

EVOLUTIONARY ENGINEERING OF SULPHUR DIOXIDE RESISTANT
Saccharomyces cerevisiae

M.Sc. Thesis by
Aziz Kaan KORKMAZ

Department : Advanced Technologies

Programme : Molecular Biology - Genetics and
Biotechnology

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**M.Sc. Thesis by
Aziz Kaan KORKMAZ
(521071052)**

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Supervisor (Chairman) : Assoc. Prof. Dr. Zeynep Petek ÇAKAR (ITU)

**Members of the Examining
Committee :**

**Prof. Dr. Necla ARAN (ITU)
Assoc. Prof. Dr. Ayten YAZGAN KARATAŞ (ITU)**

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İSTANBUL TEKNİK ÜNİVERSİTESİ ★ FEN BİLİMLERİ ENSTİTÜSÜ

**SÜLFÜR DİOKSİTE DİRENÇLİ *Saccharomyces cerevisiae*'nin EVRİMSEL
MÜHENDİSLİĞİ**

**YÜKSEK LİSANS TEZİ
Aziz Kaan KORKMAZ
(521071052)**

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Tezin Savunulduğu Tarih : 26 Ocak 2011**

**Tez Danışmanı : Doç. Dr. Zeynep Petek ÇAKAR (İTÜ)
Diğer Jüri Üyeleri : Prof. Dr. Necla ARAN (İTÜ)
Doç. Dr. Ayten YAZGAN KARATAŞ (İTÜ)**

OCAK 2011

FOREWORD

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Aziz Kaan Korkmaz

Biologist

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ABBREVIATIONS

CAT	: Catalase
EMS	: Ethyl Methane Sulphonate
FDA	: Food and Drug Administration
GAPDH	: Glyceraldehyde 3-Phosphate Dehydrogenase
GRAS	: Generally Regarded As Safe
MAP	: Mitogen Activated Protein
MPN	: Most Probable Number
PAPS	: Phosphoadenylyl Sulphate
PPG	: Polypropylene Glycol
SeL	: Sodium Selenite
ROS	: Reactive Oxygen Species
SOD	: Superoxide Dismutase
YMM	: Yeast Minimal Medium
YPD	: Yeast Peptone Medium

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EVOLUTIONARY ENGINEERING OF SULPHUR DIOXIDE RESISTANT *Saccharomyces cerevisiae*

SUMMARY

Sulphur dioxide, despite the ongoing debates, is one of the most preferred anti-microbial and anti-oxidant agents adopted especially in beverage industry. Besides its favourable preservative characteristics, as an air pollutant and an allergen, sulphur dioxide is accountable for acid rains along with anaphylactic responses in humans. Three forms of sulphur dioxide are present in aqueous solutions depending on the pH. The activity of SO₂ is optimum at low pH levels. Free form of SO₂, namely the molecular-SO₂, can freely pass into cells via simple diffusion where it lowers the intracellular pH levels, binds to important intracellular targets and inhibits, both directly and indirectly, functions of enzymes that have important roles in ATP production.

The eukaryotic model organism *Saccharomyces cerevisiae* is one of the most studied yeast species to understand the mechanisms involved in resistance to SO₂ as this yeast species is known to have long adapted to this chemical through ages of natural selection particularly in wineries. There are several studies on the physiological and genetic infrastructure of sensitivity to SO₂. However, the mode of action still remains unclear. Yeast species with elevated resistance to this stress are of industrial interest particularly in alcoholic beverage production. Cells with various types of resistance as such, also hold an important place in pharmaceutical research. In this study, the aim was to improve SO₂ tolerance of *Saccharomyces cerevisiae* by evolutionary engineering strategy and to gain insight into the SO₂ tolerance mechanism by analyzing phenotypic alterations of the mutants. For this purpose, batch selection protocols were applied to a genetically diverse, chemically mutagenized initial population of *Saccharomyces cerevisiae*. Mutant populations were exposed to gradually increasing SO₂ levels at each successive batch cultivations. SO₂ is the most active at low pH levels, thus selections were performed in a bioreactor at a constant pH of 3.5 and in culture tubes with an initial pH of 3.5, which was to decrease as the cells grow. Mutant generations were obtained from both selections in the bioreactor and culture tubes. Individual mutants were randomly selected from the final generations of each selection. These mutants were analyzed for their SO₂ resistance along with resistance to various other stresses utilizing Most Probable Number method. Results showed that mutants obtained under different pH conditions did not differ significantly in SO₂ resistance, however, they all exhibited different cross-resistance patterns in addition to the initially haploid bioreactor derived mutants becoming diploid.

SÜLFÜR DİOKSITE DİRENÇLİ *Saccharomyces cerevisiae*'nin EVRİMSEL MÜHENDİSLİĞİ

ÖZET

Süregelen tartışmalara rağmen, sülfür dioksit, özellikle içecek endüstrisinde benimsenmiş en önemli antioksidan ve antimikrobiyal ajanlardan biridir. Avantajlı koruyucu özelliklerine rağmen, hava kirletici bir unsur ve alerjen olarak, sülfür dioksit asit yağmurlarından ve insanda anafilaktik tepkilerden sorumludur. pH değerine bağlı olarak, sulu çözeltilerde sülfür dioksitin üç formu mevcuttur. SO₂'in serbest formu olan moleküler SO₂, basit difüzyonla pH seviyesini düşürdüğü hücre içi ortama geçerek burada önemli hücre içi hedeflere bağlanır; doğrudan ya da dolaylı olarak ATP üretiminde görevli enzimlerin çalışmasını engeller. Özellikle şarap üretiminde kullanılan mayaların uzun zamandır doğal seçim ile bu kimyasala karşı direnç sağladığı bilinmektedir.

Ökaryotik model organizma *Saccharomyces cerevisiae*, sülfür dioksit direnç mekanizmasının anlaşılmasında en yaygın olarak kullanılan maya türüdür. SO₂ duyarlılığının genetik ve fizyolojik altyapısı ile ilgili birçok çalışma yapılmıştır. Fakat bunların etki şekli henüz tam olarak ortaya çıkarılamamıştır. Bu strese dayanıklı maya türleri endüstriyel açıdan, özellikle alkollü içecek üretiminde, büyük ilgi uyandırmakla beraber ilaç araştırmaları açısından da önem taşımaktadırlar. Bu çalışmanın amacı, evrimsel mühendislik yöntemi ile *Saccharomyces cerevisiae* mayasını sülfür dioksite dirençli hale getirerek, elde edilen mutantların fenotipik değişikliklerinin analizi yoluyla, SO₂ tolerans mekanizmasının anlaşılmasına çalışılmasıdır. Bu amaçla, kimyasal olarak mutasyona uğratılmış, genetik çeşitliliğe sahip *Saccharomyces cerevisiae* başlangıç popülasyonuna kesikli kültürde seçim protokolleri uygulanmıştır. Mutant popülasyonlar, birbirini takip eden her kesikli kültürde kademeli olarak arttırılan SO₂ seviyelerine maruz bırakılmıştır. Sülfür dioksit en yüksek etkinliği düşük pH seviyelerinde gösterdiği için seçimler pH değerinin 3.5'te sabit tutulduğu bir biyoreaktörde ve başlangıç pH değeri 3.5'e ayarlanmış kültür tüplerinde yapılmıştır. Hem biyoreaktör hem de kültür tüplerinden mutant nesiller elde edilmiştir. Elde edilen son nesillerden rasgele olarak mutant bireyler seçilmiştir. Bu mutantlar, Most Probable Number metodu yardımıyla, SO₂ direnci ve çeşitli başka streslere karşı geliştirdikleri dirençler için analiz edilmiştir. Sonuç olarak, farklı pH değerlerinde elde edilen mutantların SO₂ direnci bakımından fazla değişiklik göstermedikleri; fakat başlangıçta haploid olan biyoreaktör mutantlarının diploid hale gelmelerine ek olarak farklı çapraz direnç modelleri gösterdikleri görülmüştür.

1. INTRODUCTION

1.1 Brief Information About Yeast: *Saccharomyces cerevisiae*

Yeasts are unicellular fungi whose acquaintance with humankind can be dated to the Neolithic era long before scientific knowledge about microorganisms was available as the evidence dates from 4-5 millennium BC when the arts of leavening, brewing and wine-making were well known. The yeast that has been most closely associated with humankind, *Saccharomyces cerevisiae*, has long been used for brewing, wine-making and baking bread. It is by far the most studied and best understood species of the yeast domain and a featured model organism for fundamental biological research, which is the first eukaryote whose genome is entirely sequenced. A typical image of budding yeast cells is shown in Figure 1.1 (Satyanarayana & Kunze, 2009).

