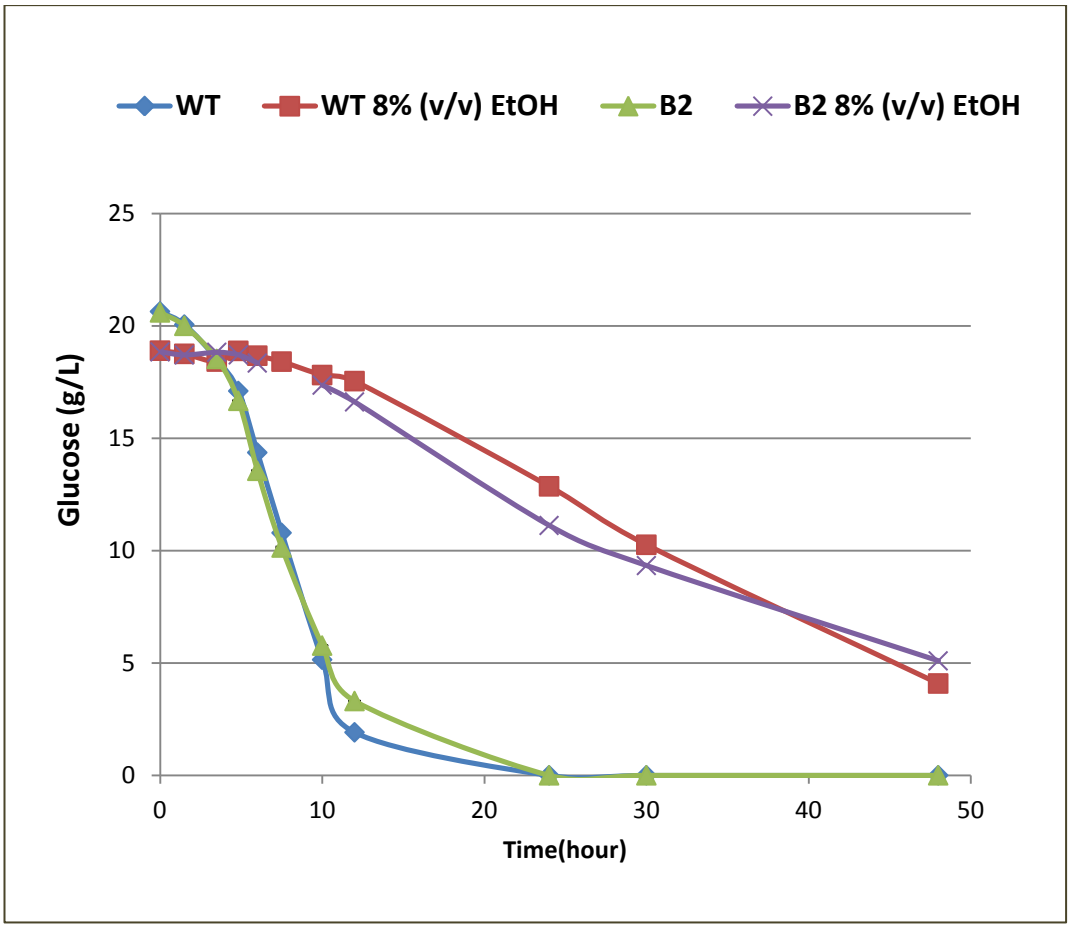

Figure 3.22 : Utilization of glucose by wild type and B2 strains at control and stress conditions after 48 hour incubation at 30 ºC, 150 rpm.

Figure 3.22 shows utilization of glucose (g/L) by time. It is obvious that, under ethanol stress, rate of glucose utilization decreased. At control condition, both wild type and mutant strains consumed all of the glucose by 24th hour. However, under 8 % (v/v) ethanol stress neither wild type nor B2 could not consume glucose by 48th hour. By the way, wild type and B2 strain showed similar growth characteristics at these culture conditions. The reason of this similarity under stress condition results from ethanol concentration level. 8% ethanol (v/v) is not so high for wild type strain. This situation confirms these data.

As expected, at control condition both wild type and B2 reached 80% of theoretical ethanol yield by producing 7.94 (g/L) and 7.64 (g/L) ethanol, respectively (Figure 3.23). However, under stress condition production of ethanol decreased. Interestingly, after 30 hour incubation wild type produced more ethanol compared to B2. It is possible for B2 to metabolize ethanol under such high concentration of ethanol. But, wild type strain produced ethanol instead of metabolizing it in the cell.

