ISTANBUL TECHNICAL UNIVERSITY ★ GRADUATE SCHOOL OF SCIENCE ENGINEERING AND TECHNOLOGY

EVOLUTIONARY ENGINEERING of PHENYLETHANOL-RESISTANT *Saccharomyces cerevisiae*

M.Sc. THESIS

Can HOLYAVKİN

Molecular Biology and Genetics Department

Molecular Biology & Biotechnology and Genetics Programme

Thesis Advisor: Prof. Dr. Zeynep Petek Çakar

JANUARY 2013

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İSTANBUL TEKNİK ÜNİVERSİTESİ ★ FEN BİLİMLERİ ENSTİTÜSÜ

EVRİMSEL MÜHENDİSLİK YÖNTEMİ İLE FENİLETANOLE DİRENÇLİ *Saccharomyces cerevisiae* SUŞLARININ ELDESİ

YÜKSEK LİSANS TEZİ

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OCAK 2013

Can Holyavkin, a **M.Sc.** student of ITU **Graduate School Of Science, Engineering And Technology** student ID **521101102** successfully defended the **thesis** entitled "**Evolutionary Engineering of Phenylethanol-Resistant *S. 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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Date of Submission : **December 2012**
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To my mother,

FOREWORD

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ABBREVIATIONS

DNA	**:** Deoxyribo Nucleic Acid
EMS	**:** Ethyl Methane Sulfonate
ESR	**:** Environmental stress response
h	**:** Hour
µg	**:** Microgram
µL	**:** Microliter
µm	**:** Micrometer
mM	**:** Micromolar
mg	**:** Milligram
mL	**:** Milliliter
min	**:** Minute
MPN	**:** Most Probable Number
PCR	**:** Polymerase Chain Reaction
PEA	**:** Phenylethanol
RNA	**:** Ribonucleic Acid
RPM	**:** Revolution per minute
RT-PCR	**:** Real-Time Polymerase Chain Reaction
SD	**:** Synthetic Defined
w/t	**:** Wild Type
YMM	**:** Yeast Minimal Medium
YPD	**:** Yeast Extract- Peptone - Dextrose

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EVOLUTIONARY ENGINEERING OF PHENYLETHANOL-RESISTANT Saccharomyces cerevisiae

SUMMARY

Saccharomyces cerevisiae is one the most widely used model organisms in genetics, molecular biology and metabolic studies. In addition to its use in scientific research, it is one of the oldest microorganisms used for ages for industrial applications. *S. cerevisiae* is a unicellular eukaryotic organism, which can be found in haploid and diploid form, and can induce meiosis to generate new progeny of haploid from diploids (so called sporulation event) or reproduce asexually by budding. It shares high degree of homologies with higher eukaryotes like human. Due to these functional similarities, *S. cerevisiae* can be used in research related to cancer, aging and other human diseases.

In natural environment and in industrial applications, *S. cerevisiae* cells are often under stress resistance that results them environmental changes. These changes can be named as osmotic, high or low temperature, dehydration, starvation, metal ion stresses etc. Researchers are interested in the microbial resistance mechanisms to these different types of stresses. Additionally, they are searching for strategies to increase stress tolerance. Producers are also interested in increasing yield and for this reason; they are searching for stress-resistant microorganisms.

The aim of the present study was to obtain phenylethanol (PEA) resistant yeast strains via evolutionary engineering approach and perform transcriptomic and metabolic characterization to identify responsible pathways ans molecular factors in this resistance.

In this thesis study, firstly, phenylethanol-resistant *S. cerevisiae* mutants were obtained by evolutionary engineering approach. Phenotypic and genetic characterization was then carried out to identify the molecular principles of phenylethanol resistance in *S. cerevisiae*.

To apply evolutionary engineering to wild type *S. cerevisiae* cells, these cells were treated with a chemical mutagen EMS (Ethyl Methane Sulfonate) to increase the genetic diversity of the initial population to which selection would be applied. This mutagenized culture was cultivated at increasing phenylethanol concentrations in the culture medium along with the wild type to determine the initial stress level to be applied. Phenylethanol stress was then applied to this mutagenized culture. The phenylethanol concentration was increased gradually for each successive population. The first population was obtained upon 1.5 mL/L exposure to phenylethanol and the final 56^{th} population was obtained upon exposure to 3.6 mL/L PEA. The final population was used for randomly selecting ten individual mutants. Those ten

individual mutants, wild type and the final population were tested for phenylethanol resistance and it was observed that the evolved strain and the final population could grow at high phenylethanol concentrations at which the wild type could not show any sign of survival. One of the individual mutants which showed highest phenylethanol-resistance was chosen and genetic stability assay was applied. It was shown that phenylethanol-resistance was a genetically stable trait in the mutant tested. This evolved strain was termed C9.

In this study, PEA-resistant strain C9 was analyzed according to its cross-resistance abilities against various metals and organic compounds and compared with the wild type. Different concentrations of phenylethanol (2.5 mL/L and 3 mL/L), ethanol (8%, 10% and 12% v/v), acetate (0.004% v/v), cobalt (1 mM and 3 mM), boron (80 mM), copper (0.5 mM), hydrogen peroxide (0.5 mM) and nickel (0.2 mM) were used. It was observed that, phenylethanol-resistant mutant also show had cross-resistance to ethanol. Besides, C9 had increased sensitivity to cobalt stress.

To investigate the molecular mechanisms of phenylethanol resistance of the evolved strain, whole genome transcriptomic analysis was conducted for wild type and C9. Sampling for microarray analysis was carried out when the cultures were in their exponential phase of growth. The expression profile of the mutant was compared to that of the wild type.

The results showed that, phenylethanol-resistant C9 strain had immense amount of upregulated and downregulated genes in its genome under control conditions without any external stress. DNA microarray analysis showed that C9 had about 1000 upregulated and 800 downregulated genes which make up about 30% of whole genome. Such large scale changes in transcription levels indicate that some global expression response was always active in C9. That genome-wide expression program resembles a highly known large-scale stress reaction called “environmental stress response” (ESR).

DNA microarray analysis results indicated that there were about 1000 upregulated genes in C9 compared to wild type and majority of these genes were responsible for carbohydrate metabolism. With upregulated 166 genes, carbohydrate metabolism contributes to about 20% of all upregulated genes in C9. Following with 98 genes responsible for oxidative stress response, 63 genes for general stress response, 35 genes for cell wall reorganization and renewal, 21 genes for degradation of mitochondria and cell itself were found to be upregulated.

With 20% contribution, genes in carbohydrate metabolism were shighly upregulated in phenylethanol resistant C9 strain. In addition to increased activity of genes involved in glycolysis, many other genes associated with hexose transport, alternative carbon source utilization were also over-expressed. Same cellular states were also observed under ESR conditions which may indicate that C9 strain apparently induces ESR actively and continuously. Additionally, many putative genes involved in cell wall biosynthesis, autophagy, DNA damage response were up-regulated.

Same similarities were also observed in repression profile of C9 compared to wild type. Interpretation of downregulated genes showed that C9 strain selectively repressed major nucleic acid metabolism and ribosome synthesis. More than 81% of 821 downregulated genes were related to synthesis and binding of rRNA and tRNA, initiation of translation, RNA-DNA binding, and helicase activity. Additionally, similar regulations have also been observed previously during ESR in stressed-wild type strains upon initial stress exposure.

C9 also showed unique stress responses against alcohol stress. In comparison with wild type, phenylethanol-resistant C9 strain showed 234-fold higher expression of *ALD3* gene. This gene might be related to main resistance mechanisms against phenylethanol and ethanol. Increased *ALD3* gene expression may prepare cells to overcome excess amounts of aldehyde byproducts of alcohol degradation.

In this thesis study, a phenylethanol hyper-resistant *S. cerevisiae* mutant was obtained and characterized at transcriptomic level. Duw to the complexity and the large size of change in the transcriptomic response of the resistant mutant, it is not likely to point out one or a few genes that are crucial for phenylethanol resistance. However, it was shown that continuous induction of ESR genes may provoke specific resistance mechanisms. It could therefore be recommended to continue molecular research to enlighten the mechanism of phenylethanol resistance, for example, by overexpression/deletion of genes that were highly upregulated/ downregulated according to transcriptomic analysis results.

EVRİMSEL MÜHENDİSLİK YÖNTEMİ İLE FENİLETANOLE DİRENÇLİ *Saccharomyces cerevisiae* SUŞLARININ ELDESİ

ÖZET

Saccharomyces cerevisiae, genetik ve moleküler biyoloji çalışmalarında çok sık kullanılan, özellikleri iyi bilinen model organizmalardan biridir. Bilimsel araştırmalardaki kullanım alanlarının yanı sıra, *S. cerevisiae* endüstriyel üretimde de önemli bir yere sahiptir. Özellikle etanol üretimi ve ekmek yapımında yaygın olarak kullanılmaktadır.

S. cerevisiae, tek hücreli bir ökaryotik mikrorganizma olup, tomurcuklanma yolu ile hem eşeysiz, hem de mayoz bölünme gerçekleştirerek eşeyli olarak çoğalabilmektedir. *S. cerevisiae*'nin yüksek ökaryotların genomu ile gösterdiği yüksek homoloji de bir çok bilimsel çalışmada yarar sağlamaktadır. Özellikle insan genomu ile olan benzerliği sebebiyle, kanser, yaşlanma ve birçok hastalık mekanizmaları *S. cerevisiae* hücreleri kullanılarak araştırılmaktadır.

Mikroorganizmalar, doğal ve endüstriyel ortamlarda sıkça stres koşullarına maruz kalmaktadır. Bunlar, yüksek yada düşük sıcaklık, ozmolarite, oksidatif stres, mekanik stres ve metal stresi gibi streslerdir. Araştırmacılar, mikrobiyel stres direnç mekanizmalarını araştırmakta ve aynı zamanda çeşitli streslere karşı direnç düzeylerini arttırmaya çalıştırmaktadırlar. Aynı zamanda, endüstriyel verimin arttırılması amacıyla, üreticiler de stres direnci yüksek mikroorganizmalar aramaktadırlar.

Bu tez çalışmasında, feniletanole dirençli maya hücreleri elde edilerek feniletanole karşı geliştirilen direncin moleküler mekanizmalarının incelenmesi amaçlandı. Bunun için ilk olarak evrimsel mühendislik yöntemi ile feniletanole dirençli *S. cerevisiae* mutantları elde edildi. Ardından, feniletanole dirençli *S. cerevisiae* mutantlarında, feniletanol direncinin moleküler mekanizmasını anlamak amacıyla fenotip analizleri, fizyolojik ve transkriptomik analizler gerçekleştirildi.

Çalışma başlangıcında evrimsel mühendislik yaklaşımı yaban tip *S. cerevisiae* hücreleri üzerinde gerçekleştirildi. Bu amaçla ilk olarak başlangıç popülasyonunda genetik çeşitliliği arttırmak için kimyasal bir mutajen olan etil metan sülfonat (EMS) yaban tip maya hücrelerine uygulandı. Elde edilen mutajenize edilmiş maya kültürü, sonrasında seçilime maruz bırakılarak, kültür içinden istenen fenotipteki bireylerin seçilmesi planlandı. Seçilim süresince, ilk başta düşük konsantrasyonlarda (1.5 mL/L) feniletanol kültüre uygulandı ve inkübasyon gerçekleştirildi. Sonraki basamakta, hayatta kalan maya hücreleri, daha yüksek bir feniletanol konsantrasyonunda tekrar inkübe edildi. Her basamakta, OD_{600} değerleri ölçüldü ve hayatta kalma oranları

kritik bir seviyeye düşene kadar bu seçilim işlemleri devam edildi. En son 3.6 mL/L feniletanol konsantrasyonuna kadar gelindi ve 56. nesilde seçilim işlemi durduruldu. Bu elde edilen son popülasyondan rastgele 10 birey seçildi ve direnç yeteneklerine göre kıyaslandı. On mutant birey, yaban tip ve son popülasyonun feniletanol dirençleri damlatma ve en muhtemel sayı (MPN) yöntemleri ile ölçüldü ve karşılaştırıldı. Elde edilen 10 birey arasından en yüksek direnci gösteren birey seçildi ve "C9" olarak adlandırıldı. C9 bireyinde genetik kararlılık testi uygulandı. Bu test ile maya mutantının feniletanol direncinin kalıcı olup olmadığı belirlendi. Damlatma ve MPN çalışmaları, bu bireyin feniletanol direncinin değişmediğini gösterdi. İlgili mutantta feniletanol direnci genetik olarak kararlı bulundu.

Feniletanole dirençli maya mutantının çapraz direnç özellikleri de incelendi. Bunun için çapraz direnç testi uygulandı. Bu testte, seçilen mutant ve yaban tip, farklı konsantrasyonlarda feniletanol (2.5 mL/L ve 3 mL/L), etanol (8%, 10% ve 12% v/v), asetat (0.004% v/v), kobalt (1 mM ve 3 mM), bor (80 mM), bakır (0.5 mM), hidrojen peroksit (0.5 mM) ve nikel'e (0.2 mM) maruz bırakıldı, hayatta kalma oranları kıyaslandı. Tüm bu stres faktörleri içinde, feniletanol dirençli mutant, etanole karşı da direnç gösterdi. Etanol ve feniletanol'ün hücresel etki mekanizmalarının muhtemel benzerliklerinden dolayı bu iki stres faktörünün çapraz dirence neden olması beklenen bir durum olarak nitelendirilebilir. Feniletanol dirençli C9 mutantı, aynı zamanda kobalt'a karşı belirgin bir hassasiyet göstermektedir.

Feniletanole dirençli mutantın direnç mekanizmasının moleküler düzeyde incelenmesi için transkriptomik analiz gerçekleştirilmiştir. Bu amaçla, DNA mikroarray yaklaşımı kullanılmış ve C9 ile yaban tip arasında, kontrol koşullarındaki transkripsiyon profilleri karşılaştırılmıştır. Analiz sonucunda, C9'un genel transkripsiyon profilinde ilgi çekici sonuçlara rastlanmıştır. Bu sonuçlardan biri, çok yüksek sayıda gende transkripsiyon artışı görülmesidir. *S. cerevisiae* genomunda bulunun yaklaşık 6000 gen içerisinde 1000 kadar genin anlatımı artarken 800'e yakın gende de anlatımda azalış olmuştur. Tüm bu genler, maya genomunun yaklaşık %30'una denk gelmektedir. Bu yüksek transkripsiyon profili, maya hücrelerinin stres anında gösterdiği kısa süreli cevaplar ile benzerlik göstermektedir. Normalde kısa süren ve çok sayıda kendini gösteren bu reaksiyonlar çevresel stres cevabı (Environmental stress response, 'ESR') olarak bilinmektedir. Feniletanole dirençli mutantta ESR'den sorumlu genler önemli düzeyde aktif durumdadır.

Feniletanole dirençli mutanta ait transkripsiyon profilinde ilk göze çarpan anlatımı artan 1000 kadar gen arasında, karbonhidrat metabolizması ile ilgili genlerin önemli bir yer kaplamasıdır. Anlatımı artan 166 gen ile karbonhidrat metabolizmasından sorumlu genler, C9'un anlatımı artmış tüm genlerinin yaklaşık %20'sini oluşturmaktadır. Bunu 98 gen ile oksidatif stres cevabı izlemektedir. Aynı zamanda anlatımı artmış genler arasında 63 tanesi genel stres cevabından, 35 tanesi hücre duvarı organizasyonundan, 21 gen ise otofaji ve mitokondri yıkımından sorumludur.

Belirtilen %20'lik katkı karbonhidrat metabolizmasının, C9 mutantında önemli bir şekilde tetiklenmiş olduğunu göstermektedir. Benzer durum, daha önce tanımlanan ESR koşullarında da görülmüştür. Hücreler, stres altında kısa süreliğine glikoz

metabolizmasını hızlandırmaktadır. Ancak, C9 mutantında bu genlerin anlatımları ortamda stres koşulları bulunmasa da aralıksız olarak gerçekleşmiştir.

Benzer durum, anlatımı azalan genlerde de görülmüştür. Analiz sonuçlarına göre, C9 bireyinde özellikle nükleik asit metabolizması ve protein, ribozom sentezinde görev alan çoğu genin anlatımı ciddi oranda azalmıştır. Anlatımı azalan 821 genin %81'i rRNA ve tRNA'ların sentezi ve bağlanmasında, translasyonun başlamasında, RNA-DNA bağlanmasında, helikaz aktivitesinde görev almaktadır. C9'da protein sentezini azaltacak yönde görülen bu değişiklikler aynı zamanda genel ESR koşullarında da görülmektedir. Bu sonuçlar da feniletanol dirençli C9 bireylerinin sürekli bir ESR durumunda olduğu görüşünü desteklemektedir.

C9'un aynı zamanda, özelleşmiş stres cevapları da verdiği görülmüştür. Yaban tipe kıyasla, aldehit dehidrogenaz 3 adlı genin 234 kat daha fazla anlatımı gerçekleşmiştir. Alkolün yıkılması sırasında ortaya çıkan bir toksik madde olan aldehidin yıkılmasından sorumlu bu genin yüksek şekilde anlatılması, C9'un sahip olduğu feniletanol direnci için önemli olabilir.

Bu tez çalışmasında, feniletanol dirençli *S. cerevisiae* hücreleri evrimsel mühendislik yöntemleri ile elde edilmiş ve transkripsiyon seviyesinde karakterizasyonu gerçekleştirilmiştir. Dirençli maya mutantının yüksek seviyede gösterdiği gen anlatımı, yaban tip hücrelerin stres anında verdiği anlık tepkilerle benzerlik göstermektedir. Anlatımın yüksek ve karmaşık olması, feniletanol direncinin tek bir gen veya gen grubu ile ilişkilendirilmesini zorlaştırmaktadır. Bu sebeple, çevresel stres cevaplarının daha iyi anlaşılması ve feniletanol direncinin temel kökeninin bulunması için anlatımı önemli ölçüde artmış ya da azalmış genlerin delesyonu ya da aşırı anlatımı gibi ilave moleküler araştırmaların yapılması önerilebilir.

1.INTRODUCTION

1.1 General Information about *Saccharomyces cerevisiae*

Saccharomyces cerevisiae is “the yeast” that has been highly used as a primary ethanol producer in food industry and as an important model organism in molecular biology research (Dickinson and Schweizer, 2004).

S. cerevisiae, which is also known as brewer’s yeast, baker’s yeast or budding yeast, is a unicellular organism that is found in wide dispersion of natural habitats such as plant leaves and flowers, soil and salt water. *S. cerevisiae* is a strongly fermentative yeast. It is member of the fungi kingdom, under ascomycota phylum, saccharomycetes class (Kurtzman and Fell, 1998).

Table 1.1: Taxonomic location of *Saccharomyces cerevisiae*.

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

Cell structure is mainly oval-shaped; however it’s size is highly variable that changes according to environmental status (e.g. stress factors or availability of nutrients) and the age of organism. Overall, its size varies between 5 to 12 μm length and about 5 to 10 μm in width (Walker *et al.*, 2002).

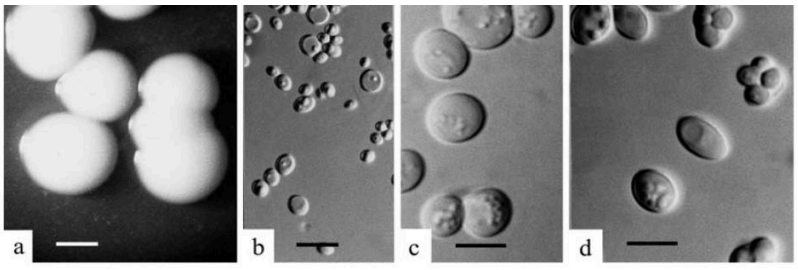

Figure 1.1: *Saccharomyces cerevisiae* a.) Colonies under rich media. Bar: 1 mm , b.)Vegetative cells. Bar: 10 μm, c.) Vegetative cells. Bar: 5 μm, d.) Ascospores. Bar: 5 μm. (Kurtzman and Fell, 1998)

Cell size and shape are mainly determined by characteristic cell wall. *S. cerevisiae* spends a significant amount of metabolic energy in cell wall construction. Its mass in terms of dry weight may account for about 10–25% of the total cell mass. The inner layer of wall consists of mechanical-resistant polysaccharides (such as branched 1,3-β-glucan), which also function as scaffold for outer layer. Outer layer includes mannoproteins which have main protective properties. Mannoproteins constitute the cell wall mass of about 30-50%, glucan polysaccharides is of about 35-50% and chitin is 1.5-6% (Klis *et al.*, 2006).

The cell wall of yeast has also important functions such as stabilization of internal osmotic conditions. The osmolarity of cytoplasm of *S. cerevisiae* and other fungi species is generally higher than the outside (Klis *et al.*, 2006). Cell wall limits excessive water influx toward cytoplasm and cell lysis. Cell wall also maintain sphysical resistance to cell via its high elastic properties and mechanical strength (Kollar *et al.*, 1995).

S. cerevisiae plasma membrane shares some common properties both with prokaryotes and eukaryotes. Like prokaryotes, *S. cerevisiae* cells are unable to synthesize polyunsaturated fatty acids, thus yeast membrane includes only mono-saturated or monounsaturated fatty acids. On the other hand, like other eukaryotes, their membrane contain large proportions of phosphatidylcholine and sterols, which are absent in most of the prokaryotes. Yeast lipid bilayer has also some unique properties such as presence of ergosterol rather than cholesterol, high proportions (70-80%) of unsaturated fatty-acyl residues (Ingram and Buttke, 1984).

The unsaturated fatty acid (UFA) composition of *S. cerevisiae* is relatively simple, consisting the mono-UFAs palmitoleic acid (C16:1) and oleic acid (C18:1). Both UFAs are formed in *S. cerevisiae* by the oxygen and NADH-dependent desaturation of palmitic acid (C16:0) and stearic acid (C18:0), respectively, catalyzed by a single integral membrane desaturase encoded by the OLE1 gene (You *et al.*, 2003).

Optimal growth temperature of *S. cerevisiae* is between 33 and 35°C in 10-30% (w/v) glucose; minimum growth temperature is about 4°C in 10% (w/v) glucose and 13°C in 50% (w/v) glucose. Its maximum growth temperature has been reported as

38-39°C (Jermini *et al.*, 1987). *S. cerevisiae* is naturally resistant to low pH conditions; it is capable to survive down to pH 1.6 in HCl (Bergman, 2001).

The yeast *S. cerevisiae* is capable of existing in both haploid (one copy of each chromosome) and diploid (two homologous or heterologous copies of each chromosome) stage. Both forms can divide through mitosis, with daughter cell budding of mother cell. Haploid cells could be 'a' or 'α' mating type depending on the allele (MATa and MATα) at MAT locus. These types differ at their cell surface receptors that detect opposite pheromone. MATa cells produces mating pheromone "a factor" that make able to mate with MATα cells. Different mating types detect each other and fuse when present in the same media. Cell proliferation of *S. cerevisiae* on rich medium is robust with a doubling time of 90 min (Esslinger, 2009). Diploid cells undergo meiosis under stressful conditions such as stress and absence of carbon and nitrogen sources (Dickinson and Schweizer, 2004).

Spore containing ascus is formed by vegetative cells without conjugation. Spores may be formed from ascus after prolonged incubation. Ascus contains 1-4 spores which are generally spherical or oval-shaped (Cook, 1958).

1.2 Importance of *S. cerevisiae* as a Model Organism

S.cerevisiae is one of the most common eukaryotic model organisms in molecular biology and genetics research. Its importance comes from highly known genetic and metabolic structure and cell behavior under certain circumstances.

First, its short doubling time (1.5 to 2 h at 30 °C) make this organism easily cultured. Short doubling time and low requirements for incubation also decrease the cost of yeast based experiments (Esslinger, 2009).

S. cerevisiae is the first eukaryotic organism the whole genome of which was sequenced (Goffeau *et al.*, 1996). The genome is compactly organized in 16 chromosomes with about 6275 genes. To date, more than 90% of these genes have been deleted for functional analysis (Cherry *et al.* 1998). The availability of the whole genome sequence data and a set of deletion mutants covering 90% of the yeast

genome have further enhanced the power of *S. cerevisiae* as a model for understanding the regulation of eukaryotic cells.

30.8% of total ORFs in yeast genome have homology with mammalian genome (p-value: $1x10^{-10}$) (Botstein, 1997). Many genes that play important roles in human genomic structure have also close homology in yeast genome. So far, various yeast and human homologous gene pairs with known activities have been identified (Table 1.2).

Table 1.2: Functional homologies and disease related homologies between human and *S. cerevisiae* genome. (Botstein et al., 1997)

Yeast gene	Human homologue	% of Sequence Similarity	p-value
MSH2	Mutator gene (*MSH2*, colon cancer)	65	3.8e-255
YCF1	Cystic fibrosis conductance regulator (*CFTR*)	57	2.4e-157
GEF1	Voltage-gated chloride ion channel	58	3.4e-95
ACT1	Cytoskeletal gamma actin	94	1.4e-243
SOD1	Superoxide dismutase (*SOD-1*)	69	8.9e-56
RHO1	GTP-binding, Ras-like (bovine *RHO*)	81	3.1e-92
CDC28	Cell cycle control (*CDC2*)	78	5.0e-130

All of these close homologies make *S. cerevisiae* an important model organism to study aging, regulation of gene expression, signal transduction, cell cycle, metabolism, apoptosis, neurodegenerative disorders and many other biological processes (Botstein et al., 1997).