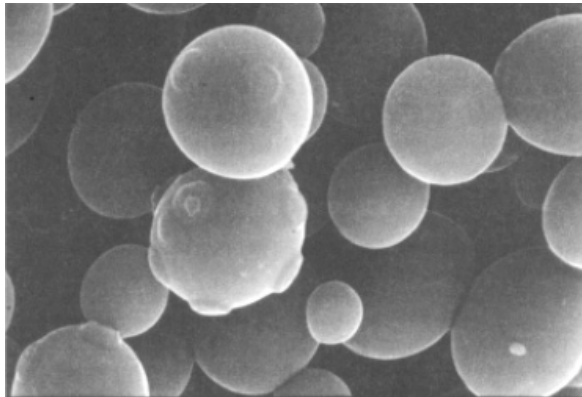


Figure 1.1: Electron micrograph of *Saccharomyces cerevisiae* (Satyanarayana & Kunze, 2009)

S. cerevisiae cells are immobile, either globose in shape [5.0-10.0] x [5-12.0] μm or ellipsoidal or cylindrical, measuring 3.0-9.5 μm x 4.5-21.0 μm . The cells may occur singly or in pairs, short chains or clusters. The cell size may vary depending on the phase of the growth cycle, cultural conditions and genome content. Being a prominent representative of the kingdom of fungi, *S. cerevisiae* is as closely related to plant cells as it is to animal cells. In this context, it reflects the main characteristics of both types of eukaryotic cells. Among the important features of yeast morphology is the cell wall, which provides both the elasticity and the robustness for coping with

the ever-changing environmental conditions. The cell wall is 150 nm-200 nm in thickness. Its composition is approximately 90% carbohydrate and 10% protein where mannose and glucans account for the biggest portion of the structure along with a small amount of chitin. The periplasm between the cell wall and the cell membrane takes place in some initial degradation processes. Enclosed in the bilayer cell membrane, the yeast cytoplasm is an acidic (pH 5.25) colloidal fluid, mainly containing ions and low or intermediate molecular weight organic compounds, and soluble macromolecules. In this set up, yeast cells harbor a system of membrane-surrounded compartments, including endoplasmic reticulum, golgi apparatus, vesicles and vacuoles, designed for biosynthesis, modification and trafficking of proteins within, into and out of the cell. Yeast cells contain mitochondria, **which** structurally resemble these organelles found in all eukaryotes; however, they do not have a chloroplast. Peroxisomes perform a variety of metabolic functions in eukaryotic cells. In yeasts, peroxisomes contain several oxidases, which serve in oxidative utilization of specific carbon and nitrogen sources. A schematic description of a yeast cell is shown in Figure 1.2 (König et al., 2009).

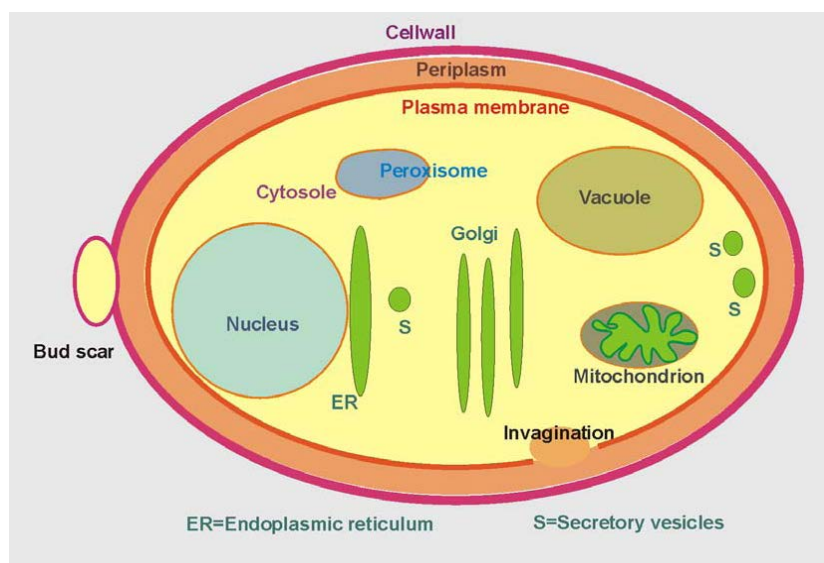


Figure 1.2: Scheme of organelles and compartments in a yeast cell (Walker, 1998).

S. cerevisiae is a heterotroph, i.e. it requires organic compounds readily present in the media for growth. Faithful to its name, the genus *Saccharomyces*, where the name stands for “the sugar fungus”, is known for their common occurrence in sugary substrates such as nectar and fruit. A limited yet easily obtainable range of sugars is

utilized as a carbon source by *S. cerevisiae*. While the monosaccharides such as glucose and fructose are readily utilized along with the disaccharides, sucrose and maltose, *S. cerevisiae* cannot utilize pentoses, other hexoses and the disaccharide lactose. Inorganic nitrogenous compounds such as ammonium sulphate as nitrogen source together with necessary major minerals and vitamins are of preferred nutrients for optimum growth. *S. cerevisiae* grows optimally at pH 4.5-5.0, although it can tolerate a pH range of 3.6-6.0. It is also a mesophile, growing best between the temperatures ranging from 25 to 40°C (Walker, 1998).

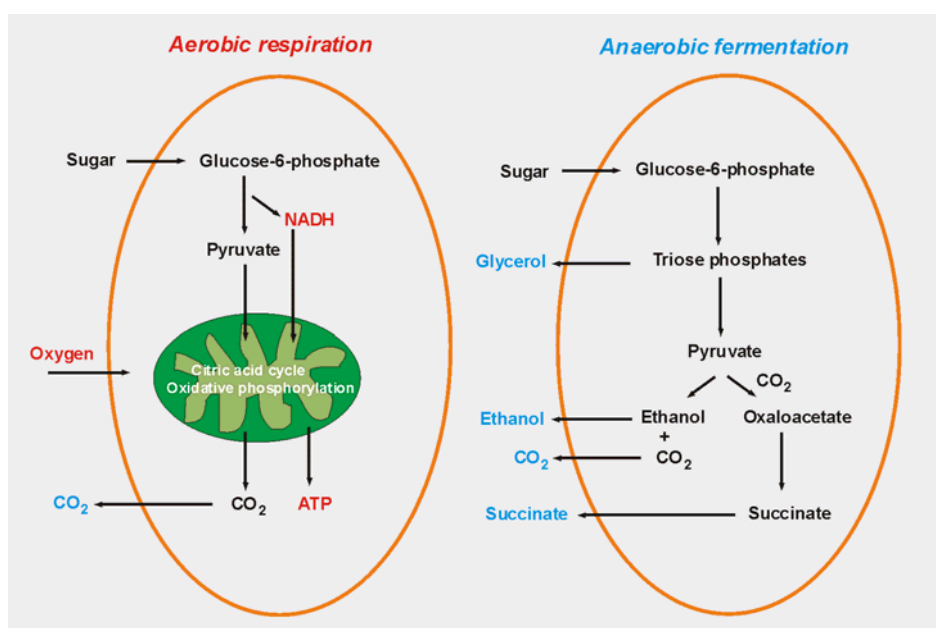


Figure 1.3: Metabolism of yeast under aerobic and anaerobic conditions. (Feldmann, 2010).

S. cerevisiae possesses a remarkable ability to thrive in varying levels of available O₂ (Figure 1.3). In very low levels of O₂, its metabolism responds by shutting off the respiratory enzymes. The yeast then leads a fermentative life, in which sugar is partially and nonoxidatively utilized for energy and the waste product is ethanol. In contrast, when adequate O₂ is available, sugar is converted by the respiratory enzymes to CO₂ and H₂O, as well as to intermediates needed for the cell biomass. Other than those conditions mentioned above, in media containing > 5% glucose, although aerated, the yeast cells fail to utilize glucose and switch to the fermentative pathway as well. This phenomenon is known as the “Crabtree effect” (Fugelsang, 1997; Jackson, 2008; Walker, 1998).

Yeast cells double in number every 100 min or so and they reproduce both asexually and sexually. Vegetative cells grow by budding, the buds arise on the ‘shoulders’ and

at either pole of the cell. The vegetative cells are haploid, diploid or polyploid, and this phase predominates in the life cycle of the yeast. Sexual reproduction involves the production of asci, within which ascospores develop after the meiosis of the diploid nucleus (Figure 1.4).