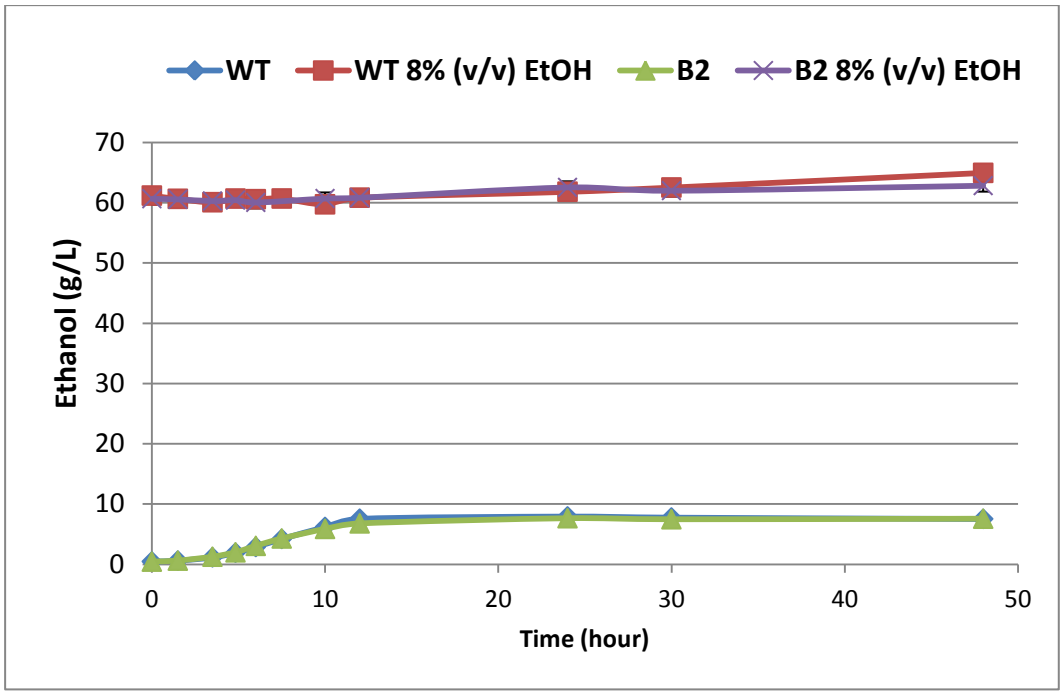

Figure 3.23 : Ethanol production levels by wild type and B2 strains at control and stress conditions after 48 hour incubation at 30 ºC, 150 rpm.

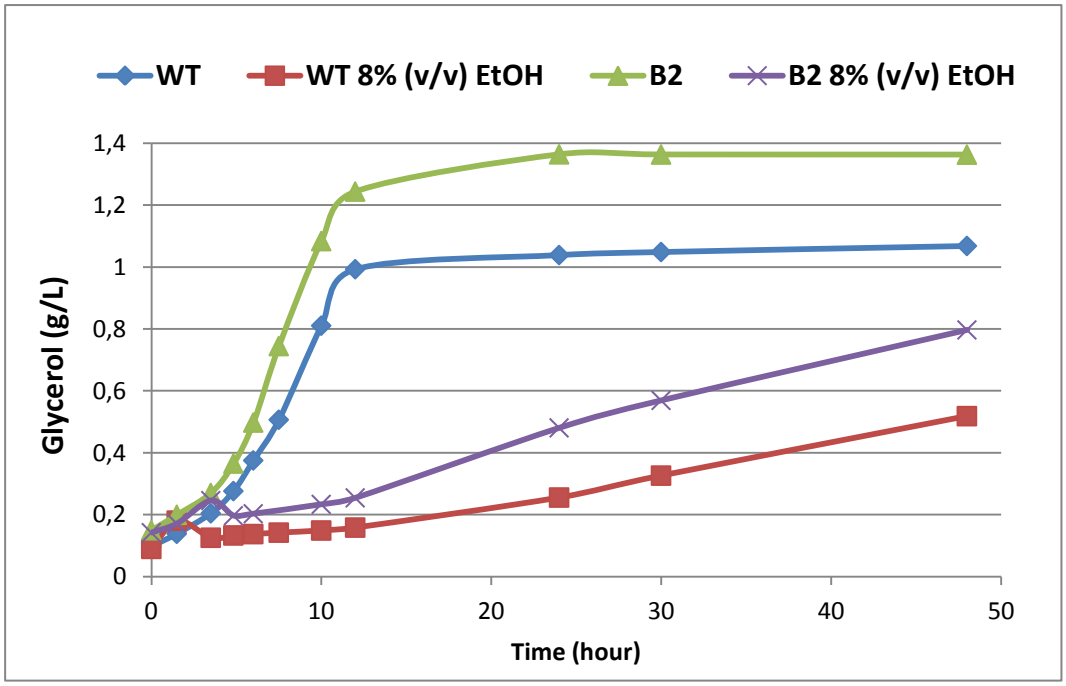

Figure 3.24 : Glycerol production levels by wild type and B2 strains at control and stress conditions after 48 hour incubation at 30 ºC, 150 rpm.

In Figure 3.24, glycerol amounts showed that B2 strain produced more glycerol compared to wild type under both control and stress conditions. It can be concluded that metabolism of evolutionary engineered B2 strain has shifted so as to produce more glycerol compared to wild type. However, under stress conditions glycerol production decreased.