S. cerevisiae allow easy transformation that makes addition and deletion of genes possible through homologous recombination. In fact, it is the first eukaryotic organism to have its DNA transformed in 1978 (Hinnen *et al.*, 1978). Currently, there are various type of transformation protocols available that produce transformants very efficiently, such as lithium-acetate method, spheroblast method, ballistic method or electroporation (Kawai *et al.*, 2010). Genetic manipulation of yeast is easy and cheap, whereas such manipulation, even when possible in mammalian systems, is neither easy nor cheap. Additionally, *S. cerevisiae* cells may grow as a haploid that makes working with knock-out strains easier.

1.3 General Effects of Alcohols on Organism

One of the most important challenges in alcohol production industry is obtaining high concentration alcohol with low cost (Lin, 2006). Current production techniques require costly purification steps (such as distillation) to produce high-titer ethanol. The main reason of this limitation is the alcohol sensitivity of yeast that it produced. Although some strains of yeast can tolerate up to 20% (v/v) ethanol concentration (Ogawa *et al.*, 2000), many industrial strains cannot efficiently continue fermentation at over 13% (v/v) ethanol concentration (Bai *et al.*, 2004). Increasing of alcohol sensitivity threshold of yeast arouses great interest in the industry, since it will possibly decrease the distillation cost.

However, improving the alcohol tolerance of yeast strains is a quite difficult task because alcohols have many damaging effects on multiple levels of cellular structure and pathways. These effects vary from DNA damage to distribution of membrane structure.

1.3.1 Effects on membrane

The primary interaction site of the cells that comes into contact with alcohols is the plasma membrane. As an amphiphile molecule, alcohols have both hydrophilic (-OH : hydroxyl group) and hydrophobic (acyl group) sites. Similar amphiphilic structure is also observed in phospholipids which are the basic building blocks of plasma membrane.

Under alcohol exposure, alcohol molecules are integrated into plasma membrane structure because of the similar amphiphile structure. Previous studies showed that hydroxyl group of alcohols interact with polar head of phospholipids at lipid-water interface through hydrogen binding with lipid phosphate groups (Barry and Gawrisch, 1994), (Patra *et al.*, 2006). Moreover, alcohols can also penetrate into zone of upper chain segments through Van der Waals attraction between ethyl group and upper chain segments (Feller, 2002). This integration affects both membrane properties and functions.

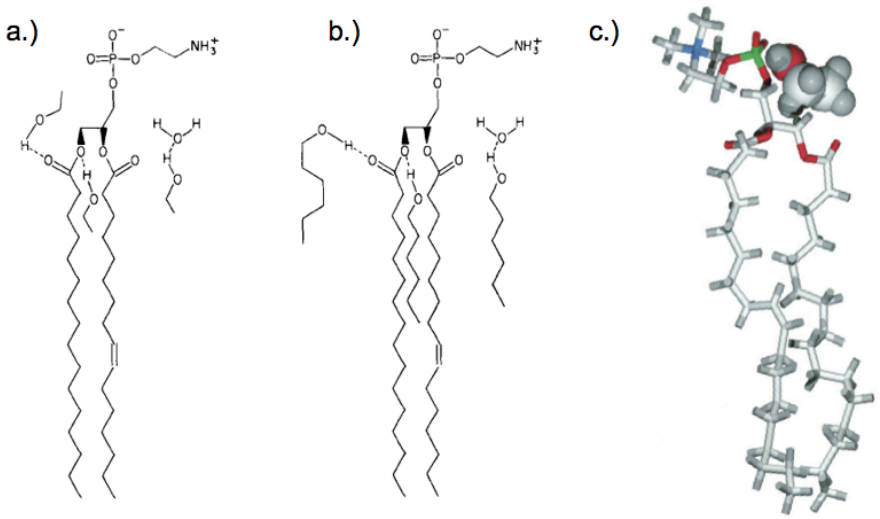

Figure 1.2: Relative sizes of phospholipids with ethanol and hexanol, a.) Ethanol-lipid, and Ethanol-water binding by hydrogen bonding b.) Hexanol-lipid and Hexanol-water binding formed by hydrogen bonds (Ingram and Buttke, 1984). c.) Representative conformation of association of ethanol with a phospholipid molecule. Ethanol prefers to form hydrogen bonds with the lipid phosphate group whereas the ethyl residue is directed toward the bilayer hydrophobic core (Feller, 2002).

1.3.1.1 Effects on lipid ordering

Structure and motion characteristics of biological membranes are explained by "fluid mosaic model". According to this model, membranes contain heterogeneously dispersed different kinds of lipid molecules that move in fluid-like motion. Fluidic properties of membranes are quantified by term of the "temperature of transitions state" (T_M) which is the required temperature for transition between two forms of membranes (gel and liquid-crystalline phases). In terms of fluidity, lower T_M indicates that the membrane can turn into less-ordered liquid form in lower temperatures. Decrease in transition temperature generally causes loss of rigidity (Weber and de Bont, 1996).

Many studies showed that alcohol-membrane interactions decrease the gel to liquid transition temperature (T_M) which lead to more disordered lipid structure (Chin and Goldstein, 1977). The binding of ethanol to lipid molecule blocks nearby lipids to come closer and inhibit formation of tight structures between lipids via steric hindrance (Ingolfsson and Andersen, 2011). In absence of attached alcohols, lipid

molecules are sticking together in bilayer more than those with an attached alcohol. Alcohol attached lipids are shifted into center of bilayer, eventually leads to thinner and disordered bilayer (Patra, 2006). Consequently, alcohols decrease the membrane rigidity and lower the T_M.

1.3.1.2 Effects on bilayer stability

As explained in "fluid mosaic model", biological membranes consist of different kind of macromolecules. Phospholipids constitute great majority of this diversity with various sizes and structures. Every type of phospholipids has specific functions which are proper to their structures. The characteristics of their structures are determined by their relative dimensions which is the phospholipid head group water interfacial area (a), hydrocarbon chain length (l) and hydrocarbon chain volume (v) (Sikkema *et al.*, 1995). These lipids pack together in different forms according to these parameters. For example, lipids with bigger head group area (a) have tendency to form micellar or hexagonal structure, which have important functions in cell division, membrane movement, and phagocytosis (Seddon, 1990).

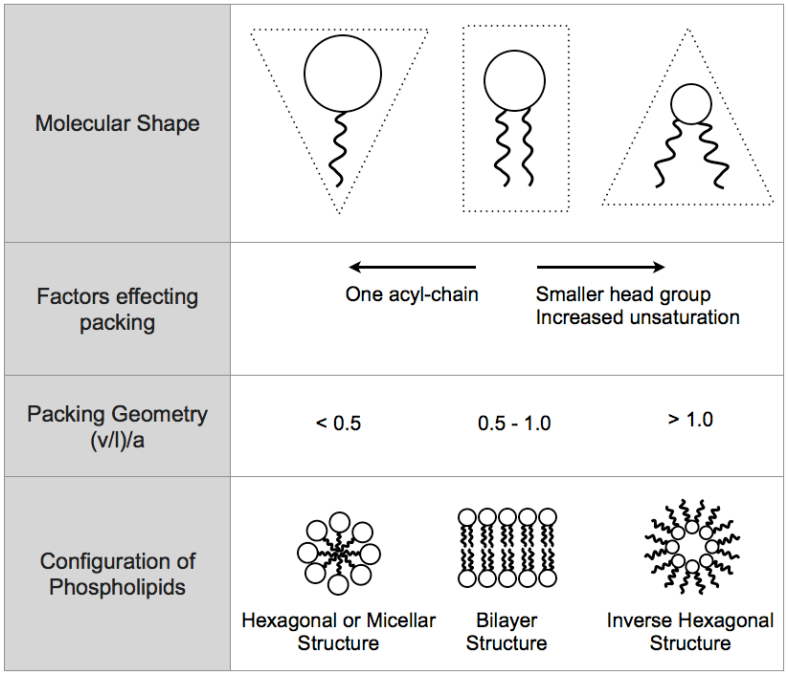

Figure 1.3: Molecular shape of various phospholipids and their corresponding polymorphic lipid phases (Weber and de Bont, 1996)

NMR studies showed that, alcohols that bind polar head groups of lipids, generally increase the surface area of head groups (a) relatively to baseline area (v/l) that produce inverted cone shape. Under alcohol exposure, bilayer forming lipids (which have similar 'a' and 'v/l' value) shifted toward micelle forming lipids (Weber and de Bont, 1996). Likewise, cone shaped lipids shifted to bilayer forming lipids. As a result, alcohol binding fully changes the mosaic structures of membranes and disturb the functions of each kind of lipids (Figure 1.3).

1.3.1.3 Effects on membrane permeability

Changes in lipid order and bilayer stability impair the influx and efflux control systems on membrane. Weakening permeability barrier of membrane is important because it regulates the passage of important solutes between cell and environment. Permeability has also vital importance in energy transduction (Nicholls, 1982).

Alcohol-dependent permeability increases the leakage of ions (e.g. protons) and small metabolites (Ingram and Buttke, 1984). Loss of ion gradient leads to reduction in proton motive force that is used in influx and efflux systems (Eddy, 1982). Thus, alcohol-based leakage leads to loss of ion gradient on both sides of membrane that diminishes nutrient uptake and accumulation that leads eventually to growth inhibition in yeast cells (Ingram and Buttke, 1984). Increased permeability to ions also critically alters pH levels of cell or causes loss of important metabolites (Sikkema *et al.*, 1995).

1.3.1.4 Effects on membrane bound proteins

Cellular membrane harbours many enzymes involved in various functions including transport, reception, electron transport chains, etc. Many studies have shown that these membrane-bound enzymes are affected by composition and structure of membrane. Interaction with solvents such as alcohols changes the physiochemical properties of the membrane and therefore affect the activity of membrane-bound enzymes (Veld *et al.*, 1991). Especially, transmembrane carrier proteins are highly affected from bilayer thickness which is a factor changed under alcohol exposure (Pope *et al.*, 1984).

1.3.2 Aldehyde stress

Upon entry into cytoplasm, great majority of alcohols are metabolized in oxidative pathways. In oxidative pathway, alcohols are converted into aldehydes by cytoplasmic alcohol dehydrogenases (ADH), catalases or cytochrome p450 enzymes (Beier *et al.,* 1985) (Aranda and Olmo, 2003). Aldehyde, a metabolite of alcohol, is further metabolised to carboxylic acids by aldehyde dehydrogenase enzymes (ALD) (Oyesanmi *et al.*, 2010).

The enzymes alcohol dehydrogenase (ADH), cytochrome P450 2E1 (CYP2E1), and catalases contribute to oxidative metabolism of alcohol. ADH converts alcohol to aldehyde. This reaction involves nicotinamide adenine dinucleotide (NAD+), which is reduced by two electrons to form NADH. Catalase, located in peroxisomes, requires hydrogen peroxide (H_2O_2) to oxidize alcohol. CYP2E1 presents predominantly in the cell's microsomes, assumes an important role in metabolizing ethanol to acetaldehyde at elevated ethanol concentrations. Acetaldehyde is metabolized mainly by aldehyde dehydrogenases (ALD) to form acetate and NADH.

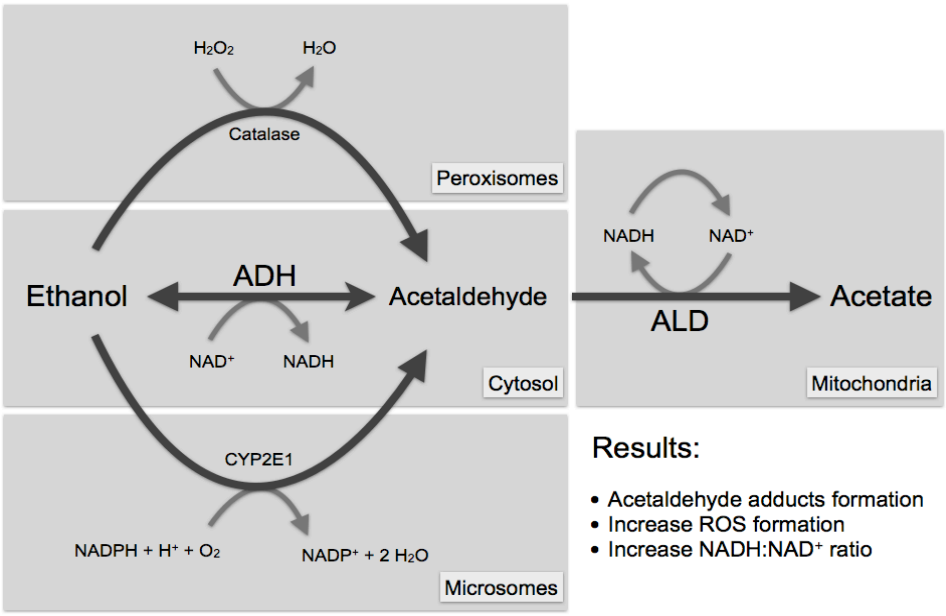

Figure 1.4: Oxidative pathways of alcohol metabolism.

Aldehydes, intermediate products of alcohol metabolism are highly toxic and reactive molecules. In human, they are responsible for damage in liver and other

tissues (ECRI, 2010). Acetaldehyde, a major metabolite of ethanol metabolism is a known carcinogen (Woutersen *et al.*, 1984) and molecule that leads to cell death and apoptosis through DNA damage (Singh and Khan, 1995). Aldehyde-induced damage to DNA occurs by different ways including strand breaks, free radical generation and DNA cross-links by modification of proteins and DNA (Ewald and Shao, 1993). Additionally, acetaldehyde covalently binds to DNA and form adducts, interferes at many sites with DNA synthesis and repair (Yu *et al.*, 2010). DNA adducts that are formed in genome may cause polymerase errors and lead to mutation in critical genes (Matter *et al.*, 2007). Some of the adducts that formed by acetaldehyde also block translation DNA synthesis (DNA repair by polymerases) and induces mutations (Singh *et al.*, 2009).

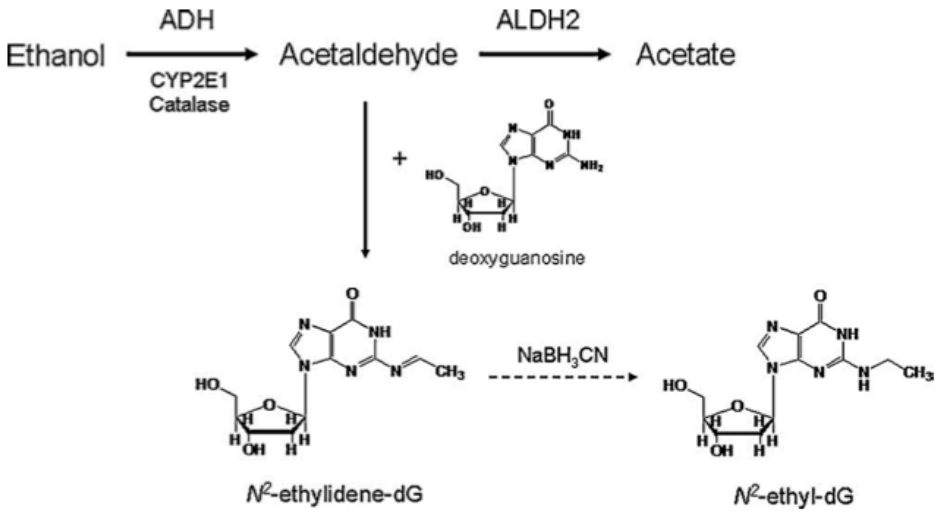

Figure 1.5: Formation of the DNA adducts (N2-ethylidene-dG and N2-ethyl-dG). Alcohol is converted to acetaldehyde by ADH, CYP2E1, and catalase, and then to acetate by ALDH2. Acetaldehyde can interact with deoxyguanosine to form a Schiff base N2 ethylidene-dG. (Yu *et al.*, 2010)

Inter-strand cross links are other results of aldehyde stress. Two molecules of acetaldehyde bind both strands of DNA covalently and block many vital processes such as transcription, recombination and DNA replication (Liu *et al.*, 2006).

Previous studies showed that aldehydes also bind to proteins. Especially acetaldehyde has high tendency to interact with specific amino acids such as lysine (Tuma and Casey, 2003). In general, enzymes which have lysine-rich domains in

outer surfaces are under threat of reactive aldehyde attack that diminishes the enzyme activity before the irreversible binding (Zakhari, 2006).

1.3.3 Oxidative stress

Once alcohol enters the cytoplasm of cell, it is immediately metabolized into other compounds to prevent further alcohol-related damage. These metabolic processes include the oxidation of alcohols into aldehydes and carboxylic acids. Whole process is managed by several enzymes (such as alcohol dehydrogenase, aldehyde dehydrogenase) and electron carriers (such as NAD^+) (Lieber, 2005).

During the oxidation steps, electrons originated from alcohol are transferred to nicotinamid adenine dinucleotide (NAD^+) and form NADH. Later then, electrons stored in NADH are transferred to last electron acceptor oxygen molecule via electron transport system (ETS) in mitochondria. Electron transfer to oxygen must be carefully controlled in cells to prevent production of reactive oxygen species (ROS). Alcohol metabolism leads to small yet significant increase in mitochondrial activity in parallel with higher superoxide production (Koop, 2006).

In addition to oxidation of alcohols by alcohol dehydrogenase, there is another oxidation pathway which is regulated by cytochrome family enzymes. Although cytochrome contribution to the alcohol oxidation is low, it still produces significant amounts of ROS (Koop and Coon, 1986).

In cytochrome-based metabolism same products are formed by different chemical pathways. These pathways use additional oxygen to metabolize alcohol that can lead to ROS production (Kopp, 1992).

1.3.4 Water stress

Extracellular water has the tendency to interact with low molecular mass solute molecules in the environment. Strength of this interaction with solute molecules determines the availability of water to the cell. Even small amounts of solutes may greatly lower the available water and eventually inhibit cell growth. Low water availability may affect the structures of hydrated enzyme and membrane molecules (Hallsworth, 1998).

Water availability is measured with water activity (a_w) which is accepted as 1 for pure water. Presence of solutes decreases water activity. Majority of yeast strains grow in narrow water activity range which is between 0.9 and 1.0. Most strains are unable to survive under 0.92 a_w (Jones and Greenfield, 1986).

Water stress is seen as critical decrease in water availability to cell. This decrease leads to disruption of hydrogen bonds of important proteins. Additionally, functions and structures of phospholipid bilayers are disrupted when hydrogen bonds are broken.

The structure of membrane is mainly maintained by lipid molecules that are bound each other with hydrogen bonding. Hydrogen-bonded network damaged when these lipid molecules move too far or come close to each other. That ordered structure is preserved by water which maintains a relatively constant distance between lipid molecules. Intra-membrane water have critical role in stabilization of this hydrogen bonded network. Water soluble alcohols replace and disrupt role of intra-membrane water. Under presence of alcohol, distances between lipid molecules are not maintained and that leads to more disordered bilayer (Hallsworth, 1998).

Water soluble alcohols are agents that decrease water activity. A small increase in concentration of these agents sharply decreases the water activity. For example, medium containing 20% (v/v) ethanol has 0.895 a_w which is below the growth limit of yeast. Even low concentrations (5% ‘v/v’) of ethanol affects yeast metabolism and growth (Jones and Greenfield, 1986).

1.4 Effects of Phenylethanol on Yeast

Phenylethanol (or phenyl ethyl alcohol - PEA) is an aromatic alcohol compound widely found in flora. It is naturally found in essential oils in many plants such as rose. With formula $C_6H_5CH_2CH_2OH$, PEA carries basic characteristics of alcohols with its amphipathic structure. It has one polar (-OH hydroxyl) group and one non-polar (phenyl-ethyl) group. The phenyl group of PEA gives aromatic properties to the molecule.

Phenylethanol has been widely used in the cosmetic, perfume, and food industries and is mainly produced by chemical synthesis (Hua and Xu, 2011). PEA is structurally very similar to ethanol, that makes this chemical important chemical to understand the effects of ethanol. Current production of phenylethanol is mainly based on chemical synthesis which is competetively cheaper than biological production. However, the raw materials used in chemical synthesis is hiaghly toxic for human health. Creating alternative production line may prevent the usage of these harmful materials. More importantly, there is no extensive studies about effects of phenylethanol. Current literature about phenylethanol is highly limited. Also, phenylethanol metabolism pathways are quite unclear (Hua and Xu, 2011).

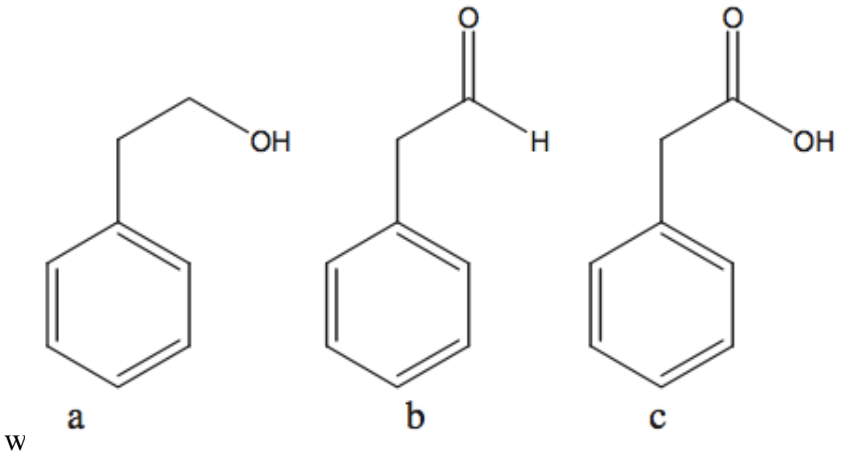

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Figure 1.6: Chemical structure of a.) 2-phenylethanol, (b) 2-phenylacetaldehyde, (c) 2-phenylacetic acid (Zhu *et al.*, 2011).

PEA is metabolized by alcohol dehydrogenase to form phenylacetaldehyde. This intermediate byproduct is then further metabolized into phenylacetic acid via aldehyde dehydrogenase (Çelik, 2004).

Like other alcohols, PEA disrupts the order between molecules, reduces acyl chain order and causes increased fluidization in membrane (Silver and Wendt, 1967). PEA also alters the helix-helix interactions of proteins in membrane structure which may lead to detrimental effects in faulty protein folding or changed transmembrane signaling (Anbazhagan *et al.*, 2010).

As a rule (Traube's rule), for every additional methyl groups, an alcohol becomes three times more effective in decreasing interfacial tension of the bilayer (Ly and

Longo, 2004). Considering the principle, every additional methyl group increases the partition of alcohol into the interface three times more. Alcohols that have bigger hydrophobic regions are more susceptible to penetrate and pass across the membrane (Patra *et al.*, 2006). With bigger hydrophobic tail, effect of PEA is likely to be more significant than smaller alcohols such as ethanol.

It is reported that, PEA causes increased membrane fluidization (Ingram and Buttke, 1984), ion leakage (Seward *et al.*,1996) and reduced ion-coupled amino acid, glucose intake (Lester, 1995). It was also shown that PEA inhibits the growth of *S. cerevisiae* by causing respiratory deficiency (Wilkie and Maroudas, 1969). It is proposed that, respiration deficiency is due to direct inhbition of respiration through increased mitochondrial permeability. There are also reports showed that PEA inhibits DNA, RNA synthesis (Bostock, 1970) and some cytoplasmic enzymes (Zhu *et al.*, 2011). In addition, Lutthini *et al.* (1993) reported that the main effect of PEA is due to production of highly toxic molecule phenylacetaldehyde during PEA degradation.

All of these damaging effects make PEA an efficient bactericide in pharmaceutical industry. Concentrations of 2 mL/L and 3 mL/L completely inhibit growth of many bacteria and fungi species (Lester, 1995), (Ingram and Buttke, 1984). *S. cerevisiae* growth rate decreases by 75% in 2.5 g/l PEA (Seward *et al.*,1996).

1.5 General Stress Responses in *S. cerevisiae*

For all living organisms, keeping internal homeostasis is one of the most important requirements for survival. However, homeostatic balance is always under threat by sudden or extreme changes in environmental conditions. Excessive fluctuations in environment may severely damage cell structure and homeostasis of organisms in various ways. For survival, organisms should resist to effect of these environmental shifts. These rapid changes can be observed in different terms such as temperature, pH, osmotic changes, oxidative stress pressure, cold/hot shock, alteration/absence of carbon/nitrogen source. Under such stress conditions, cells reorganize their physical and metabolic structure to keep internal homeostasis. In general, such stress conditions strictly initiate complex internal signals that lead to specific

reprogramming of genetic expression (Gasch and Werner-Washburne, 2002). These genomic level adjustments induce the stress-specific responses in cell.