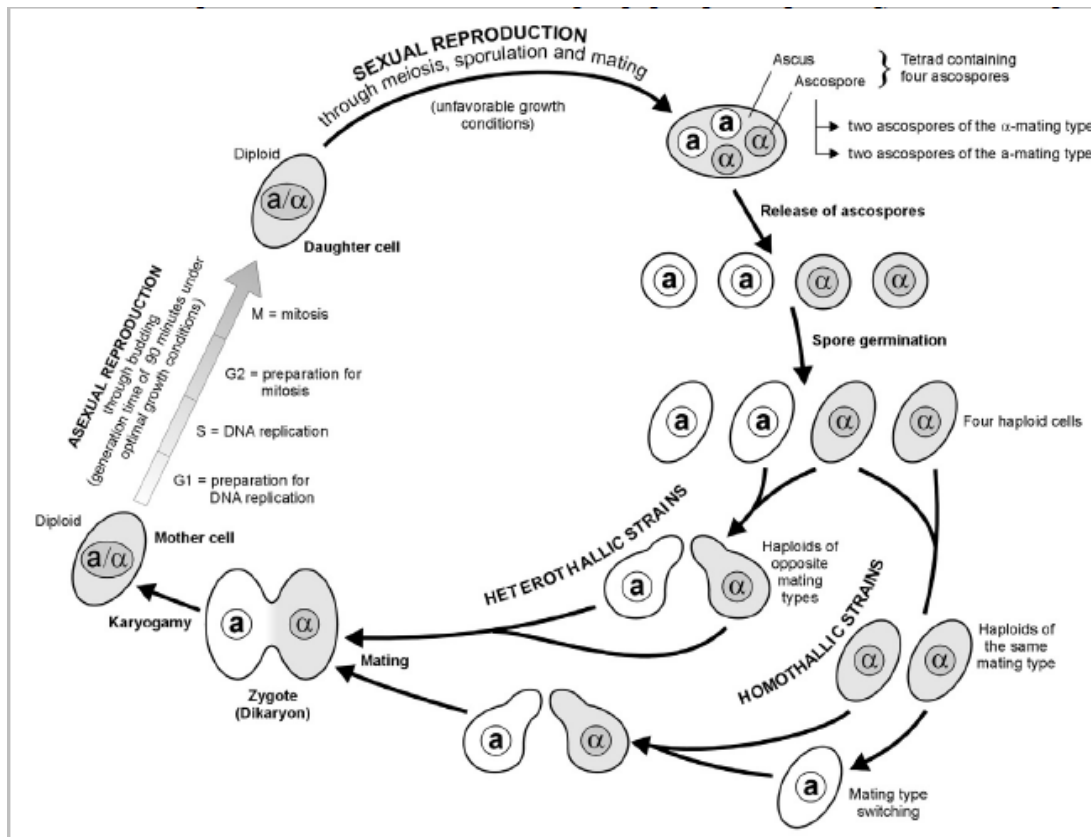


Figure 1.4: Life cycle of *S. cerevisiae* (Winde, 2003).

Yeast cells stop proliferation under certain environmental circumstances. For example, if they run out of nutrients, they arrest as unbudded cells in the G1 phase of the cell cycle. The other environmental influence that interrupts the proliferation is the presence of another yeast cell in the vicinity with which it can mate (Feldmann, 2010; Fugelsang, 1997; Herskowitz, 1988).

If we were to look closely at the events of mating, specific signalling molecules and receptor systems of a and α cell types, stand out in the facilitation of mating process. Cells of each haploid type produce a peptide mating factor that prepares cells for mating. These signalling molecules are responsible for communication between organisms and are properly termed pheromones. The mating factors cause cells to arrest in the G1 phase of the cell division cycle, that is, before the initiation of DNA replication. Considering this suppression, the mating factors could be regarded as

negative growth factors as they inhibit cell growth. As a result of cell cycle arrest, cell and nuclear fusion of the mating partners occurs when cells have exactly one copy of each chromosome and, hence, leads to formation of a diploid. The mating factors also activate synthesis of proteins essential for mating. The precise sequence of events that occurs when cells respond to the mating factors is not known, but some of the components have been identified.

In addition to the system touched upon above, some yeast cells reserving the ability to switch mating types, offers an alternative mechanism. As opposed to the self-sterile heterothallic cell types which is either a or α mating type, a homothallic haploid yeast cell is adept to changing the initial mating type after a mitosis which results in two different mating types capable of fusing. Having the active HO gene a homothallic yeast cell could manage to induce the transposition of previously silent mating type allele from a silent locus to an active locus thus leading to the switching of mating types (Figure 1.5) (Feldmann, 2010; Fugelsang, 1997).

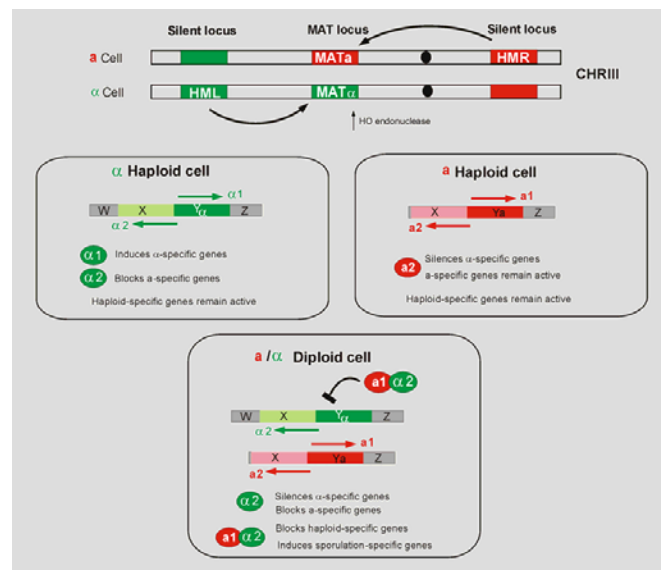


Figure 1.5: Switching of mating types (Feldmann, 2010).

Saccharomyces cerevisiae carries nearly 6000 annotated genes, on 16 chromosomes. Functional nature of only 40 – 45 % is known. *S. cerevisiae* contains an autonomously replicating plasmid in its nucleus which is present in about 60 – 100 copies. The plasmid has an origin of replication but does not appear to confer any selective advantage to the host. The yeast genome is highly compact and 72 % of DNA codes for genes (Satyanarayana & Kunze, 2009; Winde, 2003).

1.2 Biotechnology of the Yeast *S. cerevisiae*

Biotechnology is the exploitation of biological processes for industrial, health care and environmental purposes besides the potential implementations for everyday routines. It is generally defined as “the application of microorganisms/cells or components thereof (e.g., enzymes) for the production of useful goods and services’. In accordance with this definition, despite its close association in our day with the advancements in medical therapeutics and pharmaceuticals, this technology has an important place in many fields.

Constituting the main element of biotechnology, microbes have contributed to industrial science for over 100 years. More and more genomes of industrial microorganisms are being sequenced giving valuable information about the genetic and enzymatic makeup that potentially will lead to novel and improved processes. Major tools such as functional genomics, proteomics, and metabolomics are being exploited for the discovery of new valuable strains and thus products. The main reasons for the use of microorganisms to produce compounds instead of plants and animals or synthesis by chemists are: (i) a high ratio of surface area to volume, which facilitates the rapid uptake of nutrients required to support high rates of metabolism and biosynthesis; (ii) a wide variety of reactions that microorganisms are capable of carrying out; (iii) ease of adaptation to a wide range of different environments, allowing a culture to be transplanted from nature to the laboratory flask or the factory fermentor, where it is capable of growing on inexpensive carbon and nitrogen sources and producing valuable compounds; (iv) ease of genetic manipulation, both in vivo and in vitro, to scale up the amount of production, to modify structures and activities, and to make entirely new products; (v) simplicity of screening procedures allowing thousands of cultures to be examined in a reasonably short time; and (vi) a wide diversity, in which different species produce somewhat different enzymes catalyzing the same reaction, allowing one flexibility with respect to operating conditions in the reactors (Branduardi et al., 2008; Patnaik, 2008).

Among the valuable microorganisms used in the industry, yeasts combines the advantages of unicellular organisms with the ability of a protein processing resembling eukaryotic organisms together with the absence of endotoxins as well as oncogenic or viral DNA. *Saccharomyces cerevisiae*, accounting for the 90% of the biotechnological products acquired from yeasts, offers certain advantages over

bacteria as a cloning host. This yeast can be grown rapidly in simple media, can secrete heterologous proteins into the extracellular broth, and its genetics are more advanced than any other eukaryote. Additionally, because of negative consumer reaction, the only metabolically engineered yeasts strains that have been approved for commercial utilization are the ones in which the heterologous DNA utilized for strain improvement derives from the host species, a sort of self-cloning. Despite these advantages, *S. cerevisiae* is sometimes disregarded as an optimal host for large scale production of mammalian proteins because of drawbacks such as hyperglycosylation, presence of α -1, 3-linked mannose residues that could cause antigenic response in patients, and absence of strong and tightly-regulated promoters. The most commercially important yeast recombinant process has been the production of the genes encoding surface antigens of the hepatitis B virus resulting in the first safe hepatitis B vaccine (Demain & Adrio, 2008).

Saccharomyces cerevisiae is best known for its domesticated role in the production of breads and fermented alcoholic beverages and for positive contribution to the flavour. *Saccharomyces cerevisiae* converts hexose sugars, to ethanol, CO₂ and a variety of compounds, including alcohols, esters, aldehydes and acids that contribute to the sensory attributes of the food or beverage. In the baking industry, *S. cerevisiae* is generally inoculated into bread dough to ensure the swelling and the formation of crumb. Addition to that, it contributes to the acid fermentation of a wide range of bread and pancake doughs.

Saccharomyces cerevisiae is the principal yeast species involved in the production of best understood processes of wine, beer and cider fermentations. Wine is fermented either by natural fermentation or by inoculation with a starter strain of *S. cerevisiae*. Beer is produced from alcoholic fermentation of extracts from cereal grains. Although there is a complex microflora associated with the process, in most commercial beer production the alcoholic fermentation is conducted by an inoculated strain of *S. cerevisiae*. Brewing strains are selected for their influence on the flavour of beer, and for their ability to flocculate and sediment at the end of fermentation, assisting in clarification of the beer. Ethanol is the main constituent targeted in these processes where both the production capacity and the ability to resist accordingly are the most sought-after traits. Ethanol is not only a beverage associated chemical it also is a source of energy, easily attainable from widely accessible agricultural

products with a process catalyzed by *S. cerevisiae*. Apart from food, medical and chemical industry; *S. cerevisiae* has become a well established eukaryotic model organism to study fundamental biological processes such as aging, mRNA transport the cell cycle, and many more. *S. cerevisiae* also serves as a model organism for studying human diseases such as cancer and has been used as a tool for drug research (Demain & Adrio, 2008; Patnaik, 2008; Rogers et al., 2005).