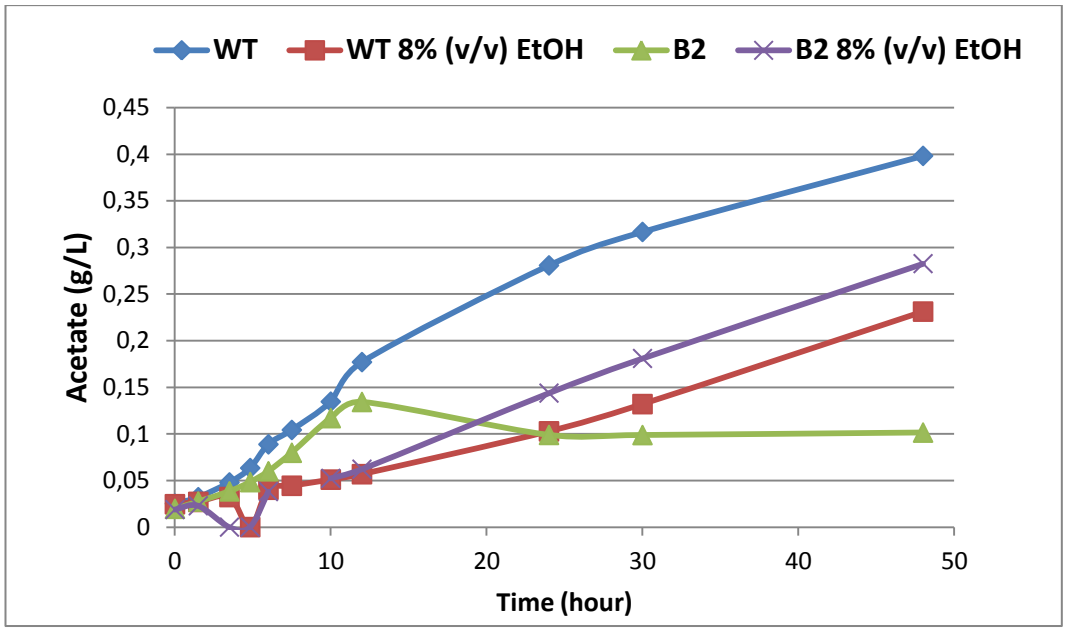

Figure 3.25 : Acetate production levels by wild type and B2 strains at control and stress conditions after 48 hour incubation at 30 ºC, 150 rpm.

Figure 3.25 shows acetate production levels of wild type and B2 strain at control and 8% ethanol (v/v) concentration. From the figure it can be seen that acetate metabolism of wild type and mutant is exactly opposite with regard to each other. At stress condition, acetate production by wild type decreased ~2 fold compared to production at control condition, but increased by B2 ~3 fold at stress condition compared to control condition.

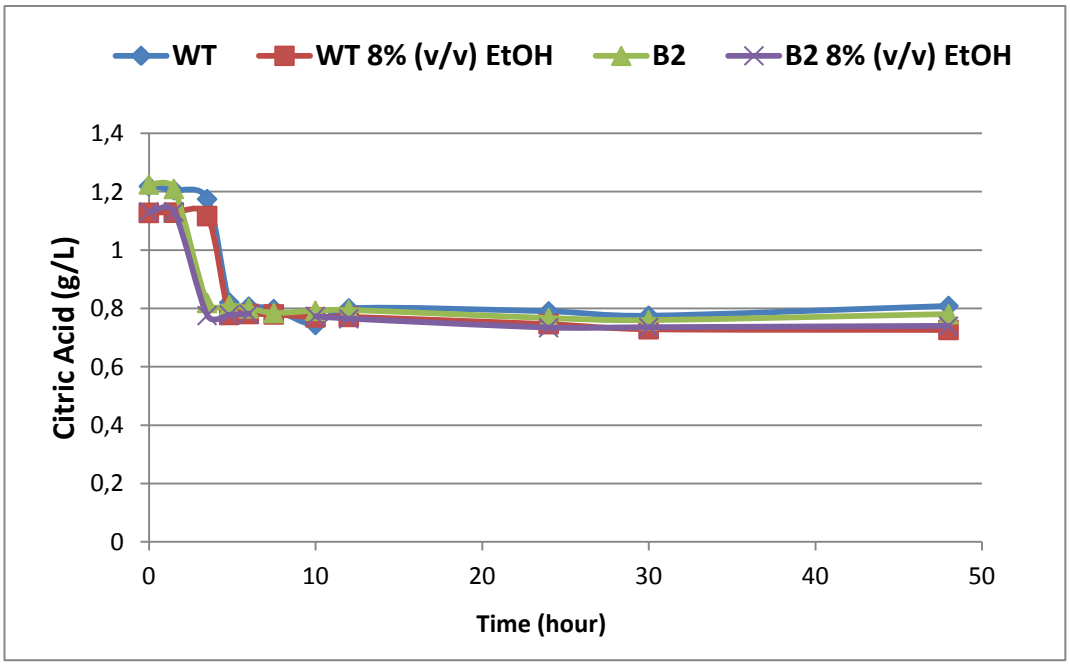

Figure 3.26 : Citric acid production levels by wild type and B2 strains at control and stress conditions after 48 hour incubation at 30 ºC, 150 rpm.