S. cerevisiae is one of the organisms that uses such protective mechanisms and several defensive measures which are evolved to resist such stresses (Botstein *et al.*, 1997).

Previous large scale experiments showed that, under stress conditions approximately 900 genes in yeast altered in expression manner. These genes, called as "environmental stress response" (ESR) genes, constitute about 14% of the whole genome of yeast (Chen *et al.*, 2003). Functional analyses indicate that great majority of these genes are associated with cellular growth and protein synthesis (Gasch and Werner-Washburne, 2002). These changes in transcription profile are possibly due to energy conservation strategy of cells during stress exposure.

Even expression of ESR genes are seen in any suboptimal conditions, regulation of ESR is highly stress-specific. Yeast cells are able to detect external stress factors simultaneously yet individually. Cells give different responses to different stress factors. Depending on environmental conditions, different transcription factors regulate ESR system in terms of magnitude of expression and duration of response. Also, usage of different transcription factors lead to more specialized gene expression (Gasch and Werner-Washburne, 2002).

Understanding these behavioural changes of cells has vital importance in industry, especially regarding the use microorganisms in production. Improving of cellular resistance to stress conditions will greatly enhance the efficiency of microbial production process, despite harsh conditions of the industry. However, improving cellular stress resistance requires extensive knowledge about the underlying molecular mechanisms. Characterisation of environmentally triggered gene expression changes provides insights into when, where, and how each gene is expressed.

1.6 Stress Responses Against Alcohols in *S. cerevisiae*

Alcohols affect cell viability in various ways through oxidative damage, ion leakage or water stress. On the other hand, *S. cerevisiae* has natural alcohol-resistance mechanisms, it also induces various counter-stress mechanisms under alcohol exposure that lowers the alcohol-related damage.

1.6.1 Change in membrane composition

Many reports showed that, the primary target of alcohol in cell is the plasma membrane (Ingram and Buttke, 1984), (del Castillo Agudo, 1992), (Weber and de Bont, 1996). Alcohol exposure may lead to both excessive fluidization, leakage and disorder on membrane, and disrupt structures of membrane proteins, as mentioned previously.

Under alcohol stress, *S. cerevisiae* induces many adaptations in membrane structure to counteract the detrimental effects of those organic solvents. One of the adaptations in membrane lipid composition against ethanol stress is to increase the proportion of unsaturated fatty acids (Beaven *et al.*, 1982). Same kind of adaptations are also observed in other alcohol-resistant organisms such as *Escherichia coli, Clostridium thermocellum* and *Lactobacillus heterohiochii* (Vollherbst-Schneck *et al.*, 1984), (Lepage *et al.*, 1987), (Herrero *et al.*, 1982).

Especially short alcohols bind to polar head group area of lipids and change the membrane structure to have more tendency to form micelles and hexagonal structures compared to formation of bilayer (Weber and de Bont, 1996). Changes in membrane structure causes disorder and increased permeability in membrane. *S. cerevisiae* increases the ratio of unsaturated lipids to counter-act to such disordering effect of alcohols.

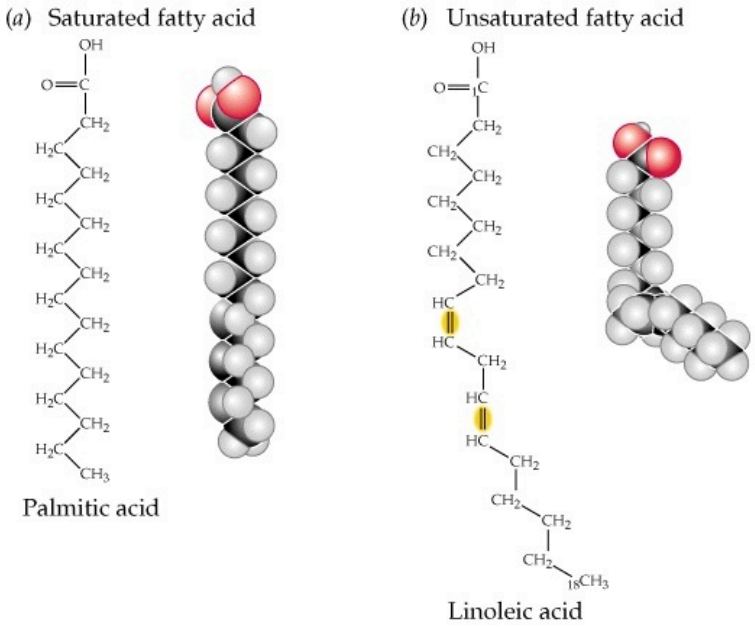

Figure 1.7: Chemical structures of a.) saturated palmitic acid and b.) unsaturated linoleic acid (del Castillo Agudo, 1992).

Ethanol adaptation leads to increase of unsaturated fatty acids (palmitoleic acid and linoleic acid) on membrane (Rattray, 1975). Increase of unsaturated lipids in yeast membranes is an adaptation to optimize ratio of water surface area (a) to baseline area (v/l) of lipids to keep the ratio of bilayer-forming lipids (Figure 1.3).

Unsaturated fatty acid synthesis is regulated by fatty acid desaturase which is encoded by *OLE1* gene. Although expression of *OLE1* is inhibited by ethanol, ethanol-resistant yeast strains show significantly higher expression for this gene (del Castillo Agudo, 1992).

Membrane bound sterols have also important roles in plasma permeability. It is showed that yeast cell also induce the production of sterols, especially ergosterol. Biosynthesis of ergosterol is associated with various genes, *ERG2, ERG3, ERG5, ERG6, ERG24,* and *ERG28* (Ma and Liu, 2012). A decrease in ergosterol content in *S. cerevisiae* membrane was shown to be directly linked with an increase in ethanol sensitivity (del Castillo Agudo, 1992).

1.6.2 Antioxidant Systems

Great majority of short chain alcohols enter cytoplasm after initial exposure. After entry, alcohol is immediately metabolized to other compounds such as acetic acids. However, this conversion may lead to production of undesirable reactive oxygen species. As an adaptive mechanism, yeast cells induce the production of antioxidant systems to prevent oxidative damage. It was shown that, under alcohol stress, *S. cerevisiae* cells induce the mitochondrial superoxide dismutase (*SOD1 and SOD2*) and catalase T (*CTT1*) (Costa *et al.*, 1997), which are both used for avoiding damaging effects of ROS.

1.6.3 Protein Refolding

Structures of many cellular proteins supported with hydrogen bonds between internal amino acids or with external water molecules. Additionally, weak Van der Waals interactions have major roles in many proteins. Especially polar groups of alcohols disrupt these bonds as in the membrane. Additionally, alcohols critically decrease the water availability (a_w) to cell and its components. All of these effects may change the structure of proteins.

It has been reported that *S. cerevisiae* cells induce the production of heat shock proteins (HSP) to compensate the structural change of proteins regarding alcohol exposure. At least 10 HSP genes, *HSP12, HSP26, HSP30, HSP31, HSP32, HSP42, HSP78, HSP82, HSP104,* and *HSP150* were identified as upregulated under alcohol stress (Piper and Talreja, 1994). HSPs, mainly acting as chaperones, insure proper folding or refolding of other nascent or denatured proteins and enzymes to maintain a functional conformation (Ma, 2012). Since ethanol alters protein formation and causes aggregation of denatured proteins, protein repairing functions over time by multiple chaperones appear to be critical for yeast tolerance to ethanol.

1.7 Obtaining PEA Resistant *S.cerevisiae* Strains by Evolutionary Engineering

Alcohols are among the primary stress factors to which industrial yeast strains are exposed to. Altough yeast cells have great potential for protection from alcohol, their

alcohol resistance is limited. Damaging effects of alcohol, generally limit the microbial alcohol production in industry. Production of high-titer alcohol requires more resistant yeast strains which need to be metabolically engineered. To redesign the microbial metabolism, several engineering methods have been developed such as metabolic engineering, inverse metabolic engineering and evolutionary engineering (Çakar, 2009), (Çakar *et al*., 2012).

Metabolic engineering is used to change the cellular regulations for the purpose of increasing the production of natural metabolite. Rational, metabolic engineering first identifies target systems, and then redesigns the related metabolic pathways. In other words, metabolic engineering highly needs to know the genetic basis of the phenotypic property of interest. However, inverse metabolic engineering and evolutionary engineering do not require this preliminary information about related metabolic pathways (Nevoigt, 2008). These methods are more useful to identify and improve characteristics with unknown and complex molecular basis, such as stress resistance mechanisms.

Evolutionary engineering basically follows the ways of natural evolution. In nature, the gene pool of an organism is generally not stable because of the environmental effects such as mutagenic agents. These agents diversify the related gene pool. In next step, nature applies a selective pressure on this diversified gene pool which makes some members of the gene pool more advantageous against the changing environment. Consequently, environmentally adapted organisms are developed (Barton, 2007).

In evolutionary engineering, the same steps of natural evolution are used. In laboratory conditions, mutagenesis and selection processes are highly controlled to shape generated organisms (Nevoigt, 2008). It is based on applying selective pressure to obtain desired phenotypes. This approach begins with application of mutagens to produce random mutagenesis on the gene pool of selected organism. Then, a selective pressure is applied to obtain organisms with the targeted phenotype (Hahn-Hägerdal *et al.*, 2007). After obtaining an organism with desired phenotype, genetic basis of that phenotype is identified through transcriptomic and metabolic analyses.

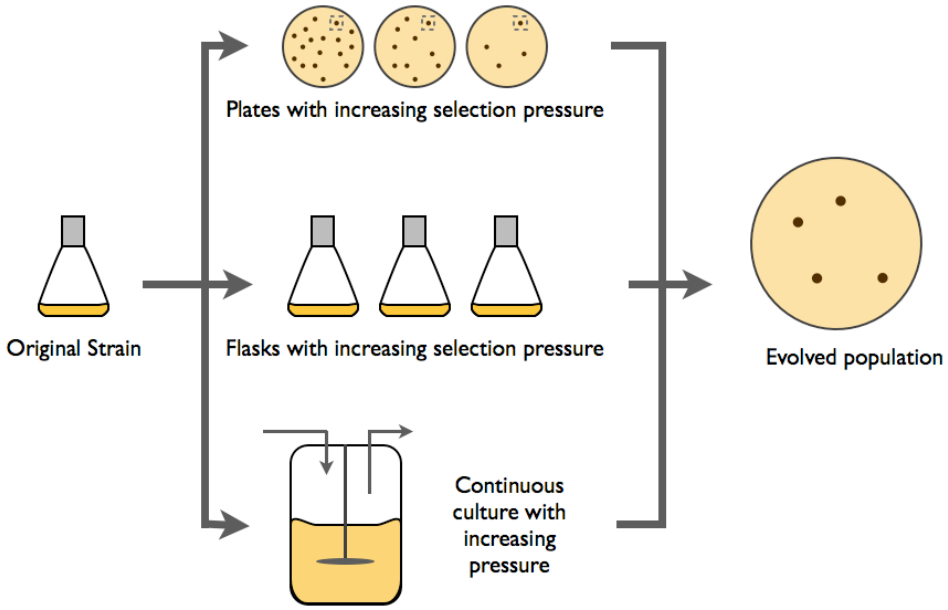

Figure 1.8: Principle of Evolutionary Engineering (Hahn-Hägerdal, 2007)

Yeast is a highly used organism as the subject of evolutionary engineering. There are several strains that are developed by evolutionary engineering approach. These strains have an ability of increased utilization of glucose, xylose and arabinose mixture (Wisselink *et al.*, 2009), xylose fermentation (Sonderegger and Sauer, 2003), L-arabinose fermentation (Wisselink *et al.*, 2007) and lactose consumption (Guimaraes et al. 2008) and resistance to multiple stresses (Çakar *et al.*, 2005), cobalt (Çakar *et al.*, 2009). Altough there are evolutionary engineered PEA-resistant *Escherichia coli* strains (Lucchini *et al.*, 1993), PEA-resistant yeast strains have not developed yet by evolutionary engineering.

1.8 Aim of the Study

Despite the fact that phenylethanol has more detrimental effects on cell structure as compared to other small-chain alcohols, its targets are generally considered the same (e.g cell membrane). For this reason, it is probable that under PEA exposure, cells induce similar protective mechanisms to those induced under ethanol stress. Obtaining PEA-resistant mutants may help us understand the common protective mechanisms under alcohol stress.

The aim of the present study was to obtain phenylethanol-resistant yeast via evolutionary engineering approach and perform transcriptomic and metabolic analyses to identify the molecular mechanisms underlying PEA resistance. The results obtained in this study might also be useful for understand the other common stress mechanisms in *S. cerevisiae*, such as ethanol, freeze-thaw, and H_2O_2 stress.

2. MATERIALS AND METHODS

2.1 Materials and Laboratory Equipments

2.1.1 Yeast strain and Mutagenesis

The wild type *Saccharomyces cerevisiae* CEN.PK 113.7D was kindly provided by Dr. Laurent Bendabis (INSA-Toulouse, Toulouse University, France). *S. cerevisiae* CEN.PK113.7D strain was renamed as "905" and used as the wild type strain.

Chemical mutagenesis was applied to the wild type strain by using ethyl methane sulphonate (EMS) on wild type strain 905 as described previously (Lawrence, 1991). Briefly, culture of *Saccharomyces cerevisiae* CEN.PK 113.7D was cultivated overnight at 30 °C. Cultures were washed and diluted with potassium phosphate buffer. EMS added into yeast culture and cultivation continued for 90 minutes. After cultivation, EMS deactivated with sodium thiosulphate. Culture was taken through centrifugation and inoculated into yeast minimal medium (YMM).

2.1.2 Cultivation and conservation conditions

Incubation of both wild type and mutant strains was carried out at 30 °C, 150 rpm in minimal medium (YMM) or complex medium (YPD). Stock cultures were stored in 1.5 mL microcentrifuge tubes, in a -80°C deep freezer after glycerol solution addition. To do this, 1000 µL of cell cultures were placed in 1.5 ml microcentrifuge tubes and centrifuged at 10000 rpm for 1 min. Cultures were washed with yeast minimal medium (YMM). Then, 500 µL of culture supernatant was removed by micropipette. 500 µL of 60% glycerol (v/v) was added onto the cell pellet and gently mixed with a micropipette. Later, glycerol culture mixture was placed in -80°C deep freezer for extended storage.

Prior to any cultivation both wild type and PEA-resistant strains were incubated in YMM after removal from -80 °C freezer. First, 50 µL of cell suspension was transferred to 10 mL YMM in 50 mL test tubes. Cells were incubated overnight at 30°C, 150 rpm. Next day, cultures were inoculated into fresh medium at an initial OD_{600} of 0.3.

2.1.3 Yeast culture media compositions

2.1.3.1 Yeast minimal medium (YMM)

In this study, yeast minimal medium (YMM) was used before stress exposure and for transcriptomic analysis.

Table 2.1: Ingredients of Yeast minimal medium (YMM)

Chemical	Supplier	Amount
Yeast Nitrogen Base without amino acids	Fluka BioChemika	6.7 g
Dextrose	Riedel-de Haen	20 g
Agar (only for solid media)	Applichem	20 g
Water		to 1 L.

2.1.3.2 Yeast extract peptone dextrose medium (YPD)

Yeast extract peptone dextrose medium is a complex medium used for regular growth of cultures.

Table 2.2: Ingredients of Yeast extract-peptone-dextrose medium (YPD)

Chemical	Supplier	Amount
Yeast Extract	Fluka BioChemika	10 g
Dextrose	Riedel-de Haen	20 g
Peptone	Riedel-de Haen	10 g
Agar (only for solid media)	Applichem	20 g
Water		to 1 L.

2.1.4 Laboratory equipment

The instruments that were used during experiments are shown in Table 2.3.

Table 2.3: Instruments that are used during experiments

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)
NanoDrop2000 Spectrophotometer	Thermo Fischer Scientific
Deep Freezer (-80°C)	Sanyo Ultra Low MDT-U40865
Refrigerators	Arçelik (Turkey)
Vortex mixer	Heidolph (Germany)
pH meter	Mettler Toledo MP220 (Switzerland)
BioAnalyzer 2100	Agilent (Provided by SEM-Limited)
Incubator	Nüve EN400 - EN500

2.1.5 Chemicals, buffers, solutions, kits and enzymes

Table 2.4. The chemicals used during experiments.

Chemical	Supplier
Phenylethanol	Sigma-Aldrich
Ethanol	J.T.Baker (Holland)
Potassium acetate	Carlo Erba Reagents (Italy)
Cobalt chloride ($CoCl_2$)	Merck (Germany)
Ammonium iron (II) sulfate	Carlo Erba Reagents (Italy)
Boron (II) Sulfate pentahydrate	Merck (Germany)
Chrome (II) chloride hexahydrate	Acros Organics (USA)
Copper (II) Sulfate pentahydrate ($CuSO_4.5H_2O$)	Merck (Germany)
Hydrogen Peroxide (H_2O_2) (35%, v/v)	Merck (Germany)
Nickel (II) chloride hexahydrate ($NiCl_2.6H_2O$)	Merck (Germany)
Zinc Sulfate heptahydrate ($ZnSO_4.7H_2O$)	Merck (Germany)
Glycerol	Carlo Erba Reagents (Italy)
Ethyl methane sulphonate	Alpha-Aeasar (Germany)

Table 2.5. The kits used for transcriptomic analysis

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)

2.2 Methods

2.2.1 Obtaining phenylethanol-resistant strain through evolutionary engineering

Phenylethanol-resistant *Saccharomyces cerevisiae* mutants were obtained by using EMS-treated wild type (906) via evolutionary engineering approach, based on batch selection under continuous exposure to phenylethanol stress.

To test the phenylethanol stress tolerance of wild type (905) and EMS-mutagenized culture (906), overnight cultures of these cells were first incubated in YMM containing 0.5 mL/L, 1.0 mL/L, 1.5 mL/L, 2.0 mL/L, 2.5 mL/L and 3.0 mL/L phenylethanol. Incubation was performed in 50 ml culture tubes containing 10 ml YMM. After 24 h of cultivation at 30°C and 150 rpm, the optical density values at 600 nm were determined. Survival ratio of the cultures was determined by dividing OD_{600} of stress-treated cultures to those of the non-treated ones.

Selection was carried out simply by exposure to increasng PEA concentrations and then picking survived mutants. The same procedure was repeated by gradually increasing PEA concentrations at each succesive cultivation.

The initial population for the selection procedure was the EMS-treated wild type. This culture was inoculated into YMM and YMM containing 1.5 mL/L PAE in a 50 ml culture tube with 10 mL culture volume. Cultures were incubated at 30°C and 150 rpm, for 24 h. At the end of the incubation, OD_{600} values of the cultures were measured, and stress-treated culture was named as the 1st PAE-resistant population. This culture was inoculated into YMM with 1.6 mL/L PEA and the cultivation was repeated for the 2nd PAE-resistant population. Selection experiments to obtain more resistant *S. cerevisiae* mutant populations under phenylethanol stress was continued by increasing phenyl ethanol concentrations gradually throughout successive populations. Successive populations were obtained until the survival ratio of the last population decreased below 0.2.

The final PEA-resistant population was diluted and inoculated to YMM-agar plate to have distinct colonies on the surface of the plate. Ten individual mutant colonies

were randomly selected and further analyses were performed on individual mutants for determination of the genetic and metabolic changes relevant to stress resistance.

2.2.2 Estimation of stress resistance

2.2.2.1 Spot assay

After selection process, 10 colonies were randomly picked from the final population grown on agar plate which contained 3.4 mL/L PEA. These PEA-resistant mutants were compared to each other according to their PEA resistance levels. Comparison was carried out by spot assay and Most Probable Number (MPN) assay.

In first, all of the selected PEA-resistant mutants, 905 and the last (56.) batch generation cultures were inoculated into YMM medium with 1 mL/L, 3 mL/L and 5 mL/L PEA also control medium which does not contain any PEA. Cultures were inoculated at different dilution factors from 10^{-1} to 10^{-5}. After 48 h of incubation, more resistant mutants were determined and selected for further analysis.

2.2.2.2 Most Probable Number (MPN) assay

After spot assay, 4 different PEA-resistant mutants were selected for further screening to compare their survival ratio under stress conditions. For this comparison, MPN assay was used. MPN assay is a statistical technique to quantify cell density from positive/negative data. In this study, MPN method included serial dilution of sample cultures and application of same stress factor (PEA) to all these dilutions and then detection of possible growth in these dilutions. It is possible to find the number of survived cells by statistical analysis of presence/absence of growth in these dilutions. The analysis was performed by using MPN tables which are based on Poisson regression.

In this study, cells were inoculated into YMM with and without PEA stress conditions in 96-well plates with five replicates and serially diluted up to 10^8 fold dilution. After incubation for 72 h, presence/absence of growth in wells was noted and converted into concentration data via MPN table. According to concentration

data, the mutant which showed higher survival ratio (C9) under PEA exposure is selected for further tests.

2.2.3 Genetic stability test

Genetic stability test was applied to the selected PEA-resistant mutant (C9) which showed the highest survival ratio in screening test. The main aim of this test was to determine the of persistence of PEA resistance capabilities of C9, and find out if the PEA resistance was permanent or not.

In genetic stability test, PEA resistance of C9 was measured during five succesive batch cultivations in the absence of the selective pressure, namely PEA stress. For each cultivation, a culture sample of C9 was stored at -80 °C freezer. Finally, all 5 samples were compared according to their PEA resistance values.

In this test, firstly PEA-resistant C9 was taken from the -80°C freezer. 100 µL of culture was inoculated into YMM for pre-culture. After overnight incubation at 30°C and 130 rpm, cultures were inoculated into fresh YMM again. After incubation for 5-6 h, 1 ml of culture was withdrawn and stored in -80°C freezer. Next, remaining culture was inoculated into fresh YMM again. For following 5 cultivations, 5 culture samples were taken which represent the last 5 cultures.

After 5 cultivations, all of frozen cultures were inoculated into fresh YMM medium as pre-cultures. After overnight incubation, all cultures were inoculated into YMM with 2.5 mL/L phenylethanol in 96-well plates with five replicates and serially diluted up to 10^8 fold dilution for MPN assay. After 72 hours, MPN data was analysed to find possible changes in survival ratio during five succesive cultivations.

2.2.4 Cross resistance test

Different stress factors may damage cell metabolism in similar ways, and in that case these stress factors may induce common stress responses in cell (Estruch, 2000). So, strains that gain resistance to a specific stress factor may also gain resistance to another stress factor. For example, oxidative stress damages cells by increasing the ROS production. Similarly, one of the detrimental effects of freeze-thaw stress is to

increase the ROS production during thawing (Gasch and Werner-Washburne, 2002). As, two different stresses affect cell in the oxidative way, the cells that have resistance against oxidative damage would also be resistant to freeze-thaw stress. These cross-resistance abilities give important clues about moleculat pathways that are responsible for related resistances.

In this study, PEA-resistant strain C9 was analysed according to its cross-resistance abilities against various metals and organic compounds and compared with the wild type.

To do this, pre-cultures of wild type and C9 were collected at 4 OD_{600}. For 4 optical density unit culture, 1 mL culture was transferred to microfuge tube. Tubes were centrifuged at 10,000 rpm for 1 min and the supernatants were removed. 50 µL sterile distilled water was added to the pellet.

The culture was serially diluted until 10^{-5} level by adding 20 µL culture to 180 µL YMM. All dilutions of both strains were placed on petri dishes with different stress factors.

In cross-resistance test, serial dilutions of C9 and 905 cultures were incubated at different concentrations of phenylethanol (2.5 mL/L and 3 mL/L), ethanol (8%, 10% and 12% v/v), Potassium acetate (CH_3CO_2K 0.004% v/v), Copper (II) Sulfate pentahydrate (CuSO4.5H2O 1 mM and 3 mM), Boron (II) Sulfate pentahydrate (80 mM), Copper (II) Sulfate pentahydrate (CuSO4.5H2O) (0.5 mM), hydrogen peroxide (0.5 mM) and Nickel (II) chloride hexahydrate (NiCl2.6H2O) (0.2 mM) are used.

2.2.5 Microarray analysis

Transcriptomic analysis of PEA resistant mutant was done by microarray method and compared with the wild type strain.

2.2.5.1 RNA isolation

Both wild type and C9 cultures were inoculated into 100 ml YMM medium at an initial OD600 of 1. Incubation was maintained at 30°C and 130 rpm until the cell

cultures reached OD_{600} value of 1 (10^7 cells/ml). Total RNA was isolated from both 905 and C9 by using RNeasy Mini Kit (QIAGEN). Sample preparation and RNA isolation was performed 4 times separately.

2.2.5.2 RIN detection of RNA samples

Integrity of isolated RNA samples was measured with BioAnalyzer 2100 (Agilent) by using RNA 6000 Nano Assay Kit (Agilent) according to the manufacturers instructions. Before the measurement, RNA concentrations of samples were measured with NanoDrop 2000 (ThermoScientific) and concentrations set to 10 µg/ µL by proper dilutions.