1.3 Strain Development and Metabolic Engineering

Metabolic engineering, the redirection of metabolic fluxes, has played an exceptional role in improving yeast strains for all industrial applications mentioned above. In contrast to classical methods of genetic strain improvement such as selection, mutagenesis, mating, and hybridization metabolic engineering has conferred two major advantages: (i) the directed modification of strains without the accumulation of unfavorable mutations and (ii) the introduction of genes from foreign organisms to endow *S. cerevisiae* with novel traits.

The goal of metabolic engineering is the directed modification of metabolic fluxes. It aims at improving the organism towards enhanced production of native metabolites or introduction of novel machineries into the organism to utilize atypical substrates as well as to form heterologous metabolites. Metabolic engineering was introduced by Bailey in 1991 as a subdiscipline of engineering and was first defined as “the improvement of cellular activities by manipulation of enzymatic, transport, and regulatory functions of the cell with the use of recombinant DNA technology”. Metabolic engineering is always based on genetic engineering, i.e., the targeted manipulation of a cell’s genetic information (Stephanopoulos et al., 1998). However, in the face of the increasing importance of informatics, the advancements in technologies for modifying, analyzing, and modeling metabolic fluxes, urges a holistic view of the entire cell rather than the isolated metabolic pathways. This holistic view and techniques involved in the complementary disciplines combined with the necessity of handling vast amount of data have paved the way for omics based yeast strain development.

Emerging as a powerful alternative approach to recombinant technology, metabolic engineering has expanded its arsenal of tools with related disciplines such as inverse metabolic engineering and evolutionary engineering. These new studies have

facilitated the primary form of metabolic engineering; also known as the rational metabolic engineering (Bailey, 1991; Cakar et al., 2005; Patnaik, 2008; Sonderegger & Sauer, 2003).

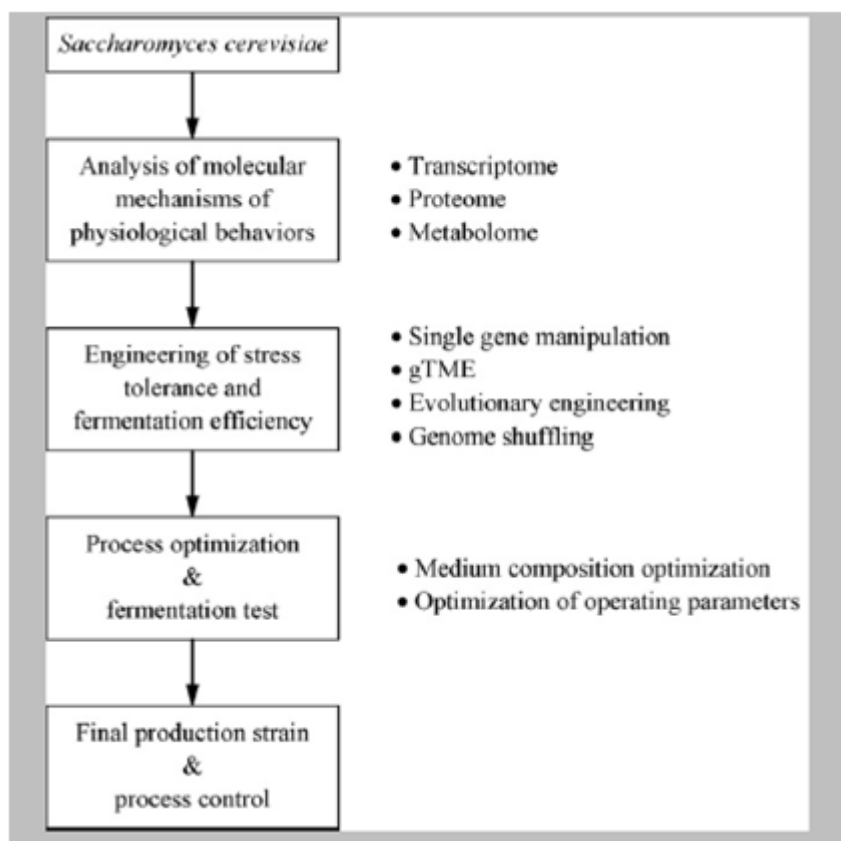


Figure 1.6: Algorithm for strain improvement and process optimization of *S. cerevisiae* (Patnaik, 2008).

1.3.1 Rational Metabolic Engineering

Traditional metabolic engineering is rational and deductive and has been referred to as “rational, constructive” or “reductionistic”. Rational metabolic engineering refers to the engineering of enzymes, metabolites or regulatory proteins based on available information about the pathways, enzymes, and their regulation. Moving from this knowledge, a strategy is designed to optimize these protein activities in order to achieve the desired metabolic flux or phenotypic trait. Rational metabolic engineering has been very successful but obviously many attempts failed or end up less successful than expected. Moreover, there have been cases where a metabolic engineering approach worked well under laboratory conditions but not when transferred to industrial scale. Setbacks, as such, and the models based on the

experimental data has led to the discipline of systems biology (Kohlstedt et al., 2010).

1.3.2 Inverse Metabolic Engineering

Bailey et al. later introduced the approach of “inverse metabolic engineering”, providing a new standpoint, as a subdiscipline of metabolic engineering. They described “the elucidation of a metabolic engineering strategy by: first, identifying, constructing, or calculating a desired phenotype; second, determining the genetic or the particular environmental factors conferring that phenotype; and third, endowing that phenotype on another strain or organism by directed genetic or environmental manipulation”. The idea behind this discipline is assuming the opposite starting point of traditional metabolic engineering. According to this, a system with two or more different manifestations of the desired phenotype, resulting from exposure to different environmental conditions or exploitation of random genetic manipulation, is the first step. The next step is the identification of the genetic basis for the differing trait values; this is the biggest challenge in inverse metabolic engineering. However, global methods of gene expression analysis such as transcriptomics, proteomics, metabolomics, and even fluxomics have strongly facilitated the identification of differences at various molecular levels. The advantages of inverse metabolic engineering could be summarized as follows:

- (i) No need for preliminary knowledge regarding the proteins/enzymes of a pathway and their regulation.
- (ii) One can directly utilize industrial strains and real production conditions to identify crucial genetic players.
- (iii) Genetic traits, conferred via multigenic systems could be studied thanks to the holistic approach.
- (iv) In general, the final strain improvement strategy is based on homologous genes (i.e., the modified strain can be considered “self-cloned,”) which, in turn, is an important issue for consumer perception especially in the food area.
- (v) There is a good chance of discovering novel genetic targets for strain improvement, which would have never been found by a rational method.

As a result, inverse metabolic engineering comes as a very powerful set of tools for strain development (Bailey et al., 1996; Randez-Gil et al., 1999; Stephanopoulos, 1999).

1.3.3 Evolutionary Engineering

The term evolutionary engineering encompasses all methods for empirical strain improvement, such as mutagenesis (natural or induced) and recombination or shuffling of genes, pathways, even whole cells, followed by selection of cells with the desired phenotype. These techniques are inspired from natural design principles and very effective in strain development as evident in strains developed with tolerance to multiple stressors. Yet, thoroughly designed screening procedures are required to render this method effective. Evolutionary engineering separates from metabolic engineering as it relies on random methods instead of the directed genetic modifications. It can be very difficult to determine which genetic modifications are responsible for improved traits in an evolutionarily evolved strain, particularly if random mutations are spread over the entire genome. This bottleneck, however, might well be the point where inverse metabolic engineering picks up. Evolutionarily evolved strains, could provide useful starting points for inverse metabolic engineering approaches.

Basically, an evolutionary engineering procedure begins with the mutagenesis of a monoclonal initial strain or introduction of a heterologous genomic fragment library into this strain in order to increase genetic diversity. EMS (ethyl methane sulfonate), for instance, is an alkylating agent which causes mispairings via nucleotide modifications throughout the genome and used extensively to introduce single nucleotide mutations. This step of diversification is then followed by serial subcultures for the enrichment of clones or individuals exhibiting the desired phenotype. Not always, however, the targets are random as it is the case in global transcription machinery engineering, where random manipulations are inflicted on protein factors of basal transcription machinery. This application comes with its advantage as the predesignated traceable protein target forms the basis for revealing the underlying genetical background (Cakar et al., 2005; Nevoigt, 2008; Sonderegger & Sauer, 2003).

