İSTANBUL TECHNICAL UNIVERSITY ★ INSTITUTE OF SCIENCE AND TECHNOLOGY

IMPROVEMENT OF ETHANOL RESISTANCE OF COMMERCIAL YEAST STRAIN BY IN VIVO EVOLUTIONARY ENGINEERING

M.Sc. Thesis by
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Department : Advanced Technologies

Programme : Molecular Biology Genetics and Biotechnology

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JANUARY 2011

İSTANBUL TEKNİK ÜNİVERSİTESİ ★ FEN BİLİMLERİ ENSTİTÜSÜ

İN VİVO EVRİMSEL MÜHENDİSLİK YÖNTEMİ İLE TİCARİ BİR MAYA SUŞUNUN ETANOL DİRENCİNİN GELİŞTİRİLMESİ

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FOREWORD

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Garbis Atam Akçeoğlu
Biologist

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ABBREVIATIONS

EMS **:** Ethyl methane sulphonate
YPD **:** Yeast complex medium
YMM **:** Yeast minimal medium
MPN **:** Most probable number
EtOH **:** Ethanol

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IMPROVEMENT OF ETHANOL RESISTANCE OF A COMMERCIAL YEAST STRAIN BY IN VIVO EVOLUTIONARY ENGINEERING

SUMMARY

The aim of the present study was to obtain ethanol resistant commercial yeast strain (*Saccharomyces cerevisiae*) cells by using evolutionary engineering strategy. For this purpose wild type (Hef) strain was exposed to a chemical mutagen Ethyl methane-sulfonate in order to obtain a randomly mutagenized population (Hef1). Genetically diverse population (Hef1) was used to select the ethanol resistant individuals. Continuous selection strategies were designed. Continuous stress selection strategy was based on exposing cells continuously to ethanol stress to obtain survivors resistant to that stress level. Individuals surviving the first stress level were transferred to the next stress level. The stress levels were increased for each successive generation of selection stress.

A screening procedure was applied in order to detect the initial ethanol stress level. Depending on the screening results, 10 % ethanol (v/v) for the initial stress level with a 0.1 % increasing range for both selection strategies were determined. Twenty eight generations were obtained with continuous stress selection strategy. Ten different individuals were selected. A screening procedure was applied to individuals in order to determine the ethanol resistence. Mutant *11* was more resistant to ethanol. Cross-resistance to several different stresses of four mutants *6*, *8*, *11* and *12* were determined by using most probable number (MPN) method. Mutants obtained from continuous selection strategy had cross resistance to metal, sorbitol, heat, osmotic, freezing-thawing, 20 % ethanol pulse stresses.

To summarize, by applying evolutionary engineering, ethanol resistant individuals were successfully obtained. In order to understand the mechanism of ethanol resistance and relationship with stress factors, detailed transcriptomic and proteomic analyses would be necessary for future studies.

İN VİVO EVRİMSEL MÜHENDİSLİK YÖNTEMİ İLE TİCARİ BİR MAYA SUŞUNUN ETANOL DİRENCİNİN GELİŞTİRİLMESİ

ÖZET

Yapılan çalışmanın amacı, evrimsel mühendislik stratejisi kullanılarak etanole dirençli *Saccharomyces cerevisiae* hücreleri elde etmektir. Bu amaçla yaban tip suş (Hef) rastgele mutasyona uğramış (Hef1) popülasyonunu elde etmek amacıyla kimyasal mutajen etil metan sülfonat'a maruz bırakılmıştır. Etanole dirençli bireylerin seçiminde genetik çeşitliliği olan Hef1 populasyonu kullanılmıştır. Sürekli stres seçilim stratejisi geliştirilmiştir. Sürekli stres seçilim stratejisi kültürü strese sürekli maruz bırakarak o stres seviyesine dayanıklı bireyleri elde etme temeline dayanmaktadır. Hayatta kalmayı başarabilen bireylerin metabolizmalarını yüksek stres seviyelerine adapte olmaya eğilimli genetik diziye sahip olmaları beklenir. İlk nesilde hayatta kalmayı başarabilen bireyler bir sonraki stres seleksiyon basamağına geçerler ve stres seviyesi bir sonraki basamakta arttırılır.

Başlangıç etanol konsantrasyonunu ve artış aralığını belirlemek amacıyla tarama prosedürü gerçekleştirilmiştir. Tarama sonuçlarına göre başlangıç stres seviyesi için 10 % etanol (v/v) ve artış aralığı için 0.1% etanol (v/v) olmasına karar verilmiştir. Sürekli stres seleksiyon stratejisi ile 28 nesil elde edilmiştir. Seleksiyon stratejisi için 10 farklı birey seçilmiştir. Seçilen bireylere tarama prosedürü, etanole dirençliliklerini ölçmek amacıyla tekrar uygulanmıştır. Mutant *11*'in etanole karşı daha fazla direnç geliştirdiği görülmüştür. *6, 8, 11,* ve *12* mutantlarının farklı birçok strese çapraz dirençlilikleri en muhtemel sayı metoduyla (MPN) tespit edilmiştir. Sürekli stres seçilim yöntemi ile elde edilen bireylerin metal, sorbitol, yüksek sıcaklık, ozmotik, donma-erime, ve hacmen %20 etanol ani şok stresine çapraz direnç gösterdiği gözlemlenmiştir.

Sonuç olarak evrimsel mühendislik yöntemi ile etanole dirençli bireyler elde edilmiştir. İleriki çalışmalarda da etanol direnç mekanizmasının ve bunun diğer stres faktörleri ile ilişkisini anlamak üzere detaylı transkriptomik ve proteomik analizlerin yapılması.

1. Introduction

1.1. Brief Information about *Saccharomyces cerevisiae*

Saccharomyces cerevisae is one of the earliest domesticated organism all around the world. It has been utilized for several millennia and it has a past as old as mankind. Not only the great abilities to leaven dough in baking or generate ethanol for beverage production, it is also has a wide spread usage on the industrial and on the biological studies (Briggs, 2004).

1.2. Morphology

Individual yeast cells are not visible to human eye and they become evident only when proliferation produces a mass of many millions of cells. When this occurs, yeast cells take on the appearance of surface pellicles, sediments or hazes on or within the body of liquids (Briggs 2004). Yeast cells are mostly round and ovoid. Partly elliptical or cylindrical cells can also be observed. They are of very regular shape and size. Cells measure 5-10µm in diameter, 3-10µm in width, and 4-14µm in length. The values are imprecise because the size of the cell depends greatly on the physiological state (e.g. prior tobudding yeast cells can reach 3 times the volume of regular cells) (Esslinger, 2009). The size and the shape of cells and the patterns of vegetative propagation are characteristic of individual yeast species and may be used as aids to identification (Briggs 2004). Considerable information about a yeast cell can be obtained under the microscope. Young cells show a clear interior and a thin cell wall. Deeper insights into the cell require higher resolutions, which can be achieved with an electron microscope or through confocal microscopy. The yeast cells contain the typical organelles of other eukaryotic cells (Esslinger 2009). A typical budding cell and the bud scars on the parent cell are shown in Figure 1.1.

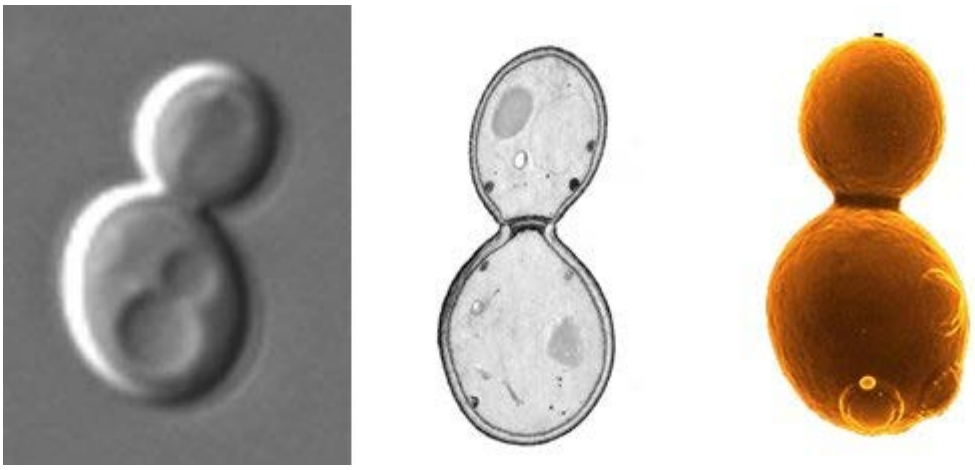


Figure 1.1: Three different views of budding yeast (url.1).

1.3. Taxonomy

The *Saccharomyces cerevisiae* are Protists. *The Protista* are organisms which possess characteristics of cells of higher organisms, but show a simpler level of biological organization. The *Saccharomyces cerevisae*'s are higher protists because they possess a true nucleus (i.e, eukaryotic), cytoplasmic organelles (plastids) and mitochondria. Higher Protist comprise the algae, protozoa, fungi and slime moulds (Hough, 1982). Yeasts are define as being fungi with vegetative states that reproduce by budding or fission resulting in growth that is frequently in the form of single cells (Kurtzman&Fell, 1998). Although all the yeasts are generally accepted fungi which are predominantly unicellular, there are various borderline "yeast like fungi" which are difficult to classify.

Classification of fungi is largely on the form of vegetative growth and the nature of the spores, if formed. *Saccharomyces cerevisae* are ascomycetous types classified within the genus *Saccharomyces.*The precise taxonomy of the fungi in general and the *Saccharomyces* in particular is still subject to debate and continental revision. A current version is given in Table1.1.

Table 1.1: The current taxonomic version of *S. cerevisiae* (Briggs, 2004).

Taxon	Name	Comments
Kingdom	Fungi	
Phylum	Ascomycotina	Teliomorphic forms characterized by formation of ascospores enclosed within ascus
Sub-Phylum	Saccharomycotina (Syn. Hemiascomycotina)	
Class	Saccharomycetes (Syn. Hemiascomycetes)	Single ascus not enclosed in ascosarp developing directly from zygotes
Order	Saccharomycetales (Syn. Endomycetales)	Yeast like cells, rarely developing hyphae
Family	Saccharomycetaceae	
Genus	Sacchaeomyces	Globose, ellipsoidal or cylindroidal cells. Vegetative reproduction by multilateral budding. Pseudohyphae may be formed but hyphae are not septate.The vegetative form is predominantly diploid, or of higher ploidy. Diploid ascopores may be formed that are globose toshort ellipsoidal with a smooth wall. There are usually 1-4 ascopores per ascus
Type species	S. cerevisiae	

1.4. Ecology

Yeasts are predominantly saprophytes so they depend strictly on the presence of organic carbon compounds as energy and carbon sources. The organic carbon may be in the form of carbohydrates or polyols or acid derivates of them (Hough 1982). In nature yeasts are widely distributed where they are found in both terrestrial and aquatic habitats (Rose&Harrison, 1987). In the terrestrial habitats; contrary to expectation, non-fermentative obligately aerobic yeast types are most common. Typically, they occupy niches that provide a particular oxidizable substrate that they are capable of assimilating. Fermentative yeast are able to take advantage of habitats where there is a source of sugar but no oxygen. Since such yeasts are facultative anaerobes the result of their own metabolic activity would be to remove oxygen from aerobic environments. They would then be able to continue to grow under conditions of anaerobiosis, where purely oxidative yeasts could not. In aquatic habitats containing a source of fermentable sugar the result would be that aerobic yeast would be restricted to the surface layers, possibly resulting in the formation of a pellicle. The population of fermentative yeasts would be capable of growth throughout the body of the liquid. Many yeasts are species are found in specialized plant habitats, which reflect theis biochemical capabilities (Briggs 2004).

The rain and plant deaths are the main reason that yeast can survive at the soil and passed on to other hosts because the soil acts like a reservoir. The transfer of yeasts between plants is most often accomplished by intermediary of insect vectors. Symbiosis of some yeasts with certain insects is also well documented (Hough 1982).

Yeasts are able to grow at very low temperature and such *psychrophiles* are common in arctic soil and waters and they could grow in the range of (-10ºC) and (10ºC). Similar adaptation to low temperatures has led to yeasts being important spoilage agents of frozen foods and they are very common contaminants of fruits and are potential spoilage organisms in extracted fruit juices, purées and concentrates. However they are rarely plant pathogens, instead they are commonly found on damaged fruits, in flowers and exudates associated with wounds.

1.5. Life cycle

Proliferation of unicellular organisms involves coordination of the biochemical processes that together underpin growth of individual cells and those specific events that culminate in cellular multiplication. The combination of events that occur during the intervals between the seperation of successive daughter cells are as seen in Figure 1.2 termed cell cycle. It requires coordination of continous processes of DNA replication, mitosis and daughter cell excision (Briggs 2004).

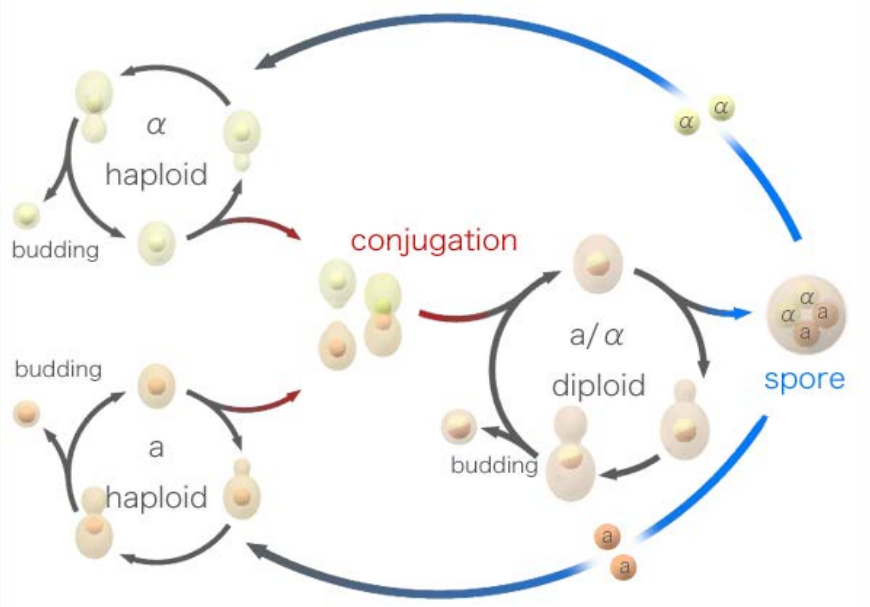


Figure 1.2: Cell cycle of budding yeast (url.2).

Progression through the cell cycle can be considered from three standpoints. Firstly, the morphological changes that occur as a mother cell gives birth to a daughter. Secondly the biochemical events that underpin the process of cellular proliferation. Thirdly, the molecular mechanisms that regulate the coordinated processes of cellular growth and multiplication. The cell cycle is divided into a number of phases (Figure 1.3). These are termed G1, which is the pre-synthetic gap phase; S, the synthetic phase during which DNA is replicated; G2, the post-synthetic gap phase, M, the mitotic phase and cytokinesis, the phase during which the daughter cell seperates from the mother and the division of the nucleus is completed. So long as reproduction is continuous, the process of budding and mitosis will proceed and the cells are said to reproduce in the vegetative state in the haplophase of the life cycle (Hough 1982).