2.2.5.3 Sample preparation

Prior to labelling, One-color RNA Spike-in Kit (Agilent) was used for spike mix preparation. cDNA master mix, transcription mix and T7 promoter Primer mix were prepared according to the protocol of One-Colour Microarray-based Gene Expression Analysis. Lastly, by using Absolutely RNA NanoPrep Kit (Agilent Technologies) RNA samples were purified.

2.2.5.4 Hybridization

The labeled cRNAs were hybridized to Agilent yeast microarrays. For 20 hours at 65 °C, the microarrays were incubated in a hybridization chamber. Washing procedure was applied with gene exprssion wash buffers at the end of the hybridization process.

2.2.5.5 Scanning and data analysis

Microarray raw data was gathered from Agilent Laser scanner. Primary analysis of microarray data was done using Agilent Feature Extraction software. Additionally, GeneSpring GX 12.00 was used for interpretation of raw data.

3. RESULTS

3.1 Obtaining PEA-Resistant Mutants

To determine starting phenylethanol concentration for selection, both mutagenized yeast culture (906) and the wild type yeast strain (905) were incubated in 10 mL YMM including 0.5 mL/L, 1.0 mL/L, 1.5 mL/L, 2.0 mL/L, 2.5 mL/L and 3.0 mL/L phenylethanol.

Incubation was performed at 30°C and 150 rpm at an initial OD_{600} of 0.1 for 24 h. After 24 h of incubation, final OD_{600} values were measured. The results are given in Table 3.1.

Table 3.1: OD_{600} values of wild type (905) and EMS-mutagenized culture (906) after 24 h cultivation in the presence of different PEA stress levels

	OD_{600}	
Concentration of phenylethanol (mL/L)	***905***	***906***
Control	5.84	5.92
0.5	5.69	5.48
1.0	5.65	5.77
1.5	5.24	5.23
2.0	4.74	4.98
2.5	4.02	4.04
3.0	2.54	2.74

According to results shown in Table 3.1, OD_{600} values of cultures started to decrease significantly after 1.5 mL/L phenylethanol. Therefore, initial stress concentration for selection was set as 1.5 mL/L phenylethanol.

EMS-mutagenized culture (906) was exposed to increasing concentrations of phenylethanol in succesive batch cultures starting from 1.5 mL/L. In each passage, cultures were inoculated to phenylethanol containing YMM with an initial OD_{600} of

0.3. OD_{600} measurements were performed and survival ratios were calculated after 24 h incubation. The cultures were then inoculated to fresh media with higher phenylethanol concentration and the whole process was repeated until survival ratios decreased critically.

In this study, phenylethanol concentrations was increased from 1.5 mL/L to 3.4 mL/L where survival ratio decreased significantly. The whole process of selection took 56 passages.

Table 3.2: Phenylethanol concentrations of each passage, OD_{600} values of stress and non-stress conditions, incubation times and growth ratios.

Passage Number	Phenylethanol concentration (mL/L)	OD_{600} value of stress condition	OD_{600} value of non-stress condition	Incubation time (h)	Survival Ratio
1	1.5	5.16	5.84	24	0.88
2	1.6	4.80	5.92	24	0.81
3	1.7	5.05	5.77	24	0.88
4	1.8	4.85	5.25	24	0.92
5	1.9	4.50	5.44	24	0.83
6	2.0	4.31	5.11	24	0.84
7	2.1	4.38	5.54	24	0.79
8	2.2	4.10	5.15	24	0.80
9	2.3	4.00	5.80	24	0.69
10	2.4	3.87	5.16	24	0.75
11	2.5	3.58	6.52	24	0.55
12	2.6	3.34	5.13	24	0.65
13	2.7	3.03	5.06	24	0.60
14	2.8	3.45	5.75	24	0.60
15	2.8	3.40	5.54	24	0.61
16	2.8	1.91	5.59	72	0.34
17	2.8	2.01	5.47	72	0.37
18	2.8	2.92	5.44	48	0.54
19	2.8	3.15	6.13	48	0.51
20	2.8	2.61	5.04	24	0.52
21	2.8	2.85	5.13	24	0.56
22	2.9	2.70	5.15	48	0.52
23	2.9	2.91	5.13	48	0.57
24	2.9	4.03	5.82	24	0.69
25	2.9	3.03	4.70	24	0.64
26	3.0	2.77	5.70	48	0.49

Passage Number	Phenylethanol concentration (mL/L)	OD_{600} value of stress condition	OD_{600} value of non-stress condition	Incubation time (h)	Survival Ratio
27	3.0	2.63	5.30	48	0.50
28	3.0	2.50	5.74	72	0.44
29	3.0	2.00	6.98	72	0.29
30	3.0	3.25	6.02	24	0.53
31	3.0	3.03	6.52	24	0.46
32	3.1	2.38	5.20	24	0.45
33	3.1	2.64	5.20	24	0.50
34	3.2	2.90	7.17	48	0.40
35	3.2	3.23	5.80	48	0.55
36	3.2	3.00	6.64	48	0.45
37	3.2	2.97	5.26	48	0.56
38	3.2	3.40	5.33	24	0.63
39	3.2	2.70	5.69	48	0.47
40	3.2	3.19	5.80	48	0.55
41	3.2	2.46	4.90	24	0.50
42	3.3	2.13	4.73	48	0.45
43	3.3	3.05	4.64	48	0.65
44	3.3	2.83	4.01	72	0.70
45	3.3	2.98	4.67	48	0.63
46	3.3	2.62	5.30	48	0.49
47	3.3	2.82	4.60	72	0.61
48	3.3	2.72	5.20	24	0.52
49	3.3	4.12	5.23	48	0.79
50	3.3	2.44	5.39	48	0.45
51	3.3	3.40	4.62	48	0.74
52	3.3	2.83	5.42	24	0.52
53	3.3	2.80	4.88	48	0.57
54	3.3	3.05	5.11	24	0.60
55	3.4	4.11	5.67	72	0.72
56	**3.4**	**2.90**	**4.90**	**72**	**0.59**

Phenylethanol concentration was gradually increased at each passage for strain 906. Table 3.2 shows the phenylethanol concentrations of each passage, OD_{600} values of stress and non-stress conditions, incubation times and growth ratios. At 56^{th} generation, cell growth decreased critically to 0.59 even after 72 h incubation, and therefore selection was stopped at that point. 56^{th} PEA-resistant population was used for further individual selection.

The final 56th population was incubated on solid YMM plate with 3 mL/L phenylethanol to isolate the individual mutant. After 48 h of incubation, 10 different colonies were picked randomly for individual mutant selection.

3.2 Phenylethanol Resistance of Mutants and Wild Type

To select the mutant with the highest PEA resistance capability, 10 mutants and the wild type strain were compared according to their PEA resistance levels. Spot assay and MPN method were used to estimate stress resistance levels of the cultures.

3.2.1 Stress resistance analysis through spot assay

Stress resistance capabilities of selected mutants were measured firstly by spot assay. In this assay, serial dilutions of mutant and wild type cultures were inoculated onto solid YMM plate with different concentrations of phenylethanol. The incubation was maintained at 30°C for 72 h. Plate images were taken at 42th hour and 72th hour.

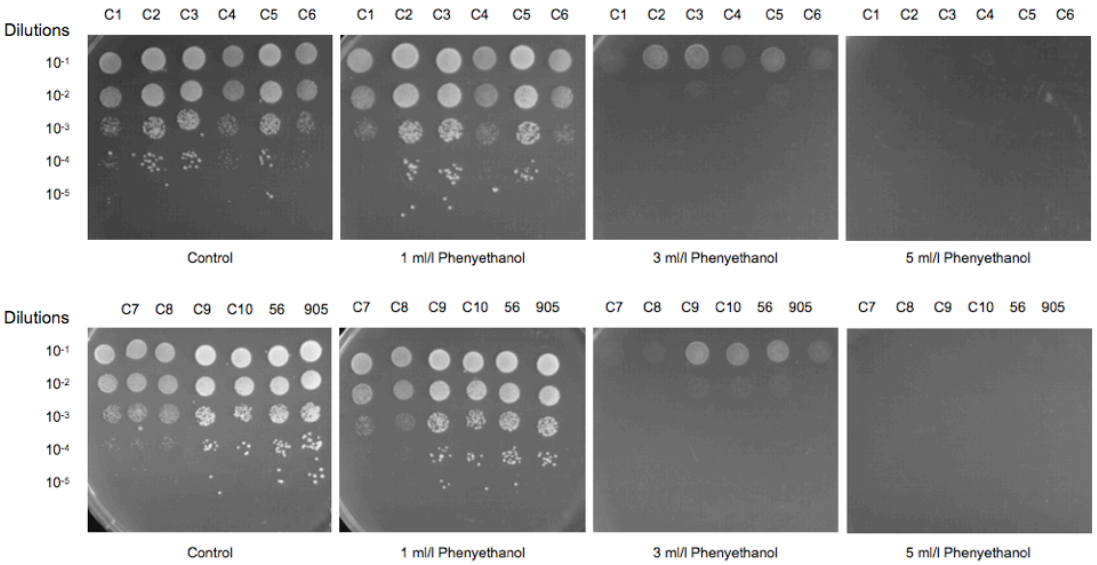

Figure 3.1: Spot assay results of individual mutants (C1 to C10), 56th generation and wild type (905) after 48 h incubation.

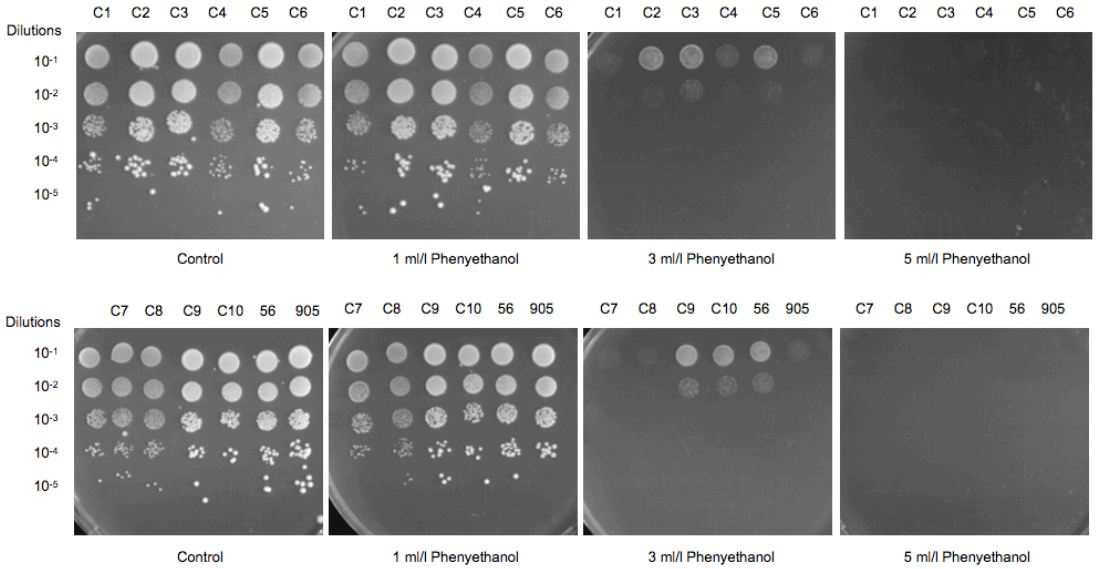

Figure 3.2: Spot assay results of individual mutants (C1 to C10), 56th generation and wild type (905) after 72 h incubation.

Wild type and individual mutants were shown similar growth at control and 1 mL/L PEA stress exposure conditions. At 3 mL/L PEA concentration, growth of wild type and some mutants were severely inhibited. However, it was observed that mutant strains C2, C3, C9 and C10 survived better at higher dilutions compared to other individual mutants which indicated that these mutants were more resistant to phenylethanol. On the other hand 5 mL/L phenylethanol had detrimental effect on growth of all mutants and th wild type.

3.2.2 Stress resistance analysis through MPN method

The selected individual mutants in spot assay (C2, C3, C9 and C10) were further compared according to their survival ratio under phenylethanol stress by using the most probable number (MPN) assay.

For MPN assay individual mutants which were obtained by selection were incubated at 3 mL/L and 4 mL/L phenylethanol in 96-well plates via MPN method to compare the phenylethanol resistance levels. Using MPN data conversion table, relative viable cell concentrations of mutants and wild type strains were obtained. Additionally, the survival rate values were calculated by dividing cell numbers of stress treated samples to that of the non-treated cells.

Table 3.3: The survival ratios of phenylethanol resistant mutants and 905 (48 h).

	Cell/ml			Survival Ratio		Fold of WT	
	Control	3 mL/L PEA	4 mL/L PEA	3 mL/L PEA	4 mL/L PEA	3 mL/L PEA	4 mL/L PEA
905	1100000	2400	240	0.000218	0.000022	1	1
56th	5400000	70000	240	0.012963	0.000044	59.46	2.02
C2	1700000	22000	240	0.012941	0.000141	59.37	6.42
C3	16000000	140000	240	0.00875	0.000015	40.14	0.68
C9	7000000	1100000	240	0.157143	0.000034	**720.84**	1.56
C10	9200000	220000	240	0.023913	0.000026	109.69	1.19

According to the MPN assay results, all mutants showed higher survival ratios compared to wild type (Table 3.3). Among mutant individuals, C9 strain had significiantly higher survival ratio under 3 mL/L phenylethanol stress and used for further analysis.

3.3 Genetic stability analysis

The stability test was performed by using MPN method. The stability results of phenylethanol resistant mutant C9 after 48 h incubation are indicated in Table 3.4.

Table 3.4: Genetic stability results of phenylethanol resistant mutant *C9* (48 h).

Cycles	Control (Cell/ml)	Stress (Cell/ml)	Survival Ratio
C9_1	5400000	5400000	1.00
C9_2	2400000	540000	0.23
C9_3	2400000	700000	0.29
C9_4	3300000	1700000	0.52
C9_5	3500000	350000	1.00

The genetic stability test was also performed by using spotting assay. The stability results of phenylethanol resistant mutant C9 after 48 h incubation are indicated in Figure 3.3.

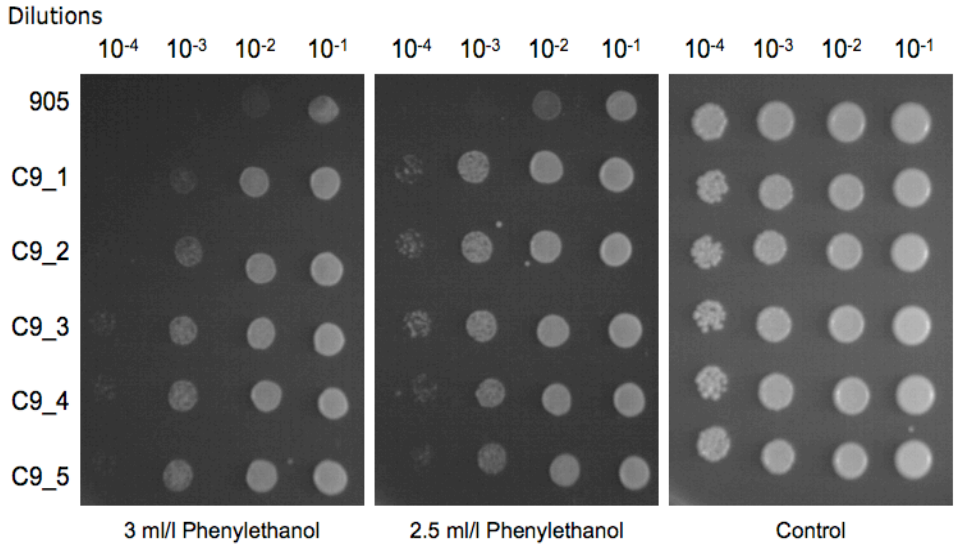

Figure 3.3: Genetic stability results of phenylethanol resistant mutant C9 (48 h)

3.4 Cross Resistance Test

Selected C9 strain and wild type strain were incubated in YMM with various metal and non-metal stress factors. After 72h, images of colonies were taken.

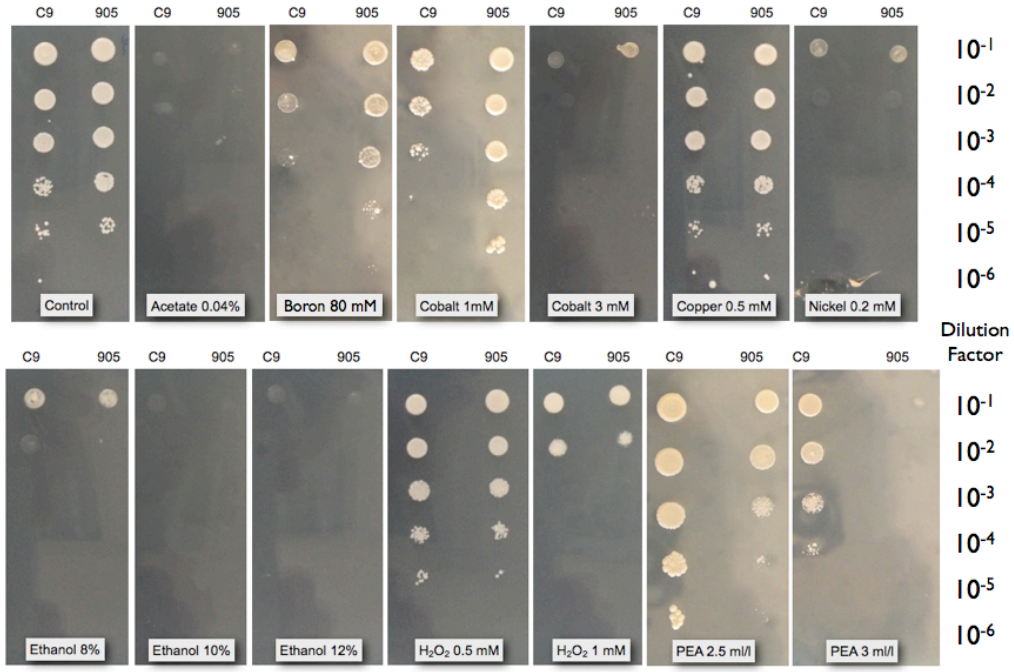

Figure 3.4: Cross resistance test results (72 h) of PEA-resistant C9 and wild type

C9 strain had higher phenylethanol resistance in both 2.5mL/L and 3 mL/L PEA concentrations compared to wild type (Figure 3.4). C9 strain also show higher

resistance to ethanol at 8% ‘v/v’ and 10% ‘v/v’ containing plates. The high resistance phenotype of C9 to ethanol was more clearly observed after 72 h incubation. On the other side, C9 clearly showed sensitivity to cobalt in both 1 mM and 3 mM concentrations and bromine at 80 mM (Figure 3.4).

3.5 Microarray Analysis

The molecular mechanism of phenylethanol resistance of C9 was investigated by global analysis of transcription profile via DNA microarrays. During microarray analysis, transcriptomic levels of nearly 6000 genes in both wild type and C9 were measured and compared with each other. To find upregulated and downregulated genes, *S. cerevisiae* CEN.PK 113.7D (905) was used as reference strain. Both phenylethanol-resistant ‘C9’ and wild type strain were incubated in non-stress conditions in YMM for microarray analysis as triplicates. RNA samples of the cultures were taken at their exponential growth phase by setting the cell concentration equal for both strains. Isolated RNA samples were analysed according to their integrity level.

Table 3.4.1: Initial OD_{600} of the cultures and OD_{600} of the cultures just before the RNA purification

Name	Initial OD_{600} of the cultures	OD_{600} of the cultures just before the RNA purification				
		Culture 1	Culture 2	Culture 3	Culture 4	Culture 5
w/t	0.12	1.16	1.08	1.09	1.12	1.13
C9	0.13	1.12	1.09	1.13	-	-

Next, Agilent 2100 BioAnalyzer was used to identify RNA Integrity Number ‘RIN’.

Table 3.4.2: RIN values for the parallel sets of cultures

Name	RIN of the cultures				
	Culture 1	Culture 2	Culture 3	Culture 4	Culture 5
w/t	9.9	10.0	9.70	9.60	9.40
C9	8.2	7.00	8.40	---	**---**

RNA concentration was determined by using Nano Drop instrument (Nano Drop 2000, Thermo Scientific).

For both upregulated and downregulated genes, those genes whose expression changed more than two fold in C9 as compared were to reference was accepted as meaningful.

Table 3.5: The upregulated genes in C9 under control conditions. The genes which have been upregulated by less than 20 fold are not represented.

Fold Change	Upregulated Genes
Between 200 and 250	***ALD3***, ***HXK1***, *GPH1, TLK2, MAL12, MAL32*
Between 150 and 200	-
Between 100 and 150	***HSP12***, *MAL11, FMP45, PGM2*
Between 50 and 100	*RTN2,* ***HSP26***, ***TSL1***, ***DDR2***, ***CTT1***
Between 20 and 50	*YMR206W,* ***YNL194C***, *PHM7, PIR3, GAC1, TMA10,YNR034W-A, YFL052W,* ***GLC3***, ***ALD4***, *MSC1,* ***HXT7***, ***SOL4***, ***YGP1***, *YER067W,* ***SED1***, ***ISF1***, *BDH2, INO1,* ***GSY1***, *DCS2, HXT6,* ***XBP1***

Table 3.6: The downregulated genes in C9 under control conditions. The genes which have been downregulated by less than 5 fold are not represented.

Fold Change	Downregulated Genes
Between 15 and 20	*SUL1, ZRT1, PHM6, PHO84*
Between 10 and 15	*YDL241W, STE3, ARO3, AAH1, FET3, RAS1, SSP1*
Between 5 and 10	*DBP2, ECM1, PNO1, OPT2, SPL2, DHR2, FCY2, YOL014W, ATO3, NSR1, BNA2, RSA4, MMP1, YER187W, CIC1, YCR087C-A, HFM1, NIP7, DAL1, KRE33, GFD2, YBR141C, MRT4, YNR062C, BFR2, RKI1, ADH4, AI3, MAK16, IMD4, FRM2, HES1, RLP24, YIL096C, YGL101W, YLR460C, PGA3, RCL1, CTP1*

In Table 3.7 and 3.8, upregulated and downregulated genes are demonstrated with known functions, respectively to gain insight into mechanisms that yeast cells use to cope with these stresses. Data intrepretation was carried out by using Funspec database.

Table 3.7: Biological processes and fold change of highest upregulated genes. The genes that are represented as bold are responsible in environmental stress response. The genes which have been upregulated by less than 10 fold are not represented.

Process	*Gene*	Fold of Wt	*Gene*	Fold of Wt
Carbohydrate Metabolic Process (p value: 1.239e-09)	*MAL12*	207.4	***AMS1***	15.6
	MAL32	198.7	*NQM1*	17.7
	GLK1	10.8	***SOL4***	26.0
	EMI2	18.3	*GPH1*	217.1
	GLC3	28.1	***PGM2***	100.2
	HXK1	223.6		
Glycogen Biosynthetic Process (p value: 2.654e-09)	***GLC3***	28.1	*GSY2*	17.7
	GSY	20.7	***PGM2***	100.2
	GAC1	36.8		
Response To Stress (p value: 3.678e-09)	*RSB1*	10.9	*FMP43*	14.2
	FMP45	101.6	***GRE3***	11.8
	TPS2	12.8	***XBP1***	19.8
	HSP78	10.3	***TSL1***	67.2
	SSA4	16.1	***DDR48***	10.0
	HSP12	136.6	***DDR2***	58.8
	CTT1	52.4		
Maltose Metabolic Process (p value: 1.138e-07)	*MAL31*	18.0	*MAL11*	127.9
	MAL32	198.7	*MAL12*	207.4
Trehalose Biosynthetic Process (p value: 7.519e-07)	***TPS2***	**12.8**	***TSL1***	67.2
	UGP1	**12.3**	***PGM2***	100.2
Glucose 6-P Metabolic Process (p value: 7.32e-06)	***GLK1***	10.8	***PGM2***	100.2
	EMI2	18.3		
Mannose Metabolic Process (p value: 0.0001524)	***GLK1***	10.8	***AMS1***	15.6
	HXK1	223.6		

Table 3.7 (continued): Biological processes and fold change of highest upregulated genes. The genes that are represented as bold are responsible in environmental stress response. The genes which have been upregulated by less than 10 fold are not represented.