Figure 3.26 shows citric acid production levels of wild type and B2 strain at control and 8% (v/v) ethanol concentration. Although utilization of citric acid by B2 occured at 3rd hour, wild type started to utilize citric acid at 4.5th hour. After that time point, there is not any difference in terms of citric acid utilization of both B2 and wild type under control and stress conditions. That is, these cultures did not utilize citric acid.

3.8 Microarray Analysis of Wild Type, B2 and B8

Microarray analysis was carried out by using wild type and two ethanol-resistant mutants B2 and B8 which were incubated at control condition. Taken B2 into consideration, expression levels of 228 genes were found to be significantly different compared to wild type. 82 of these genes were up-regulated, and 146 of them were down-regulated. B2 genes whose expression level changed at least two fold compared to wild type were given on the Table 3.10 (up-regulation) and Table 3.11 (down-regulation), respectively.

Table 3.10 : Up-regulated genes of B2 compared to wild type (Fold change stands for the B2 fold change of corresponding gene expression level to that of wild type).

Fold Change	Gene Symbol
179	IME4
18	SPL2, YAR068W
17	YAR068W, HPF1
16	YIL169C
14	PHO12
13	OPT2
12	GIT1, PHO3
10	YDR366C, PHO11, PHO84
7	PGM2
6	YLR053C, MAL32, MAL12
5	PHM6
4	VTC3, YNR034W-A, MAL11, HXK1, YER067W, TSL1
3	GPH1, GAD1, ARO9, DAL5, RTN2, HSP78, VTC1, PHO89, BTN2, YPK2, VTC4, MF(ALPHA)1, RTS3, GPM2, DCS2
2	BSC5, HSP82, VTC4, GLC3, CTF19, GAC1, PDH1, TMT1, STB2, VBA1, CAR2, AIM17, TPK1, MEP2, YCR100C, SOL4, CIT1, MET28, STI1, UIP4, YMR291W, YJL163C, GSY1, PYK2, YNR066C, YCT1, GDH1, DLD3, OPT1, ALD6, GSY2, IML2, TIS11, PTK1, KAP95, CIT2, FET3, YAP5

Table 3.10 shows that IME4 gene is the most up-regulated gene of B2 compared to wild type at control condition.

Table 3.11 : Down-regulated genes of B2 compared to wild type (Fold change stands for the B2 fold change of corresponding gene expression level to that of wild type).

Fold Change	Gene Symbol
517	HO
183	MFA2
116	STE2
109	MFA1
61	FUS3
60	AGA2
54	STE18
40	BAR1, YGL193C
32	FAR1
23	GPA1
17	NEJ1
16	STE6
15	STE4
10	ASG7, YLR031W, STE5
8	YLR161W, YLR159W, YLR156W
7	YKR106W
6	YBR298CA, YCL021WA, RDH54
5	YLR042C, PTR2, SRD1, FUS1, SPO19 SOR2, YCL073C, YLR030W
4	SST2, DDR2, SOR1, UTR5, THI22, AXL1, YIG1, PRM1, HES1, LEE1, VAM10, SNZ2, SPS1, AGA1, SNZ3, YER138WA
3	SMA1, RME1, NCA3, VBA2, ASP34, REG2, YOL163W, SNO2, SNO3, POT1, ASP33, ASP32, YOL162W, ASP31, FIG1, IZH4, YIL055C, YLR040C, YKL070W, YPL113C, PEX18, YNL155W, IZH1, MUP1, YER138WA, YGR035C, PRM7, GNP1, PRM7
2	MUM3, FIT2, YHR214W, MAG1, STE12, YCL001WB, UBC11,YSW1 STE14, PRM3, YBR284W, ICS2, YNL024C, STE3, MET31, NDT80, POX1, REC102, UFD1, MTC7, DBP2, YHL012W, DIT2, CTA1, IRC18, AMN1, YOR338W, XBP1,RRT12, PRR2, RRT5, TEC1 YJL045W, FRM2, YDR042C, IZH2, PUT4

As shown in Table 3.11 that there are 17 B2 genes whose expression level decreased at least ten fold compared to wild type. Among them, HO gene is the most down-regulated gene with 517 fold change.

FunSpec database was utilized for classifying up-regulated genes in terms of molecular functions. On the Table 3.12, those genes and molecular classifications were shown.

Table 3.12 : Molecular functions of the up-regulated genes of B2.