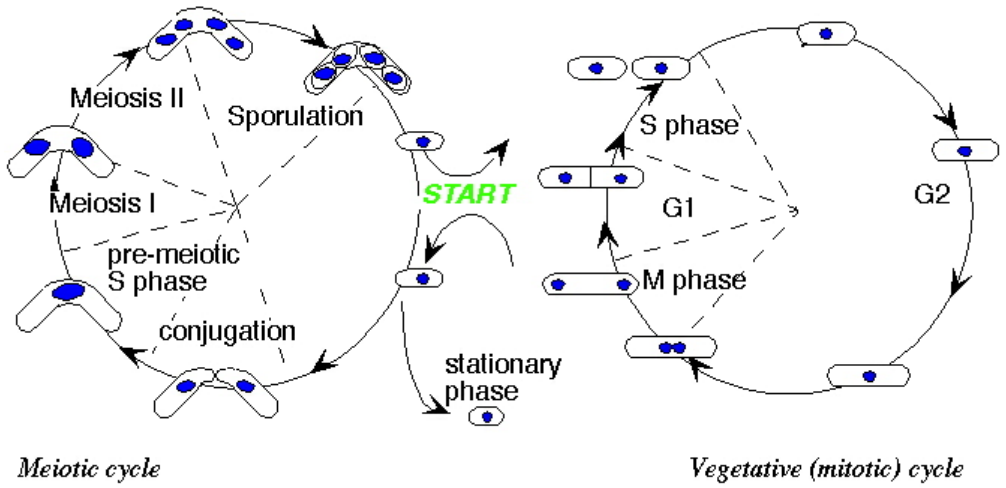


Figure 1.3: Phases of cell cycle of budding yeast (url.3)

Many yeast genera are capable of sexual reproduction. *S. cerevisiae* is a perfect member of the *Ascomycetae* and is included in this group. Wild strains of *S. cerevisiae* are usually diploid. Under appropriate conditions, yeasts can be induced to undergo meiotic division and produce ascospores that are borne in fruiting body, an ascus.

Industrial strains of *S. cerevisiae*, including brewing strains, are typically polyploid and do not normally have sexual cycle (Figure 1.4). But this feature may gain them a number of benefits from being polyploid. For instance, extra copies of important genes such as those responsible for maltose utilization (*MAL*) could improve their fermentation performance. Indeed, production of *a*-glucosidase and hence the rate of maltose fermentation is increased with the dosage of *MAL* genes (Mowshowitz, 1979); (Stewart, 1981). It has also been argued that polypoid yeasts are more stable than haploid yeasts since multiple mutational events are required in order to change them. However, because of their very nature, polyploid yeasts can harbour non-functional recessive mutations (Delgado&Zurita, 1983). Nevertheless, the sexual cycle of yeast has been used widely, as a method for exploring the genome.

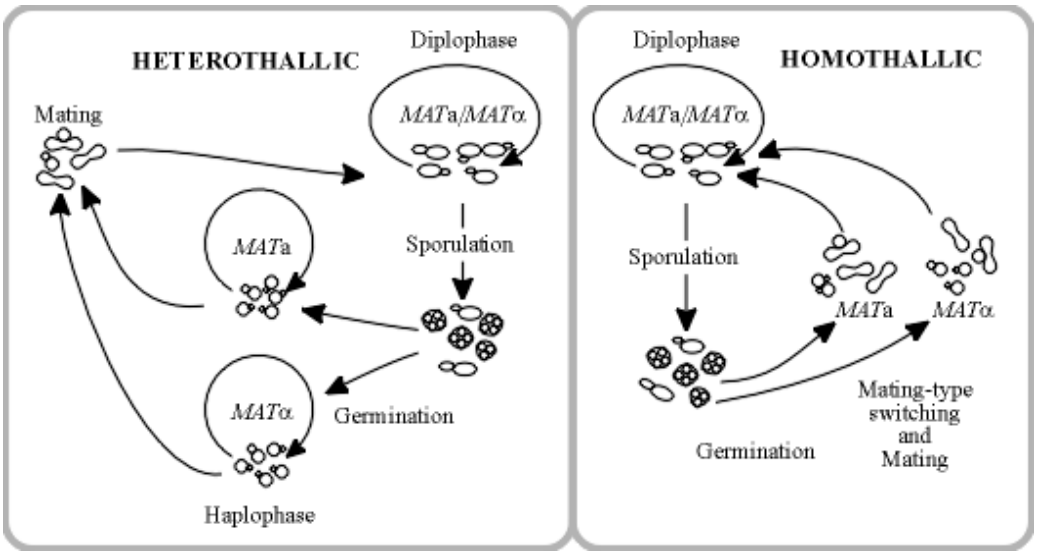


Figure 1.4: The sexual cycle of budding yeast (url.4).

S. cerevisiae has both haploid and diploid modes of existence. Strains in which haploid is stable and can be maintained for many generations are termed heterothallic. The haploids from such strains exist as one of two mating types, *a* and α are mate to form dipliods when a cell of one mating type comes into contact with a cell of the other mating type. Strains in which cell fusion and diploid formation occur among cells derived from a single spores are termed homothallic. The presence of the *HO* gene in such strains brings about a high frequency of switching between mating types during vegetative growth. Under the influence of this gene, the mating type locus MAT, of such strains readily changes from MAT*a* to MATα or vice versa. The MAT gene is found on chromosome 3 of the yeats genome together with two silence genes, HMLα and HML*a* which provide the information to allow the switch of mating type at the MAT locus (Strathern et al., 1981).

In both homothallic and heterothallic strains, mating takes place when cells of opposite mating type come into closer proximity. Cells of α mating type produce an oligopeptide called α factor which stops the growth of *a* cells and causes *a* and α cells to adhere each other. Cells of mating type *a* and *a* factor which has similar efects on *a* cells. In the presence of these factors, the cells adhere and cytoplasmic fusion takes place to form a heterokaryon (Strathern et al., 1981). Nuclera fusion follows rapidly to give a zygote (Priest&Campbell, 1996). By subsequent cell division this forms the diploid phase of the yeast life cycle which can be stably maintained for many generations. Meiosis and sporulation of diploid cells is triggered by nitrogen deprivation in thr presence of a non-fermentable carbon source

but will only occur if both MAT*a* and MATα genes are present. Following entry into meiosis the chromosomes in the yeasts nucleus undergo DNA synthesis, pairing, recombination and segregation. Spore walls grow and envelop the four haploid genomes (two each of α and *a* mating types) forming the characteristic four spored ascus. The spores, when placed in suitable nutrient media, germinate to form haploids and begin the whole cycle once more (Strathern et al., 1981).

1.6. Genetics

Genetics is the study of the relationship between the structure of the genotype and phenotypic expression. Genetics analyses provide a means of exploring the evolutionary and taxonomic relationship between individual strains. An understanding of the make up of the genotype is prerequisite for phenotypic modification. Thus, with knowledge of the nature of the genotype opportunities may present themselves by which undesirable characteristic can be deleted and desirable characteristic acquired. The comparatively rapid cell cycle of *S. cerevisiae,* its ease of cultivation and relatively compact genome has made these organism a common choice for the study of eukaryotic genetics, consequently the scientific literature is enormous. Nevertheless, the majority of these studies employ haploid starins of *S. cerevisiae* (Briggs 2004).

Genetic studies with *S. cerevisiae* were pioneered by Winge and colleagues at the Carlsberg Laboratories in Copenhagen and subsequently developed by Lindegren and co-workers. Yeasts, mainly of industrial origin, were extensively interbred to produce strains which could be mated to give healthy diploid cells capable of sporulating to produce four viable ascospores. Strain S288C and its diploid derivative X2180 provide the source of most genetically marked strains used in laboratory studies througout the world (Priest&Campbell, 1996).

Genetic analysis sets out to answer three fundamental questions about the genetic determinant for a given phenotypic character:

- Is it located on a chromosome or in the cytoplasm? (nuclear or cytoplasmic)
- To which chromosome does it belong and what is its position relative to other genes on the chromosome? (linkage and map position)

- How many different genes give the same genotype?(complementation analysis)(Hough 1982)

The chromosomes of *S. cerevisiae* are located in the nucleus and make-up between 80% and 85% of the total cellular DNA (Petes, 1980). Haploid cells of *S. cerevisiae*, there is sufficient DNA to account for about 15.000 genes at 16 chromosomes, ranging in size from 140 to 2500 kilobase pairs (kbp) together with basic histone protein molecules. All have been sequenced and 1996 saw publication of the complete sequence of the entire genome of a strain of *S. cerevisiae* (Briggs 2004). Most genes of the haploid genome are present as single copies, the major exceptions being the ribosomal RNA genes present as about 100 copies and the approximately 15 copies of each transfer RNA gene (Strathern et al., 1981). The magnitude of this task reflected by the fact that in the case of *S. cerevisiae*, some twelve million nucleotide bases were sequenced. Comparison of this sequence with those obtained from other cells and with knowledge of gene structure allows identification of sequence that encode for specific proteins. Such sequences are termed open reading frames (ORFs). Some 6,217 potential potential open reading frames have been identified in the yeast genome (Mewes et al., 1997) (Table 1.2).

Compared to many other cells, the yeast genome is very compact. Approximately 72% of chromosomal DNA codes for actual genes The average size of the yeast genome is 1,456 kb or 483 codons representing 40 to nearly 5,000 codons. Approximately 4% of yeast genes contain introns (non-coding regions). Genes are not evenly distributed throughout the chromosomal DNA, instead there are gene-rich clusters. In haploid strains, approximately half of the genes are dublicated. This has led to suggestion that this species arose from the fusion of two ancestral diploid strains, each with eight chromosomes.The resultant tetraploid cell was reduced to a 16 chromosome diploid by deletion (Wolfe&Shields, 1997).

Table 1.2: Yeast genome analysis of identified genes based on function (Mewes et al., 1997)

Gene Function	Propotion of identified genome (%)
Cellular organization and biogenesis	28
Intracellular transport	5
Transport facilitation	5
Protein trafficking	7
Protein synthesis	5
Transcription	10
Cell growth, division and DNA synthesis	14
Energy transduction	3
Metabolism	17
Cell rescue	4
Signal transduction	2

Much of the work, which has resulted in the current level of understanding of the yeast genome, has been performed on laboratory haploid strains. The genomes of industrial strains are vey different. The most notable difference is the fact that brewing yeast strains are polypoid or aneuploid. Commonly, three or four sets of chromosomes are present (triploid, tetraploid). Often the sets of chromosomes are not present in matched sets rather one or more chromosomes is present as an extra or one less copy (aneuploid) (Priest&Campbell, 2003). Polyploid strains of *S. cerevisiae* may have been selected for an industrial processes since they may have very very stable phenotypes. Thus, the chance of a single point mutation having an effect on the phenotype is reduced where multiple copies of the gene are present on the chromosomes. In addition, it is possible that multiple copies of some genes and concomitant increased expression might confer a selective advantage. For example, it has been claimed that multiple copies of maltose utilizing genesproduces a phenotype where maltose utilization rates are higher than comparable haploid strains (Priest&Campbell, 1996).

1.7. Industrial importance:

S. cerevisiae has utilized in many areas since discovery of its brewing and baking abilities in the middle east at some time between 6000 and 8000 BC. It seams that the use of cereals for baking and brewing developed simultaneously. Clearly this must also have included the discovery of malting and the use of yeast for leavening of dough and fermentation (Corran, 1975). After many years of production of wine, beer, alcohol and bread, economic importance is clearly understood by mankind. After the scientific developments at biotechnological areas, application of *S. cerevisiae* increases in industrial processes.

In assessing a yeast strain for industrial use, specific physiological properties are required (Ekunsanmi&Odunfa, 1990). Ethanol tolerance, sugar tolerance and invertase activities are some of the important properties for use in industrial ethanol production (Jiménez&Benítez, 1986). Yeasts have been isolated from many sources for industrial purposes. Such include yeasts isolated from palm wine for industrial production of ethanol, for single cell protein, for leaving of dough for bread-making and for wine production (2002). Yeasts have also been isolated from many fermenting sources including fermenting cassava tubers (Ofuya et al., 1990).

Acquirirng one simple ability make the main reason that *S. cerevisiae* is commonly used in a variety of commercial fermentation and biomass conversion processes. That ability is as seen in figure 1.5, convert sugars and other carbon sources into ethanol in the absence of oxygen, and into CO_2 and water in presence of oxygen (Ratledge&Kristiansen, 2001). Leaving dough is a good example for *S. cerevisiae*'s ability. *Saccharomyces cerevisiae* cells produce alcohol and carbon dioxide during fermentation. These carbon dioxide bubbles give the bread a lighter and finer texture (Black, 2002).

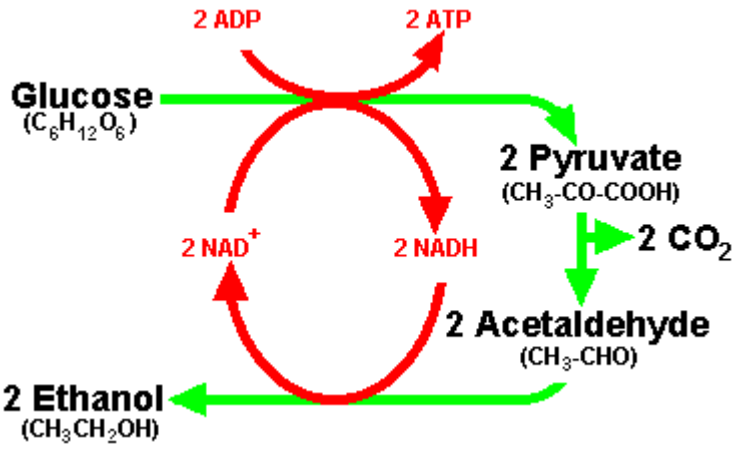


Figure 1.5: Conversion of glucose as a carbon source to the ethanol (url.5).

Other application area of *S. cerevisiae* is brewing industry. *S.cerevisiae* is known as the ale-fermenting yeast in the brewing practice. The important properties of good fermenting yeast are listed below (Priest&Campbell, 1996).

• A rapid fermentation rate without excessive yeast growth,

• An efficient utilization of maltose and maltotriose with good conversion to ethanol,

• An ability to withstand the stresses imposed by the alcohol concentrations and osmotic pressures encountered in breweries,

• A reproducible production of the correct levels of flavor and aroma compounds,

• An ideal flocculation character for the process employed,

• Good 'handling' characteristics (e.g. retention of viability during storage, genetic stability).

S. cerevisiae has become a very important organism for biotechnological applications. It can be concluded that it is one of the most studied microorganisms on the planet(Murphy&Kavanagh, 1999). In addition to its widespread use in food technology, *S.cerevisiae* has also been used as a cloning tool in the biotechnology sector. The most important advantage of this is the fact that *S.cerevisiae* can secrete the protein of interest into the growth medium which makes the downstream processing easier (Schreuder et al., 1996).

The other advantage of *S. cerevisiae* is its belong to phylum Ascomycetes, so it can produce ascospores in ascus by meiosis and sporulate under stress. After meiosis diploid nucleus produce four haploid nuclei which than incorporate into four stress-resistant ascospores, encapsulated in ascus. This packaging of meiotic products makes analysis simple. They can proliferate when they are haploid and mutations can be easily studied because they can easily be isolated due to having dispersed cells (Snustad&Simmons, 2000).