Process	*Gene*	Fold of Wt	*Gene*	Fold of Wt
Metabolic Process (p value: 3.165e-06)	*BDH2*	2.7	*NQM1*	17.7
	TKL2	208.5	***GSY1***	20.7
	MAL32	198.7	***GSY2***	17.7
	GPM2	13.9	***UGP1***	12.3
	TPS2	12.8	***ALD3***	234.0
	HXK1	223.6	***ALD4***	28.0
	AMS1	15.6		
Maltose Catabolic Process (p value: 0.0001524)	*MAL32*	198.7	*MAL11*	127.9
	MAL31	18.0	*MAL12*	207.4
Glycolysis (p value: 0.0003605)	***GLK1***	10.8	***GPM2***	13.9
	EMI2	18.3	***HXK1***	223.6
Galactose Catabolic Process (p value: 0.0004534)	***GRE3***	11.8	***PGM2***	100.2
Sucrose Catabolic Process (p value: 0.0004534)	*MAL32*	198.7	*MAL13*	13.1
Glucose Import (p value: 0.0004534)	***GLK1***	10.8	***HXK1***	223.6
Carbohydrate Transport (p value: 0.0005386)	*MAL31*	18.0	*HXT7*	26.2
	HXT6	19.8	*MAL11*	127.9
Pentose-Phosphate Shunt (p value: 0.0006088)	***TKL2***	208.5	*NQM1*	17.7
	SOL4	26.0		
D-Xylose Catabolic Process (p value: 0.0008995)	***GRE3***	11.8	*GCY1*	11.0
Arabinose Catabolic Process (p value: 0.0008995)	***GRE3***	11.8	*GCY1*	11.0
Cellular Response To Heat (p value: 0.005183)	***HSP78***	10.3	***HSP12***	136.6
Response To Oxidative Stress (p value: 0.009362)	***HSP12***	136.6	***GRE3***	11.8
	GAD1	19.3	*GCY1*	11.0

It was observed that highly upregulated genes in C9 are mainly responsible in carbohydrate metabolism, glucose import and alternative carbon source utilization. There were also some upregulated gene clusters that are responsible in some specific stresses (e.g heat, oxidative stress). Interestingly, more than half of the upregulated genes in C9 were also associated with environmental stress response (ESR).

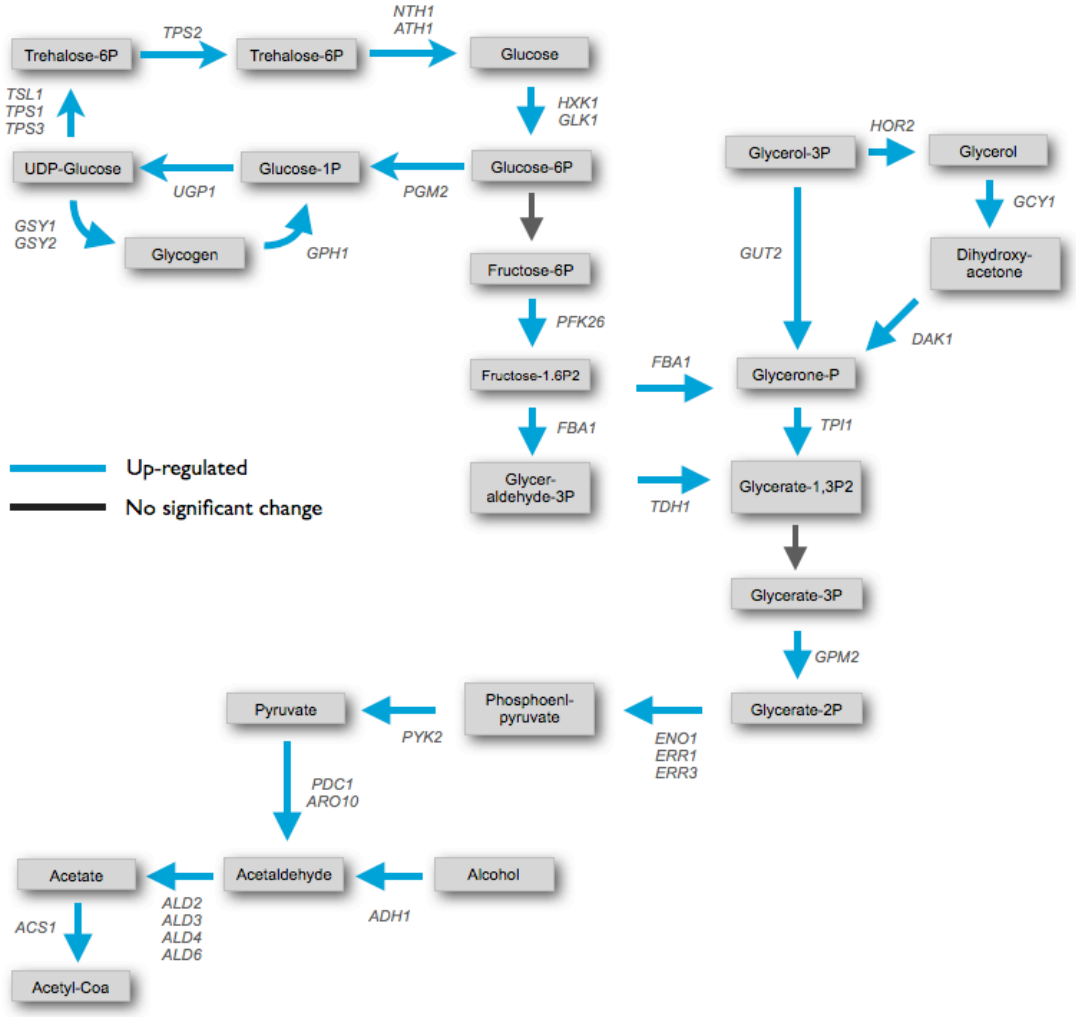

Figure 3.5: Significantly upregulated genes that are responsible in carbohydrate metabolism. Blue arrows indicate the metabolic reactions of upregulated genes. Grey arrows indicate the reactions of genes whose expression change is under 2 fold.

Table 3.8: Biological processes and fold change of highest downregulated genes. The genes that are represented as bold are responsible in environmental stress response. The genes which have been upregulated by less than 4 fold are not represented.

Process	Gene	Fold of Wt	Gene	Fold of Wt
Ribosome Biogenesis (p value: <1e-14)	***CIC1***	6.7	*IPI1*	5.2
	NOP10	4.6	*MRT4*	6.1
	DBP9	5.4	***DHR2***	7.5
	RRB1	4.8	***EBP2***	5.0
	IPI3	5.3	***ESF2***	2.1
	RCL1	5.5	***PNO1***	8.5
	RRS1	4.7	***NIP7***	6.4
	IMP4	3.7	***RIX7***	5.1
	DBP2	9.3	***RLP24***	5.7
Ribosomal Subunit Biogenesis (p value: <1e-14)	***MAK16***	5.9	***PUF6***	4.7
	ARX1	4.8	***CIC1***	6.7
	RLP24	5.7	***RRS1***	4.7
	NIP7	6.4	*MRT4*	6.1
	RIX7	5.1		
Ribosomal Large Subunit Assembly (p value: 2.999e-11)	***RSA4***	6.9	***DBP9***	5.4
	IPI1	5.2	***IPI3***	5.3
	YVH1	5.3	***NIP7***	6.4
	MRT4	6.1	***RPF2***	5.1
	BFR2	6.0	*MRT4*	6.1
rRNA Processing (p value: <1e-14)	***EBP2***	5.0	***IPI1***	5.2
	DHR2	7.5	*NOP10*	4.6
	NSR1	7.2		

Table 3.8 (continued): Biological processes and fold change of highest downregulated genes. The genes that are represented as bold are responsible in environmental stress response. The genes which upregulated by less than 4 fold are not represented.

Process	Gene	Fold of Wt	Gene	Fold of Wt
Transmembrane Transport (p value: 0.001754)	***CTP1***	5.5	***ENB1***	4.8
	PHO84	16.8	***ATR1***	5.2
	ATO3	7.4	***SUL1***	19.7
	ZRT1	18.6	***YJR124C***	5.2
	FTR1	4.7	***FCY2***	7.4
	OPT2	8.1	***SSU1***	5.4
	YOL163W	4.8	***MMP1***	6.8
Mitochondrial Citrate Transport (p value: 0.0008948)	***CTP1***	5.5	***YHM2***	5.1
Iron Assimilation By Reduction (p value: 0.0008948)	*FTR1*	4.7	*FET3*	10.8
Ribosomal Large Subunit Export From Nucleus (p value: 1.891e-07)	*ECM1*	9.2	*RRS1*	4.7
	RIX7	5.1	*BCP1*	5.4
High-Affinity Iron Ion Transport (p value: 0.005158)	*FTR1*	4.7	*FET3*	10.8
Iron Ion Homeostasis (p value: 0.005158)	*FTR1*	4.7	*FET3*	10.8
	ENB1	4.8		

Downregulated genes in C9 were involved in RNA metabolism (mainly in ribosome synthesis) and iron transport/metabolism. Nearly all of the genes responsible in RNA metabolism also have role in ESR. Although ESR genes are expressed upon most of the environmental stress exposure, they might show differences depending on properties of the stress type. These differences generally involve stress-specific responses. For example, under hyperosmotic shock, in addition to induction of ESR genes, some other genes (which are responsible in synthesis of cellular osmolites) are also super-induced.

Using FunCat database, both upregulated and downregulated genes were analyzed according to their functional categories (Ruepp *et al.,* 2004).

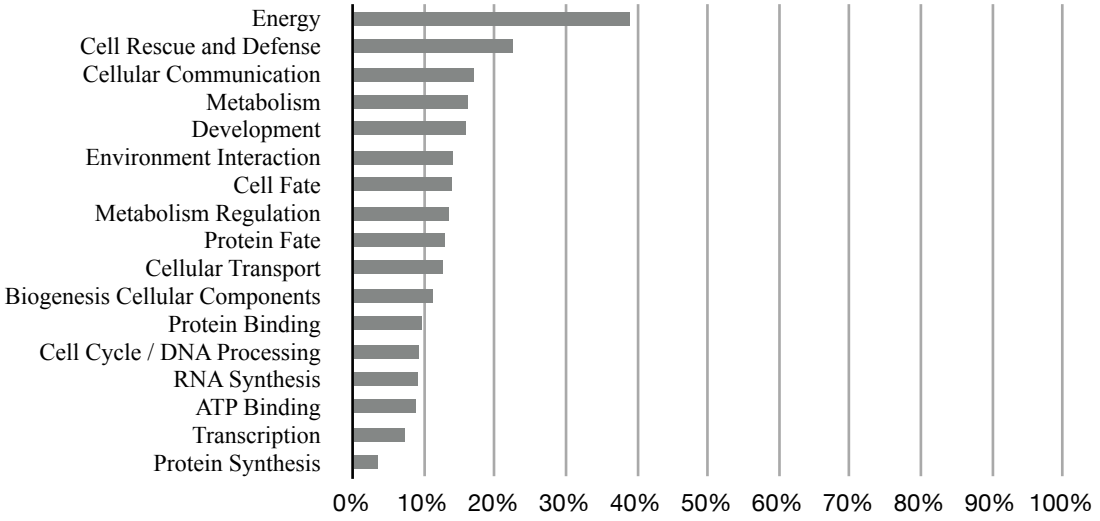

Figure 3.6: Functional categories of upregulated genes and ratio of these genes in related category (Ruepp *et al.,* 2004).

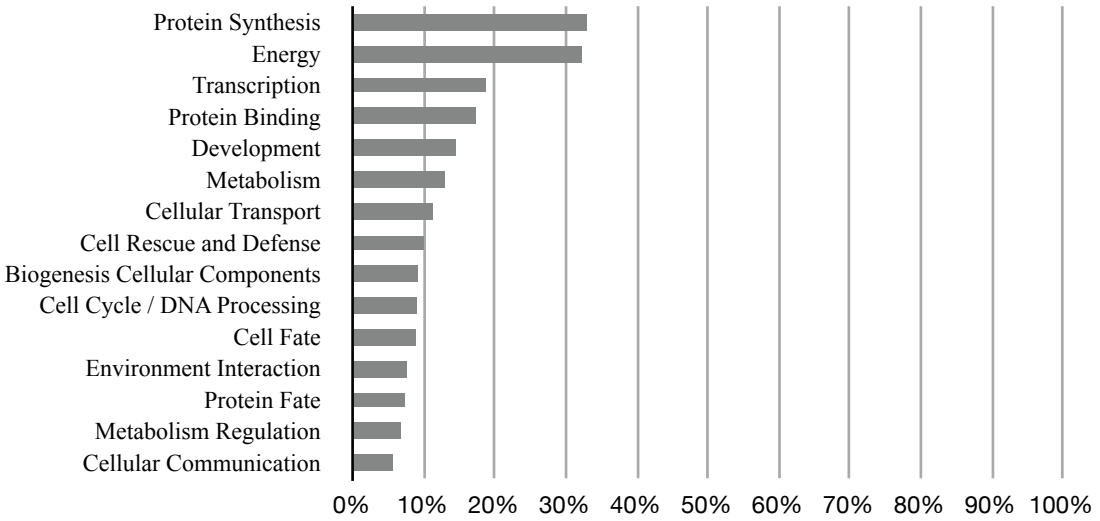

Figure 3.7: Functional categories of downregulated genes and ratio of these genes in related category (Ruepp *et al.,* 2004).

Energy metabolism seems to be highly activated in phenylethanol-resistant mutant compared to wild type. Nearly 40% of genes in this category were significantly upregulated. In addition, C9 induced nearly 25% of genes that are related with cell defense. On the other hand, about 35% of genes in protein synthesis were downregulated. Changes in these three categories were also observed under ESR

conditions which might support that C9 might constitutively express environmental stress response genes, even when there are no stress conditions present.

The expression of genes in the ESR is regulated by different transcription factors depending on the conditions, and the response is governed by several different upstream signaling pathways (Gasch *et al.*, 2000).

In this study, using Yeastract database, the transcription factors that regulate upregulated genes of C9 were also determined and are shown in Table 3.9.

Table 3.9: Transcription factors that affect upregulated genes in C9 and their contribution percentage (Abdulrehman *et al.*, 2011)

Transcription Factor	Percentage of Contribution to Upregulated Genes
Msn2p	67.0 %
Aft1p	59.4 %
Rpn4p	57.1 %
Ste12p	56.1 %
Sok2p	54.7 %
Msn4p	53.3 %
Yap1p	50.9 %

Main transcription factors that are responsible for regulation of the upregulated genes of C9 are Msn2p, Aft1p, Rpn4p, Ste12p, Sok2p, Msn4p and Yap1p. Previous studies showed that the main regulators of ESR genes Msn2p, Msn4p and Yap1p are highly active under stress conditions (Gasch *et al.*, 2000). Analysis of microarray data showed that, high proportion of upregulated genes of C9 is controlled by ESR-related transcription factors. However, only MSN4 showed increased expression by 3 fold. Other transcription factors may be upregulated too, However, in the main list their expression could be lower than the 2-fold threshold, and they may not have been included.

4. DISCUSSION

In this study, to investigate mechanisms of phenylethanol resistance in *Saccharomyces cerevisiae*, first PEA-resistant *S.cerevisiae* mutants were obtained by evolutionary engineering and then transcriptomic analysis was carried out for one of the most phenylethanol resistant yeast mutants "C9".

To obtain phenylethanol resistant yeast, principles of evolutionary engineering were employed in controlled laboratory conditions. EMS-treated yeast culture was exposed in batch cultures to continuously increasing phenylethanol concentrations. After the whole batch selection process, only a group of yeast mutants with improved phenylethanol resistance survived and used for further analysis. During this study, highly phenylethanol resistant and genetically stable mutants were obtained by following basic principles of evolutionary engineering which makes this approach very efficient to obtain yeast strains with desirable properties.

The PEA resistant strain (C9) showed very high tolerance to phenylethanol stress in comparison with wild type strain. It was also observed that C9 had also developed moderate resistance to ethanol. Although PEA-resistant *Escherichia coli* strains have been reported before (Lucchini *et al.*, 1993), there are no reports on PEA-resistant yeast strains obtained by evolutionary engineering in literature. For this reason, PEA-resistant C9 strain is important for clarifying PEA resistance mechanisms in yeast.

In this study, to understand transcriptomic differences between phenylethanol-resistant strain and the wild type, DNA microarray analysis was used.

DNA microarray analysis showed that a high number of genes were up-regulated and down-regulated in phenylethanol-resistant C9 under control conditions without any external stress. When 2.0 fold change was set as a lower limit, C9 had about 1000 up-regulated and about 800 down-regulated genes that make up in total nearly 30% of the whole genome. Such broad changes in transcription levels may indicate that some global expression response might have been continously active in C9. That

genome-wide expression program resembles a highly known large-scale stress reaction called “environmental stress response” (ESR). Detailed studies showed that in ESR, there are about 900 genes that change at transcriptomic level after stress exposure (Gasch *et al.*, 2000). Most stress factors induce the regulation of some common genes in ESR immediately after exposure. These regulation leads to large but transient expression changes in ESR genes. Microarray analyses of C9 under control conditions showed that resistant mutant showed similar transcription profile that seen during ESR (Table 3.7 and 3.8). C9 strain could be under ESR state even absence of stress factors. Thus, in any moment this strain might be ready for external stress conditions that could partly explain its phenylethanol resistance.

Analyses showed that the significant majority of these continuously up-regulated genes in C9 were also observed commonly during ESR in yeasts after stress exposure. About one-third of up-regulated genes of C9 are in common with ESR genes, besides more than 90% down-regulated genes in C9 were also similarly down-regulated during ESR. Transcriptomic characters of non-stressed wild type C9 and stress-treated *S. cerevisiae* indicate important similarities regarding activation of the same ESR system.

DNA microarray analysis showed that there were about 1000 up-regulated genes in C9 and the majority of these genes were responsible for carbohydrate metabolism. With up-regulated 166 genes, carbohydrate metabolism contributed to about 20% of general up-regulation of C9. There were also 98 genes responsible for oxidative stress response, 63 genes for general stress response, 35 genes for cell wall reorganization and renewal, 21 genes for degradation of mitochondria and cell itself, among up-regulated genes.

With 20% contribution, genes in carbohydrate metabolism were widely upregulated in phenylethanol resistant C9 strain. Similar upregulation in this gene clusterwas also observed during environmental stress response. In ESR, stress exposure triggers up-regulation of large amounts of genes responsible in resistance mechanisms such as HSP’s (Ogawa *et al.*, 2000). Synthesis and utilization of these response elements use high amount of energy that leads to rapid depletion of cytoplasmic ATP source. Cells have to buffer the ATP levels in their cytoplasm to keep response systems working.

The main adaptation against this drawback is induction of energy production pathways, such as glycolysis and oxidative phosphorylation (Gasch *et al.*, 2000).

Glycolysis was one of the most induced pathways in C9 according to microarray analysis results. Nearly all genes involved in catabolism of glucose were highly up-regulated (Figure 3.5). Also various genes such as aldehyde dehdyrogenases (*ALD2, ALD3, ALD4, ALD6*), a hexokinase that catalyzes the first step in glycolysis and responsible for priming reaction (*HXK1*), and phosphoglucomutase (*PGM2*) that catalyzes the conversion from glucose-1-phosphate to glucose-6-phosphate were highly upregulated. In addition to catabolic pathway, several genes associated with hexose import mechanism (*HXT1, HXT11, HXT3, HXT5, HXT6, HXT7, HXT8*) were up-regulated.

Contrary to highly activated glycolysis, C9 induced less genes that have been involved in respiration. Induced genes were involved in catalysis of the rate-limiting step of the TCA cycle (*CIT1*), an alternate isoform of cytochrome c (*CYC7*), various subunits of cytochrome c oxidase (*COX1, COX2, COX3, COX5B* and *COX20*), and genes responsible in ubiquinone (Coenzyme Q) biosynthesis (*COQ4, COQ5, COQ6, COQ9*). Genes encoding subunits of mitochondrial ATP synthase (*ATP6, ATP10, ATP18*) were also up-regulated. Although these genes are involved in oxidative phosphorylation, they are also associated with oxidative stress response. Over-expression of these genes might indicate increased resistance to oxidative stress rather than oxidative phosporylation.

Increased glucose catabolism of C9 might be due to increased energy requirements in ESR state. Gasch *et al.* (2002) previously reported that ESR-related protection results in increased energy consumption. To compensate energy depletion, cells have to up-regulate the catabolic pathways. Although, highly induced transcription profile was observed in C9, this mutant had no significant growth defect on control conditions. That might imply that C9 balanced the increased energy consumption through increased catabolic reactions, mainly by glycolysis.

Surprisingly, genes associated with maltose metabolism were also highly up-regulated. Genes encoding maltose transporter (*MAL11, MAL33*) and maltases

(*MAL12, MAL32*) which hydrolyze the disaccharides (e.g maltose) were up-regulated more than 100-fold. Additionally, some genes associated with galactose metabolism were up-regulated. The genes encoding the enzyme that catalyzes the interconversion of UDP-galactose and UDP-D-glucose in galactose (*GAL10*), galactose permease (GAL2) and DNA-binding transcription factor required for activating GAL genes (GAL4) were over-expressed. This might indicate that C9 cells also used alternative carbon sources such as maltase and galactose. Generally, in the presence of exogenous glucose, maltose and galactose metabolisms are normally repressed in yeast cells, because of catabolite repression (Federoff *et al.*, 1983). However, C9 might have overcome that repression to use alternative carbon sources.

Alcohol may damage cells through oxidative stress due to increased ROS production during its metabolism. ROS generation is generally attributed to electron leakage from mitochondria during oxidative phosphorylation reactions (Scandalios, 1997). That outflow is further increased under alcohol stress because of increasing mitochondrial permeability. It can lead to a chain of oxidation reactions in the cell, which damages cellular structures such as proteins, lipids, and DNA and prevent proper enzymatic activity by perturbing the internal redox potential (Ma and Liu, 2012).

Yeast cells use a number of enzymes associated with the detoxification of ROS. C9 also showed over-expression of these genes. Most significant ones in this group were genes encoding the cytosolic superoxide dismutase (*SOD2*) and cytosolic catalase (*CTT1*), which degrade superoxide and hydrogen peroxide, respectively. While catalase specifically reduces hydrogen peroxide, the glutathione peroxidase (*GPX1*) also uses organic peroxides as substrates. Another antioxidant enzyme, ubiquinone is an essential redox component of the aerobic respiratory chains. This lipid-soluble antioxidant prevents lipid peroxidation. In C9, many genes involved in ubiquinone biosynthesis (*COQ4, COQ5, COQ6, COQ9*) were up-regulated. The same genes are also over-expressed under ESR (Gasch *et al*., 2002).

One of the detrimental effects of short chain alcohols is increasing the membrane permeability for water (Weber and de Bont, 1996). Alcohols bind to fatty acid molecules and disrupt the stability of bilayer structure (Sikkema et al., 1995) that

leads to water uptake and swelling. As a physical barrier, cell wall is one of the protective mechanisms against swelling-related cell lysis. Outer layer includes mannoproteins which have main protective properties. Most abundant molecules (30-50% dry mass) are mannoproteins, stabilizing molecule of wall. Ma and Liu (2012) reported that under ethanol stress genes involved in cell wall structure (such as mannoproteins) are upregulated. This regulation possibly further increase the stability of cell wall. In C9 many known and putative genes were up-regulated which might contribute to PEA-resistance. According to microarray data, genes encoding mannoproteins (*CWP1, CWP2, FIT1, FIT2, CCW12*) were over-expressed. Also, some putative genes involved in cell wall biosynthesis (*ECM12, ECM27, ECM30, ECM4, ECM8*) were up-regulated.

Additionally, some putative genes of PAU family were up-regulated as a whole in C9 mutant. PAU gene family contains 24 different genes. In C9, 20 of them were over-expressed. PAU genes are found on all of the sixteen yeast chromosomes and their function is not completely known (Luo, Z. , & van Vuuren, H., 2009). PAU genes are known to bring about the synthesis of the seripauperines, a group of almost identical serine-poor proteins with unknown function (Goffeau, A., 1996). PAU genes are expressed during stress conditions and play a role in the stress response of *Saccharomyces cerevisiae*. 22 of PAU genes contain at least one copy of the anaerobic response element and the aerobic repression motif. Therefore, PAU genes are negatively regulated by oxygen (Rachidi *et al.,* 2000). Over-expression of these genes in C9 might indicate presence of some kind of repression in aerobic reactions.

Genes associated with autophagy were also induced in C9. Autophagy, the breakdown of cellular components can ensure cellular survival during starvation by maintaining cellular energy levels (Lin *et al*., 2012). In C9, ATG genes (*ATG13, ATG14, ATG15, ATG17, ATG19, ATG29, ATG33, ATG7, ATG8, ATG9*) that encodes proteins involved in vesicle formation during autophagy were over-expressed. It might indicate that C9 mutant strains might be in starvation or induce system related with starvation response.

ESR is a general response system and is activated after various stresses; however, this genomic expression program is customized for each environment. Additional to general stress responses, specialized and unique responses may also observed (Gasch *et al.*, 2000). In this study, unique response of phenylethanol resistant yeast strain might be attributed to super-induced aldehyde dehydrogenase gene (*ALD3*) which is responsible for the conversion of toxic acetaldehyde compounds to less toxic carboxylic acid forms. This reaction can occur in two different sub-cellular localizations in yeast: the mitochondria and the cytosol. The cytosolic ALDHs are encoded by the *ALD6*, *ALD2* and *ALD3*, while the mitochondrial counterparts are encoded by ALD4 and *ALD5* (Navarro-Aviño *et al.*, 1997).