1.4 Stress Response and Molecular Basis of Adaptation

Systems that exist, be it inorganic or organic, has a close relation with its surroundings. This relation often defines the current composition and the course of a habitat driven by the dynamics amongst the elements within. One of the most determinative factors that play an important role with regard to these dynamics is stress. If we were to approach this factor from a cellular standpoint; environmental conditions stand out in biological systems. Environmental changes may be of a physical or chemical nature: temperature, pressure, radiation, concentration of solutes and water, presence of certain ions, toxic chemical agents, pH and nutrient availability. They threaten the survival of a cell or at least prevent it from performing optimally, and are commonly referred to as cell stress. All cell types, even individual cells in multi-cellular organisms, have the ability to respond to changes in environmental conditions. Such responses require a complex network of sensing and signal transduction leading to adaptations of cell growth and proliferation as well as to adjustments of the gene expression programme, metabolic activities, and other features of the cell (Winde, 2003).

As a nonmotile, unicellular organism, yeast relies on physiological mechanisms to cope with these environmental changes. These mechanisms consist of the sensing and the response phases in a cell. Global gene and protein expression analysis are used in an attempt to elucidate these mechanisms. However, it often turns out to be difficult to demonstrate a role of a protein in a given stress response since there are systems that overlap and are interlinked during stress response as evident in those mechanisms studied up to now. Not only the impact of interactions between cells within a population but differences between the individual cells in terms of stress response may also play an important role in the manifestation of phenotypes. Control of cell proliferation and cellular stress responses are very much interrelated and in some respect seem to be two sides of the same coin. This fact is apparent in stress treatments causing a transient arrest of the cell cycle. This blocking of cell growth may be needed to prevent damage during cell cycle phases in which the cell is specifically vulnerable (S and M) and allow adaptation while cells are in G1. In the regulation of such a common response, widely-studied Msn2p and Msn4p proteins play an important role. However, the phenomenon that these proteins are involved in more than one responsive pathway besides the cell arrest not being the only action

they are responsible for, leads us to the idea of cross protection. This phenomenon of cross-protection suggests that different stress conditions require common cellular responses, such as adjustment of energy metabolism and production of protective proteins. Many studies show that cells exposed to a mild dose of one stress become resistant to large, normally lethal doses of other stresses. With the further transcriptional analysis, another fundamental aspect in the stress response enters the picture which suggests that underlying molecular mechanisms also play an important role in normal unstressed cells such as the heat shock proteins functioning as chaperones. These findings, hinting at a general fundamental system, subsequently gave rise to the identification of a sequence element common to the promoters of the stress-induced genes, referred to as the Stress Response Element (STRE)(Martinez-Pastor et al. 1996; Schmitt and McEntee 1996 Estruch). During exposure to stress, these genes are regulated by “general stress transcription factors”, following induction by an accompanying sensing mechanism which involves protein kinase A or MAP kinases (Brauer et al., 2008; Estruch, 2000; Tibbles & Woodgett, 1999; Zhao & Bai, 2009).

Stress tolerance is possible through the activation of general and specific stress responses. A common procedure could be summarized as follows.

- (i) Upon the introduction of stress condition, these unfavourable changes are sensed by membrane associated proteins.
- (ii) Subsequently, a relay system is activated with the signal received from membrane. This relay system is generally achieved through sequential phosphorylation of related activators which creates a cascade effect. Ras adenylate cyclase pathway, MAP kinase pathways are the most important transduction systems in most of the cells including yeast.
- (iii) Following phosphorylation, stress related transcription factors are translocated to nucleus. Here they bind to the corresponding elements on DNA and lead to cellular adaptation.
- (iv) Once the new homeostasis is reached and the cell has survived the stress, cell cycle arrest is lifted and the cell resumes normal operation under these conditions.

Reaching to this new state ensuing the above summarized mechanism is referred to as adaptation. Evolution itself, being one of the most prominent results of such a network of relations, is greatly influenced by the ability of adaptation that organisms are adept to exhibit. In other words, the more responsive the organisms, the more they are fit. As well as the unicellular organisms in nature, some of these stresses also applies for industrially important microorganisms during production processes. Brewing strains of yeasts, for instance, are exposed to fluctuations in oxygen concentration, osmotic potential, pH, ethanol concentration, nutrient availability and temperature. All of these stress factors have both general and specific impacts on cellular responses resulting in important protective molecules (Figure 1.7). Although these molecules may vary greatly in their structure they have overlapping effects with regard to different industrial stress conditions. Studies about these stress factors have a great potential to yield in multiple-resistant industrial strains (Gibson et al., 2007; Tibbles & Woodgett, 1999; Zhao & Bai, 2009).

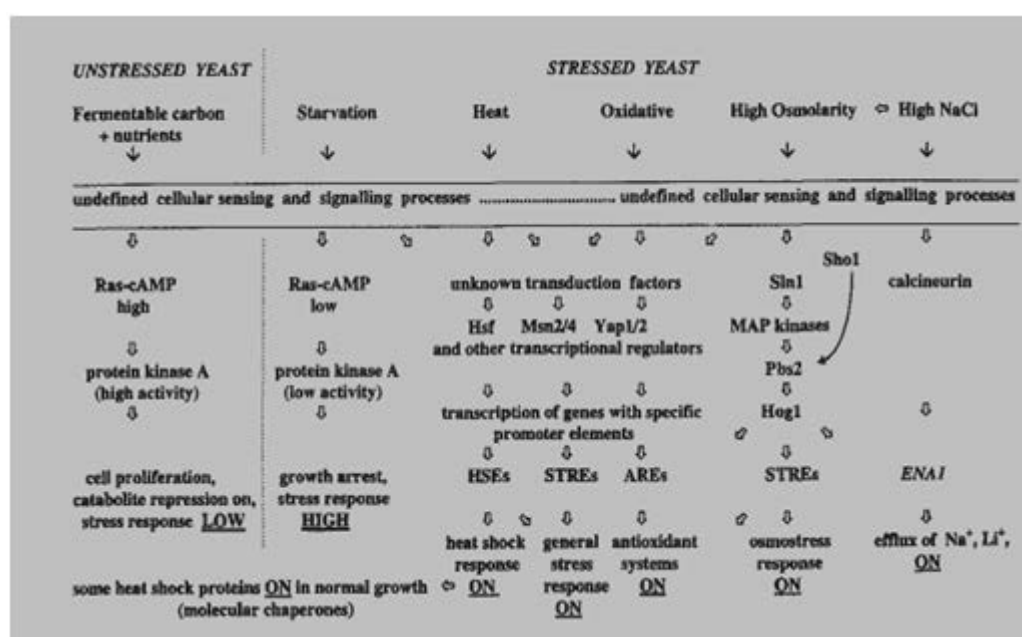


Figure 1.7: A simplified summary of stress response in *S. cerevisiae* (Attfield, 1997).

1.5 Sulphur Dioxide

Sulfur dioxide (SO₂) is a non-flammable, non-explosive, colorless gas that is readily soluble in water. It stands out as an indispensable food additive besides its impact on air quality and human health (Ziemann, 2010). Sulfur dioxide is used in large quantities as a captive intermediate in the production of sulfuric acid and in the pulp and paper industry. Other common uses of sulfur dioxide include the following: fumigant, preservative, bleach, and steeping agent for grain in food processing; catalyst or extraction solvent in the petroleum industry; flotation depressant for sulfide ores in the mining industry; intermediate for bleach production; and reducing agent in several industrial processes (IARC, 1992). Sulphur dioxide has been produced commercially from: elemental sulfur; pyrites; sulfide ores of non-ferrous metals; waste sulfuric acid and sulfates; gypsum and anhydrite; hydrogen sulfide containing waste gases; and flue gases by the combustion of fossil fuels (WHO, 1992). Although it is known that ancient Egyptians, Greeks and Romans burned sulphur incenses into the containers in order to preserve their food and beverage, the first clear reference of sulfur dioxide use in history comes from a wine production report published in Rotenburg, Germany in 1487 (Jackson, 2008).

Once dissolved in water, sulfur dioxide exists in equilibrium between molecular SO₂ (SO₂•H₂O), bisulfite (HSO₃⁻), and sulfite (SO₃²⁻) species as illustrated in Figure 1.8 below:

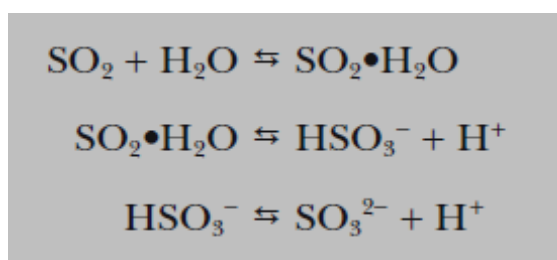


Figure 1.8: Equilibrium of SO₂ in aqueous solutions.

This equilibrium is dependent on pH (Figure 1.9). Besides being in equilibrium with the molecular and sulfite species, bisulfite also exists in “free” and “bound” forms. Here, the molecule will react with carbonyl compounds (e.g., acetaldehyde), forming addition products or adducts such as hydroxysulfonic acids (Ribéreau-Gayon et al., 2006).