Single cell protein production also makes use of *S. cerevisiae* because of its high biomass yield and GRAS (generally regarded as safe) organism status. Starch and industrial fermentation media are used in biomass production for single cell protein applications (Ogden&Tubb, 1985).

Additionally, *S.cerevisiae* is reconstructed for the utilization of lactose in whey for ethanol production, and treatment of dairy industry wastewaters. For this purpose laboratory strains have been constructed by expressing the *Kluvyeromyces lactis* genes *LAC4* and *LAC12* (Rubio-Texeira et al., 2000).

1.8. Importance of Ethanol tolerance in *Saccharomyces cerevisiae*

Yeast cells are usually exposed to some environmental changes during the course of production process, such as increase in osmotic pressure or accumulation of ethanol and/or carbon dioxide (Attfield, 1997). Since ethanol is one of the main stress factors in industrial production process using yeast, high level of ethanol tolerance for a yeast strain is a prerequisite for a high efficiency of fermentation and in turn for a high yield of ethanol (Hirasawa et al., 2007).

The rising ethanol level during batch fermentation on high concentrations of sugar substrates acts initially to reduce growth and fermentation rates and adversely affects cell viability (Piper, 1995). The fermentation efficiency of *S. cerevisiae* at supra-optimal temperatures is very low and cooling is necessary. Moreover, in the summer time, more cooling is needed and the cost increases because of this. High osmotic stress brought by high sugar concentrations also affects yeast metabolism and leads to decrease in final ethanol production (Attfield, 1997). Therefore, improving the multiple stress tolerance of ethanologenic yeast, particularly tolerance of ethanol, heat, and osmotic stresses, has attracted great attention among researchers (Wei et al., 2007).

While the mechanisms of ethanol tolerance are of fundamental scientific interests, they are also of significant economical interest. At present, there is an increasing demand for alternative energy sources. Such a demand has been driven by dwindling fossil fuel reserves and ever increasing gas prices (Hill et al., 2006). One of the alternatives is the renewable biofuel. Aside from the obvious advantages of being renewable, biofuel promises to alleviate the problems brought by the fossil fuels, especially in greenhouse gas emissions. Among the many types of biofuels, unblended ethanol has shown the greatest promise. Thus, detailing the mechanisms of ethanol stress responses or tolerance may be helpful for the increase of ethanol concentration during bioethanol production and extensive application of this biofuel (Ding et al., 2009).

1.9. Ethanol stress in *Saccharomyces cerevisiae*

Ethanol is an inhibitor of yeast growth at relatively low concentrations, inhibiting cell division, decreasing cell volume and specific growth rate, while high ethanol concentrations reduce cell vitality and increase cell death (Birch&Walker, 2000). Ethanol also influences cell metabolism and macromolecular biosynthesis by inducing the production of heat shock-like proteins, lowering the rate of RNA and protein accumulation, enhancing the frequency of petite mutations, altering metabolism, denaturing intracellular proteins and glycolytic enzymes and reducing their activity (Hu et al., 2007).

The main sites for ethanol effects in yeast are cellular membranes, hydrophobic and hydrophilic proteins and the endoplasmic reticulum. Lipid composition of yeast cell membranes and ethanol tolerance are strictly related (Piper, 1995). In particular, the ability to operate acyl chain unsaturation and ergosterol biosynthesis are essential for ethanol tolerance, in other words cell viability is related to the presence of ergosterol and of specific fatty acids in plasma membranes. Oleic acid is the most important UFA in counteracting the toxic effects of ethanol through its effect on plasma membrane fluidity. However, also the presence of shorter (C16) monounsaturated fatty acids are leading to ethanol tolerance due to the possibility to allocate ethanol molecules in the hydrophobic core of the membranes (Mannazzu et al., 2008).

Ethanol is a stress factor for wine strains growing under fermentative conditions (Piper, 1995). It modifies the polarity of membranes and the hydration of polar head-groups of membrane surfaces (plasma membrane and organelles), thus affecting the efficiency of membrane functions, e.g. uptake of nutrients and excretion of ethanol. Moreover, at concentrations above 15 g l^{-1}, it leads to cell death by increasing membrane permeability (Mannazzu et al., 2008).

One of the main factors affecting yeast survival in the presence of ethanol is the ability to modulate the lipid composition of cell membranes (Thomas&Rose, 1979). Thomas et al., (1978) have reported that in the exposure of yeast to ethanol results in increased membrane fluidity and consequential decrease in membrane integrity (Mishra&Prasad, 1989). A decrease in water availability due to the presence of ethanol causes the inhibition of key glycolytic enzymes and these proteins may be denatured (Hallsworth et al., 1998). The main effects of ethanol on the yeast are shown in Table 1.3.

Table 1.3: The main effects of ethanol on the yeast cells (Stanley et al., 2010).

Cell function and ethanol influence	Source
Cell viability and growth	
Inhibition of growth, cell division and cell viability	Stanley *et al.* (1997)
Decrease in cell volume	Birch and Walker (2000)
Metabolism	
Lowered mRNA and protein levels	Chandler *et al.* (2004), Hu *et al.* (2007)
Protein denaturation and reduced glycolytic enzyme activity	Hallsworth *et al.* (1998)
Induction of heat shock proteins and other stress response proteins	Plesset *et al.* (1982)
Intracellular trehalose accumulation	Lucero *et al.* (2000)
Cell structure and membrane function	
Altered vacuole morphology	Meaden *et al.* (1999)
Inhibition of endocytosis	Lucero *et al.* (2000)
Increased unsaturated/ saturated fatty acid ratio in membranes	Alexandre *et al.* (1994)
Increase in ergosterol content of membranes	Sajbidor *et al.* (1995)
Loss of electrochemical gradients and proton-motive force	Petrov and Okorokov (1990)
Inhibition of transport processes	Leao and van Uden (1984)
Inhibition of H^+-ATPase activity	Cartwright *et al.* (1986)
Increased membrane fluidity	Mishra and Prasad (1989)

Yeasts however have evolved to become more resilient to environmental stresses.Yeast survival and growth under stress conditions is achieved through a series of stress responses that depend on a complex network of sensing and signal transduction pathways leading to adaptations in cell cycle, and adjustments in gene expression profiles and cell metabolic activities (Stanley et al., 2010).

1.10. The response of *Saccharomyces cerevisiae* to ethanol stress:

The yeast stress response is a transient reprogramming of cellular activities to ensure survival in challenging conditions, protect essential cell components and enable resumption of 'normal' cellular activities during recovery.The response of yeast to environmental stress is complex, involving various aspects of cell sensing, signal transduction, transcriptional and posttranscriptional control, protein-targeting, accumulation of protectants, and increased activity of repair functions (Mager&Ferreira, 1993).

The efficiency of these processes in a given yeast strain determines its robustness and, to a large extent, ability of a given strain to perform well in industrial processes. A better understanding of the cellular consequences of microbial ethanol stress and of the underlying ethanol stress defence mechanisms is crucial for improving the performance of yeast strains during stress (Stanley et al., 2010).

Yeast cells have developed appropriate mechanisms to deal with several types of damages caused by increased ethanol concentration (Table1.4). First, ethanol increases the fluidity of the plasma membrane and destroys the normal membrane structures. In response, yeast cells may change membrane compositions to antagonize membrane fluidization and stabilize plasma membrane. Specifically, it has been shown that the levels of unsaturated fatty acids (UFAs) (You et al., 2003) and ergosterol (Daum et al., 1998; Swan&Watson, 1998) increase in response to the high concentration of ethanol. Furthermore, the addition of some types of amino acids (Takagi et al., 2005) and inositol (Kelley et al., 1988) can enhance ethanol tolerance when provided as a supplement, most likely through enhanced membrane stability. Second, the expression of factors that stabilize and/or repair denatured proteins in yeast cells, such as trehalose and induced heat shock proteins (HSPs), have been revealed to correlate with the capabilities to tolerate alcohol (Swan&Watson, 1998). Third, some candidate proteins involved in the expression of stress-related genes like the zinc finger protein, and the recently reported alcohol sensitive ring/PHD finger 1 protein (Asr1p) also play a role in ethanol tolerance in *Saccharomyces cerevisiae* (Ding et al., 2009).

Table 1.4: Appropriate mechanism developments on yeasts against increased ethanol concentration (Ding et al., 2009).

Factors involved in ethanol tolerance	Functions	Reference
Composition of yeast plasma membrane		
Unsaturated fatty acids (UFAs)	Increase membrane stability and antagonize the fluidity caused by ethanol	You et al. (2003); Xiao et al. (2008); Ingram (1990); and Weber and de Bont (1996)
Ergosterol		Inoue et al. (2000) and Swan and Watson (1998)
Amino acid		Hu et al. (2005) and Takagi et al. (2005)
Inositol and H^+-ATPase		Cartwright et al. (1987); Furukawa et al. (2004); and Fernandes and Sa-Correia (2003)
Factors that stabilize or repair denatured proteins		
Trehalose	Stabilize or repair denatured proteins caused by ethanol	Gomes et al. (2002); Ogawa et al. (2000); Barry and Gawrisch (1995); and Lucero et al. (1997)
Heat shock proteins (HSPs)		Craig et al. (1993); Seymour and Piper (1999); Quan et al. (2004); and Piper et al. (1994, 1997)
Stressful genes		
Transcriptional factor interact with stress response element (STRE)	Through the genes transcriptional expression, a large number of genes can be up-or down-regulated to restore the cellular normal functions upon the ethanol stress	Schuller et al. (1994); Marchler et al. (1993); Moskvina et al. (1998); Watanabe et al. (2007); Gorner et al. (1998, 2002); and Hirata et al. (2003)
Heat shock factors (HSFs)		Hahn et al. (2004); Wu (1995); Takemori et al. (2006); Sorger and Pelham (1988); Guo et al. (2008); and Liu et al. (2008)
Alcohol sensitive ring/PHD finger 1gene (*Asr1*)		Daulny et al. (2008); Betz et al. (2004); Izawa et al. (2006); Aravind et al. (2003); Capili et al. (2001); Kubota et al. (2004); and van Voorst et al. (2006)
Some other genes		

1.10.1. Unsaturated fatty acids:

The changes of unsaturated fatty acids have often been observed due to the presence of alcohol. The compositions of UFAs in *S. cerevisiae* consist mainly of the mono-UFAs palmitoleic acid (∆9z-C16:1) and oleic acid (∆9z -C18:1) with the former dominating. Both of them can be catalyzed by a single integral membrane desaturase encoded by OLE1 gene. Experimental data suggested that among the two common types of UFAs, oleic acid is more efficacious in overcoming the toxic effects caused by ethanol in growing yeast cells (You et al., 2003).

1.10.2. Ergosterol:

Ergosterol is the major sterol in *S. cerevisiae* membranes and performs roles similar to those of cholesterol in mammalian cells. It is required for the normal structure and function of cellular membranes by regulating the delicate balance among membrane components such as lipids and proteins (Bagnat et al., 2000). These essential cellular functions of ergosterol suggest that it likely plays a critical role in ethanol resistance in *S.cerevisiae* (Daum et al., 1998; Swan&Watson, 1998). Mutant of *S. cerevisiae* defective in ergosterol biosynthesis could not proliferate under normal conditions and not ferment sugars in the presence of a moderate concentration of ethanol that normal cells usually tolerate. In contrast, yeast cells that had the highest content of ergosterol in the plasma membrane showed the highest ethanol resistance (Inoue et al., 2000). This was due to increased membrane rigidity by ergosterol to particularly antagonize the fluidity caused by the high concentrations of ethanol, as well as the changes of UFAs. Furthermore, the role of ergosterol in stress tolerance is independent of HSPs or trehalose, since the mutant with variable alcohol tolerance synthesized HSPs and accumulated trehalose to the same levels as the wild-type cells (Swan&Watson, 1998).

1.10.3. Amino acids:

In many studies it was founded that when yeast cells were exposed to 20% (v/v) ethanol for 9 h at 30ºC, all cells would die. However, 57% of the cells would remain viable if the ethanol solution contained three amino acids: isoleucine, methionine, and phenylalanine (Hu et al., 2005). Based on the analysis of amino acid compositions of plasma membrane proteins and plasma membrane fluidity, it was revealed that the significant increased ethanol tolerance in yeast cells was due to the incorporation of the supplementary amino acids into the plasma membranes. Similarly, another study identified that the accumulation of L-proline in the yeast cells can also improve ethanol tolerance. Being an osmoprotectant, L-proline helps protect yeast cells from damage incurred by freezing, desiccation, or oxidative stress (Takagi et al., 2005).

1.10.4. Inositol and H+-ATPase:

It has been demonstrated that when yeast cells were cultivated in the presence of a sufficient amount of inositol, the tested cells showed a higher level of ethanol tolerance than those grown in a limited amount of inositol, indicating that the cellular inositol content might be positively correlated to ethanol tolerance (Ding et al., 2009). Further analysis revealed that when exposed to ethanol, yeast cells grown in limited amount of inositol would leak more intracellular components such as nucleotide, phosphate, and potassium than those grown in high concentrations of inositol. The leakage of intracellular components also affected the intracellular pH and lowered the activity of H+-ATPase, which functions in ensuring the homeostasis of ions in the cytoplasm and resultantly affects the permeability barrier of the yeast membrane (Furukawa et al., 2004). Thus, when the yeast cells cultivated with sufficient inositol were exposed to ethanol, their ATPase activity increased, which likely offset the proton inflow induced by ethanol and triggered by a change in lipid composition, and eventually, enhanced ethanol tolerance (Cartwright et al., 1987).

1.10.5. Trehalose:

Trehalose has remarkable stress protection properties and may determine the survival response of yeasts under extreme environmental conditions. Trehalose is present in the yeast cell as a storage carbohydrate and as a stress protectant. One of the main functions of trehalose is to protect cells against the denaturation and aggregation of proteins during periods of stress. Consistent with this function, yeast cells accumulate trehalose when exposed to adverse conditions, such as heat stress or ethanol stress (Novo et al., 2004).

In contrast, cells unable to accumulate trehalose show retarded growth in the presence of high ethanol (Ogawa et al., 2000). During ethanol stress, trehalose functions as a chemical co-chaperone, which means that the increased trehalose prevents the aggregation of the misfolded proteins on the membrane.

Water plays an important role in the structure of biological membranes because they can penetrate the lipid bilayer and form hydrogen bonds with the polar groups of phospholipids. Ethanol can substitute for water in this role and in doing so, alter the positioning of molecules on the membranes, influence the interactions between lipids and proteins, and ultimately, damage the structure and functions of membrane. A high concentration of trehalose displaces water and ethanol on yeast membranes, and the subsequent formation of hydrogen bonds between the hydroxyl groups of trehalose and the polar groups of lipids stabilizes the membrane (Ding et al., 2009).

1.10.6. HSPs:

Heat shock proteins are the most common protective proteins and have a similar function to trehalose, as molecular chaperones in response to stress stimuli in most cells. Briefly, while the molecular chaperones do not themselves take part in the final assembly of new or refolded structures, under stressful conditions, HSPs can mediate the correct folding of other proteins to prevent further damage and repair intracellular injury (Ding et al., 2009). Under laboratory conditions, the yeast heat-shock protein Hsp104p has been found to be responsible for tolerance to several stress conditions such as heat, ethanol, arsenite and long-term cold storage, and its expression has been shown to be sufficient for thermotolerance. Recently, Hsp12p has been shown to protect membranes against desiccation and ethanol-induced stress (Carrasco et al., 2001).