In comparison with wild type, phenylethanol-resistant C9 strain showed 234-fold higher expression for *ALD3* gene. Various ALD genes (*ALD2, ALD3, ALD4, ALD6*) were also up-regulated. This might be related to main resistance mechanisms against phenylethanol and ethanol. Aranda and Olmo (2003) previously reported that aldehyde dehydrogenase activity is higher in ethanol-growing flor yeasts. Increased ALD3 and other ALDs gene expression, might prepare cells to overcome excess amount of aldehyde byproducts of alcohol degradation. However, there are no extensive studies about metabolism of PEA and its metabolic pathway is quite unclear. Although ALD3 might aid in its resistance mechanism; overall response against phenylethanol should be more complex and contain bigger network and action mechanism.

Interpretation of down-regulated gene results showed that C9 mutant had significantly repressed nucleic acid metabolism and ribosome synthesis. More than 81% of 821 down-regulated genes were related to synthesis and binding of rRNA and tRNA, initiation of translation, RNA-DNA binding, helicase activity. Again same regulations were also observed during ESR in stressed-wild type strains during initial stress exposure (Gasch *et al.*, 2000). This was correlated with the observed decrease in cellular translation that occurs in response to stressful environmental transitions (McAlister and Finkelstein, 1980).

Because ribosome synthesis requires substantial energy and cellular mass, it is predictable that transcript levels of genes encoding rRNA and ribosomal proteins are

inhibited under stressful conditions. As demonstrated by previous studies, the ribosomal protein genes are among the most strictly co-regulated genes in the yeast genome (Eisen et al. 1998). Causton *et al*. (2001) reported that, the transcript levels of all the ribosomal protein genes are rapidly reduced under environmental stresses, in some cases more than 80-fold. Expression of the rDNA encoding genes are known to be down-regulated in response to various stresses, including heat shock, starvation, secretion defects, and drug treatments (Shulman *et al*. 1977), (Veinot-Drebot *et al*. 1989).

In C9, many genes responsible for rRNA production, modulation and regulation were repressed significantly. Besides, expression of the tRNAs is known to be repressed following a variety of stresses, including amino acid and nitrogen starvation, progression into stationary phase, defects in secretion, and DNA damage (Gasch, 2003). Similarly, in C9 mutant, many genes in rRNA and tRNA processing, modification and synthesis were significantly downregulated. Similar to the repression of rDNA and ribosome protein genes, inhibition of tRNA synthesis is a general feature of the ESR (Gasch *et al.*, 2000).

C9 strain highly inhibits the transcription and translation processes under control conditions (Figure 3.6 and 3.7). This repression might be related with energy conservation strategy under stress conditions.

Surprisingly, some of the non-ESR genes (*FET3, FTR1*), which are responsible for iron metabolism were repressed significantly. These genes are normally related with general iron uptake under iron-depletion state or under cobalt stress (Stadler and Schweyen, 2002). The gene *FET3* codes for ferro-O_2-oxidoreductase and is required for high-affinity iron uptake and involved in resistance to copper toxicity, and *FTR1* codes high affinity iron permease which is involved in the transport of iron across the plasma membrane. DNA microarray analysis showed that C9 mutant generally downregulated the iron uptake genes that may lead to decreased intracellular iron ion levels. This outcome could explain cobalt sensitivity of C9: it is known that, cobalt toxicity is generated by a competition with iron. Cobalt shares several atomic structure similarities and can bind iron-containing enzymes (Thorgersen, 2007). As an adaptation process, cells increase the intracellular iron ion uptake to repress the

competitive properties of cobalt (Stadler and Schweyen, 2002). In C9 mutant, continuous repression of iron uptake genes seemed to make this mutant sensitive to cobalt stress.

Gasch *et al.* (2000) reported that, the main regulators of ESR genes Msn2p, Msn4p and Yap1p are highly active under stress conditions. The transcription factors responsible for regulation of upregulated genes were Msn2p, Aft1p, Rpn4p, Ste12p, Sok2p, Msn4p and Yap1p. Analysis of microarray data showed that, high proportion of up-regulated genes of C9 were also controlled by ESR-related transcription factors.

To sum up, evolutionary engineered C9 strain showed high tolerance to PEA and intermediate level ethanol resistance. DNA microarray analysis showed that PEA-resistant C9 up-regulated many stress response genes that also found in ESR system, even in the absence of external stresses. C9 seemed to have that it induced energy production and conservation pathways to balance increased energy requirement resulting from continuous expression of wide range of genes. C9 showed many resistance mechanisms mainly based on oxidative stress. Many genes involved in oxidative stress resistance were also up-regulated in C9. However, in cross resistance test, mutant strain did not showed higher resistance to oxidative stress (hydrogen peroxide). These genes might be strictly controlled in post-transcriptional level and over-expression might not indicate actual induction of related enzymes. C9 mutant also showed alcohol-specific stress responses such as cell wall modification and aldehyde metabolism. Many known and putative genes involved in synthesis of mannoproteins which are components of cell wall were up-regulated. Additionally, genes encoding ALDs which catalyze toxic byproducts of alcohol metabolism were highly up-regulated.

5. CONCLUSION & FUTURE REMARKS

In this study, by evolutionary engineering approach, a highly phenylethanol-resistant *S. cerevisiae* mutant (C9) was successfully obtained. The obtained phenylethanol-resistant strain also developed ethanol cross resistance, owing to possibly common stress response mechanisms between ethanol and phenylethanol. Additionally, the mutant showed increased sensitivity to cobalt that could be result from iron uptake repression in C9. DNA microarray data showed that C9 induced a global gene expression program under non-stress conditions which resemble to cell's environmental stress response (ESR). Both up-regulated and down-regulated genes in C9 showed high similarities to those that are expressed during ESR. Although ESR is a broad but transient response system during stress exposure, C9 seems to have continuously induce that system even under non-stress conditions possibly due to mutation-related changes in ESR regulation system. Even, DNA microarray data gave important clues about resistance mechanism, ESR and other systems might be highly regulated at post-transcriptional level. For deeper understanding of underlying mechanism, proteomic studies can be performed. Additional verification can be done by quantitative real time PCR. Also, whole genome sequence analysis can be carried out to detect DNA level changes in phenylethanol-resistant mutant C9. Overeexpression/deletion of genes that were highly upregulated/downregulated accordig to transcriptomic analysis results could also be done to gain insight into the complex molecular mechanism of phenylethanol resistance. The results will help produce highly phenylethanol-resistant strains that can ultimately be used in industrial applications with improved efficiency.

APPENDICES:

Table A.1: Biological processes and systematic names of highest upregulated genes. The genes that are represented as bold is responsible in ESR. The genes which upregulated lower than 10 fold change are not represented.

Table A.2: Biological processes and systematic names of highest downregulated genes. The genes that are represented as bold is responsible in environmental stress response. The genes which upregulated lower than 4 fold change are not represented.

Table A.3: List of all up-regulated and down-regulated genes in PEA resistant C9 mutant

Table A.1: Biological processes and systematic names of highest upregulated genes. The genes that are represented as bold is responsible in ESR. The genes which upregulated lower than 10 fold change are not represented.

Process	*Gene*	Systematic Name	*Gene*	Systematic Name
Carbohydrate Metabolic Process (p value: 1.239e-09)	*MAL12*	YGR292W	***AMS1***	YGL156W
	MAL32	YBR299W	*NQM1*	YGR043C
	GLK1	YCL040W	***SOL4***	YGR248W
	EMI2	YDR516C	*GPH1*	YPR160W
	GLC3	YEL011W	***PGM2***	YMR105C
	HXK1	YFR053C		
Glycogen Biosynthetic Process (p value: 2.654e-09)	***GLC3***	YEL011W	*GSY2*	YLR258W
	GSY	YFR015C	***PGM2***	YMR105C
	GAC1	YOR178C		
Response To Stress (p value: 3.678e-09)	*RSB1*	YOR049C	*FMP43*	YGR243W
	FMP45	YDL222C	***GRE3***	YHR104W
	TPS2	YDR074W	***XBP1***	YIL101C
	HSP78	YDR258C	***TSL1***	YML100W
	SSA4	YER103W	***DDR48***	YMR173W
	HSP12	YFL014W	***DDR2***	YOL052C-A
	CTT1	YGR088W		
Maltose Metabolic Process (p value: 1.138e-07)	*MAL31*	YBR298C	*MAL11*	YGR289C
	MAL32	YBR299W	*MAL12*	YGR292W
Trehalose Biosynthetic Process (p value: 7.519e-07)	***TPS2***	YDR074W	***TSL1***	YML100W
	UGP1	YKL035W	***PGM2***	YMR105C

Table A.1 (continued): Biological processes and systematic names of highest upregulated genes. The genes that are represented as bold is responsible in ESR. The genes which upregulated lower than 10 fold change are not represented.

Process	*Gene*	Systematic Name	*Gene*	Systematic Name
Metabolic Process (p value: 3.165e-06)	*BDH2*	YAL061W	*NQM1*	YGR043C
	TKL2	YBR117C	***GSY1***	YFR015C
	MAL32	YBR299W	***GSY2***	YLR258W
	GPM2	YDL021W	***UGP1***	YKL035W
	TPS2	YDR074W	***ALD3***	YMR169C
	HXK1	YFR053C	***ALD4***	YOR374W
	AMS1	YGL156W		
Maltose Catabolic Process (p value: 0.0001524)	*MAL32*	YBR299W	*MAL11*	YGR289C
	MAL31	YBR298C	*MAL12*	YGR292W
Glycolysis (p value: 0.0003605)	***GLK1***	YCL040W	***GPM2***	YDL021W
	EMI2	YDR516C	***HXK1***	YFR053C
Galactose Catabolic Process (p value: 0.0004534)	***GRE3***	YHR104W	***PGM2***	YMR105C
Sucrose Catabolic Process (p value: 0.0004534)	*MAL32*	YBR299W	*MAL13*	YGR288W
Glucose Import (p value: 0.0004534)	***GLK1***	YCL040W	***HXK1***	YFR053C
Carbohydrate Transport (p value: 0.0005386)	*MAL31*	YBR298C	*HXT7*	YDR342C
	HXT6	YDR343C	*MAL11*	YGR289C
Pentose-Phosphate Shunt (p value: 0.0006088)	***TKL2***	YBR117C	*NQM1*	YGR043C
	SOL4	YGR248W		
D-Xylose Catabolic Process (p value: 0.0008995)	***GRE3***	YHR104W	*GCY1*	YOR120W
Arabinose Catabolic Process (p value: 0.0008995)	***GRE3***	YHR104W	*GCY1*	YOR120W
Cellular Response To Heat (p value: 0.005183)	***HSP78***	YDR258C	***HSP12***	YFL014W
Response To Oxidative Stress (p value: 0.009362)	***HSP12***	YFL014W	***GRE3***	YHR104W
	GAD1	YMR250W	*GCY1*	YOR120W

Table A.2: Biological processes and systematic names of highest downregulated genes. The genes that are represented as bold is responsible in environmental stress response. The genes which upregulated lower than 4 fold change are not represented.

Process	*Gene*	Systematic Name	*Gene*	Systematic Name
Ribosome Biogenesis (p value: <1e-14)	***CIC1***	YHR052W	*IPI1*	YHR085W
	NOP10	YHR072W-A	*MRT4*	YKL009W
	DBP9	YLR276C	***DHR2***	YKL078W
	RRB1	YMR131C	***EBP2***	YKL172W
	IPI3	YNL182C	***ESF2***	YNR054C
	RCL1	YOL010W	***PNO1***	YOR145C
	RRS1	YOR294W	***NIP7***	YPL211W
	IMP4	YNL075W	***RIX7***	YLL034C
	DBP2	YNL112W	***RLP24***	YLR009W
Ribosomal Subunit Biogenesis (p value: <1e-14)	***MAK16***	YAL025C	***PUF6***	YDR496C
	ARX1	YDR101C	***CIC1***	YHR052W
	RLP24	YLR009W	***RRS1***	YOR294W
	NIP7	YPL211W	*MRT4*	YKL009W
	RIX7	YLL034C		
Ribosomal Large Subunit Assembly (p value: 2.999e-11)	***RSA4***	YCR072C	***DBP9***	YLR276C
	IPI1	YHR085W	***IPI3***	YNL182C
	YVH1	YIR026C	***NIP7***	YPL211W
	MRT4	YKL009W	***RPF2***	YKR081C
	BFR2	YDR299W	*MRT4*	YKL009W
rRNA Processing (p value: <1e-14)	***EBP2***	YKL172W	***IPI1***	YHR085W
	DHR2	YKL078W	*NOP10*	YHR072W-A
	NSR1	YGR159C		

Table A.2 (continued): Biological processes and systematic names of highest downregulated genes. The genes that are represented as bold is responsible in environmental stress response. The genes which upregulated lower than 4 fold change are not represented.

Process	Gene	Fold of Wt	Gene	Fold of Wt
Transmembrane Transport (p value: 0.001754)	***CTP1***	YBR291C	***ENB1***	YOL158C
	PHO84	YML123C	***ATR1***	YML116W
	ATO3	YDR384C	***SUL1***	YBR294W
	ZRT1	YGL255W	***YJR124C***	YJR124C
	FTR1	YER145C	***FCY2***	YER056C
	OPT2	YPR194C	***SSU1***	YPL092W
	YOL163W	YOL163W	***MMP1***	YLL061W
Mitochondrial Citrate Transport (p value: 0.0008948)	***CTP1***	YBR291C	***YHM2***	YMR241W
Iron Assimilation By Reduction (p value: 0.0008948)	*FTR1*	YER145C	*FET3*	YMR058W
Ribosomal Large Subunit Export From Nucleus (p value: 1.891e-07)	*ECM1*	YAL059W	*RRS1*	YOR294W
	RIX7	YLL034C	*BCP1*	YDR361C
High-Affinity Iron Ion Transport (p value: 0.005158)	*FTR1*	YER145C	*FET3*	YMR058W
Iron Ion Homeostasis (p value: 0.005158)	*FTR1*	YER145C	*FET3*	YMR058W
	ENB1	YOL158C		

Table A.3: List of all up-regulated and down-regulated genes in PEA resistant C9 mutant

Fold Change (Up-regulation)	Gene Symbol	Fold Change (Down-regulation)	Gene Symbol
234,00	ALD3	19,77	SUL1
223,64	HXK1	18,64	ZRT1
217,14	GPH1	17,90	PHM6
208,60	TKL2	16,84	PHO84
207,44	MAL12	14,47	YDL241W
198,75	MAL32	13,67	STE3
136,62	HSP12	12,33	ARO3
127,94	MAL11	11,92	AAH1
101,69	FMP45	10,90	FET3
100,23	PGM2	10,82	RAS1
91,99	RTN2	10,73	SSP1
71,31	HSP26	9,38	DBP2
67,29	TSL1	9,26	ECM1
58,87	DDR2	8,52	PNO1
52,48	CTT1	8,19	OPT2
45,38	YMR206W	8,08	SPL2
43,37	YNL194C	7,58	DHR2
43,32	PHM7	7,49	FCY2
38,39	PIR3	7,49	YOL014W
36,84	GAC1	7,42	ATO3
32,96	TMA10	7,22	NSR1
30,58	YNR034W-A	7,11	BNA2
29,07	YFL052W	6,98	RSA4
28,15	GLC3	6,85	MMP1

Fold Change (Up-regulation)	Gene Symbol	Fold Change (Down-regulation)	Gene Symbol
28,09	ALD4	6,80	YER187W
27,45	MSC1	6,76	CIC1
26,28	HXT7	6,66	YCR087C-A
26,03	SOL4	6,54	HFM1
25,39	YGP1	6,44	NIP7
24,21	YER067W	6,40	DAL1
23,71	SED1	6,35	KRE33
21,38	ISF1	6,22	GFD2
21,13	BDH2	6,17	YBR141C
21,09	INO1	6,15	MRT4
20,74	GSY1	6,08	YNR062C
20,53	DCS2	6,02	BFR2
19,89	HXT6	6,00	RKI1
19,82	XBP1	5,98	ADH4
19,39	GAD1	5,93	AI3
19,14	FMP16	5,91	MAK16
18,39	EMI2	5,84	IMD4
18,03	MAL31	5,83	FRM2
17,76	NQM1	5,82	HES1
17,72	GSY2	5,79	RLP24
16,49	PNS1	5,70	YIL096C
16,36	YGR287C	5,65	YGL101W
16,17	YFR017C	5,64	YLR460C
16,16	SSA4	5,61	PGA3
15,74	SDS24	5,58	RCL1

Fold Change (Up-regulation)	Gene Symbol	Fold Change (Down-regulation)	Gene Symbol
15,64	AMS1	5,50	CTP1
15,38	MAM1	5,48	GCD10
15,07	YIL060W	5,47	MIP6
14,41	YLR149C	5,46	DCG1
14,20	FMP43	5,46	SSU1
14,04	PRM10	5,46	BCP1
13,92	GPM2	5,45	DBP9
13,69	YLR042C	5,43	IMP4
13,16	GPG1	5,36	YVH1
13,00	YMR090W	5,33	IPI3
12,80	TPS2	5,30	IPI1
12,63	UIP4	5,29	ATR1
12,32	GUT2	5,28	YJR124C
12,32	CWP1	5,24	YBR271W
12,23	UGP1	5,18	FYV7
11,87	GRE3	5,18	FUR1
11,73	SPI1	5,17	TOD6
11,67	HBT1	5,15	RRT14
11,29	TFS1	5,14	RIX7
11,02	GCY1	5,13	TRM13
10,93	RSB1	5,12	RPF2
10,85	GLK1	5,12	YHM2
10,77	STF1	5,11	ESF2
10,70	YLR312C	5,06	MHT1
10,39	YNL200C	5,06	EBP2

Fold Change (Up-regulation)	Gene Symbol	Fold Change (Down-regulation)	Gene Symbol
10,38	HSP78	5,02	YPR157W
10,37	YNL195C	5,01	BUD23
10,15	PIG2	4,99	YBL107C
10,05	DDR48	4,98	ERG20
10,00	YJL107C	4,93	ATC1
9,76	OM14	4,90	ENB1
9,57	YLR162W	4,90	YLR413W
9,55	CYB2	4,88	YOL163W
9,38	MTH1	4,87	RRB1
9,15	IKS1	4,86	SEE1
9,00	RTC3	4,86	URA7
8,96	LEE1	4,83	RPB5
8,92	HAL1	4,80	ARX1
8,90	ERR3	4,75	PUF6
8,68	PRX1	4,72	RRS1
8,60	YJR115W	4,71	IMD2
8,50	SSE2	4,71	FTR1
8,48	AFR1	4,69	NOP10
8,44	YDR379C-A	4,66	YLR363W-A
8,42	HXT5	4,65	RLP7
8,40	SDP1	4,63	DRS1
8,34	YLR030W	4,61	REI1
8,27	YKL151C	4,59	NSA2
8,23	OM45	4,57	EFG1
8,22	HSP33	4,57	FAL1

Fold Change (Up-regulation)	Gene Symbol	Fold Change (Down-regulation)	Gene Symbol
8,16	GDB1	4,55	FAF1
8,15	TPS1	4,55	TSR2
7,86	YJL163C	4,50	KRE29
7,67	SYM1	4,50	AAT1
7,66	HSP104	4,49	CYB5
7,64	STB2	4,49	NCS2
7,58	FMP33	4,46	TSR1
7,52	RNY1	4,45	NMD3
7,40	YOR289W	4,45	EHD3
7,30	YHR097C	4,44	RFU1
7,30	GSC2	4,43	RRP8
7,29	CYC7	4,42	HMT1
7,27	YMR196W	4,41	RPC53
7,27	MGA1	4,40	YGR283C
7,25	TDH1	4,39	MRD1
7,11	YGR205W	4,39	HAS1
7,09	YML131W	4,38	PEX21
7,07	SRL3	4,37	REX4
6,91	MEK1	4,37	LEU9
6,89	SSA1	4,37	RPG1
6,82	GND2	4,36	YTM1
6,79	MRK1	4,35	SDA1
6,78	ERR1	4,35	NOP53
6,78	SNO4	4,34	BRX1
6,76	COX5B	4,31	SEO1

Fold Change (Up-regulation)	Gene Symbol	Fold Change (Down-regulation)	Gene Symbol
6,70	SGA1	4,30	PPT1
6,69	YNL305C	4,30	AQY2
6,68	HSP82	4,30	POR2
6,67	VHS3	4,26	URA1
6,67	ERR2	4,24	SSF1
6,65	SWH1	4,24	SPS4
6,64	HSP32	4,20	TGS1
6,63	KIN82	4,17	HIM1
6,62	YMR291W	4,16	HIT1
6,57	PFK26	4,12	DUS3
6,55	YLR345W	4,12	DBP8
6,52	YKL091C	4,11	YLL053C
6,49	HSP42	4,11	NOG1
6,45	SOL1	4,10	ENP1
6,29	OYE3	4,08	RRN11
6,29	YGR066C	4,08	APT1
6,29	VID30	4,08	GRC3
6,28	URA10	4,06	DAS2
6,22	YNR014W	4,04	FCF2
6,21	YKL161C	4,01	TAT2
6,18	TPK1	4,01	HIS1
6,09	PST1	3,99	SOR1
6,02	AGP2	3,99	MTO1
5,99	CRG1	3,98	PGA2
5,98	YPR127W	3,98	YGR079W

Fold Change (Up-regulation)	Gene Symbol	Fold Change (Down-regulation)	Gene Symbol
5,88	TPS3	3,97	RIO2
5,88	GPD1	3,94	RPC17
5,86	BBC1	3,93	MTR4
5,86	GPT2	3,93	NOG2
5,84	THI4	3,91	NRP1
5,83	TMA17	3,89	FPR4
5,71	GGA1	3,88	HCA4
5,65	HOR2	3,86	MUP1
5,64	RTS3	3,84	YCR051W
5,63	YPT53	3,81	TIF6
5,61	YER079W	3,81	YHB1
5,60	XKS1	3,80	TRM8
5,58	PRY1	3,80	RRP1
5,57	GIP2	3,77	NOP13
5,56	YPK2	3,76	SHM1
5,55	ECM8	3,76	DIP2
5,53	YOR152C	3,76	NOP2
5,52	PBI2	3,75	TMA46
5,48	YLR053C	3,75	IMP3
5,48	YJR096W	3,75	YDR161W
5,42	SIP18	3,73	UTP18
5,38	ETR1	3,73	YCR016W
5,36	LAP4	3,71	MSH1
5,36	YLR415C	3,71	YNL019C
5,33	YER121W	3,71	TRM9

Fold Change (Up-regulation)	Gene Symbol	Fold Change (Down-regulation)	Gene Symbol
5,29	ROM1	3,71	MAG1
5,28	AIM17	3,70	RMT2
5,24	YOR186W	3,69	DPH1
5,21	GRE2	3,69	NOC4
5,21	IRC15	3,68	PRP24
5,19	NCA3	3,67	YNL022C
5,19	PRR2	3,67	YCR102C
5,12	UBI4	3,66	TRM10
5,06	YNL144C	3,66	PRM9
4,99	IML2	3,64	CDA1
4,97	GRX1	3,63	NAF1
4,92	YAR064W	3,61	PES4
4,92	FLO9	3,57	AQR1
4,90	SUE1	3,57	MAK11
4,88	CIN5	3,56	RPC37
4,86	YRO2	3,56	RPA14
4,84	AIM26	3,54	BNA4
4,82	PRM5	3,53	EMG1
4,80	DAK1	3,52	RAI1
4,78	PRM6	3,52	NOC3
4,78	ATG8	3,51	SNU13
4,77	VRP1	3,51	NOB1
4,75	ZRG8	3,51	TRM44
4,74	PSK1	3,50	NOP1
4,73	YNR066C	3,49	FUS1

Fold Change (Up-regulation)	Gene Symbol	Fold Change (Down-regulation)	Gene Symbol
4,68	BAG7	3,49	YML108W
4,67	PRB1	3,48	PDC5
4,66	PRY2	3,48	RGS2
4,65	JIP4	3,47	LIA1
4,64	ADR1	3,46	NUG1
4,62	YDR034C-A	3,45	DIT1
4,61	CRH1	3,45	SFG1
4,58	PTK2	3,44	TIF5
4,53	NAB6	3,44	DBP6
4,53	GPX1	3,44	SPE4
4,49	ATG13	3,42	UTP21
4,48	DCS1	3,42	KRI1
4,48	STF2	3,42	HSP31
4,46	YKR075C	3,42	URK1
4,44	CWP2	3,41	PPH3
4,43	PHR1	3,40	YBL081W
4,42	PAI3	3,40	DPH2
4,42	YIR014W	3,39	TRM1
4,40	ATH1	3,39	RIX1
4,40	YJR008W	3,38	YIL091C
4,40	YDR018C	3,37	SAM1
4,37	YLR446W	3,36	YNL024C
4,36	MNN4	3,35	RPP1
4,36	NTH1	3,35	RPA12
4,34	ACH1	3,35	EFG1