Category	Gene name
acid phosphatase activity	PHO11 PHO3 PHO12
catalytic activity	MAL32 GPM2 GLC3 DLD3 GSY1 HXK1 MAL12 ARO9 GSY2 CAR2 TSL1 GAD1 DCS2 PYK2 GDH1
glycogen (starch) synthase activity	GSY1 GSY2
maltose alpha-glucosidase activity	MAL32 MAL12
citrate (Si)-synthase activity	CIT2 CIT1
oligopeptide transporter activity	OPT1 OPT2
sucrose alpha-glucosidase activity	MAL32 MAL12
alpha-glucosidase activity	MAL32 MAL12
transferase activity	CIT2 GLC3 TMT1 GSY1 HXK1 IME4 ARO9 TPK1 PTK1 GSY2 CAR2 YPK2 TDA1 CIT1 PYK2 GPH1
inorganic phosphate transmembrane transporter activity	PHO89 PHO84
pyridoxal phosphate binding	ARO9 CAR2 GAD1 GPH1
cation binding	MAL32 GLC3 MAL12
intramolecular transferase activity, phosphotransferases	GPM2 PGM2
transferase activity, transferring acyl groups, acyl groups converted into alkyl on transfer	CIT2 CIT1
symporter activity	PHO89 MAL11
phosphatase activity	PHO11 PHO3 PHO12

On the Table 3.13, down-regulated genes of B2 and their corresponding molecular functions were shown.

Table 3.13 : Molecular functions of the down-regulated genes of B2.

Category	In Category from Cluster
asparaginase activity	ASP3-1 ASP3-2 ASP3-3 ASP3-4
signal transducer activity	STE2 GPA1 STE18 STE3 SST2 STE4
mating-type factor pheromone receptor activity	STE2 STE3
L-iditol 2-dehydrogenase activity	SOR2 SOR1
cell adhesion molecule binding	AGA2 AGA1
G-protein coupled receptor activity	STE2 STE3
transmembrane transporter activity	YOL162W YOL163W
L-proline transmembrane transporter activity	GNP1 PUT4
pheromone activity	MFA1 MFA2
receptor activity	STE2 STE3 IZH2
solute:hydrogen antiporter activity	GEX1 GEX2
protein binding	AMN1 SNZ3 PEX18 SNZ2 IZH2
mating pheromone activity	MFA1 MFA2
oxidoreductase activity, acting on the CH-CH group of donors	POX1 YJL045W
amino acid transmembrane transporter activity	GNP1 MUP1 PUT4

Taken B8 into consideration, expression levels of 396 genes were found to be significantly different compared to wild type. 176 of these genes were found as up-regulated, and 220 of them were found as down-regulated. B8 genes whose expression level changed at least two fold compared to wild type were given on the Table 3.14 (up-regulation) and Table 3.15 (down-regulation), respectively.

Table 3.14 : Up-regulated genes of B8 compared to wild type (Fold change stands for the B8 fold change of corresponding gene expression level to that of wild type).

Fold Change	GeneSymbol
190	IME4
43	YPR002C-A
14	YAR068W
12	YIL169C
11	HPF1, BTN2, PGM2, MAL32
10	YDR366C, YLR053C, MAL12
9	OPT2
8	TSL1, HXK1
7	YNR034W-A, GPH1
6	MAL11, YER067W, RTN2, YJL144W, HSP78, BSC5, HSP82
5	GAD1, HSP26, YDR034C-A, RTC2, RTS3
4	SOL4, GPM2, DCS2, YNL194C, SNO1, CTT1, HSP42, CTR3, YJL163C, TMA10, YNR068C, TPK1, MF(ALPHA)2, APJ1, GLC3, YCR100C, HSP30, MF(ALPHA)1
3	GAC1, OPT1, FMP33, MLS1, TMT1, MET28, UIP4, GSY1, AIM17, YRO2, ICY2, YPK2, MSC1,CUR1, SSE2, YCR101C, PDH1, SSA1 YFR017C, MBF1, YPT53, SSA4, GSY2, IML2, YCR099C, HSP104, ADH5, HSP12, MUP3, SDP1, STB2, STI1, OPI10, GRE3, RTC3, GCY1, TOS8, TPS2, YGP1, CIT1, FMP23, CAR2, ATG15, HXT7, AGP2, PIG2, MGA1, ATG29, SDS24, YBR285W
2	EMI2, FES1, GTO1, CPR6, YKL151C, XKS1, STR3, MET2, SPI1, GPG1, COX5B, GLG1, YNR066C, SYM1, MTH1, MTL1, STF2, HXT6, YBR085C-A, YCT1, CIT2, YHR022C, ECL1, UBI4, RNY1, PHM7, BNA3, TFS1, MAL13, COB, GLG2, ISF1, YGR287C, YDR379C-A, YNR065C, YER079W, YGL117W, RSB1, SAP4, SAF1, MRP21, SNZ1, RIB5, PIN3, KAP95, OM14, ATG7, COX3, RRI2, YNR014W, AHA1, MAL31, FMP16, ZTA1, PRX1, YLR345W, PYK2, YBR074W, YBR056W, YJR154W, UGP1, YPL264C, YBL029C-A, MOH1, FYV10, ALD3, YGR205W, YLR149C, YAP1801

On the Table 3.14, up-regulated genes of B8 were given. 11 genes were found as 10 fold up-regulated compared to wild type. According to these results, IME4 is the most up-regulated gene of B8 likewise in the B2.