Initial studies showed that heat shock pretreatment of yeast cells, the condition to induce synthesis of heat shock proteins, could result in a significant increase in ethanol tolerance, which implied the relationship between HSP induction and ethanol tolerance. Meantime, it was observed that ethanol at concentrations of 4–10% strongly induced HSP synthesis (Piper et al., 1994; Kubota et al., 2004). The molecular genetic studies with HSP mutants in yeast indicated the direct contribution of HSPs to ethanol tolerance. Mutation of HSP104 greatly reduced both ethanol-induced tolerance to heat and heat-induced tolerance to ethanol (Eckert et al., 1992).

Similar results were also obtained with HSP30 mutation. This mutant would reduce biomass yields and extend the time required for yeast cells to adapt to growth at 10% ethanol and other stress conditions. Furthermore, it was also demonstrated that HSP30 could down-regulate the stress-activation of plasma membrane H+-ATPase so that the ATPase does not deplete the energy reserves (Piper et al., 1994). The yeast HSP70 family protein SSA4p was specifically accumulated in the nuclei under ethanol stress, which was suggested to be involved in the repair of the nuclear proteins vulnerable to damage by ethanol (Quan et al., 2004).

1.10.7. Alcohol sensitive ring/PHD finger 1 gene

The alcohol sensitive ring/PHD finger 1 protein (Asr1p) was recently identified to be related to ethanol tolerance in yeast cells (Betz et al., 2004). This protein changes its subcellular localization especially upon exposure to alcohol stress. Asr1p constitutively shuttles between the nucleus and the cytoplasm but accumulates in the nucleus upon exposure to ethanol. This accumulation is rapid, reversible, and requires a functional Ran/Gsp1p gradient. Recently, protein Asr1 was a RING finger ubiquitin ligase that could bind directly to the RNA polymerase II via carboxyl-terminal domain and through the action of ubiquitylation, inactivated the RNA polymerase II by ejecting the Rpb4/Rpb7 subunits from the pol II complex. Their data supported the conclusion that ubiquitin can influence gene activity by altering the subunit composition of a core component of the transcriptional machinery such as pol II but with little experimental evidences related with Asr1p and ethanol tolerance (Daulny et al., 2008).

1.11. Obtaining ethanol resistant *Saccharomyces cerevisiae* mutant by using an inverse metabolic engineering strategy: evolutionary engineering

Metabolic engineering is the directed improvement of cellular properties through the modification of specific biochemical reactions or the introduction of new ones, with the use of recombinant DNA technology (Figure 1.6). The metabolic engineering approach examines biochemical reactions in their entirety, rather than individually, and is concerned with the construction of novel pathways, the thermodynamic feasibility of pathways, and the location of limiting branch-point(s) and enzymatic reaction(s) in a reaction network (Stephanopoulos, 1994; Stephanopoulos, 1999).

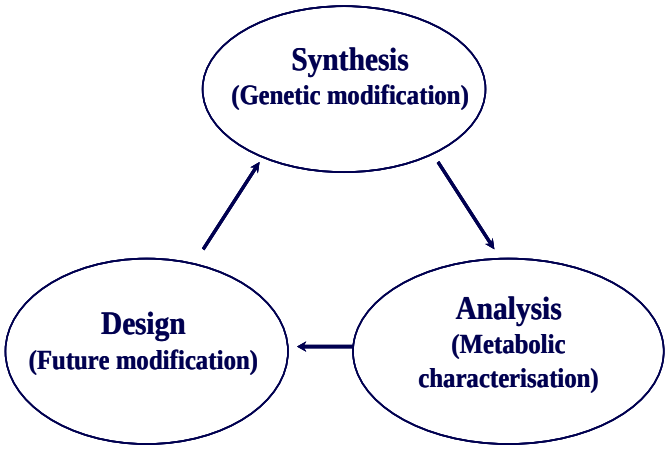


Figure 1.6: Principe of Metabolic Engineering (Nielsen, 2001).

The classical approach of metabolic engineering requires detailed knowledge of the enzyme kinetics and the system network, in order to have the desired phenotype at the end. However, the basis of inverse metabolic engineering which was defined by Bailey in 1996 is first of all, identifying a desired phenotype in a heterologous organism or in a related model system, secondly defining or hypothesizing the genetic basis for this desired phenotype and lastly constructing the desired phenotype on desired organism. If any pressure is applied in order to have this desired characteristic, the strategy is called *directed evolution* (Bailey et al., 1996). In other words, inverse metabolic engineering maintains a rapid development of new experimental methods for creating genetic diversity and for searching large populations for improved functions. Further advances in screening and selection technologies will reduce the time and cost of the experiments and will make it possible to solve more difficult problems involving multiple enzymes, multi component enzymes, and the creation of new functional molecules (Arnold et al., 1999).

The evolutionary engineering (Figure 1.7) which is an inverse metabolic engineering strategy, consists of obtaining variant cell population followed by selection for desired phenotypes. A strain with desirable properties obtained by evolutionary engineering approach can then be optimized by rational metabolic design if necessary (Petri&Schmidt-Dannert, 2004).

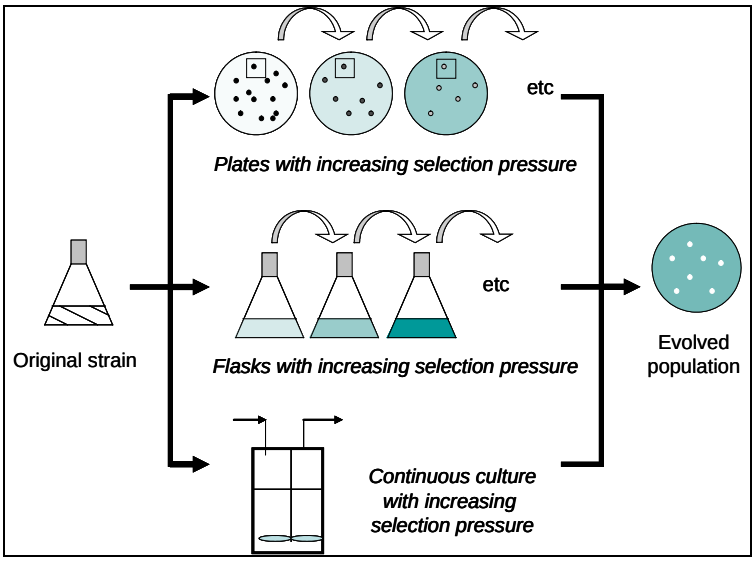


Figure 1.7: Principle of Evolutionary Engineering (Sauer, 2001).

Aim of the study:

The aim of the present study was to obtain ethanol resistant mutants by using evolutionary engineering methodology. Different selection strategies were applied to observe efficiencies in selecting ethanol resistant mutants. Survival ratios of mutants were calculated quantitatively under different ethanol stress levels and most resistant individuals were selected. Cross resistance to different metals and other stress types were determined by most probable number method and basic microbiological tests.

2. MATERİALS METHODS

2.1.Materials

2.1.1. Yeast Strain

Saccharomyces cerevisiae strain was kindly provided by Kutman winery and named as Hef. Wild type Hef was then treated with a chemical mutagen ethyl methane sulfonate which is a mutagen and the mutant population named Hef1 was obtained.

2.1.2. Yeast Culture Media

Different types of yeast culture media were used depending on the desired growth rate and physical conditions of the culture media.

2.1.2.1 Yeast Minimal Medium (YMM)

Yeast Nitrogen Base without aminoacids	6.7 g
Dextrose	20 g
Agar (for solid media)	20 g

per liter of distilled water.

2.1.2.2 Yeast Complex Medium (YPD)

Bacto Yeast Extract	10 g
Dextrose	20 g
Bacto Peptone	20 g
Agar (for solid media)	20 g

per liter of distilled water.

2.1.3. Chemicals

Chemicals and their company and country names are listed below.

- Ethanol (absolute) was purchased from J.T.Baker (Holland).
- Hydrogen peroxide (35%, v/v) was obtained from Merck (Germany).
- Sodium Thiosulphate was purchased from J.T.Baker (Holland).
- D(+)-Trehalose dihydrate was obtained from Riedel-de Haën (Germany).
- Kobalt (II)-chloride-hexahydrate was purchased from Merck (Germany).
- Glycerol (Sigma, Germany).
- Sodium chloride (Riedel-de Haën, Germany).

2.1.4. Buffers and Solutions

Potassium phosphate buffer (pH7)	50mM
Sodium thiosulfate solution	10 % (w/v)
H_2O_2 solution	5 M
$CoCl_2$ solution	1 M
Glycerol	60 %

2.1.5 Laboratory Equipment

The equipments used in this study are listed below.

- Thermomixer (Eppendorf, Thermomixer Comfort 1.5-2 ml, Germany)
- Rotor (Beckman Coulter JA-30.50i rotor, USA)
- Vortex mixer (Heidolph REAX top, Germany)
- UV-Visible Spectrophotometer (Shimadzu UV-1700, Japan)
- Ultrapure Water System (USF-Elga UHQ, USA)
- Micropipettes (10000 µl, 5000µl, 1000µl, 200µl, 100µl, 20µl, Eppendorf; Germany)
- pH meter (Mettler Toledo MP220, Switzerland)
- Water Bath (Julabo SW22, Germany)
- Balances (Precisa BJ 610C, XB 620C and XB 220A, Switzerland)
- Laminar Flow (Faster BH-EN 2003, Italy)

- Autoclaves (Tomy SX 700E, China)
- Deep Freezes (-80˚C Sanyo Ultra low, Japan) and (-20˚C Arçelik, Turkey**)**
- Refrigerators (+4˚C Arçelik, Turkey**)**
- Orbital Shaker Incubators (Forma Orbital Shaker, USA)
- Centrifuge (Eppendorf Micro-centrifuge 5424, Germany)
- Light Microscope (Olympus CH30, Japan)
- Magnetic Stirrer (Magnetic stirrer standard unit, Germany)

2.2. Methods

2.2.1. API ID 32C Yeast Identification System

API ID 32C identification test kit for yeast strains was used for contamination control. Strips were inoculated with samples according to manifacturer's instructions. Strips were incubated at 30ºC. Positive growth wells were marked on the result sheets. Result were analyzed by PC software.

2.2.2. Obtaining ethanol-resistant mutant *Saccharomyces cerevisiae* strains

2.2.2.1. EMS application to wild type *Saccharomyces cerevisiae* strains

Saccharomyces cerevisiae, approximately at a concentration of $1x10^6$ cells/ml; was inoculated into 10 ml YPD, and incubated overnight at 30ºC and 150 rpm in order to have the cell concentration of approximately $2x10^8$ cells/ml. 2.5 ml of this culture was washed twice with 50 mM potassium phosphate buffer (pH 7) and resuspended in the same buffer to obtain a final concentration of $5x10^7$ cells/ml. 300 µl of EMS was added into each 10 ml of cell suspension in a 3 screw-cap glasses tubes. The tubes were vortexed and then incubated for 60, 90, 120 minutes at 30º C. In order to stop EMS mutagenesis, an equal volume of freshly made and filter-sterilized sodium thiosulfate solution (10%, w/v) was added into the tubes at three different time periods. The solution was mixed well with vortex and the cells were centrifuged at 10,000 rpm for 10 min (Beckman Coulter, JA 30.50i rotor). The supernatant was discarded and the cells were washed twice with yeast minimal medium without dextrose.

The mutated cells were then inoculated into YPD and the death ratio of each culture was analyzed by calculating the number of CFU in reference to control group. The optimum death rate was determined to be ≈ 90 % at 120 minutes. The survival ratio of the cultures after 120 minutes of EMS application was 4 % (death ratio: ≈ 90 %). Thus, the culture exposed to EMS for 120 min was found to be the optimal chemically mutagenized culture for further studies and named as “HEF1”.

2.2.2.2. Selection of mutant population

Obtaining the generations is based on applying the initial stress condition to the mutated culture Hef1 and transferring the survivors of this present stress condition to the next stress condition. Fresh mutated culture Hef1 was used for the first stress application and the rest of the steps continued over the newly obtained generations.

HEF1 was used as the initial culture for applying increasing ethanol stress for several generations. The required incubation time for ethanol stress was considered to be 48 h. The constant stress to be applied on each generation was 10% ethanol and the selection at increasing stress levels started at 10% and was gradually increased by 0.1% at each level of stress (10% → %10,1 → 10.2). *Continuous stress selection strategy* is adopted for obtaining the generations. The increasing stress application strategies are both shown in Figure 2.1.

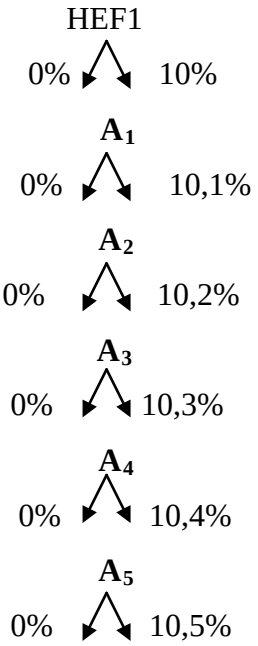


Figure 2.1: Increasing stress application strategy. A_1 refers to the initial culture exposed to %10 ethanol. In every step new generations were obtained from different ethanol concentrations. This first 4 generations are shown as A_2, A_3, A_4, A_5.

The control groups were used to obtain the survival ratio of the determined generations at each stress selection step. The optical density values of both the generations and the control groups were determined at 600 nm at 48. h of incubation.

2.2.2.3. Stock Culture Preparation

Frozen stock cultures were prepared after obtaining each new generation and mutant selection for long term storage. Frozen stock cultures were kept in -80ºC deepfreezer. Unless the culture is not in a stressful condition, 500 µl of culture was added to microfuge tubes and same amount of 60 % (v/v) glycerol was added. If the starting culture is in a medium with stress, the culture was then washed twice with YMM by centrifuging at 10’000 rpm for 5 min and discarding the supernatant.

2.2.2.4. The optical density measurements and survival ratio determination of the mutant generations

Optical density measurements were done by UV-Visible Spectrophotometer at 600 nm. The survival ratios of the populations were calculated by dividing the OD_{600} values of the cultures that were exposed to ethanol stress to the OD_{600} values of their corresponding control groups. Survival values as fold of wild type values were calculated by dividing the OD_{600} value of wild type (Hef) to OD_{600} value of the mutant (Hef1) strain.

2.2.2.5. Selection of ethanol-resistant individual mutant strains

Overnight liquid cultures of the selected highly resistant population were inoculated into agar plates. Either diluting the culture by streaking method or spreading 100 µl of the overnight culture in a dilution ratio of $1:10^6$ was applied for selection of individual colonies. The individuals were transferred into fresh 10 ml of YMM by using sterile toothpicks and used for further investigations.

2.2.3. Phenotypic characterization of ethanol-resistant individual mutant strains

Selected individual mutants were characterized by screening under various stress conditions such as heavy metal stress, heat stress, ethanol stress, and oxidative stress.