Fold Change (Up-regulation)	Gene Symbol	Fold Change (Down-regulation)	Gene Symbol
4,33	FMP46	3,35	SGD1
4,32	ECL1	3,34	PRS3
4,31	YJL144W	3,34	YJL045W
4,29	SSD1	3,34	RPL21A
4,28	TSA2	3,34	UTP13
4,28	PHM8	3,34	RPL41B
4,28	HOR7	3,33	DUS1
4,26	GTO3	3,33	UTP23
4,25	ATG17	3,32	LCP5
4,22	GOR1	3,32	SPS22
4,22	USV1	3,32	ALG3
4,21	GLG1	3,31	TRM7
4,20	SVS1	3,31	GGC1
4,18	COB	3,30	TNA1
4,17	SDH1	3,30	PRP28
4,14	YAP6	3,30	HGH1
4,14	ARK1	3,30	VBA4
4,13	RNP1	3,28	PHO88
4,11	YGR130C	3,28	BUD22
4,11	YSC84	3,27	SLX9
4,09	YFL051C	3,27	MST28
4,06	YBR085C-A	3,27	YDL063C
4,05	YBR139W	3,26	LTV1
4,04	PCD1	3,26	YMR310C
4,04	TPK2	3,25	ADE1

Fold Change (Up-regulation)	Gene Symbol	Fold Change (Down-regulation)	Gene Symbol
4,03	GSP2	3,25	YHL039W
4,02	MBR1	3,25	BUD27
3,98	YLR177W	3,25	RPS11A
3,97	SPG4	3,23	MZM1
3,97	YNL092W	3,22	FAP7
3,96	YHR210C	3,22	CAF20
3,95	AMA1	3,22	UTP30
3,95	ATG14	3,21	SWC5
3,95	PDC6	3,21	RPP2B
3,94	YLR445W	3,20	YML082W
3,93	HUL4	3,19	TRM2
3,91	SSH4	3,18	UTP9
3,89	ATG15	3,18	DAD2
3,88	CIS3	3,18	ASP1
3,88	PIC2	3,17	SOF1
3,87	VMR1	3,16	UFD1
3,85	CIT1	3,16	RPL8B
3,83	YJL070C	3,16	SMA1
3,82	SPO73	3,16	PSF2
3,81	PNC1	3,16	YHR122W
3,81	ULA1	3,15	RPS9B
3,79	ATG19	3,15	FIG1
3,78	YBR056W	3,15	HXT9
3,78	YCR101C	3,15	GIR2
3,78	SRX1	3,15	RPS8B

Fold Change (Up-regulation)	Gene Symbol	Fold Change (Down-regulation)	Gene Symbol
3,74	YFL054C	3,15	SHQ1
3,73	GAT4	3,14	RSM26
3,72	UGX2	3,14	ALB1
3,72	YOR008C-A	3,11	PUS7
3,70	PKH1	3,11	MEU1
3,68	VPS73	3,10	RRP5
3,67	MER1	3,10	FRE7
3,66	PAM1	3,09	SRL4
3,66	YHR022C	3,09	NAR1
3,66	YJL132W	3,08	YNL033W
3,65	MPH3	3,08	IKI1
3,64	ALD6	3,08	FUI1
3,64	COX2	3,06	RRP36
3,64	YNR065C	3,05	YGR093W
3,63	UBC8	3,05	GIT1
3,63	YBL112C	3,04	SPB4
3,63	ECM12	3,03	TCP1
3,62	RFX1	3,03	BCD1
3,61	EMP46	3,03	UTP4
3,60	RSF1	3,02	SUI1
3,59	YPL191C	3,01	YHI9
3,59	UBP9	3,00	SWD3
3,58	YBR053C	3,00	RPC34
3,57	YDL025C	2,99	ADE5,7
3,55	SAP4	2,99	OTU2

Fold Change (Up-regulation)	Gene Symbol	Fold Change (Down-regulation)	Gene Symbol
3,55	YBR285W	2,99	FAP1
3,55	CYR1	2,99	ALP1
3,53	MATALPHA1	2,99	SAD1
3,52	GIS1	2,99	BI2
3,51	YMR160W	2,98	AUS1
3,48	YPS3	2,97	NSG1
3,48	YJL185C	2,97	MSS116
3,47	SOD2	2,96	ELP2
3,47	GPB1	2,95	RPL1A
3,46	JSN1	2,95	YNL313C
3,45	YGL157W	2,95	MTC3
3,44	ICS2	2,94	DUT1
3,44	ATO2	2,94	RPC19
3,42	MAM3	2,93	YOR021C
3,42	SAF1	2,93	CDC6
3,42	BSC4	2,93	UTP11
3,41	FMP40	2,93	MRPS9
3,41	YEF1	2,93	YBR028C
3,40	ROD1	2,92	TPA1
3,39	MCR1	2,90	RPS28B
3,39	NCE103	2,90	RPS12
3,38	HER1	2,89	YDR514C
3,36	MTL1	2,89	SUI3
3,35	SPG3	2,89	NOP9
3,35	HMLALPHA1	2,88	YLR063W

Fold Change (Up-regulation)	Gene Symbol	Fold Change (Down-regulation)	Gene Symbol
3,34	ZTA1	2,88	ARO7
3,34	YLR159W	2,88	VHT1
3,34	RAV1	2,88	RRP40
3,34	NCE102	2,87	RPP1A
3,33	MOH1	2,87	YML096W
3,33	AIM41	2,87	NOP8
3,32	YNL115C	2,87	RRT5
3,32	YGR149W	2,87	ATF2
3,31	YLR156W	2,87	SUA5
3,31	YLR161W	2,86	FCF1
3,30	YKL106C-A	2,85	GPI2
3,30	MSS11	2,85	RPL1B
3,29	YHR138C	2,85	ZRC1
3,29	ECM4	2,85	SLF1
3,28	YLR281C	2,85	HPT1
3,27	ECM30	2,84	SSF2
3,26	RPI1	2,84	RPS19A
3,26	MYO3	2,83	UTP8
3,26	PSD2	2,83	AFG2
3,24	MRP8	2,83	HAM1
3,23	PTP2	2,82	BIO2
3,23	YIL055C	2,82	MET8
3,22	YPL088W	2,82	UTP14
3,21	FRT2	2,82	RPL41A
3,20	YMR181C	2,82	UBC11

Fold Change (Up-regulation)	Gene Symbol	Fold Change (Down-regulation)	Gene Symbol
3,20	NFT1	2,81	DAL7
3,19	PIN2	2,81	RBG2
3,18	PRM8	2,81	NDE1
3,16	UBP11	2,81	RPL30
3,16	RMD5	2,81	RPA49
3,15	YPL247C	2,80	LYS4
3,15	FMT1	2,80	YPR071W
3,14	MUC1	2,80	DAL2
3,13	SCO2	2,80	YHR127W
3,12	RGM1	2,80	MPP10
3,12	OPI10	2,79	BAP3
3,12	FRA1	2,79	SNO3
3,11	YDR034W-B	2,79	QDR1
3,11	NDE2	2,79	FUR4
3,11	FUN14	2,77	YBL028C
3,11	HFD1	2,77	RPS22A
3,10	MAL13	2,77	TIF35
3,10	YMR087W	2,77	YER156C
3,10	YPL141C	2,76	RKM4
3,09	GIC2	2,76	RPS17A
3,08	NDI1	2,75	NSA1
3,07	AHP1	2,75	RPL19B
3,07	YPR015C	2,75	YCL073C
3,06	YDL199C	2,75	YML018C
3,06	MPH2	2,74	FRE4

Fold Change (Up-regulation)	Gene Symbol	Fold Change (Down-regulation)	Gene Symbol
3,06	PAU22	2,74	DBP7
3,05	YML003W	2,74	RTT10
3,05	YCL042W	2,74	YMC1
3,05	PIL1	2,73	FOB1
3,05	PEP4	2,73	RPS11B
3,03	YER039C-A	2,73	TSR4
3,03	STL1	2,72	YLR065C
3,03	FBP26	2,71	ALG3
3,03	YLR408C	2,71	DTR1
3,02	SEC31	2,71	BAP2
3,02	PAU3	2,70	CCT7
3,01	VID28	2,70	RMD9
3,01	ADY3	2,70	HFI1
3,00	PAU21	2,70	FET4
3,00	REC114	2,69	SWM2
3,00	UBX3	2,69	PWP2
3,00	ALD2	2,69	RPA43
2,99	HXT1	2,69	LHP1
2,98	SPO20	2,68	SUI2
2,98	GID7	2,68	MCH5
2,98	SPS100	2,68	RHB1
2,96	FDH1	2,68	RPS13
2,95	PTH1	2,67	NOC2
2,95	NGR1	2,67	RPS26B
2,95	OXR1	2,67	YER064C

Fold Change (Up-regulation)	Gene Symbol	Fold Change (Down-regulation)	Gene Symbol
2,94	YHR213W	2,67	CCT5
2,93	ESC1	2,66	SSB1
2,93	PAU19	2,66	YLF2
2,92	BSC5	2,66	PPR1
2,92	YHR213W	2,66	SSB2
2,92	YBR241C	2,66	TRM12
2,92	YOR292C	2,66	HNM1
2,91	PAU16	2,65	RPC25
2,91	SNF3	2,65	UPS2
2,91	PAU15	2,65	POL5
2,90	YDL027C	2,64	SAP185
2,90	ATG29	2,63	GUA1
2,89	YMR118C	2,63	SAS2
2,88	YOR062C	2,62	SKG6
2,88	YLR271W	2,62	HTA1
2,88	YKL171W	2,62	UTP5
2,88	APE2	2,62	SCD6
2,87	SIP2	2,62	KEL3
2,87	GTT1	2,61	SLP1
2,86	MPM1	2,61	YGR125W
2,86	YMR262W	2,60	RPC31
2,85	BNI1	2,60	TRM82
2,84	SMF1	2,60	CDC33
2,84	HXT11	2,60	SUN4
2,84	COQ9	2,60	PAM18

Fold Change (Up-regulation)	Gene Symbol	Fold Change (Down-regulation)	Gene Symbol
2,83	YMR258C	2,59	LSG1
2,82	RRT8	2,59	PRE7
2,82	YLR218C	2,59	YDR222W
2,82	SCW4	2,58	SNO1
2,81	FYV10	2,58	SRY1
2,81	YBR204C	2,57	SNO2
2,79	YBL111C	2,57	RRP4
2,78	GIP4	2,57	SAS10
2,78	YHR218W	2,57	EMP70
2,77	PAU17	2,57	HIS3
2,77	CAT8	2,56	AAD16
2,77	YIR007W	2,56	WSS1
2,77	YCL049C	2,56	RNA14
2,76	FLO5	2,56	HXT17
2,76	GLO1	2,56	RPS6B
2,76	YIL108W	2,56	FRE1
2,76	PDE1	2,55	VTS1
2,75	PPE1	2,55	ENA2
2,75	YBP1	2,55	TIF34
2,75	BDH1	2,55	THI11
2,73	GDE1	2,54	IZH2
2,72	KIN1	2,54	YHR020W
2,71	ATG7	2,54	CNE1
2,71	PAU12	2,54	UTP6
2,71	STE18	2,54	UTP10

Fold Change (Up-regulation)	Gene Symbol	Fold Change (Down-regulation)	Gene Symbol
2,71	YMR122W-A	2,54	MRH4
2,71	GRE1	2,54	YKR045C
2,70	YFR045W	2,53	SIR2
2,70	SLM1	2,53	PMU1
2,69	SSH4	2,53	ZUO1
2,69	MPT5	2,53	RPL27B
2,69	TAX4	2,53	TMA16
2,68	YBR284W	2,53	YGR210C
2,68	HXT8	2,52	YJR141W
2,68	COX3	2,52	GCV2
2,68	ICS3	2,52	CIN4
2,68	COP1	2,51	MCH2
2,67	CLD1	2,51	CDC8
2,67	RRI2	2,51	URA5
2,67	PAU14	2,51	RPL16B
2,67	MET13	2,50	DAL3
2,67	RAD16	2,50	RPA135
2,67	PAR32	2,50	SEN34
2,66	MDH2	2,50	AIR1
2,66	YGR126W	2,50	YOL162W
2,66	MUM3	2,50	BRE2
2,66	PAU7	2,50	RRP9
2,65	HUR1	2,49	BER1
2,64	RRT6	2,49	RPL23A
2,64	FAA1	2,49	MEP2

Fold Change (Up-regulation)	Gene Symbol	Fold Change (Down-regulation)	Gene Symbol
2,63	PAU1	2,49	MRPS35
2,63	ATP6	2,49	RPL12A
2,63	VHS1	2,48	TRM3
2,63	HOS3	2,48	RPS25B
2,63	SWC7	2,48	HEM12
2,62	COX1	2,48	RPC82
2,62	AIM23	2,48	HUT1
2,62	RGA2	2,48	TAD3
2,62	YET2	2,47	SNZ2
2,62	SSL2	2,47	DFR1
2,61	YPR1	2,47	ZPR1
2,60	TRR2	2,47	RPS4B
2,60	YBL029W	2,47	SAM4
2,60	GYP7	2,47	APQ12
2,60	ATG33	2,47	ANB1
2,60	PAU9	2,46	RPL16A
2,60	AIM14	2,46	NOP15
2,60	PAU24	2,46	YNL035C
2,59	YKR011C	2,46	XDJ1
2,59	GTO1	2,46	RPO26
2,59	COQ6	2,45	DAT1
2,58	PAU13	2,45	MRPL19
2,58	MHP1	2,45	NOP12
2,58	ARO10	2,44	SRP102
2,58	ENO1	2,44	CDC123

Fold Change (Up-regulation)	Gene Symbol	Fold Change (Down-regulation)	Gene Symbol
2,58	PCM1	2,44	RPL25
2,58	ENT2	2,44	TSR3
2,57	PRC1	2,44	RRG1
2,57	STB3	2,44	ENP2
2,57	IDP2	2,44	SVF1
2,56	LSP1	2,44	RPT6
2,56	PGM3	2,43	ARO4
2,56	SRL1	2,43	CCT2
2,56	YCL056C	2,43	RKM2
2,56	PIN4	2,43	LYS1
2,55	OCH1	2,43	GCN3
2,55	MSN4	2,43	RRP45
2,54	SAE2	2,42	NHP6A
2,54	ENA5	2,42	PEX11
2,54	YKL162C	2,42	RPL42B
2,54	SSY5	2,42	AGA1
2,54	YCR050C	2,41	RBD2
2,54	COS1	2,41	PRM7
2,53	RHO5	2,41	RPS7A
2,53	SIP5	2,41	PAC2
2,53	GRX2	2,41	ELP3
2,53	PUF2	2,41	UGA4
2,53	AIM19	2,40	RBA50
2,53	RIM11	2,40	URB2
2,53	CMK1	2,40	PFA4

Fold Change (Up-regulation)	Gene Symbol	Fold Change (Down-regulation)	Gene Symbol
2,53	PUN1	2,40	SNZ1
2,52	CSH1	2,40	RPS0B
2,52	PMD1	2,39	SNZ3
2,52	GAL4	2,39	HIF1
2,52	CRF1	2,39	RPL21B
2,52	NUT2	2,39	UAF30
2,52	NAT4	2,39	AIM36
2,51	CAR2	2,39	FUN12
2,51	PYK2	2,39	YMC2
2,51	DOG2	2,38	AAD15
2,51	YHR202W	2,38	COG1
2,51	ARG82	2,37	PRS4
2,50	MET28	2,37	ERG3
2,49	YCR061W	2,37	ARG8
2,49	YAK1	2,36	MTD1
2,49	RCN2	2,36	BUD20
2,49	VPS64	2,36	IMD3
2,48	YGR053C	2,36	YHR214W
2,48	BSC1	2,36	YDR374C
2,48	MUP3	2,36	HIS6
2,48	PAU10	2,35	YLR050C
2,48	PAN2	2,35	YLR287C
2,48	PKH2	2,35	MRPL8
2,48	SNF7	2,35	YMR244W
2,47	YGR237C	2,34	SMM1

Fold Change (Up-regulation)	Gene Symbol	Fold Change (Down-regulation)	Gene Symbol
2,47	ICY1	2,34	YLH47
2,47	KNS1	2,34	TUB4
2,47	HUA1	2,33	PAC1
2,46	SCS22	2,33	NOP16
2,46	YMR317W	2,33	MNI1
2,46	FUN19	2,33	BIO4
2,45	PAU2	2,32	YKR041W
2,45	OLI1	2,32	PUS6
2,44	CBP4	2,32	YLR179C
2,43	YPK9	2,32	SWM1
2,43	ICT1	2,32	RPS1B
2,43	COQ5	2,32	RPS18A
2,43	GRS2	2,32	SPE3
2,43	PEP3	2,32	PEX2
2,42	UGA2	2,32	TRF5
2,42	CUR1	2,32	PKR1
2,42	RAD59	2,31	RPL40B
2,41	PMT6	2,31	MND1
2,41	APC4	2,31	SFH5
2,41	YMR114C	2,31	RSA1
2,41	TOS6	2,31	YHK8
2,40	YIL172C	2,30	YIL165C
2,40	YLR281C	2,30	SAS5
2,40	YTP1	2,30	BNA1
2,40	FPK1	2,29	YDR352W

Fold Change (Up-regulation)	Gene Symbol	Fold Change (Down-regulation)	Gene Symbol
2,39	YIR016W	2,29	PUS4
2,39	SGN1	2,29	CSL4
2,39	MPS3	2,29	DPB4
2,39	YML037C	2,29	RRP42
2,39	YPR045C	2,29	MEP1
2,39	TGL1	2,28	FAU1
2,39	YJL016W	2,28	CDC40
2,39	FSP2	2,28	UTP22
2,38	NYV1	2,28	RNH70
2,38	ETP1	2,28	GCD1
2,38	RRD2	2,28	KEI1
2,38	YMR086W	2,27	IRC7
2,37	CTR2	2,27	BUD16
2,37	AUA1	2,27	RPL14A
2,37	YJL216C	2,27	KIN3
2,37	PAU8	2,27	SPT4
2,36	YMR252C	2,27	VBA2
2,36	KRE1	2,26	MRI1
2,36	SIS1	2,26	URA3
2,36	UFD2	2,26	RTC6
2,36	CCW12	2,25	ADE4
2,35	MDJ2	2,25	YPL108W
2,35	FMP27	2,25	YOR012W
2,35	DAS1	2,25	YBR242W
2,35	ATG9	2,25	YPL113C

Fold Change (Up-regulation)	Gene Symbol	Fold Change (Down-regulation)	Gene Symbol
2,35	YMR084W	2,24	YBR220C
2,35	YIL024C	2,24	CMC2
2,34	CDC34	2,24	SDO1
2,34	AVO2	2,24	PRS1
2,33	CPR6	2,24	MIC17
2,33	ABM1	2,23	ERG24
2,33	COS6	2,23	ATP11
2,33	SWH1	2,23	RPL34B
2,33	YOL075C	2,23	PWP1
2,33	KKQ8	2,23	MAS2
2,32	YFL067W	2,23	RPL4B
2,32	SIR1	2,22	PRO1
2,32	GRX6	2,22	YDR179W-A
2,32	YNL011C	2,22	TOM5
2,32	APS1	2,22	SPB1
2,31	PKH3	2,22	ASN1
2,31	MDG1	2,22	RFC5
2,31	ARC18	2,22	OGG1
2,30	RPR2	2,22	PRP19
2,30	CDC15	2,22	RNT1
2,30	MDS3	2,22	POP4
2,30	LCB3	2,22	IZH1
2,30	YKL107W	2,22	MEP3
2,30	YDL124W	2,21	PUG1
2,30	CHS5	2,21	FIT2

Fold Change (Up-regulation)	Gene Symbol	Fold Change (Down-regulation)	Gene Symbol
2,30	SIC1	2,21	PRP39
2,29	JIP4	2,21	MRM1
2,29	TCB1	2,21	RKM3
2,29	HYM1	2,21	YAR1
2,28	YKL105C	2,20	RPS16A
2,28	VNX1	2,20	AUR1
2,27	HVG1	2,20	ERB1
2,27	CLN3	2,20	GAP1
2,27	IPK1	2,20	MNN9
2,27	GLC7	2,20	GRX8
2,27	RPN4	2,20	CCS1
2,27	MDH1	2,20	GLY1
2,27	CAT2	2,19	MET22
2,27	UFD4	2,19	KAP123
2,27	GAL10	2,19	MRPL35
2,27	LAT1	2,19	KTR3
2,26	YPS1	2,19	YNL095C
2,26	RRT13	2,19	RPF1
2,26	SKN1	2,18	SGT2
2,26	TUL1	2,18	MRPL33
2,26	RIM8	2,18	YPL162C
2,26	TRX2	2,18	SPE2
2,25	IMP2'	2,18	RPL42A
2,25	PEX19	2,18	RRP46
2,25	YMR253C	2,17	PRE1

Fold Change (Up-regulation)	Gene Symbol	Fold Change (Down-regulation)	Gene Symbol
2,25	CDD1	2,17	CCT3
2,24	COS10	2,17	URA6
2,24	RAD55	2,17	RRP15
2,24	YGL138C	2,17	POP6
2,24	PGC1	2,17	SPO7
2,24	AAR2	2,17	YIL169C
2,23	PAU18	2,16	RRP12
2,23	FIT1	2,16	COX23
2,23	UTR5	2,16	RPL19A
2,23	RLM1	2,16	YDL144C
2,23	YLR257W	2,16	DCD1
2,23	PSA1	2,15	RPL17B
2,22	GYP5	2,15	SQT1
2,22	PAU5	2,15	SHM2
2,22	COS111	2,15	TOA2
2,22	PCL1	2,15	SOL3
2,22	VPS8	2,15	RPS27A
2,22	CCR4	2,15	YMR010W
2,21	FBA1	2,15	BIO5
2,21	IME4	2,15	TAT1
2,21	YML053C	2,14	RLI1
2,21	PKP1	2,14	SCC2
2,21	AI5_ALPHA	2,14	SEC66
2,21	GUD1	2,14	GUK1
2,21	PEX29	2,14	NAT2

Fold Change (Up-regulation)	Gene Symbol	Fold Change (Down-regulation)	Gene Symbol
2,21	GAL2	2,14	EMC6
2,20	GRX7	2,14	ARO2
2,20	VPS15	2,14	SOR2
2,20	YNL208W	2,14	BDF2
2,19	YNL176C	2,14	ECM16
2,19	HBN1	2,14	ILV1
2,19	YJR039W	2,13	YKT6
2,19	YLR352W	2,13	ADI1
2,18	ATP18	2,13	YDL121C
2,17	MET2	2,13	HCR1
2,17	HAP4	2,13	HIP1
2,17	APL3	2,13	NAN1
2,16	NKP1	2,12	ESF1
2,16	YJL016W	2,12	URA4
2,16	SCS3	2,12	PRM4
2,16	SNX3	2,12	PAP2
2,16	GPB2	2,12	NTR2
2,16	YPL260W	2,12	TOS3
2,16	YKL133C	2,12	ILV3
2,15	YAP1801	2,12	YJL213W
2,15	YGL081W	2,12	YGR054W
2,15	LAP2	2,12	PHO90
2,15	GDH2	2,11	RPS10A
2,15	HMX1	2,11	URM1
2,14	MLF3	2,11	SPT8

Fold Change (Up-regulation)	Gene Symbol	Fold Change (Down-regulation)	Gene Symbol
2,14	DIN7	2,11	DSK2
2,14	ACC1	2,11	RPL13A
2,14	CTS1	2,11	ANT1
2,13	MGA2	2,11	YLR126C
2,13	PSO2	2,11	RED1
2,13	CHL4	2,11	FMP52
2,13	MMS4	2,11	VBA1
2,13	AKL1	2,10	MRP7
2,13	AVT6	2,10	TRZ1
2,13	TWF1	2,10	RNH201
2,13	ADH1	2,10	YCL001W-B
2,13	RTS1	2,10	CMS1
2,13	PDC1	2,10	YKR106W
2,13	RRI1	2,09	MED6
2,13	VAM7	2,09	TMA22
2,12	STE13	2,09	SPS1
2,12	YNK1	2,09	YDL129W
2,12	OSH6	2,09	RPS27B
2,11	SER3	2,08	TRM5
2,11	YDR391C	2,08	MAK5
2,11	SMF3	2,08	PHO11
2,11	YHR112C	2,08	RPC40
2,11	CHC1	2,08	PPA2
2,11	YKR104W	2,08	ADE6
2,11	SEC27	2,08	ADE13

Fold Change (Up-regulation)	Gene Symbol	Fold Change (Down-regulation)	Gene Symbol
2,11	FIR1	2,08	SUV3
2,11	FMS1	2,08	ANP1
2,10	CDC13	2,08	FRE3
2,10	TVP15	2,08	GEP3
2,10	DPM1	2,08	YNL162W-A
2,10	SMT3	2,08	YHR003C
2,10	YFL066C	2,08	SER2
2,09	YPR003C	2,07	ERG5
2,09	COS5	2,07	RPB7
2,09	SIM1	2,07	CAB1
2,09	APJ1	2,07	IFH1
2,09	HXT3	2,07	YMR321C
2,09	PMC1	2,06	RPS16B
2,09	MSB3	2,06	STM1
2,09	UTR1	2,06	CTR86
2,09	SYP1	2,06	MRPL7
2,09	YGL242C	2,06	RPS14A
2,08	TIS11	2,06	YKR051W
2,08	GUF1	2,06	RRN7
2,08	LCB2	2,06	RPL33A
2,08	PLB1	2,05	RPL26A
2,08	HDA3	2,05	MPS2
2,08	PHD1	2,05	PHO12
2,07	DAN4	2,05	CCT4
2,07	AIM18	2,04	MNN10