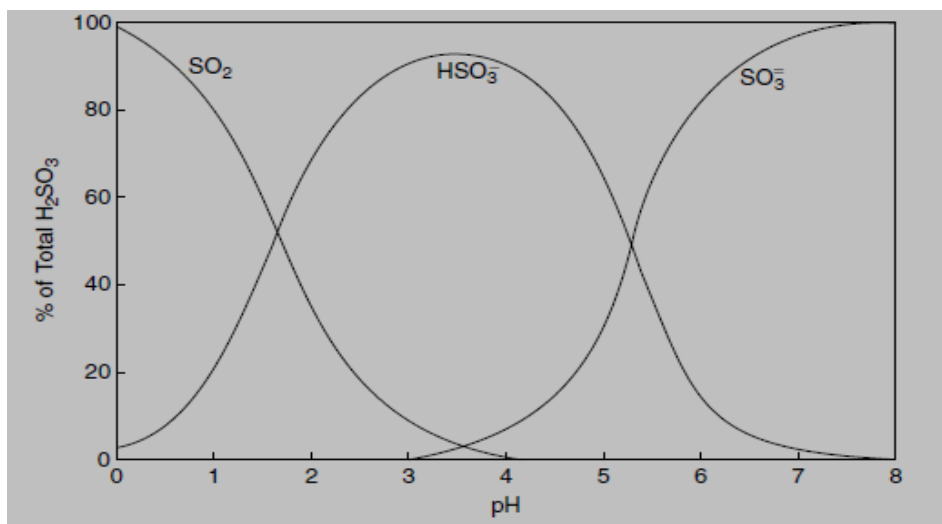


Figure 1.9: Distribution of sulphite at various pHs (Davidson et al., 2005).

Sulphur dioxide has been investigated in a multi focal manner in the literature as it both has its advantages and disadvantages for humans in many fields. It is of major concern regarding air quality and pollution all over the world as it in the air results primarily from activities associated with the burning of fossil fuels. In nature, sulfur dioxide can be released to the air, for example, from volcanic eruptions. Once released into the environment, sulfur dioxide moves to the air. In the air, sulfur dioxide can be converted to sulfuric acid, sulfur trioxide, and sulfates and leads to acid rains (WHO 2006). Sulfur dioxide dissolves in water, thus, carries a great risk of contaminating soil. Considering two major sources, foods and air, exposure to dangerous levels of sulphur dioxide might occur either by absorption of sulfur dioxide in the mucous membranes of the nose and upper respiratory tract as a result of its solubility in aqueous media or by ingestion. Many studies have linked sulfur dioxide levels in the general environment to a variety of adverse health consequences, including acute and chronic bronchitis, respiratory tract infections and mortality, particularly among people with pre-existing lung or heart disease. Ingestion of sulfites has been postulated to be a cause of rapid, acute allergic reactions, including fatal anaphylactic-like responses (WHO 1998).

Sulphites has an essential role in food and beverage industry. They are considered GRAS substances by the FDA when used in amounts that are in accordance with good manufacturing practices. They are allowed in fruit juices and concentrates, dehydrated fruits and vegetables, and wine (Davidson et al., 2005). However ,the FDA later (1986a) cancelled the GRAS status of sulfites on raw fruits or vegetables

and declared that sulfites are banned from restaurant and supermarket use on salads and vegetables as a result of serious reactions on individuals. These included unconsciousness, anaphylactic shock, nausea, diarrhea, and asthma attacks, which resulted in deaths in some incidents (Lewis, 1999). Yet, it should be recognized that many “traditional foods” cannot be prepared without this additive; including wine, beer and other fermented beverages. Sulphur dioxide is unique in its ability to control browning in food caused enzymatic or non enzymatic reactions. The term nonenzymic browning is synonymous with the Maillard reaction, i.e., the reaction between reducing sugars and amino acids, peptides, and proteins. The Maillard reaction is responsible for both the desired and the undesired features of foods like savory taste of meat and bread after cooking or unwanted pungent odors and tastes in wine. This reaction is inhibited by sulphur dioxide solutions applied on foods as well as in beverages. The major products of the irreversible combination of sulphites with food components are organic sulfonates, also known as the melanoidines. However this preventive effect lead to a most intriguing question regarding the generally accepted role of sulphite in food as an antioxidant, whereas its behavior in model systems can often be seen to be that of a pro-oxidant. On the other hand the mechanism of inhibition of enzymic browning is the reaction of sulfite ion with the *o*-quinones which are formed by the enzymatic oxidation of *o*-diphenols. Essentially the quinones are reduced to the sulfonated phenols. Addition to this type of inhibition of enzymatic browning, another mechanism via direct inhibition of enzymes involves the splitting of disulfide bonds which are often essential in maintaining the functional structure of enzymes. A summary of conservation properties of SO₂ is shown in Table 1.1 (Eskin & Robinson, 2001; Namiki, 1988).

Table 1.1: Conservation properties of different forms of SO₂ (Ribéreau-Gayon et al., 2006)

Property	SO ₂	HSO ₃ ⁻	R-SO ₃ ⁻
Fungicidal	+	low	0
Bactericidal	+	low	low
Antioxidant	+	+	0
Antioxidasic	+	+	0
Gustatory amelioration:			
Reduction-oxidation potential	+	+	0
Neutralization of ethanal	+	+	+
Gustatory role of SO ₂	biting odor, SO ₂ taste	odorless, salty, bitter taste	odorless, tasteless at normal concentrations

1.5.1 SO₂ as a Stress Factor at a Molecular Level

Sulphur dioxide and its derivatives have always been a major concern of health as mentioned above. Their purposeful uses date back to 1800s and they have been a major role player of food protection, therefore, it is understandable that the damage inflicted by sulphites has not been noticed until 90s. In recent years, studies on sulphites and their potential health effects have had a tremendous impact on the literature. Among these studies, yeast holds an important place taking a motive from the long known interrelation of the species with sulphites. Not only yeasts tolerance to sulphites is an important feature to be further investigated for the industrial concerns, it is also important to understand the mechanisms underlying sulphite toxicity for human health where such a model eukaryotic organism could serve as a suitable tool (Davidson et al., 2005).

Today most of our knowledge about sulphur dioxide on a cellular level, comes from the studies conducted on microorganisms associated with wine industry. Being most extensively used in wine industry, it has numerous effects on endogenous microfauna of must used in wine making. The most common microorganisms found in a must consists of lactic acid and acetic acid bacteria, non-saccharomyces spoilage yeasts of genus *Dekkera*, *Candida* or *Pichia* and *Saccharomyces cerevisiae*. Both wine making and laboratory practices show that bacteria are the most sensitive while *Saccharomyces cerevisiae*, brewer's yeast, has been shown to represent the highest resistance against sulphur dioxide. In this regard, effects of sulphite on the yeast and the bacteria along with the tolerance mechanisms are summerized under the following topics (Fugelsang, 1997).

1.5.2 SO₂ Effect on the Cell

Sulfite is a normal metabolite, which occurs naturally by yeast as an intermediate in the reductive sulfate assimilation pathway. It is therefore found in most wines and beers. Endogenous sulfite can be also formed during the body's normal catabolism of sulfur containing amino acids such as methionine and cysteine (Park & Hwang, 2008). However, both the exogenous and endogenous sulphite is detrimental to cell and understanding how sulphur dioxide interacts with cell is an important issue in management of sulphites.

As in all stress factors, sulphur dioxide has been shown to be more effective in certain conditions. Temperature and the physiological state of the cell are the basic parameters affecting its impact on cell. Temperature increase, as expected, results in elevated toxicity on the cell by inhibiting growth in solutions containing 1 mM SO₂. As for the physiological state of cell, when treated with low concentrations of sulphite, stationary phase cells are shown to be more resistant while high doses like 2 mM were lethal (Schimz, 1980). Nonetheless, apart from these and other environmental and physiological factors the main dynamic that directly influences sulphur dioxide action is the pH value since it is present in 3 forms in aqueous solutions depending on pH. Although it was first suggested that the transport of sulphite, where molecular SO₂ was found to be the form that passes through cell membrane, is achieved in a facilitated manner (Macris & Markakis, 1974); results of subsequent studies, countered the active transport mechanism and pointed to a simple free diffusion of molecular SO₂ by tracking the accumulation of labeled ³⁵S atoms of sulphite. Both of these studies were in agreement as to the form of the sulphite that passes into the cytoplasm as indicated by the labeled atom movement in lower pH range between 3-5. Between these pH ranges, as mentioned above, the dominant sulphite species is molecular SO₂. It was also found that the intracellular label accumulation was 60 times that of suspension. This result was also consistent with pH dependency of sulphur dioxide. Since the intracellular pH value of yeasts is about 6.5, SO₂ that passes inside is converted into HSO₃⁻ ions or binds to carbonyl containing compounds. This in turn lowers the intracellular SO₂ and allows further diffusion (Stratford & Rose, 1986).

Once inside the cell, the most prominent deleterious effect of SO₂ is exhibited in the ATP levels of the cell. A series of studies showed decreased ATP and ADP levels with increased AMP and Pi levels upon addition of sulphite at low pH. Depletion of ATP by sulphite at low pH occurs in both the presence and absence of glucose. These findings point to at least two different mechanisms for sulphite action on energy metabolism. One mechanism involving the inhibition of glyceraldehyde-3-phosphate dehydrogenase accounts for the strongest influence on cell viability. GAPDH enzymes extracted from sulphite treated cells showed nearly %80 decrease in activity. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is the first enzyme of energy extraction phase in glycolysis. It catalyses the oxidation and

phosphorylation of glyceraldehyde-3-phosphate in two coupled steps and leads to ATP generation. Inhibition of this enzyme by sulphite causes termination of AMP phosphorylation (Hinze & Holzer, 1985; Hinze & Holzer, 1986; Schimz, 1980).