2.2.3.1. Quantitative resistance determination of mutant individuals with MPN method

Five-tube MPN (most probable number) method was used to compare resistance of individuals obtained from the final population. Five-tube MPN method gives the number of cells per ml (Lindquist, J., 2001). According to the number of cells grown in YMM medium involving a selective ethanol amount, a quantative result can be obtained to select the most ethanol resistant mutant. Five columns of 96 well plates were filled with 180 µl media and 20µl culture. By the help of multiwell pipette, 20µl of the culture at row A was transferred to row B. This transferring procedure diluted the culture 1:10 times each and was continued to row H. In order to prevent errors, pipettes were used carefully. After adding cultures and media 96 well plates were kept at 30°C room for 24 h. The culture growth was observed as pellet formation on

the bottom of the well. Final 3 rows where culture was grown were identified and the number of wells at that corresponding row were detected. By the help of 5 tube MPN table, number of cells per ml for each culture was calculated.

2.2.3.2. Determination of cross resistance of ethanol-resistant individual mutant strains

2.2.3.2.1. Osmotic Stress:

Ethanol resistant individual mutant cells (Hef1), and wild type cells (Hef) were exposed to osmotic stress in minimal media involving varying percentages of NaCl (0%, 5%, 10%, 15%). The growth of the cultures was checked after overnight incubation.

2.2.3.2.2. Oxidative stress:

The number of cells/ml and percent survival values of overnight cultures of mutant individuals and wild type Hef under continuous 0.,3M H_2O_2 stress were determined by 5 tube-MPN method. The cultures without H_2O_2 stress were used as control groups.

2.2.3.2.3 Sorbitol Stress:

The number of cells/ml and percent survival values of overnight cultures of mutant individuals and wild type Hef under continuous 2M sorbitol stress were determined by 5 tube-MPN method. The cultures without 2M sorbitol stress were used as control groups.

2.2.3.2.4. Heat Stress (+60^0 C):

One ml of fresh overnight culture was exposed to temperature stress for 10 min. After this pulse stress application, the cells were harvested by centrifugation at 14,000 rpm for 5 min. The cells were washed twice by YMM without dextrose. A cell suspension of 500 µl was inoculated into 10 ml of YMM and incubated at 30^0 C and 150 rpm. The growth of cells was checked after overnight incubation.

2.2.3.2.5. Cold Stress (-20^0 C):

One ml of overnight cultures of mutant individuals and wild type Hef were washed with dextrose-free YMM and exposed to -20^0 C cold stress for 90 min. After this pulse stress application, the cells were thawed at room temperature. The number of cells per ml and the percent survival values were determined by 5 tube- MPN method. The cultures with 30 ^{0}C exposure were used as control groups.

2.2.3.2.6. Metal ($CoCl_2$) Stress:

Ethanol resistant individual mutant cells Hef1, and wild type cells Hef were exposed to metal stress by 7mM $CoCl_2$. The number of cells per ml and the percent survival values were determined by 5 tube- MPN method after 72 h of incubation in 96-well plates.

2.2.3.2.7. %20 v/w ethanol pulse stress

One ml of overnight cultures of mutant individuals Hef1 and wild type Hef were washed with dextrose-free YMM and exposed to %20 v/w ethanol pulse stress for 90 min. After this pulse stress application, the cells were harvested by centrifugation at 14,000 rpm for 5 minutes. The number of cells per ml and the percent survival values were determined by 5 tube- MPN method. The cultures without %20 ethanol pulse stress at 30 ^{0}C exposure were used as control groups.

2.2.3.2.8. Freezing-Thawing Stress

Ethanol resistant individual mutant cells Hef1 and wild type cells (Hef) were exposed to freezing-thawing stress. For this purpose, 1.5 ml of overnight cultures was frozen in liquid nitrogen for 25 min. This freezing step was followed by thawing at 30^0 C for 20 min. The number of survivors was determined by MPN-based method in reference to control groups (no exposure to freezing- thawing stress).

2.2.4. Determination of fermentation capacity of the ethanol-resistant individual mutant strains

Tubes (fioles) with a 15 ml volume were used for this experiment. They were filled up to 12 ml because above this volume the liquid can escape from the needle. The test was applied under absence and presence of ethanol so for each sample a tube with and without ethanol is prepared. 2xYPD is prepared and 6 ml delivered to tubes and for the control tubes 6 ml water was added but for the tubes that will contain 10% ethanol, 4.8 ml water is added. They were capped with the plastic and metallic materials and then autoclaved. The tubes were supplemented with Tween 80 (Merck) and Ergosterol (minimum 75%, E6510, Sigma) with those concentrations; 0,21 g/l and 7,5 mg/l respectively after autoclave. Along with this experiments 200x Tween 80 and 1000x Ergosterol stocks were used. Absolute ethanol (1,2 ml) was added to corresponding tubes.The inoculation of the samples was made by using over-night grown pre-cultures (in glass tube, 4.5 ml culture volume in YPD). The parental strains (S288C and 59A) were added to each experimental set. Before inoculation, OD_{600} of the cultures were determined after an additional sonication step. The inoculation was made with a syringe and needle. Whenever an inoculation or addition of liquid was made to the tube, a second needle was inserted on the plastic cap. Tubes weights were measured by using a precision scale starting from the first inoculation time (t0).

2.2.5 Growth Curve

We compared the optical density differences of WT(Hef) and Hef1 (*11*) at 3 different ethanol concentrations (8%, 10% and 12%) in Yeast Minimal Medium (YMM) and at control medium. Pre-cultures were inoculated to those media by setting their OD_{600} as 0,2. OD_{600} was measured at 4^{th}, 6^{th}, 9^{th}, 12^{th}, 24^{th} and 48^{th} h.

3. RESULTS

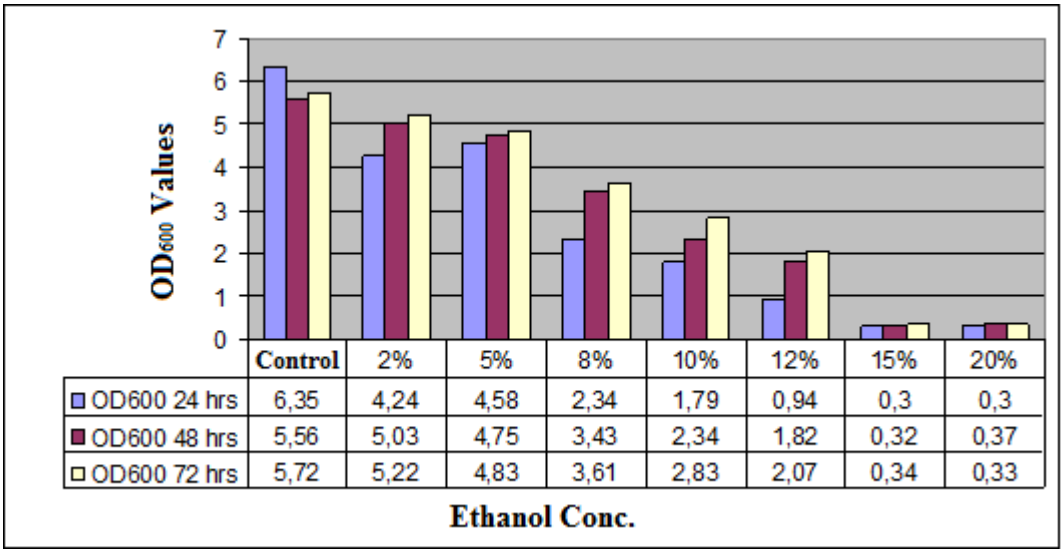
3.1 Screening for Ethanol Stress Resistance to Determine the Initial Selection Stress Levels

3.1.1 Screening for continuous stress application

Before applying the initial the stress level for obtaining the generations, the wild-type (Hef) and the mutated cell (Hef1) culture was screened under various concentrations of ethanol stress. Both strains were cultivated in YMM involving 0 (control one), 2 %, 5 %, 8 %, 10 %, 12 %, 15 % and 20 % ethanol (v/v). Ten ml cultures were prepared with starting 0.2 OD_{600} value in 50 ml test tubes. The cultures were then incubated at 30 ºC. The optical density values at 600 nm were monitored at 24th, 48th and 72nd hours and according to these results survival ratios were calculated. Optical density and survival ratio values results were shown below in Tables 3.1, 3.2, 3.3, 3.4 and Figures 3.1 and 3.2.

According to API ID 32C test kits initial generation was identified *Saccharomyces cerevisiae* and contamination was ruled out.

Table 3.1: Optical density values (OD_{600}) of cultures Hef.



	Control	2%	5%	8%	10%	12%	15%	20%
OD600 24 hrs	6,35	4,24	4,58	2,34	1,79	0,94	0,3	0,3
OD600 48 hrs	5,56	5,03	4,75	3,43	2,34	1,82	0,32	0,37
OD600 72 hrs	5,72	5,22	4,83	3,61	2,83	2,07	0,34	0,33

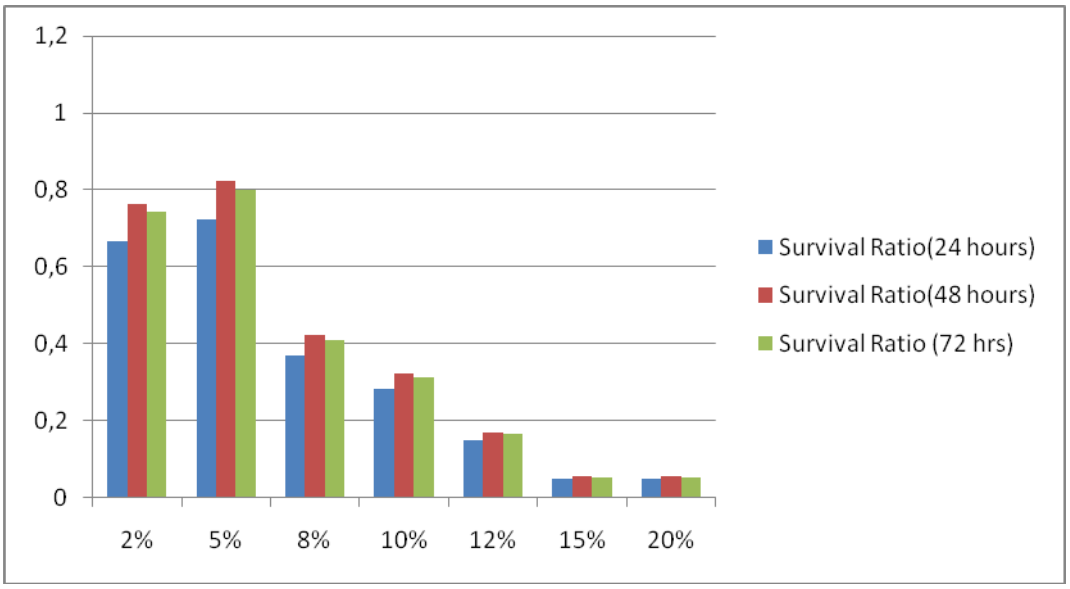
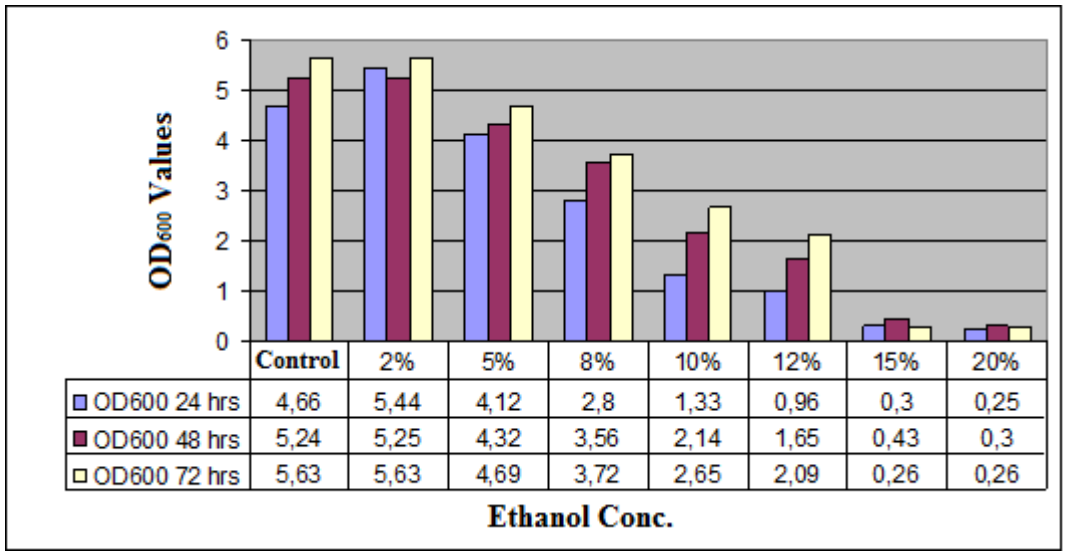


Figure 3.1: Graphics of Hef yeast survival ratio

Table 3.2: Survival ratio results of Hef yeast

	Ethanol Concentration						
	2%	**5%**	**8%**	**10%**	**12%**	**15%**	**20%**
Survival Ratio (24 hrs)	**0.66**	**0.72**	**0.36**	**0.28**	**0.14**	**0.04**	**0.04**
Survival Ratio (48 hrs)	**0.76**	**0.82**	**0.42**	**0.32**	**0.16**	**0.05**	**0.05**
Survival Ratio (72 hrs)	**0.74**	**0.8**	**0.4**	**0.31**	**0.16**	**0.05**	**0.05**

Table 3.3: Optical density values (OD_{600}) of cultures Hef1



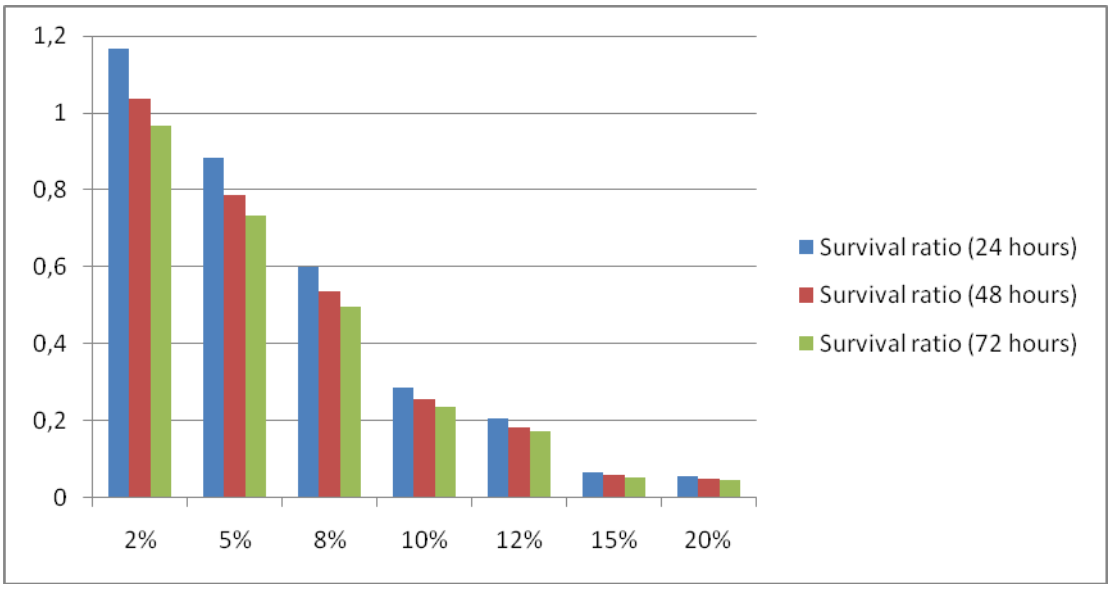


Figure 3.2: Graphics of Hef1 yeast survival ratio

Table 3.4: Survival ratio results of Hef1 yeast

	Ethanol Concentration						
	2%	**5%**	**8%**	**10%**	**12%**	**15%**	**20%**
Survival ratio (24hrs)	**1.16**	**0.88**	**0.6**	**0.28**	**0.2**	**0.06**	**0.05**
Survival ratio (48hrs)	**1.03**	**0.78**	**0.53**	**0.25**	**0.18**	**0.05**	**0.04**
Survival ratio (72hrs)	**0.96**	**0.73**	**0.49**	**0.23**	**0.17**	**0.05**	**0.04**

3.2 Stress Application and Creation of Generations

Creation of generations was based on applying the stress conditions and transferring the survivors of the previous stress step to the next stress step. Initially, the mutated culture Hef1 was used as the starting culture for selection experiments. The survivors of the stress step constituted the generation and also used to prepare stock cultures at each step. *Increasing stress generations* were obtained by increasing the level of stress at each step.