Fold Change (Up-regulation)	Gene Symbol	Fold Change (Down-regulation)	Gene Symbol
2,07	SET3	2,04	YLR099W-A
2,07	SRC1	2,04	HIS7
2,07	INO80	2,04	ARG81
2,07	YCL057C-A	2,04	RPC10
2,07	NCA2	2,04	MAK21
2,07	YPR084W	2,04	TIM23
2,07	PRP38	2,04	YLR243W
2,07	PIN3	2,04	CST26
2,06	PAU6	2,04	MTR3
2,06	ADD37	2,04	RPS1A
2,06	SNQ2	2,04	YJR054W
2,06	YJR061W	2,04	CNS1
2,06	NSP1	2,04	ATP23
2,05	ARC35	2,04	YBL055C
2,05	YCL068C	2,04	PRE5
2,05	VAB2	2,03	NEW1
2,05	TEL1	2,03	ERG2
2,05	LRO1	2,03	AMN1
2,05	SLT2	2,03	FRS1
2,05	EDE1	2,03	MET6
2,05	HTD2	2,03	FOL1
2,05	YJR116W	2,02	NDC1
2,05	NDD1	2,02	YMR209C
2,05	BGL2	2,02	SME1
2,05	AIM39	2,02	MRPL6

Fold Change (Up-regulation)	Gene Symbol	Fold Change (Down-regulation)	Gene Symbol
2,05	PHO23	2,02	RPL28
2,05	COX20	2,02	NUC1
2,05	FAB1	2,02	SDS23
2,05	COS7	2,02	RPS19B
2,05	PAH1	2,02	MRPL49
2,04	BCK2	2,02	PEX25
2,04	SCD5	2,02	DIS3
2,04	YKR096W	2,01	PRP31
2,03	ATP10	2,01	SRP68
2,03	YLR040C	2,01	HTL1
2,03	VPS53	2,01	STE4
2,03	YLR464W	2,01	ICY2
2,03	ERD2	2,01	ARD1
2,02	MAD2	2,01	TSC10
2,02	SET4	2,01	YRA2
2,02	AGE2	2,01	THI80
2,02	YOR019W	2,01	CTA1
2,02	CPR4	2,01	EAF5
2,02	YKU70	2,01	ATS1
2,02	SEC6	2,00	MCM5
2,02	YGR127W	2,00	WRS1
2,02	YIL102C		
2,02	RSF2		
2,01	ECM27		
2,01	SWI5		

Fold Change (Up-regulation)	Gene Symbol	Fold Change (Down-regulation)	Gene Symbol
2,01	SHE4		
2,01	APL5		
2,01	COQ4		
2,01	COS4		
2,00	NCR1		
2,00	AVL9		

REFERENCES

Abdulrehman D., Pedro T. Monteiro, Miguel C. Teixeira, Nuno P. Mira, Artur B. Lourenço, Sandra C. dos Santos, Tânia R. Cabrito, Alexandre P. Francisco, Sara C. Madeira, Ricardo S. Aires, Arlindo L. Oliveira, Isabel Sá-Correia, Ana T. Freitas, (2011) YEASTRACT: providing a programmatic access to curated transcriptional regulatory associations in *Saccharomyces cerevisiae* through a web services interface, Nucl. Acids Res., 39:136-140, Oxford University Press

Aguilera F, et al. (2006) Relationship between ethanol tolerance, H+ -ATPase activity and the lipid composition of the plasma membrane in different wine yeast strains. Int J Food Microbiol 110(1):34-42

Anbazhagan V, Munz C, Tome L, Schneider D (2010) Fluidizing the membrane by a local anesthetic: phenylethanol affects membrane protein oligomerization. J Mol Biol 404:773-7

Aranda A, del Olmo ML (2003) Response to acetaldehyde stress in the yeast *Saccharomyces cerevisiae* involves a strain-dependent regulation of several ALD genes and is mediated by the general stress response pathway. Yeast 20:747–759

Avalos S., and A.Saavedra-Molina (2010). Cellular and mitochondrial effects of alcohol consumption. Int J Environ Res Public Health, 7:4281-4304.

Bai, F.W.; Chen, L.J.; Zzhang, Z.; Anderson, W.A. and Moo-Young, M. (2004). Continuous ethanol production and evaluation of yeast cell lysis and viability loss under very high gravity medium conditions. Journal of Biotech, 110:287-293.

Barry JA and Gawrisch K (1994) Direct NMR evidence for ethanol binding to the lipid-water interface of phospholipid bilayers. Biochemistry 33:8082–8088.

Barton, N. H. (2007). Evolution. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Bayrock D and W.M. Ingledew. (2002) Evaluation of *Saccharomyces cerevisiae* grown in a multistage chemostat environment under increasing levels of glucose. Biotechnology Letters. 24:449-453.

Beaven, M. J., C. Charpentier, and A. H. Rose. (1982) Production and tolerance of ethanol in relation to phospholipid fatty-acyl composition in *Saccharomyces cerevisiae* NCYC 431. J. Gen. Microbiol. 128:1447-1455.

Beier DR, Sledziewski A, Young ET. (1985). Deletion analysis identifies a region, upstream of the ADH2 gene of *S. cerevisiae,* which is required for ADR1-mediated derepression. Mol Cell Biol 5:1743–1749.

Bergman L.W. (2001) Two-Hybrid Systems: Methods and Protocols, Methods in Molecular Biology, 177:449-453

Bostock CJ (1970) The effect of 2-phenyl ethanol on the DNA synthesis cycle of Schizosaccharomyces pombe. J Cell Sci. Sep;7(2):523-30.

Botstein, D., Chervitz, S.A., and Cherry, J.M. (1997) Yeast as a model organism. Science 277:1259-1260.

Brauer MJ, Huttenhower C, Airoldi EM, Rosenstein R, Matese JC, Gresham D, Boer VM, Troyanskaya OG, Botstein D (2008) Coordination of Growth Rate, Cell Cycle, Stress Response, and Metabolic Activity in Yeast. Mol Biol Cell 19: 352–367

Brecher AS, Adamu MT. (2002) Short- and long-term effects of acetaldehyde on plasma. Alcohol. 26(1):49-53

Çakar, Z. P. et al. (2012). Evolutionary Engineering of *Saccharomyces cerevisiae* for improved industrially important properties. FEMS Yeast Research, 12: 171-182.

Çakar, Z. P. (2009). Metabolic and evolutionary engineering research in Turkey and beyond. Biotechnology Journal, 4: 992–1002.

Cakar, Z. P., Seker, U. O., Tamerler, C., Sonderegger, M., Sauer, U. (2005). Evolutionary engineering of multiple-stress resistant *Saccharomyces cerevisiae*. FEMS Yeast Res, 5(6-7):569-578.

Cameron DR. (1988) The mannoprotein of *Saccharomyces cerevisiae* is an effective bioemulsifier. Appl Environ Microbiol 54:1420

Chen D, Toone WM, Mata J, Lyne R, Burns G, Kivinen K, Brazma A, Jones N, Bahler J (2003). Global transcriptional responses of fission yeast to environmental stress. MolBiolCel; 14: 214-229.

Cherry J.M., Adler C., Ball C., Chervitz S.A., Dwight S.S., Hester E.T., Jia Y., Juvik G., Roe T., Schroeder M., et al.(1998) SGD: Saccharomyces Genome Database. Nucleic Acids Res. 26:73–79.

Chin, J. H., and D. B. Goldstein. (1977) Drug tolerance in biomembranes: a spin label study of the effects of ethanol. Science. 196:684– 685.

Cook, A.H. (1958) The Chemistry and Biology of Yeasts. New York, New York. 63:251

Costa V., M. A. Amorim, E. Reis,t A. Quintanilha and P. Moradas-Ferreira (1997) Mitochondria1 superoxide dismutase is essential for ethanol tolerance of *Saccharomyces cerevisiae* in the post-diauxic phase. Microbiology, 143:1649-1

Çelik D. (2004) Biotransformation of 2-phenylethanol to phenylacetaldehyde in a two-phase fed-batch system. Biochemical Engineering Journal 17: 5–13

Daum G, Lees N. (1998)Biochemistry, Cell Biology and Molecular Biology of Lipids of *Saccharomyces cerevisiae*. Yeast 14: 1471–1510

del Castillo Agudo, L. (1992). Lipid content of *Saccharomyces cerevisiae* strains with different degrees of ethanol tolerance. Appl Microbiol Biotechnol, 37(5):647–651.

Dickinson J., Schweizer M. (2004) Metabolism and Molecular Physiology of *Saccharomyces cerevisiae*, 2nd Edition. Taylor & Francis Group.

Ding J, Huang X, Zhang L, Zhao N, Yang D, et al. (2009) Tolerance and stress response to ethanol in the yeast *Saccharomyces cerevisiae*. Appl Microbiol Biotechnol 85: 253–263.

Dombek, K . M., and L . O . Ingram. (1984). Effects of ethanol on the *Escherichia coli* plasma membrane, J . Bacteriol . 157:233-239.

Eddy, A.A (1982) Advances in Microbial Physiology 23, 1.

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

Estruch F. (2000) Stress-controlled transcription factors, stress-induced genes and stress tolerance in budding yeast. FEMS Microbiol Rev. 24(4):469-86

Ewald, S.J. and H. Shao (1993) Ethanol increases apoptotic cell death of thymocytes in vitro, Alcohol Clin. Exp. Res., 17: 359-365.

Federoff H. J., Eccleshall T. R., Marmur J. (1983) Carbon catabolite repression of maltase synthesis in *Saccharomyces carlsbergensis*. J. Bacteriol. 156:301–307

Feller, Scott (2002) Nuclear Overhauser enhancement spectroscopy cross- relaxation rates and ethanol distribution across membranes. Biophys. J. 82:1396-1404.

Gasch A.P., Spellman P.T., Kao C.M., Carmel-Harel O., Eisen M.B., Storz G., Botstein D., Brown P.O.(2000) Genomic expression programs in the response of yeast cells to environmental changes. Mol. Biol. Cell 11:4241–4257

Gasch, A.P., Werner-Washburne, M. (2002). The genomics of yeast responses to environmental stress and starvation. Funct. Integr. Genomics 2(4-5):181-192

Gasch, A.P. (2000) The environmental stress response: a common yeast response to diverse environmental stresses Mol Biol Cell 11(12):4241–4257

Goffeau A., Barrell B.G., Bussey H., Davis R.W., Dujon B., Feldmann H., Galibert F., Hoheisel J.D., Jacq C., Johnston M., et al.(1996) Life with 6000 genes. Science 274:546, 563–567

Hahn-Hagerdal B, Karhumaa K, Jeppsson M, Gorwa-Grauslund M (2007) Metabolic engineering for pentose utilization in *Saccharomyces cerevisiae*. Advances in Biochemical Engineering/Biotechnology, 108:147-177.

Hallsworth, J. E. (1998) Ethanol-induced water stress in yeast. J. Ferment. Bioeng. 85:125-137.

Harris RA (1981) Hitzemann RJ. Membrane fluidity and alcohol actions. Curr Alcohol. 8:379-404. Review.

Herrero, A.A., Gomez, R.F. and Roberts, M.F. (1982) Ethanol-induced changes in the membrane lipid composition of *Clostridium thermocellum*. Biochim. Biophys. Acta 693:195-204.

Hinnen A, Hicks JB, Fink GR. (1978) Transformation of yeast. Proc Natl Acad Sci USA; 75:1929-33.

Hori K, Miyamoto S, Yukawa Y, Muto M, Chiba T, Matsuda T. (2012) Stability of acetaldehyde-derived DNA adduct in vitro. Biochem Biophys Res Commun 423(4):642-6

Hua D, Xu P. (2011) Recent advances in biotechnological production of 2-phenylethanol . 540. Biotechnol Advances 29:654-60.

Ingólfsson HI, (2011)Andersen OS. Alcohol's effects on lipid bilayer properties Biophys J, 693:195-204.

Ingram, L. O., and T. M. Buttke. (1984) Effects of alcohols on microorganisms. Adv. Microbial Physiol, 32:25253-300.

Jan Sikkema, Jan. A.M. de Bont, and Bert Poolman, (1995) "Mechanisms of Membrane Toxicity of Hydrocarbons," Microbiological Reviews 274:546, 563–567

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

Jones, R. P . and Greenfield, P . F (1986). Role of water activity in ethanol fermentations. Biotechnol. Bioeng., 28:29-40 .

Kawai, S., H. Wataru and M. Kousaku, (2010) Transformation of *Saccharomyces cerevisiae* and other fungi, Methods and possible underlying mechanism. Bioen. Met., 1(6): 395-403.

Klis FM, Boorsma A, De Groot PWJ (2006) Cell wall construction in *Saccharomyces cerevisiae*. Yeast 23:185–202

Koffas, M., (2005) Evolutionary metabolic engineering. Metab. Eng. 7:1–3

Kollar R, Petrakova E, Ashwell G, Robbins PW, Cabib E. (1995) Architecture of the yeast cell wall. The linkage between chitin and β (1 – 3)-glucan. J Biol Chem 270: 1170 – 1178

Koop, D.R., and Coon, M.J. (1986) Ethanol oxidation and toxicity: Role of alcohol P-450 oxygenase. Alcoholism: Clinical and Experimental Research 10(Suppl. 6):44S–49S

Koop DR. (2006) Alcohol metabolism's damaging effects on the cell. Alcohol Res Health. 29(4):274–80

Kurtzman C.P., Fell J.W. (1998) The Yeasts - A Taxonomic Study. Elsevier.

Lawrence, C. W. (1991). Classical mutagenesis techniques. Methods Enzymol, 194:273-281.

Lee B., Faller R. (2004) Structural Effects of Small Molecules on Phospholipid Bilayers Investigated by Molecular Simulations. Fluid Phase Equilib 225:63–68

Lei J, Zhao X, Ge X, Bai F (2007) Ethanol tolerance and the variation of plasma membrane composition of yeast floc populations with different size distribution. J Biotechnol 131:270-275.

Lepage, C., Fayolle, F., Hermann, M. and Vandecasteele, J.-P. (1987) Changes in Membrane Lipid Composition of *Clostridium acetobutylicum* during Acetone-Butanol Fermentation: Effects of Solvents, Growth Temperature and pH. J. Gen. Microbiol. 133:103-110.

Lester G. (1995) Inhibition of growth, synthesis, and permeability in *Neurospora crassa* by phenethyl alcohol. J Bacteriol 90:29–37.

Lieber, C.S. (2005) Metabolism of alcohol. Clinics in Liver Disease 9:1–35, PMID: 15763227

Lin NY, Beyer C, Gießl A, et al. (September 2012). "Autophagy regulates TNFα-mediated joint destruction in experimental arthritis" Biophys J, 225:63–68

Lin, Y. & Tanaka, S. (2006). Ethanol fermentation from biomass resources: current state and prospects. Applied Microbiology and Biotechnology, 69:627- 642

L**iu X., Y. Lao, I.Y. Yang, S.S. Hecht, M. Moriya**, (2006) Replication-coupled repair of crotonaldehyde/acetaldehyde-induced guanine-guanine interstrand crosslinks and their mutagenicity, Biochemistry 45 (42):12898–12905.

Lucchini JJ, Bonnaveiro N, Cremieux A, Legoffic F. (1993). Mechanism of bactericidal action of phenethyl alcohol in Escherichia coli. Curr Microbiol 27:295–300.

Luo, Z. , & van Vuuren, H. (2009). Functional Analyses of PAU Genes in *Saccharomyces cerevisiae*. Microbiology-sgm, 155(185601374):4036-4049.

Ly, H. V. and Longo, M. L. (2004) The influence of short-chain alcohols on interfacial tension, mechanical properties, area/molecule, and permeability of fluid Biophysical Journal 87:345-355.

Ma, M., Liu, Z. (2012) Molecular mechanisms of ethanol tolerance in *Saccharomyces cerevisiae*. In: Liu, Z.L., editor. Microbial Stress Tolerance for Biofuels 15: 829–842

Matter B., R. Guza, J. Zhao, Z.Z. Li, R. Jones, N. Tretyakova, (2007) Sequence distribution of acetaldehyde-derived N2 -ethyl-dG adducts along duplex DNA, Chem. Res. Toxicol. 20 (10):1379–1387.

McAlister L, Finkelstein DB (1980) Alterations in translatable ribonucleic acid after heat shock of *Saccharomyces cerevisiae*. J Bacteriol 143:603-612

Navarro-Aviño J. P., Prasad R., Miralles V. J., Benito R. M., Serrano R., 1999 A proposal for nomenclature of aldehyde dehydrogenases in *Saccharomyces cerevisiae* and characterization of the stress-inducible ALD2 and ALD3 genes. Yeast 15: 829–842.

Nevoigt, E. (2008) Progress in metabolic engineering of *Saccharomyces cerevisiae*. Microbiol Mol Biol Rev 72:379-412 .

Nicholls, D.G. (1982) Bioenergetics, Academic Press, New York.

Ogawa Y, A Nitta, H Uchiyama, T Imamura, H Shimoi, and K Ito (2000). Tolerance mechanism of the ethanol-tolerant mutant of sake yeast. J. Biosci Bioeng 90:334-45

Oyesanmi O, Snyder D, Sullivan N, Reston J, Treadwell J, Schoelles KM. (2010) Alcohol Consumption and Cancer Risk: Understanding Possible Causal Mechanisms for Breast and Colorectal Cancers. Evid Rep Technol Assess. (197):1-151.

Patra M, Salonen E, Terama E, Vattulainen I, Faller R, et al. (2006) Under the influence of alcohol: The effect of ethanol and methanol on lipid bilayers. Biophysical Journal 90:423-452

Piper P. W, Talreja K. (1994) Induction of major heat-shock proteins of *Saccharomyces cerevisiae*, incluDing pIasma membrane HspSO, by ethanol levels above a critical threshold. Microbiology, 140: 1-3038

Rachidi, N. , Martinez, M. , Barre, P. , & Blondin, B. (2000). *Saccharomyces cerevisiae* PAU Genes Are Induced by Anaerobiosis. Molecular Microbiology, 35(6): 1421-1430.

Rattray J. (1975) Lipids of Yeasts. Bacter. Rev. 45:197-231

Ruepp, A., Zollner, A., Maier, D., Albermann, K., Hani, J., Mokrejs, M., Tetko, I., Guldener, U., Mannhaupt, G., Munsterkotter, M., and Mewes, H. (2004). The FunCat, a functional annotation scheme for systematic classification of proteins from whole genomes. Nucleic Acids Research, 32:5539-5545.

Scandalios JG (ed) (1997) Oxidative Stress and the Molecular Biology of Antioxidant Defenses. Cold Spring Harbor Laboratory Press

Sergent O, Pereira M, Belhomme C, et al. (1995) Role for membrane fluidity in ethanol-induced oxidative stress of primary rat hepatocytes. J Pharmacol Exp Ther. 90:334-45

Sharon A. (2010) Molecular and Cell Biology Methods for Fungi, Department of Plant Sciences, Tel Aviv University

Shulman RW, Sripati CE, Warner JR (1977) Noncoordinated transcription in the absence of protein synthesis in yeast. J Biol Chem 252:1344-1349

Singh R., J. Sandhu, B. Kaur, T. Juren, W.P. Steward, D. Segerback, P.B. Farmer, (2009) Evaluation of the DNA damaging potential of cannabis cigarette smoke by the determination of acetaldehyde derived N2 -ethyl-2′ -deoxyguanosine adducts, Chem. Res. Toxicol. 22 (6):1181–1188.

Silva MT, Sousa JC, Macedo MA, Polónia J, Parente AM. Effects of phenethyl alcohol on Bacillus and Streptococcus. J Bacteriol. 127(3):1359-69

Seward R, Willetts JC, Dinsdale MG, Lloyd D. (1996) The effects of ethanol,hexan-1-ol, and 2-phenylethanol on cider yeast growth, viability, and energy status: synergistic inhibition. J Inst Brew102: 439–43.

Silveira MG (2003) Membrane fluidity adjustments in ethanol-stressed *Oenococcus oeni* cells Appl Environ Microbiol. 90:423-452

Silver S, Wendt L. (1967) Mechanism of action of phenethyl alcohol: breakdown of the cellular permeability barrier. J Bacteriol. 93(2):560– 566

Singh N.P., Khan A. (1995). Acetaldehyde: genotoxicity and cytotoxicity in human lymphocytes. Mutat. Res., 337:1359-69

Stadler, J.A., and Schweyen, R.J. (2002). The yeast iron regulon is induced upon cobalt stress and crucial for cobalt tolerance. J.Biol. Chem. 277:39649-39654.

Stark D, Zala D. (2003) Inhibition aspects of the bioconversion of L- phenylalanine to 2-phenylethanol by *Saccharomyces cerevisiae*. Enzyme Microbiol Technol 32: 212 –223

Terama, E., Ollila, O. H. S., Salonen, E., Rowat, A., Trandum, C., Westh, P., Patra, M., Karttunen, M., and Vattulainen, (2008) Influence of Ethanol on Lipid Membranes: From Lateral Pressure Profiles to Dynamics and Partitioning. . Phys. Chem. B, 112, 4131-4139

Togashi, A. Shiraishi, M. Nishizaka et al., (2007) Antibacterial activity of long-chain fatty alcohols against *Staphylococcus aureus*. Molecules, 1:122-231

Tuma, D., and Casey, C. (2003) Dangerous byproducts of alcohol breakdown: Focus on adducts. Alcohol Res Health 27:285–90

Veinot-Drebot LM, Singer RA, Johnston GC (1989) Heat shock causes transient inhibition of yeast rRNA gene transcription. J Biol Chem 264:19473-19474

Veld, G., Driessen, A.J.M., Op den Kamp, J.A.F. and Konings, W.N. (1991) Biochim. Biophys. Acta 1065, 203-212.

Vollherbst-Schneck, K., Sands, J.A. and Montenecourt, B.S. (1984) The effects of ethanol,hexan-1-ol, and 2-phenylethanol on cider yeast growth, viability, and energy status: synergistic inhibition. Appl. Environ. Microbiol. 47:193-194.

Walker K, Skelton H, Smith K. (2002). Cutaneous lesions showing giant yeast forms of Blastomyces dermatitidis. Journal of Cutaneous Pathology 29 (10): 616–618.

Warner JR (1999) The economics of ribosome biosynthesis in yeast. Trends Biochem Sci 24:437-440

Weber FJ, de Bont JA. (1996) Adaptation mechanisms of microorganisms to the toxic effects of organic solvents on membranes. Biochim Biophys Acta. 12, 1234-55

Wilkie D, Maroudas NG. (1969) Induction of cytoplasmic respiratory deficiency in yeast by phenethyl alcohol. Genet Res 13:107–11.

Wisselink, H. W., Toirkens, M. J., del Rosario Franco Berriel, M., Winkler, A. A., van Dijken, J. P., Pronk, J. T., van Maris, A. J. (2007). Engineering of *Saccharomyces cerevisiae* for efficient anaerobic alcoholic fermentation of L-arabinose. Appl Environ Microbiol, 73(15):4881-4891.

Wisselink, H. W., Toirkens, M. J., Wu, Q., Pronk, J. T., van Maris, A. J. (2009). Novel evolutionary engineering approach for accelerated utilization of glucose, xylose, and arabinose mixtures by engineered *Saccharomyces cerevisiae* strains. Appl Environ Microbiol, 75(4):907-914.

Woutersen R.A. , Appelman L.M., A. Van Garderen-Hoetmer, V.J. Feron (1986), Inhalation toxicity of acetaldehyde in rats. III. Carcinogenicity study, Toxicology 41 (2):213–231.

You, K. M., Rosenfield, C. L., Knipple, D. C. (2003) Ethanol tolerance in the yeast *Saccharomyces cerevisiae* is dependent on cellular oleic acid App. Mic., 23: 1499–1503

Yu H.S., T. Oyama, T. Isse, K. Kitagawa, T.T. Pham, M. Tanaka, T. Kawamoto (2010), Formation of acetaldehyde-derived DNA adducts due to alcohol exposure, Chem.-Biol. Interact. 188:367–375.

Zakhari, S. (2006) Overview: How is alcohol metabolized by the body? Alcohol Research & Health 29(4): 245–254.

Zhao XQ and Bai FW (2009) Mechanisms of yeast stress tolerance and its manipulation for efficient fuel ethanol production. J Biotechnol 144(1):23-30

Zhu Y., Zhou H., Hu Y., Tang J., Su M., Guo Y., Chen Q, Liu B. (2011) Antityrosinase and antimicrobial activities of 2-phenylethanol, 2-phenylacetaldehyde and 2-phenylacetic acid. Food Chemistry 124 298–302

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