Given that, AMP levels increase after SO₂ treatment in a medium without glucose, another mechanism involving dephosphorylation of ATP might be in question. Such a mechanism is available through ATPases, i.e. the enzymes that catalyze both the phosphorylation and dephosphorylation reactions. A well-known ATPase, F₁-ATPase of bacteria which is also present on the membranes of eukaryotic cell compartments such as mitochondria and chloroplasts are shown to be sensitive to sulphite which may be the reason for elevated sensitivity in bacteria. In addition, it was shown that mitochondrial F₁-ATPase defective mutants of *Saccharomyces cerevisiae* exhibits similar decrease pattern in ATP levels with wild-type strains which in turn postulates that SO₂ action is confined to the cytosol. However, some assays on yeast vacuolar H⁺-ATPases (V-ATPase) indicates that these ATPases are both stimulated and inhibited by sulphite. Their stimulation and inhibition was found to be depending on MgATP levels. V-ATPases are responsible for the maintenance of intravacuolar acidity which in turn generates a protonmotive force to be used in proton exchange. Stimulated V-ATPases under SO₂ stress could lead to a depletion of ATP whereas it may contribute to the decrease in the cytosolic pH. This decrease in cytosolic pH dissipates the protonmotive force across the plasma membrane besides causing defects in cytosolic constituents and enzymes (Carrete et al., 2002; Hinze & Holzer, 1986; Kibak et al., 1993).

Addition to those major effects of SO₂, its reducing nature, therefore, reactivity causes other problems in the cell. Cleavage of disulfide bonds is achieved by sulphites. These bonds are very important structural components of important enzymes. Therefore conformational problems may arise from the cleavage by sulphites. SO₂ also rapidly reacts with carbonyl groups, aldehydes in particular are of concern in yeast systems. Acetaldehyde plays an important role in detoxification of SO₂ whereas it forms an adduct with sulphite in the cell which inhibits alcohol dehydrogenase via substrate inhibition thus leading to an indirect decrease in energy metabolism (Casalone et al., 1992).

Detoxification systems and responses to SO₂ involves different mechanisms according to their mode of action. As all the findings indicate, this compound is effective when passed into the cell. As a result, detoxification occurs either by rapid removal of sulphite or by converting this compound into less reactive and harmful species.

1.5.3 Sulphite Efflux

The ability to remove sulphite and reduce intracellular accumulation has an important place in resistance to SO₂. An important pathway involved in sulphite efflux was characterized in rather recent studies. A plasma membrane protein encoded by SSU1 facilitates the rapid expulsion of sulphite. (Avram & Bakalinsky, 1997; Park & Bakalinsky, 2000) This plasma protein is under strict control of a transcription factor encoded by FZF1 gene (Avram & Bakalinsky, 1996; Avram et al., 1999; Casalone et al., 1994). The importance of products of these genes has been shown in numerous studies. Remarkably, overexpression of both genes resulted in both heightened sulphite resistance and restoration of resistance in mutants that possess defects in other sulphite detoxification mechanisms. However the underlying induction mechanism is still unclear (Avram & Bakalinsky, 1996; Avram & Bakalinsky, 1997; Avram et al., 1999).

Ssu1p lacks the nucleotide binding sequence typical of ABC transporters, but resembles the general structure of facilitator/transporter proteins, suggesting that it is a member of the major facilitator superfamily involved in efflux of toxic compounds (Park & Bakalinsky, 2000). While sulphite is a normal metabolite, it is not a natural sulphur source for yeast, nor is it likely that yeast would encounter significant concentrations of sulphite in nature. Thus, the question arises as to whether sulphite is the physiologic substrate for Ssu1p. This uncertainty is supported by the increase in expression levels of FZF1 by nitrosative stress while a nitrosative stress related gene YHB1 is activated along with SSU1 through this transcription factor (Sarver & DeRisi, 2005).

Some wine strains of *S. cerevisiae* possess an allele of SSU1 which confers a high resistance against SO₂. This allele, designated as SSU1-R, has a different promoter region which is proposed to be acquired by a reciprocal translocation between

chromosomes VIII and XVI (Yuasa et al., 2004). As a result of this translocation, the *SSU1-R* allele contains four repeats of a 76-bp sequence, a promoter sequence which is a single copy of 77 bp in *ECM34*. This promoter sequence greatly increases the expression of *SSU1* (Goto-Yamamoto et al., 1998). A schematic view of translocation between chromosomes VIII and XVI is shown in Figure 1.10. Regulation of *SSU1-R* also differs from that of *SSU1*. *FZF1* is a negative regulator for *SSU1-R* while induction takes place under microaerobic conditions consistent with the environment during wine fermentation (Yuasa et al., 2005). Although in a recent study a sulphite induced allele of *SSU1-R* gene was isolated (Nardi et al., 2010), a direct relation of *SSU1* and *FZF1* to sulphite has yet to be elucidated.

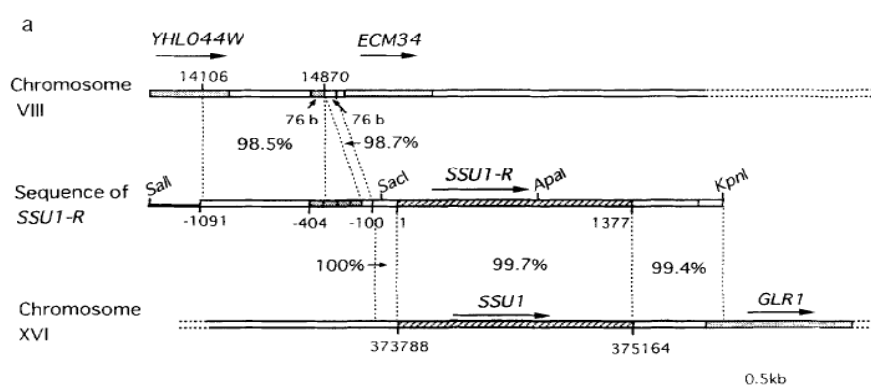


Figure 1.10: Schematic representation of *SSU1-R* (Goto-Yamamoto et al., 1998).

1.5.4 Adduct Formation

SO_2 is a reducing agent and it can readily bind to aldehydes or ketones to form α -hydroxysulphonates in an irreversible manner. Most prominent target of SO_2 in yeast is, therefore, acetaldehyde which is an intermediate produced during glycolysis. Acetaldehyde itself is toxic furthermore it can bind to proteins and DNA. Acetaldehyde forming a hydroxysulphonate adduct with sulphite is non toxic to the cell. Revealed in numerous studies, the yeast species that are capable of producing high levels of acetaldehyde usually are more resistant to sulphites. This fact is evident in direct correlation of acetaldehyde production capacities and resistance levels in *S. cerevisiae* and *S. ludwigii*. While they represent almost the same amount of ATP pools, *S. ludwigii* shows a better growth performance under moderate levels of SO_2 with an increased basal acetaldehyde excretion level. Also, in media where *S.*

cerevisiae cells are exposed to SO₂, the decrease in sulphite is directly proportional with the increase in acetaldehyde (Casalone et al., 1992; Hinze & Holzer, 1985; Stratford et al., 1987).

Transcriptional analysis, also, are in agreement with this shift of energy metabolism. It is shown that, after sulphite treatment, the functional categories of up-regulated genes belong to carbohydrate metabolism, transportprotein and cell wall biosynthesis. About 50% of the induced genes by sulphite are related to carbohydrate metabolism, suggesting that sulphite affects carbon metabolism and energy production. The genes induced in the category of carbohydrate metabolism include the genes involved in glycolysis such as TPI1 (triose phosphate isomerase), THD3 (glycealdehyde-3-phosphate dehydrogenase), FBA1 (aldolase), PDC1 (pyruvate decarboxylase), and ADH1 (alcohol dehydrogenase). Among these genes, pdc1p is the enzyme that catalyzes the oxidation of pyruvate to acetaldehyde and explains the increased acetaldehyde excretion. Besides sulphite resistance this kind of elevation in glycolysis associated mechanisms could lead the cells to a better fermentative performance which would be of industrial importance (Park & Hwang, 2008).

1.5.5 Sulphite Assimilation

In yeast, sulphite is formed by reduction of 3'-phosphoadenosine phosphosulfate (PAPS) through the action of PAPS reductase. It is then reduced to hydrogen sulfide in a sixelectron transfer catalyzed by sulphite reductase. Hydrogen sulfide condenses with *O* acetylhomoserine to form homocysteine leading directly to methionine, or to cysteine via cystathionine (Figure 1.11) (Cherest & Surdin-Kerjan, 1992) This reduction pathway aids the cell in managing exogenous sulphite as well. MET1, MET5, MET8 and MET10 genes encode for the subunits of sulphite reductase and have been of interest in sulphite resistance analysis. Nevertheless no evidence of induction by sulphite is available, mutants overexpressing these genes shows a higher resistance against SO₂ (Avram & Bakalinsky, 1996). On the other hand, substrate effect on sulphite tolerance through this pathway yields interesting results. Methionine, a product of sulphite assimilation pathway, has a negative control over the system and addition of this aminoacid causes sulphite sensitivity. This result makes sense as methionine available in the medium would repress the assimilation of sulphite. However induction of the mechanism by adenine leads us to the question as

to whether the system is controlled by a metabolite belonging to the purine synthetic pathway. These studies lack the molecular evidence, yet they have their impact on wine practices where an unfavourable product, hydrogen sulfide, excretion levels are of major concern (Aranda et al., 2006).