-80°C frozen stock culture of Hef1 was incubated overnight in YMM. This preculture was then inoculated into two 50 ml culture tubes containing 10 ml YMM with and without ethanol with an initial OD_{600} value (0.2). According to the screening results initial stress level for continuous stress application strategy was determined as 10 % (v/v) ethanol. By continuous selection strategy, 28 generations were obtained, which were resistant to ethanol stress. After 48 h of cultivation, the optical density values were measured at 600nm by using UV visible spectrophotometer and the survival ratios were calculated. Table.3.5 is showing the increasing stress

populations including their code names, stress levels in ethanol (v/v) and survival ratios.

Table 3.5: Increasing stress selection populations and their codes, stress levels as % ethanol (v/v) and survival ratios.

Increasing stress generations	Code	% Ethanol (v/v)	OD600 control	OD600 Generation	Survival Ratio
1	10	10	5.56	2.34	0.42
2	10.1	10.1	5.23	2.10	0.40
3	10.2	10.2	5.4	2.5	0.46
4	10.2.1	10.2	5.27	2.67	0.5
5	10.2.2	10.2	5.4	2.6	0.48
6	10.2.3	10.2	4.26	2.5	0.58
7	10.2.4	10.2	5.04	2.45	0.48
8	10.2.5	10.2	5.9	2.64	0.44
9	10.2.6	10.2	5.69	1.74	0.3
10	10.2.7	10.2	5.9	2.4	0.4
11	10.2.8	10.2	6.0	2.2	0.36
12	10.2.9	10.2	5.35	3.0	0.56
13	10.2.10	10.2	5.6	2.37	0.42
14	10.2.11	10.2	5.12	2.53	0.49
15	10.2.12	10.5	5.07	2.67	0.52
16	10.2.13	10.8	5.18	2.77	0.53
17	10.2.14	11	4.74	2.31	0.49
18	10.2.15	11.2	5.6	2.8	0.5
19	10.2.16	11.3	5.27	2.36	0.45
20	10.2.17	11.5	5.18	2.1	0.42
21	10.2.18	11.5	5.3	2.1	0.41
22	10.2.19	11.5	3.2	2.1	0.65
23	10.2.20	11.7	3.9	2.5	0.64
24	10.2.21	12	4.2	2.0	0.47
25	10.2.22	12	4.87	1.97	0.42
26	10.2.23	12	4.1	2.4	0.57
27	10.2.24	12.2	4.4	2.2	0.5
28	10.2.25	12.3	4.9	2.2	0.45

The survival ratios upon each generation for continuous stress application were graphed and shown in Figure 3.3.

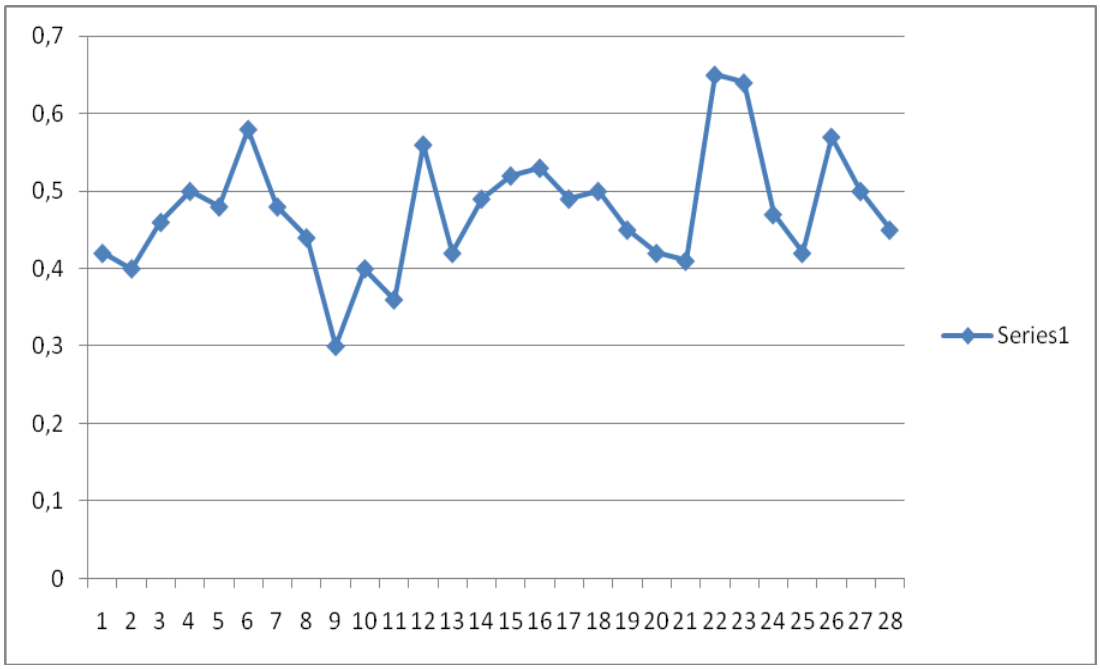


Figure 3.3: Survival ratios of continuous stress generations

3.3 Selection of individual mutants from final mutant population

The final generation (10.2.25) obtained by application of continuously increasing levels of ethanol was used for selecting the individual mutants. 28th increasing stress generation was accepted to be the final increasing stress generation, because as seen at Figure.3.4 and Table 3.6 at stress levels higher than 12.3% ethanol (v/v) almost no survival was observed .

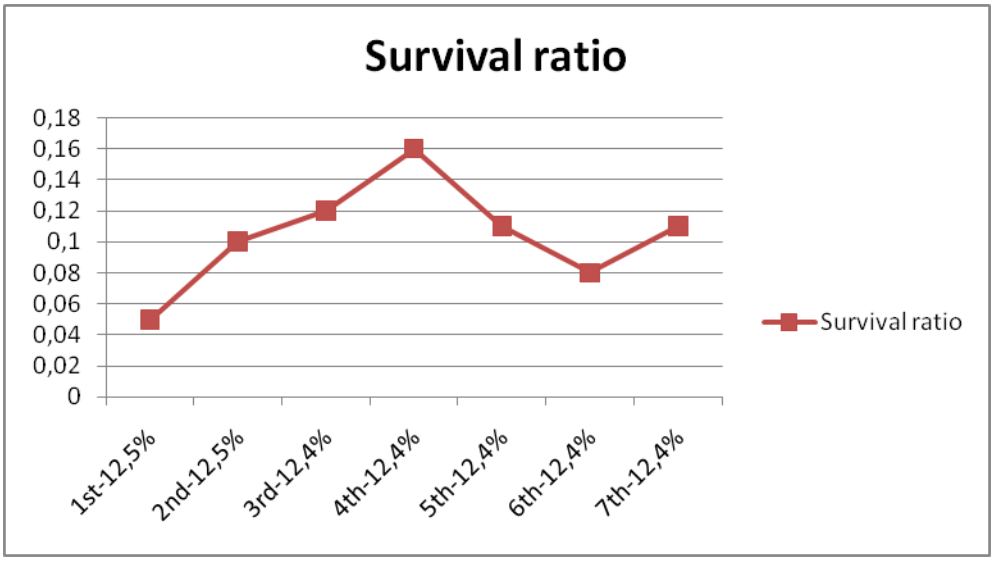


Figure 3.4: Survival ratio of the 7 attempts after 28th generation.

Table 3.6: Survival ratios, ethanol concentrations and OD_{600} values of the generations after 28^{th} generation.

	%Ethanol (v/v)	OD_{600} Control	OD_{600} Generation	Survival Ratio
1^{st} attempt	**% 12.5**	**5.2**	**0.3**	**0.05**
2^{nd} attempt	**% 12.5**	**4.9**	**0.5**	**0.1**
3^{rd} attempt	**%12.4**	**4.7**	**0.6**	**0.12**
4^{th} attempt	**%12.4**	**5.6**	**0.9**	**0.16**
5^{th} attempt	**%12.4**	**4.2**	**0.5**	**0.11**
6^{th} attempt	**%12.4**	**4.7**	**0.4**	**0.08**
7^{th} attempt	**%12.4**	**5.3**	**0.6**	**0.11**

This final generation were then diluted 10^4, 10^5, 10^6 and 10^7 times. All diluted cultures were inoculated into YMM-agar medium with spreading method and then incubated at 30 ºC for 48 h. Figure 3.5 shows 10^4, 10^5, 10^6 and 10^7 times diluted cultures. Ten distinct colonies were isolated from the final population randomly by the help of sterile toothpicks. The final population obtained from continuous selection strategy was named as 10.2.25. Individual mutants selected from each final population were named as *1, 2, 3, 5, 6, 8, 9, 10, 11, 12*.

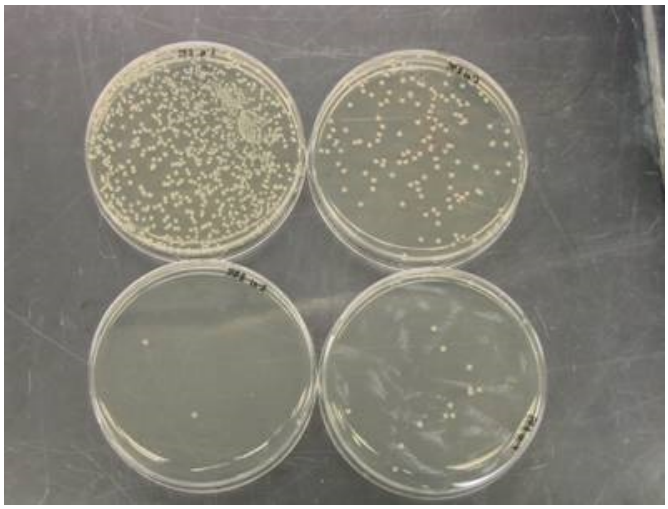


Figure 3.5: 10^4, 10^5, 10^6 and 10^7 times diluted cultures

3.4 Characterization of Ethanol stress resistance of mutant individuals according to wild type and final population

3.4.1 Determination of Ethanol stress resistance by MPN method

By using Most Probable Number (MPN) method, ethanol resistance of selected individuals from continuous selection strategy and wild type (Hef) cells were analyzed. 24, 48 and 72 h incubation results of MPN plates were visualized and MPN scores were determined. 72nd hour results, survival values and survival as fold of wild type were calculated and are shown in Table.3.7 and Table.3.8.

Table 3.7: Number of cells/ml and the survival ratio values after ethanol stress application

	Control	**7% EtOH**	**10% EtOH**	**11% EtOH**	**12% EtOH**	**7% EtOH**	**10% EtOH**	**11% EtOH**	**12% EtOH**
	(Cells/ml)					**% Survival**			
1	**49x10^5**	**54 x10^6**	**54 x10^4**	**92 x10^4**	**35 x10^4**	**1100**	**11**	**18,7**	**7.1**
2	**13x10^6**	**14 x10^6**	**2200**	**54 x10^4**	**14 x10^4**	**107**	**0.01**	**4.1**	**1**
3	**7 x10^6**	**49 x10^5**	**92 x10^6**	**24 x10^4**	**49 x10^3**	**70**	**1314**	**3.4**	**0.7**
5	**94 x10^6**	**92 x10^6**	**49 x10^5**	**79 x10^5**	**35 x10^4**	**97**	**5.2**	**8.4**	**0.37**
6	**11 x10^6**	**11 x10^6**	**28 x10^6**	**17 x10^5**	**35 x10^4**	**100**	**254**	**15.4**	**3.1**
8	**13 x10^6**	**14 x10^6**	**49 x10^5**	**35 x10^6**	**33 x10^4**	**107**	**37.6**	**269**	**2.5**
9	**7 x10^6**	**49 x10^5**	**11 x10^6**	**79 x10^5**	**17 x10^3**	**70**	**157**	**112**	**0.24**
10	**17 x10^6**	**49 x10^5**	**94 x10^5**	**79 x10^5**	**49 x10^4**	**28**	**55**	**46**	**2.8**
11	**33 x10^5**	**11 x10^6**	**11 x10^6**	**17 x10^6**	**49 x10^3**	**333**	**333**	**515**	**1.4**
12	**49 x10^5**	**14 x10^6**	**14 x10^6**	**46 x10^5**	**46 x10^3**	**285**	**285**	**93.8**	**0.93**
WT	**7 x10^6**	**22 x10^6**	**7 x10^6**	**11 x10^5**	**11 x10^3**	**314**	**100**	**15.7**	**0.15**

Table 3.8: Survival as fold of WT

Survival as fold of WT	%7 ethanol v/v	%10 ethanol v/v	%11 ethanol v/v	%12 ethanol v/v
1	**3.5**	**0.11**	**1.19**	**47.3**
2	**0.34**	**0.0001**	**0.26**	**6.66**
3	**0.22**	**13.14**	**0.21**	**4.6**
5	**0.3**	**0.05**	**0.53**	**2.46**
6	**0.31**	**2.54**	**0.98**	**20.6**
8	**0.34**	**0.37**	**17.1**	**16.6**
9	**0.22**	**1.57**	**7.13**	**1.6**
10	**0.08**	**0.55**	**2.92**	**18.6**
11	**1.06**	**3.33**	**32.8**	**9.3**
12	**0.9**	**2.85**	**5.9**	**6.2**

3.5 Determination of cross resistances upon various stress conditions

Cross resistance of mutant individuals *1, 2, 3, 5, 6, 8, 9, 10, 11* and *12* which were selected from 10.2.25 (final population obtained by continuous selection strategy); were determined by MPN method.

3.5.1 Cross resistance determination by MPN method

3.5.1.1. Osmotic Stress:

Five individual mutant cells (*11,1,6,8,12*), the final population where these individuals were selected from (10.2.25) and wild type cells (Hef) were exposed to osmotic stress by 5 % , 10 % and 15% NaCl. Table 3.9 shows the number of cells/ml after 72 hours of incubation in 96-well plates.