Table 3.15 : Down-regulated genes of B8 compared to wild type (Fold change stands for the B8 fold change of corresponding gene expression level to that of wild type).

Fold Change	Gene Symbol
420	HO
276	MFA2
113	STE2
102	MFA1
60	AGA2
59	STE18
54	BAR1, FUS3
40	YGL193C
30	FAR1
29	GPA1
20	NEJ1
16	STE4, STE6
11	STE5
9	ASG7
8	YLR031W
6	SRD1, YCL021W-A, YGR109W-A, SMA1
5	HES1, RDH54, SOR2, PTR2, SPO19, YLR042C
4	YLR030W, YGR035C, YER138W-A, SOR1, SNZ2, SST2, ANS1, STL1, SNO3, AXL1, SNZ3, PRM1, SNO2, RME1, FIT2, THI22, YLR040C, YIG1
3	POT1, LEE1, YER138W-A, YBR298C-A, IZH4, IZH1, PEX18, AGA1, GAL4, STE14, PRM7, YFL068W, YCL073C, YOL163W, DBP7, YKR106W, YPL113C, UBC11, SPS1, FUS1, VAM10, YOR214C, VBA2, MUP1, PRM9, YHL012W, STE12, YIL055C, AZR1, PRM3, YER187W, BSC4, YGR079W, YNL024C, DBP2, RRT5, NCA3, RAS1, CDA1, YJR079W, OLE1
2	GNP1, UTR5, HOP1, DDR2, PUT4, NDT80, YPR202W, REG2, REC102, FIG1, ALB1, FRM2, PUG1, MTC7, AAH1, YNL155W, IZH2, MAG1, DIT1, FCY22, GAP1, UTP23, RCK1, REV1, PPH3, MCH5, GAS4, YCL001W-B, IPI3, YCL001W-A, CTA1, YFL064C, KNH1, PRY2, YOL162W, PRM4, POX1, YKR015C, SSP1, TIR4, HXT4, PRR2, PES4, RRP36, AQR1, RGS2, SPS19, YFR012W-A, GIC2, YEL076C, YJR056C, RSA1, HXT2, SET6, DIT2, YPR078C, YLR413W, YMR018W, DIP5, ERG25, CYB5, FAL1, STE3 GFD2, HFM1, YLR462W, RLP24, YNL034W YMR244W, NSR1

As shown in Table 3.15 that there are 15 B8 genes whose expression level decreased at least ten fold compared to wild type. Among them, HO gene is the most down-regulated gene of B8 with 420 fold change likewise in the B2.

FunSpec database was utilized for classifying up- and down-regulated genes of B8 in terms of molecular functions. Those genes and molecular classifications were shown on Table 3.16 and 3.17, respectively.

Table 3.16 : Molecular functions of the up-regulated genes of B8.

Category	In Category from Cluster
cation binding	YBR056W MAL32 GLC3 IMA1 MAL12
Molecular function	YAR068W YBL029C-A MOH1 FMP23 YRO2 YBR056W YBR074W YBR085C-A RTC2 SDS24 OM14 YBR285W HSP30 YCR099C YCR100C YCR101C GPM2 RTN2 YDR034C-A FMP16 MTH1 YDR366C YDR379C-A EMI2 RGI1 YER079W SPI1 IGD1 YGL117W STF2 MTL1 ECL1 RTS3 AIM17 YHR022C RTC3 FYV10 YIL169C IML2 YJL144W FMP33 YJL163C YJR154W YKL151C YLR053C YLR149C SYM1 TMA10 MSC1 ISF1 SNO1 YGP1 YNL194C YNR014W YNR034W-A YNR065C YNR066C YNR068C BSC5 OPI10 PHM7 RRI2 ATG29 UIP4 ICY2 YPL264C PDH1 PIN3 CUR1
glycogen (starch) synthase activity	GSY1 GSY2
alpha-glucoside:hydrogen symporter activity	MAL31 MAL11
maltose alpha-glucosidase activity	MAL32 MAL12
glycogenin glucosyltransferase activity	GLG2 GLG1
catalytic activity	ZTA1 YBR056W ADH5 MAL32 GPM2 TPS2 GLC3 GSY1 HXK1 STR3 IMA1 MAL12 ATG7 BNA3 GSY2 YLR345W CAR2 TSL1 SNZ1 GAD1 MLS1 DCS2 PYK2
unfolded protein binding	SSA1 HSP26 HSP42 SSA4 HSP104 CPR6 APJ1 HSP82
oligopeptide transporter activity	OPT1 OPT2
ATPase activity, coupled	HSP104 HSP82
sucrose alpha-glucosidase activity	MAL32 MAL12
citrate (Si)-synthase activity	CIT2 CIT1
trehalose-phosphatase activity	TPS2 TSL1
alpha-glucosidase activity	MAL32 MAL12
transferase activity	RIB5 CIT2 EMI2 GLC3 TMT1 GSY1 HXK1 IME4 GTO1 XKS1 TDA10 BNA3 GLG2 TPK1 UGP1 GLG1 GSY2 YLR345W CAR2 SNO1 YPK2 MLS1 MET2 CIT1 PYK2 GPH1
pyridoxal phosphate binding	STR3 BNA3 CAR2 GAD1 GPH1
hexokinase activity	EMI2 HXK1
substrate-specific transmembrane transporter activity	MAL31 HXT7 HXT6 MAL11
alditol:NADP+ 1-oxidoreductase activity	GRE3 GCY1
adenyl-nucleotide exchange factor activity	FES1 SSE2

Table 3.17 : Molecular functions of the down-regulated genes of B8.