Table 3.9: Number of cells/ml and the survival ratio values after osmotic stress application.

Control		5 % NaCl	10 % NaCl	15 % NaCl	5 % NaCl	10 % NaCl	15 % NaCl
(Cells/ml)		**Survivors (Cells/ml)**			**%Survival**		
11	**2200000**	**2400000**	**23**	**23**	**109**	**0.001**	**0.001**
1	**3500000**	**11000000**	**23**	**23**	**314**	**0.0006**	**0.0006**
6	**7000000**	**2400000**	**23**	**23**	**34**	**0.0003**	**0.0003**
8	**5400000**	**5400000**	**23**	**23**	**100**	**0.0004**	**0.0004**
12	**2400000**	**3500000**	**130**	**23**	**145**	**0.005**	**0.0009**
WT	**2400000**	**9200000**	**23**	**23**	**383**	**0.0009**	**0.0009**

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Table 3.10: Survival as fold of WT upon 5, 10 and 15% NaCl stress.

Sample name	Survival as fold of WT (5% NaCl)	Survival as fold of WT (10% NaCl)	Survival as fold of WT (15% NaCl)
11	**0.28**	**1.1**	**1.1**
1	**0.81**	**0.6**	**0.6**
6	**0.08**	**0.3**	**0.3**
8	**0.26**	**0.4**	**1**
12	**0.37**	**5.5**	**5.5**

The results show that 12 was more resistant to osmotic stress compared to wild type.

3.5.1.2.Oxidative stress

The number of cells/ml and percent survival values of overnight cultures of mutant individuals *11,1,6,8,12* and wild type (Hef) under continuous 0.3M H_2O_2 stress were determined by 5 tube-MPN method. The cultures without H_2O_2 stress were used as control groups. The results at 72. h of incubation are given in Table 3.11.

Table 3.11: Number of cells / ml and percent survival values under 0.3M H_2O_2 stress at 72. h of incubation.

Sample name	Number of cells/ ml (0M H_2O_2)	Number of cells/ ml (0.3M H_2O_2)	% Survival (0.3M H_2O_2)	Survival as fold of wild type (0.3M H_2O_2)
11	**2400000**	**23**	**0.0009**	**1.5**
1	**2400000**	**23**	**0.0009**	**1.5**
6	**13000000**	**23**	**0.0001**	**0.16**
8	**2400000**	**23**	**0.0009**	**1.5**
12	**24000000**	**23**	**0.00009**	**0.15**
WT	**3300000**	**23**	**0.0006**	**-**

The results show that 11,1 and 8 were more resistant to oxidative stress conditions at continuous 0.3 M H_2O_2 when compared to wild-type.

3.5.1. 3. Sorbitol Stress

The number of cells/ml and percent survival values of overnight cultures of mutant individuals *11,1,6,5,12* and wild type (Hef) under continuous 2M sorbitol stress were determined by 5 tube-MPN method. The cultures without metal stress were used as control groups. The results at 72. hours of incubation are given in Table 3.12.

Table 3.12: Number of cells / ml and percent survival values under 2M sorbitol stress at 72. h of incubation

Sample name	Number of cells/ ml (0M sorbitol)	Number of cells/ ml (2M sorbitol)	% Survival (2M sorbitol)	Survival as fold of wild type (2M sorbitol)
11	**3300000**	**220**	**0.006**	**1.2**
1	**1300000**	**49**	**0.003**	**0.6**
6	**7000000**	**110**	**0.001**	**0.2**
8	**790000**	**350**	**0.04**	**8**
12	**3300000**	**350**	**0.01**	**2**
WT	**7000000**	**350**	**0.005**	**-**

The results show that 8 and 12 improved more resistance to continuous 2M sorbitol stress compared to wild-type.

3.5.1.4. Heat Stress (+60^0 C)

One ml of overnight cultures of mutant individuals *11,1,6,8,12* and wild type (Hef) were washed with dextrose-free YMM and exposed to 60^0 C temperature stress for 10 min. After this pulse stress application, the cells were harvested by centrifugation at 14,000 rpm for 5 min. The number of cells per ml and the percent survival values were determined by 5 tube- MPN method. The cultures with 30^0C exposure were used as control groups. The results at 72. h of incubation are given in Table 3.13.

Table 3.13: Number of cells / ml and percent survival values under 60 ^{0}C temperature stress at 72. h of incubation.

Sample name	Number of cells/ml at 30 ^{0}C	Number of cells/ ml at 60 ^{0}C	Survival ratio at 60 ^{0}C	% Survival at 60 ^{0}C	Survival as fold of WT at 60 ^{0}C
11	**5400000**	**110000**	**0.02**	**2**	**4**
1	**5400000**	**1300**	**0.0002**	**0.02**	**0.04**
6	**3500000**	**130000**	**0.037**	**3.7**	**7.4**
8	**3500000**	**35000**	**0.01**	**1**	**2**
12	**2400000**	**49000**	**0.02**	**2**	**4**
WT	**2200000**	**13000**	**0.005**	**0.5**	**-**

The results tells that 6, 12, 11 and 8 showed a clearly more resistance to pulse Heat stress compaerd to wild-type.

3.5.1.5. Cold Stress (-20^0 C):

One ml of overnight cultures of mutant individuals *11,1,6,8,12* and wild type Hef were washed with dextrose-free YMM and exposed to -20^0 C cold stress for 90 min. After this pulse stress application, the cells were thawed at room temperature. The number of cells per ml and the percent survival values were determined by 5 tube-MPN method. The cultures with 30 ^{0}C exposure were used as control groups. The results at 72th hours of incubation are given in Table 3.14.

Table 3.14: Number of cells/ml and the survival values after cold (-20^0 C) stress application at 72. h of incubation

Sample name	Control (Cells/ml)	Survivors (Cells/ml)	% Survival	Survival as fold of wild type
11	**5400000**	**1700000**	**31.4**	**4.06**
1	**5400000**	**350000**	**6.48**	**0.83**
6	**3500000**	**2400000**	**68.5**	**8.87**
8	**3500000**	**1100000**	**31.4**	**4.06**
12	**2400000**	**170000**	**7.08**	**0.91**
WT	**2200000**	**170000**	**7.72**	**-**

The results indicates that 6 gained a significiant resistance and also 11 and 8 improved resistences to cold stress compared to wild-type.

3.5.1.6. Metal ($CoCl_2$) Stress

Five individual mutant cells (*11,1,6,8,12*), the final population where these individuals were selected from 10.2.25 and wild type cells (Hef) were exposed to metal stress by 7mM $CoCl_2$. The number of cells per ml and the percent survival values were determined by 5 tube- MPN method. Table 3.15 shows the number of cells/ml after 72 hours of incubation in 96-well plates

Table 3.15: Number of cells/ml and the survival ratio values after $CoCl_2$ stress application

Sample name	Control (Cells/ml)	Survivors 7mM $CoCl_2$ (Cells/ml)	% Survival (7mM $CoCl_2$)	Survival as fold of WT (7mM $CoCl_2$)
11	**2800000**	**540**	**0.01**	**3.33**
1	**9200000**	**240**	**0.002**	**0.6**
6	**13000000**	**240**	**0.001**	**0.3**
8	**7000000**	**240**	**0.003**	**1**
12	**4900000**	**240**	**0.004**	**1.3**
WT	**7900000**	**240**	**0.003**	**--**

According to this results 11 showed a more resistence to continous Metal ($CoCl_2$) Stress.

3.5.1.7. 20% v/w ethanol pulse stress

One ml of overnight cultures of mutant individuals *11,1,6,8,12* and wild type Hef were washed with dextrose-free YMM and exposed to %20 v/w ethanol pulse stress for 90 min. After this pulse stress application, the cells were harvested by centrifugation at 14,000 rpm for 5 minutes. The number of cells per ml and the percent survival values were determined by 5 tube- MPN method. The cultures without 20% ethanol pulse stress at 30^0C exposure were used as control groups. The results at 72. h of incubation are given in Table 3.19.

Table 3.16: Number of cells/ml and the survival ratio values after %20 ethanol pulse stress application.

Sample Name	Control (Cells/ml)	Survivors %20 ethanol (Cells/ml)	% Survival (%20 ethanol)	Survival as fold of WT (% 20 ethanol)
11	**5400000**	**23**	**0.0004**	**0.6**
1	**5400000**	**23**	**0.0004**	**0.6**
6	**3500000**	**23**	**0.0006**	**1**
8	**2400000**	**23**	**0.0009**	**1.5**
12	**4600000**	**23**	**0.0005**	**0.83**
WT	**3300000**	**23**	**0.0006**	**--**

The results showed 8 improve more resistance to pulse 20% ethanol stress compared to wild-type.

3.5.1.8. Freezing-Thawing Stress

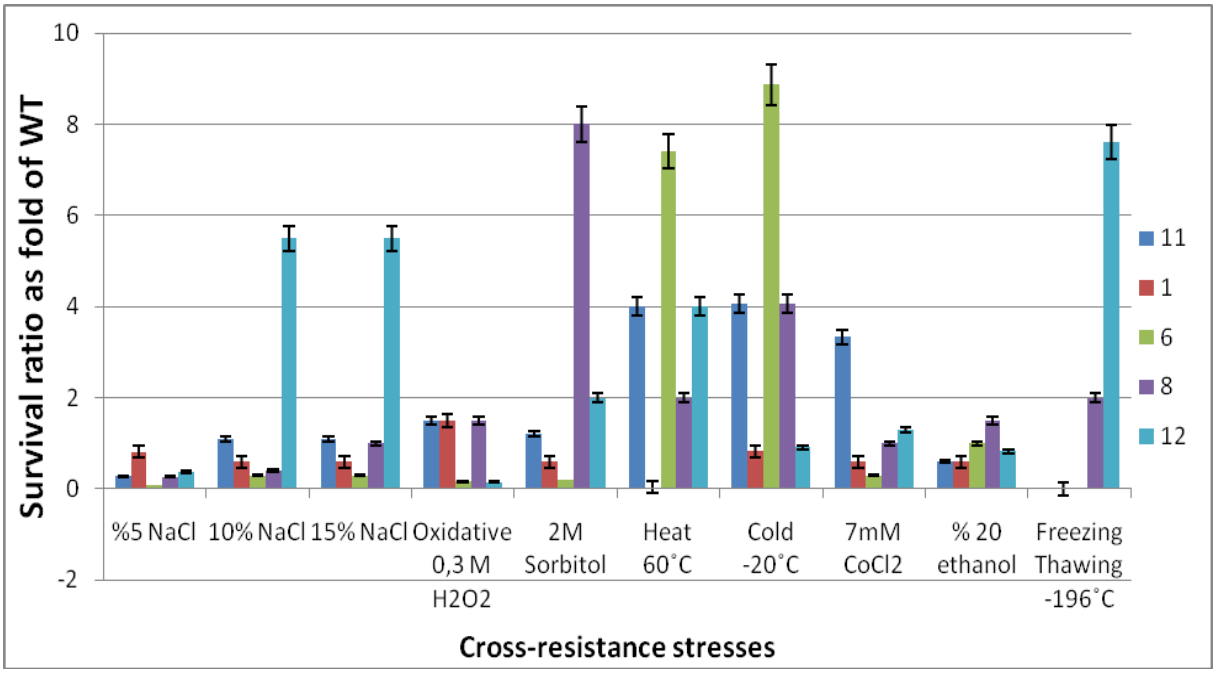
Five individual mutant cells (*11,1,6,8,12*), the final population where these individuals were selected from (10.2.25), wild type cells (Hef) were exposed to freezing-thawing stress. For this purpose, 1.5 ml of overnight cultures was frozen in liquid nitrogen for 25 min. This freezing step was followed by thawing at 30^0C for 20 min. The number of survivors was determined by MPN-based method in reference to control groups (no exposure to freezing-thawing stress). Table 3.17 shows the number of cells/ml after 72 h of incubation in 96-well plates.

Table 3.17: Number of cells/ml and the survival ratio values after freezing thawing stress application.

	Control (Cells/ml)	Survivors (Cells/ml)	%Survival ratio	Survival as fold of WT
11	**2400000**	**33**	**0.001**	**0.0007**
1	**3500000**	**23**	**0.0006**	**0.0004**
6	**9200000**	**2400**	**0.02**	**0.015**
8	**3500000**	**92000**	**2.62**	**2**
12	**2400000**	**240000**	**10**	**7.6**
WT	**3500000**	**46000**	**1.31**	**---**

This result show that 12 clearly gained more resistence to Freezing Thawing (-196˚C) compared to wild-type.

Figure 3.5: Survival ratios as fold of wild type upon different stress conditions after 72 h of incubation.



3.6 Growth Curve

The results are shown at the tables and figures below.

Table 3.18: OD_{600} values of WT strain.

Hef	Measurement time	0%	8%	10%	12%
			Ethanol Concentration		
0. hour OD_{600}	0	0,2	0,2	0,2	0,2
4. hour OD_{600}	4	0,8	0,15	0,11	0,11
6. hour OD_{600}	6	2,3	0,12	0,12	0,12
9. hour OD_{600}	9	4,6	0,25	0,22	0,24
12.hour OD_{600}	12	5,1	0,3	0,22	0,2
24.hour OD_{600}	24	5,6	1,1	0,22	0,19
48.hour OD_{600}	48	5,5	2,5	0,19	0,2

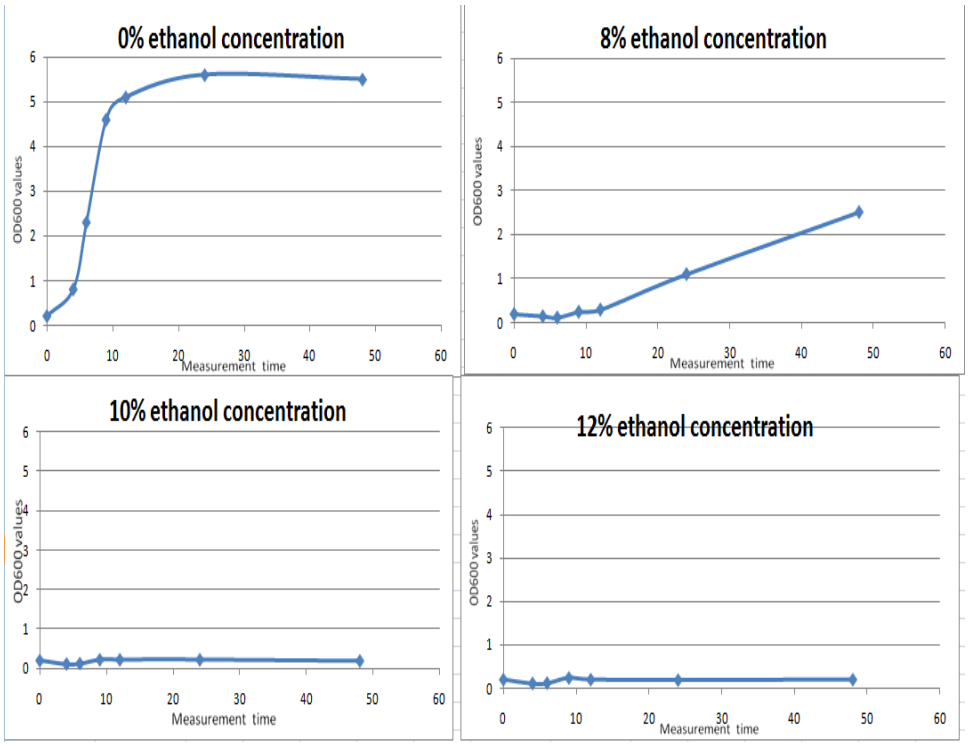


Figure 3.6: OD_{600} values of WT strains.