Category	In Category from Cluster
signal transducer activity	STE2 GPA1 STE18 STE3 SST2 RGS2 STE4
L-proline transmembrane transporter activity	GNP1 GAP1 PUT4
Molecular function	PRM9 FIG1 YBR298C-A YCL001W-A YCL001W-B YCL021W-A FUS1 GFD2 PRM7 KNH1 MTC7 UTR5 YEL076C YER138W-A PUG1 YER187W SNO3 YFL064C YFL068W YFR012W-A PES4 RRT5 YGL193C YGR035C YGR079W YHL012W ANS1 SSP1 YIL055C NCA3 ALB1 ASG7 YJR056C YJR079W PRY2 YKR015C RLP24 YLR030W YLR031W YLR040C YLR042C REC102 YLR413W YLR462W YMR018W YMR244W YNL034W YNL155W IPI3 BSC4 PRM1 SNO2 DDR2 UTP23 TIR4 VAM10 YOR214C RRP36 FIT2 SMA1 LEE1 SPO19 PRM4 SET6 PRM3 RSA1 YIG1 YPR078C YPR202W
amino acid transmembrane transporter activity	GNP1 MUP1 GAP1 PUT4 DIP5
L-iditol 2-dehydrogenase activity	SOR2 SOR1
mating-type factor pheromone receptor activity	STE2 STE3
transmembrane transporter activity	YOL162W YOL163W
G-protein coupled receptor activity	STE2 STE3
cell adhesion molecule binding	AGA2 AGA1
pentose transmembrane transporter activity	HXT4 HXT2
pheromone activity	MFA1 MFA2
receptor activity	STE2 STE3 IZH2
solute:hydrogen antiporter activity	GEX1 GEX2
heme binding	CTA1 DIT2 OLE1 CYB5
mating pheromone activity	MFA1 MFA2

4. CONCLUSIONS AND FUTURE REMARKS

The objective of this research was to perform metabolic, phenotypic and molecular characterization of the ethanol resistant *Saccharomyces cerevisiae* cells which were previously obtained by evolutionary engineering strategy. In this context, growth curve, HPLC and microarray analyses were performed. Stress resistances were determined by MPN method, viability test, and spotting assay. Also, relationship between respiratory deficient phenotype and ethanol resistance were investigated. Results obtained from these analyses were evaluated both internally and respectively in cross-analyses.

According to the MPN analysis of constant mutants at 5% (v/v) ethanol stress, it was determined that some of these mutants showed slightly higher resistance or lower compared to wild type. These findings demonstrated that 5% (v/v) ethanol stress condition, which is not so high concentration, was tolerated by both the wild type and some of the mutants. Because these mutants were selected at this stress condition, maybe it was the reason why they couldn't show improved phenotype in terms of ethanol resistance.

Viability test is a means to test the ability of vegetative growth after exposure to proper concentration of ethanol. In this test; wild type, B2 and B8 were employed. Stress condition was determined as 10% (v/v) to 14% (v/v). However, none of them could grow at condition including higher than 10% (v/v) ethanol. B2 was the sole viable culture at 10% (v/v) ethanol stress condition. This result proves that B2 was more ethanol-resistant than wild type and B8. Previously, these mutants had been obtained at continuous 11.4% (v/v) ethanol stress,but in this study stress condition was applied only for a short time. This may be the reason of decrease in the ethanol resistance level.

Stability test of B2 and B8 were performed by utilizing 5-tube MPN method. Stress condition was chosen as 10% (v/v) ethanol stress. The results obtained at 72^{nd} hours of incubation showed that both of these mutants had higher resistance values at 10%

(v/v) ethanol stress in terms of survival rate normalized to wild type. There was no steady decrease in terms of resistance after each passage. For this reason, it can be concluded that all of the mutants showed stability for five successive passages.

In spotting assay, haploid (n) wild type, diploid (2n) wild type, mutant B2 and segregants of B2 were tested for their ethanol resistance on solid ethanol plates with proper concentration of ethanol. Segregants showed higher resistance compared to other cultures. However, resistance of wild type to ethanol was the same to that of B2. This probably resulted from volatility of ethanol. As a result, concentration of ethanol was lower than the adjusted value.

To understand whether there is a relationship between ethanol resistance and respiratory deficient phenotype, petite assay was performed. In this experiment, wild type, intermediate populations and individual mutants B2 and B8 were utilized. Then, petite frequency values were determined. According to the results, it was observed that wild type culture showed the highest frequency of petite colonies. This is an expected result. Because the ethanol tolerance of *S. cerevisiae* is dependent on the maintenance of functional mitochondria under the stress (Chi and Arneborg, 1999).