Table 3.19: ln OD_{600} values of WT strains.

Hef	Measurement time	ln OD_{600} values	ln OD_{600} values	ln OD_{600} values	ln OD_{600} values
		Ethanol Concentration			
		0%	8%	10%	12%
0. hour OD_{600}	0	-1,6	-1,6	-1,6	-1,6
4. hour OD_{600}	4	-0,22	-1,89	-2,2	-2,2
6. hour OD_{600}	6	0,83	-2,12	-2,12	-2,12
9. hour OD_{600}	9	1,52	-1,38	-1,51	-1,42
12.hourOD_{600}	12	1,62	-1,2	-1,51	-1,6
24.hourOD_{600}	24	1,72	0,09	-1,51	-1,66
48.hourOD_{600}	48	1,7	0,91	-1,66	-1,6

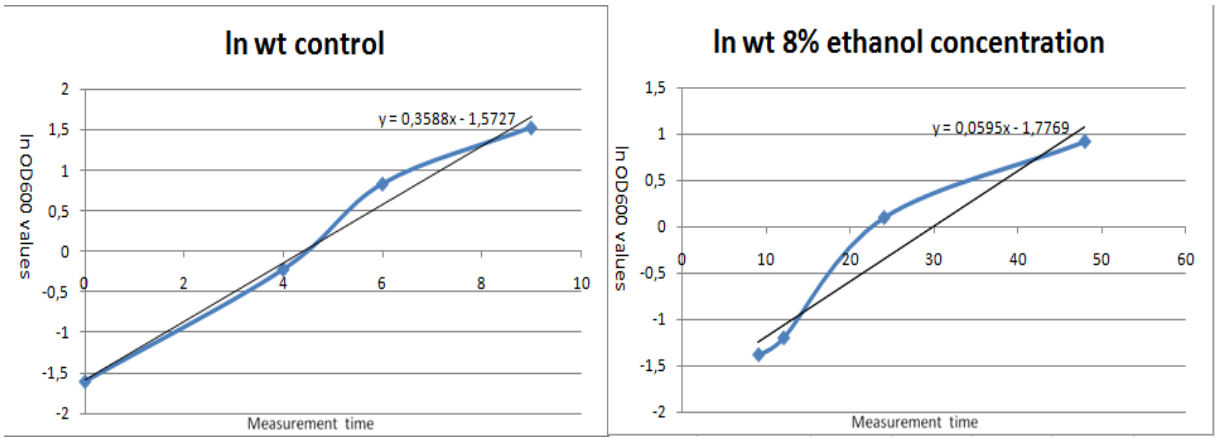


Figure 3.7: µ values of WT strain at different ethanol concentrations.

The specific growth rate 'µ' of WT in the presence and absence of ethanol was calculated by using the equation on the Figure 2. There was no significant growth for 10% and 12% ethanol supplied media so 'µ' corresponding to those cultures could not be calculated.

Table 3.20: OD_{600} values of *11* strain.

Hef	Measurement time	0%	8%	10%	12%
			Ethanol Concentration		
0. hour OD_{600}	0	0,2	0,2	0,2	0,2
4. hour OD_{600}	4	1,1	0,22	0,28	0,14
6. hour OD_{600}	6	2,8	0,34	0,36	0,16
9. hour OD_{600}	9	4,2	0,68	0,37	0,3
12.hour OD_{600}	12	4,9	1,1	0,43	0,29
24.hour OD_{600}	24	5,3	2,5	1,8	0,23
48.hour OD_{600}	48	5,4	2,5	2,1	0,27

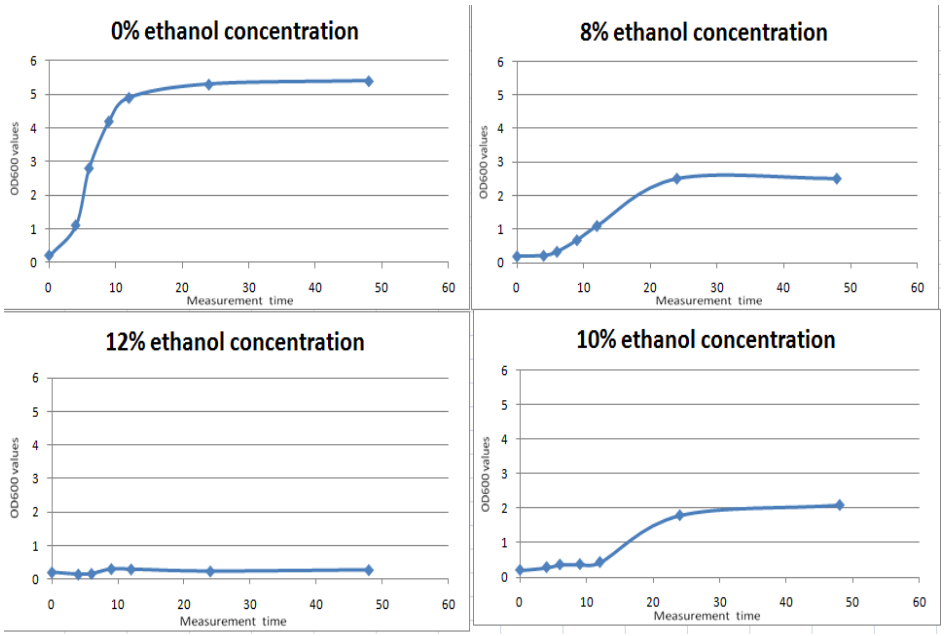


Figure 3.8: OD_{600} values of *11* strain.

Table 3.21: ln OD_{600} values of *11* strain.

Hef	Measurement time	ln OD_{600} values	ln OD_{600} values	ln OD_{600} values	ln OD_{600} values
		Ethanol Concentration			
		0%	8%	10%	12%
0. hour OD_{600}	0	-1,6	-1,6	-1,6	-1,6
4. hour OD_{600}	4	0,09	-1,51	-1,27	-1,9
6. hour OD_{600}	6	1,02	-1,07	-1,02	-1,83
9. hour OD_{600}	9	1,43	-0,38	-0,99	-1,2
12.hourOD_{600}	12	1,58	0,09	-0,84	-1,23
24.hourOD_{600}	24	1,66	0,91	0,58	-1,46
48.hourOD_{600}	48	1,68	0,91	0,74	-1,3

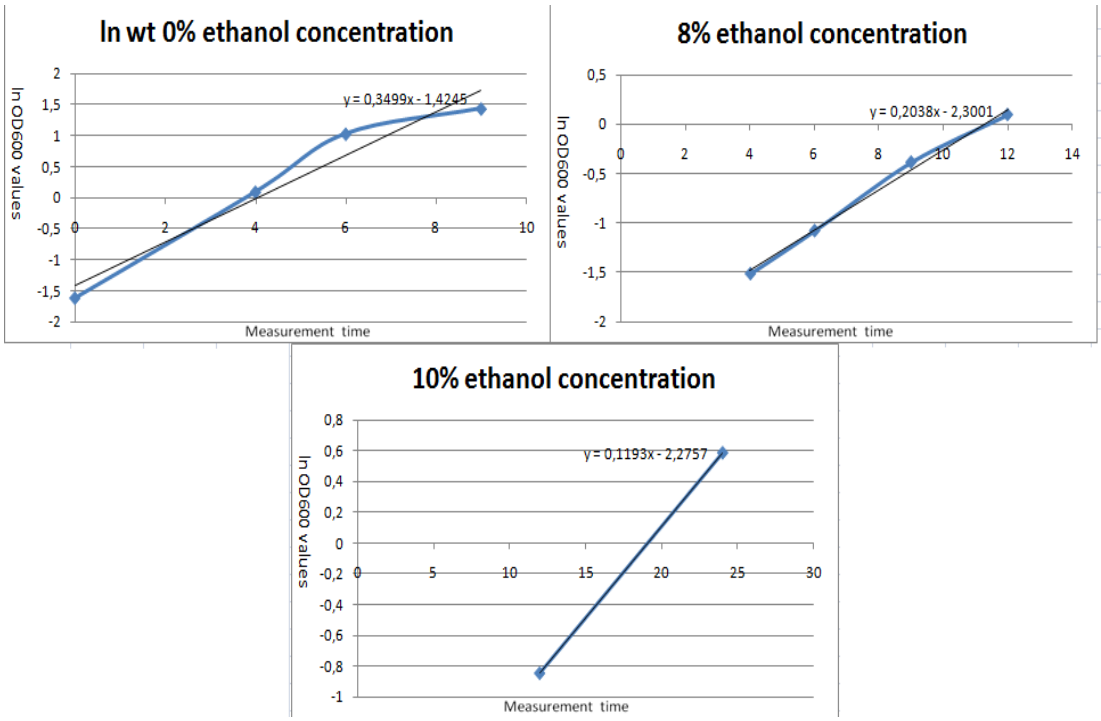


Figure 3.9: µ values of *11* strain at different ethanol concentration.

µ Values were calculated for mutant strain. WT and mutant have similar µ values for the control medium however, µ values of mutant which was grown in the presence of 8% ethanol is much more higher to that of wild type.

4. DISCUSSION AND CONCLUSIONS

The aim of this study was to increase the ethanol tolerance of an industrial strain by using evolution engineering strategies. For this purpose an industrial wine yeast strain was studied which had already been being used by a wine company. After obtaining our strains, to achieve a genetic diversity in the starting wild type culture, we applied a chemical mutagen ethyl methane sulphonate (EMS).

After obtaining our EMS-mutagenized culture, initial stress screening was performed on wild type and mutant strains in order to compare the stress resistance levels of cultures. Results showed that, (Tables 3.1, 3.3) both cultures had tolerance to ethanol at concentrations lower than 10% and we decided on the initial stress levels according to these data, to be used in selection strategies. We adopted the "continuous selection strategy" whereupon the generations of populations were obtained by passing the survivors of a stress step to the following step. Increasing range was decided to be 1% ethanol (v/v) in each step. It was observed that after the 5^{th} generation, the survival ratio decreased dramatically. This decrease may imply that the increasing range of 1% ethanol (v/v) is too high to obtain several generations. In order to increase the survival ratio of the generations, the same concentration of ethanol stress was applied for 12 generations between 3^{th}-14^{th} generations.

While applying the increasing stress selection strategy, initial stress level applied was 10% ethanol (v/v). This result was similar in the literature that commercial wine yeast strains are significantly tolerant to 10% ethanol (v/v) (Carrasco et al. 2001). The stress level was gradually increased up to 12.3% ethanol (v/v) in 28 generations. After obtaining 28^{th} generation, 12.5 and 12.4 ethanol (v/v) was applied to cells. Even after 96 hours of incubation for growth, almost no survival was obtained. Thus, 28^{th} increasing stress generation (10.2.25) was accepted as the final generation. Screening results of 10.2.25 showed that the increasing stress selection strategy was suitable for obtaining populations which were resistant to higher levels of stress conditions.

Those final populations obtained from continuous selection strategies were expected to have ethanol resistant mutants. The final populations were then inoculated to YMM-agar medium by spreading technique with different dilution rates. Cultures were diluted differently in order to obtain individual colonies on agar medium. Ten individuals (*1, 2, 3, 5, 6, 8, 9, 10, 11, 12*) were selected and coded.

Survival ratios and fold of wild type values of individuals were calculated quantitatively by MPN method, which indicated that the individual number *11* was the most ethanol resistant mutant. According to the MPN results, *11* was 3 times more resistant at 10%, 32 times more resistant at 11% and 9 times more resistant at 12% ethanol (v/v) than the wild type. *11* generally showed improved resistance at all ethanol stress levels compared to wild-type cells. These results suggest that industrial wine yeast strains can be improved for ethanol stress by evolutionary engineering. However this result was rather low when compared to other commercial yeast strains studied in the literature (Carrasco et al. 2001).

After determining the ethanol resistances; MPN assays were performed upon different stresses in order to determine any potential cross-resistances against other stresses. This stresses were; osmotic, oxidative, sorbitol, heat, cold, heavy metal, ethanol pulse and freezing thawing stresses. The results display an analogy with the work of Ogawa et al., 2000. Like their study, our commercial yeast strain's ethanol tolerant individuals exhibited resistance to other stresses including heat, high osmolarity and oxidative stress in addition to ethanol tolerance. These results indicate that the mutant exhibits multiple stress tolerance because of elevated expression of stress-responsive genes, resulting in the accumulation of stress protective substances. For example, *12* had a 5,5 fold increased survival ratio compared to the wild-type under 10% and 15% NaCl osmotic stress. *11*,*1* and *8* had a survival of 1,5-fold more than the wild-type under oxidative stress conditions at continuous 0.3 M H_2O_2. *8* survived *8*-fold of and *12* survived 2-fold more than the wild-type under 2M sorbitol stress. *11*, *6* and *8* were observed to be more resistant than the wild-type under cold stress at -20ºC. The resistance of *11* was *3* times more than wild-type under $CoCl_2$ stress. Individual *8* showed a 1,5 times improved resistance against ethanol pulse stress compared to wild-type. *12* survived 7,5-fold more under the freezing-thawing stress at -196ºC.

In previous studies it was indicated that the condition to induce synthesis of heat shock proteins, could result in a significant increase in ethanol tolerance, which implied the relationship between HSP induction and ethanol tolerance (Piper et al., 1994; Kubota et al., 2004). We obtained similar results from our MPN assays. The increasing ethanol-stress individuals *11* and *12* survived 4-fold more, *8* survived 2-fold and *6* survived 7,4-fold more than the wild type under heat stress at 60ºC. HSPs protect membranes against desiccation and ethanol stress. So we can explain that improved resistance to heat stress can be the reason of increasing expression in genes of HSP proteins (Carrasco et al. 2001).

Trehalose is present in the yeast cell as a storage carbohydrate and as a stress protectant. One of the main functions of trehalose is to protect cells against the denaturation and aggregation of proteins during periods of stress(Novo et al., 2004). That is why it cannot be overlooked in stress resistance studies. For this purpose, the trehalose contents in the population and individuals should be monitored by HPLC as a future extension of the study. It is expected to read higher trehalose content at the mutants and individuals which are already resistant to ethanol and heat shock (Swan&Watson, 1998). Additionally, a tetrad analysis accompanied by a transcription analysis could help us to gain better insight into the genetic basis of the mechanism responsible for the resistance.

In brief, ethanol resistant commercial yeast strain populations and individuals were successfully obtained by an evolutionary engineering approach. Some of the individuals show cross-resistance patterns. Further investigations of the selected individuals would explain ethanol resistance mechanism in commercial wine yeast in detail and elucidate the shared pathways in the resistance mechanisms along with other stress conditions such as heat, oxidative, osmotic and heavy metal stresses. Transcriptomic and proteomic analysis could also provide the necessary information for understanding the mechanisms in detail, which could ultimately be exploited in various applications in the fields of biomimetics and/or bioremediation.

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