Growth curves of wild type, B2 and B8 were obtained at control condition. Because there is no stress application in this experiment, all of the cultures growed efficiently. Also, trehalose, glycogen contents (normalized to cdw) were determined at proper OD values. Glycogen and trehalose are two intracellular reserve carbohydrates present in yeast cells (Parrou and Francois, 1997). Thus, intracellular trehalose and glycogen concentrations were measured within this study in order to find out whether mutant B2 and mutant B8 accumulate them as distinct from wild type *S.cerevisiae*. The result was impressive since mutants accumulated significantly high levels of glycogen and trehalose compared to wild type. Maximum trehalose and glycogen accumulations in mutant B2 were approximately 7.3 and 1.6 fold, respectively, and in mutant B8 about 5.75 and 1.5 fold, respectively, higher than wild type. Furthermore, both mutants used intracellular trehalose and glycogen whereas wild type did not. According to these results it was suggested that metabolism of these ethanol-tolerant yeast strains might have been altered via evolutionary engineering approach in terms of the production/utilization of trehalose and glycogen.

In this study, wild type and mutant strain B2 were compared in terms of their metabolism at non-stress and 8% (v/v) ethanol stress condition. The substances that were investigated in this experiment are glucose, ethanol, acetate, glycerol and citric acid. It was observed that wild type and B2 strain showed similar growth characteristics at these culture conditions. However, specific growth rate value of B2 was significantly higher than that of wild type at ethanol stress condition. This finding indicated that B2 growed better compared to wild type in the presence of 8% (v/v) ethanol. According to HPLC analysis it was found that all of the glucose was completely consumed by both wild type and B2 until 24^{th} hour incubation. However, under 8 % (v/v) ethanol stress neither wild type nor B2 could not consume glucose by 48^{th} hour. This similarity between these cultures was resulted from the stress level. Since, 8% (v/v) ethanol was not so high for wild type, it was able to grow like mutant B2. At control condition, both wild type and B2 produced 7.94 (g/L) and 7.64 (g/L) ethanol, respectively. However, under stress condition production of ethanol decreased. Also, it was determined that B2 strain produced more glycerol compared to wild type under both control and stress conditions. It can be concluded that metabolism of evolutionary engineered B2 strain has shifted so as to produce more glycerol compared to wild type. Another impressive finding was that acetate metabolism of wild type and mutant was exactly opposite with regard to each other. At stress condition, wild type produced ~2 fold less whereas B2 produced ~3 fold higher acetate compared to that produced at control condition. Also, citric acid metabolism was examined. It was found that there was not any difference between wild type and B2 in terms of citric acid utilization.

Lastly, whole genome expression profile was obtained via microarray analysis for wild type and ethanol resistant mutants B2 and B8. DNA microarrays containing approximately ~6000 genes from *S.cerevisaie* printed on glass slides were used in order to evaluate differential expression of genes in wild type, B2, and B8 cells at control conditions. Triplicate experiments were conducted on mRNA isolated from exponentially growing wild type, B2 and B8 cells of *S. cerevisiae* strain CEN.PK. By using reverse transcriptase enzyme, cDNAs were synthesized from mRNAs which were isolated previously from wild type, B2, and B8 cells, Then, in the presence of Cy3-dCTP (green) dye labeled cRNAs were prepared. This labelled cRNAs were mixed and hybridized onto microarray slides (Agilent). The fluorescence intensities

of the Cy3 fluorophore were quantified to provide a quantitative measure of the relative abundance of mRNA levels in the three cell populations. All the genes whose expression levels was changed in B2 and B8 compared to wild type were grouped according to their function by using FunSpec database (Robinson *et al.*, 2002). It was found that there was difference at least 2 fold in terms of expression levels of 228 genes between B2 and wild type. Totally, 82 genes was up-regulated and 146 genes was down-regulated in B2 culture. For B8 culture, it was found that there was 396 genes whose expression levels were at least two fold different compared to wild type. 176 of these genes were up-regulated, and 220 of them were down-regulated. The most striking result from these data was that expression level of HO gene decreased ~500 fold, and expression level of IME4 (encoding methyl transferase) gene increased ~190 fold in both of these cultures compared to wild type. The level of down-regulation in the HO gene may be associated with the formation of diploidy during evolutionary engineering of haploid strains for ethanol resistance.

Because ethanol-stress resistance mechanisms are very complex in molecular level, in the future, investigation of the relationship between genes with high expression changes will be essential. In addition, real time PCR studies for candidate genes should be performed. In the long term, this ethanol resistance phenotype can be endowed to another strain or organism by directed genetic or environmental manipulation.

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APPENDICES

APPENDIX A

Table A.1 MPN Score Table for Five Tubes

Number of Positive Tubes in			MPN in the inoculum of the middle set of tubes
First set	Middle set	Last set	
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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