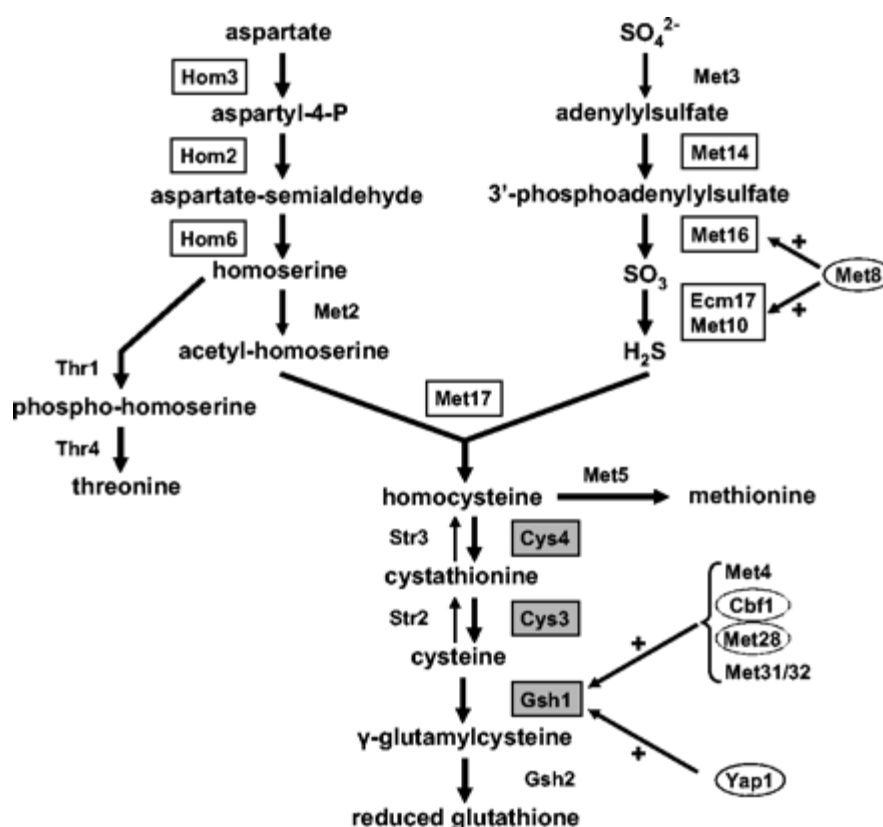


Figure 1.11: Sulphate assimilation pathway (Dilda et al., 2005).

1.5.6 General Stress Responses to SO₂

Major detoxification mechanisms mentioned above are, most of the time, plays the biggest part in sulphite resistance. Even in those mechanisms, considering the response in energy metabolism, there is a tendency of cells towards overproduction of SO₂ targets. This condition might also explain the other auxiliary responses, in particular those concerning general stress responses. Among those general stresses; oxidative stress and acid stress have significant impact on resistance to SO₂.

Oxidative stress is inflicted through reactive oxygen species (ROS) produced endogenously by cells under aerobic conditions. Among antioxidant defenses glutathione, metallothioneins, thioredoxin, superoxide dismutase (SOD) and catalase (CAT) stand out. Glutathione is a product of sulphate assimilation pathway and

alongside thioredoxin it takes part in sulphitolysis reaction which may help detoxification of SO₂. Organisms lacking thioredoxin and glutathione show sensitivity to sulphite, however, no increase in levels of these compounds is observed by introduction of sulphites. Overproduction of SOD and CAT on the other hand has been shown to confer resistance in higher eukaryotic cells (Estruch, 2000; Tseng et al., 2007).

Acid stress affects cellular physiology: proteins involved in plasma membrane integrity may be susceptible to denaturation, particularly in the presence of ethanol and pH gradient change results in poor ion exchange. Decrease in pH is one of the major effects of SO₂ in the cell and studies regarding connection between both stresses are extensively studied in bacteria. On bacterial ATPases sulphite has an inhibitory effect. Mutants constructed or strains preadapted in acidic media were shown to exhibit prolonged viability (Guzzo et al., 1998).

1.6 Aim of The Study

The aim of this study was to improve SO₂ tolerance of *Saccharomyces cerevisiae* by evolutionary engineering and to gain insight into the mechanisms taking place in the SO₂ resistance. For this purpose, batch selection protocols were applied to a genetically diverse, chemically mutagenized initial population of *Saccharomyces cerevisiae*, and the SO₂ levels were gradually increased at each successive batch cultivations. As the activity of SO₂ is optimum at low pH levels, selections were performed in a bioreactor at a constant pH of 3.5. Mutant generations were obtained from both selections in the bioreactor and culture tubes. Individual mutants were randomly selected from the final generations of each selection and were analyzed for their SO₂ resistance along with cross-resistances against other stress types.

2 MATERIALS AND METHODS

2 Materials

2.1.1 Yeast strain

Saccharomyces cerevisiae CEN.PK113.7D was kindly provided by Dr. Peter Kötter (University of Frankfurt, Germany). This strain was the wild type strain used in the study and designated as 905. The strain obtained from EMS mutagenized cultures of 905 was used for the initial stress application and designated as 906.

2.1.1 Yeast Culture Media

Yeast minimal medium (YMM) ingredients are given in Table 2.1.

Table 2.1: Yeast minimal medium (YMM) composition

Yeast Minimal Medium (YMM) 1 liter	
Components	Amount (g)
Difco Yeast Nitrogen Base without aminoacids (BD)	6.7
Dextrose (AnalaR – BDH)	20
Agar - only for solid media (Acumedia)	20

Yeast rich medium (YPD) ingredients are listed in Table 2.2.

Table 2.2: Yeast peptone medium YPD composition

Yeast Peptone Medium (YPD) 1 liter	
Components	Amount (g)
Yeast Extract (Acumedia)	10
Dextrose (AnalaR – BDH)	20
Peptone (Merck)	20
Agar - only for solid media (Acumedia)	20

Xylose medium ingredients are listed in Table 2.3

Table 2.3: Xylose medium composition

Xylose Medium (YPD) 1 liter	
Components	Amount (g)
Difco Yeast Nitrogen Base without aminoacids (BD)	6.7
Xylose	20
Agar - only for solid media (Acumedia)	20

2.1.2 Chemicals, Solutions, Kits and Buffers

The chemicals used throughout the study are listed in Table 2.4

Table 2.4: Chemicals used in the study.

Chemical	Supplier
Potassium Metabisulphite	Merck (Germany)
Ethanol (absolute)	J.T.Baker (Holland)
Hydrogen Peroxide (35%, w/v)	Merck (Germany)
Hydrogen Chloride	J. T. Baker (Holland)
Potassium Hydroxide	Merck (Germany)
Polypropylene Glycol 2000	Merck (Germany)

Solutions and buffers used in the study are listed in Table 2.5

Table 2.5 Solutions and Buffers

Chemical	Stock Concentration
SO ₂ solution	0.5 M
CoCl ₂ solution	1 M
CuCl ₂ solution	1 M
SeL solution	1 M
NiCl ₂ solution	1 M
HCl solution	3 M
KOH solution	3 M

API ID 32C Yeast Identification Test Kit (Biomérieux – France)

2.1.3 Laboratory Equipment

Thermomixer	Eppendorf, Thermomixer Comfort 1.5-2 ml, (Germany)
Microfuge	Eppendorf Centrifuge 5424 (Germany)
Ultracentrifuge	Beckman Coulter, AvantiJ-30I (USA)

Rotor	Beckman Coulter JA-30.50i (USA)
UV-Visible Spectrophotometer	Shimadzu UV-1700 (Japan), Perkin Elmer 25 UV/VIS (USA)
Ultrapure Water System	USF-Elga UHQ (USA)
Micropipettes	Eppendorf (Germany)
pH meter	Mettler Toledo MP220 (Switzerland)
Water Bath	Memmert wb-22 (Switzerland) Nüve BS402 (Turkey)
Balances	Precisa BJ 610C (Switzerland) Precisa 620C SCS (Switzerland)
Laminar Flow	Faster BH-EN (Italy)
Autoclaves	NüveOT 4060 Steam Sterilizer(Turkey)
Deep Freezes and Refrigerators	80°C Heto Ultrafreeze 4410 (Denmark), -20°C Arçelik(Turkey) +4°C Arçelik (Turkey)
Orbital Shaker Incubators	Certomat S II Sartorius (Germany)
Incubators	Nüve EN400 (Turkey)
Light Microscope	Olympus CH30 (USA)
Bioreactor	B. Braun, Biostat Q with Digital Measurement and Control System DCU 3 (Germany)

2.2 Methods

A general course of the study is summarized in Figure 2.1.

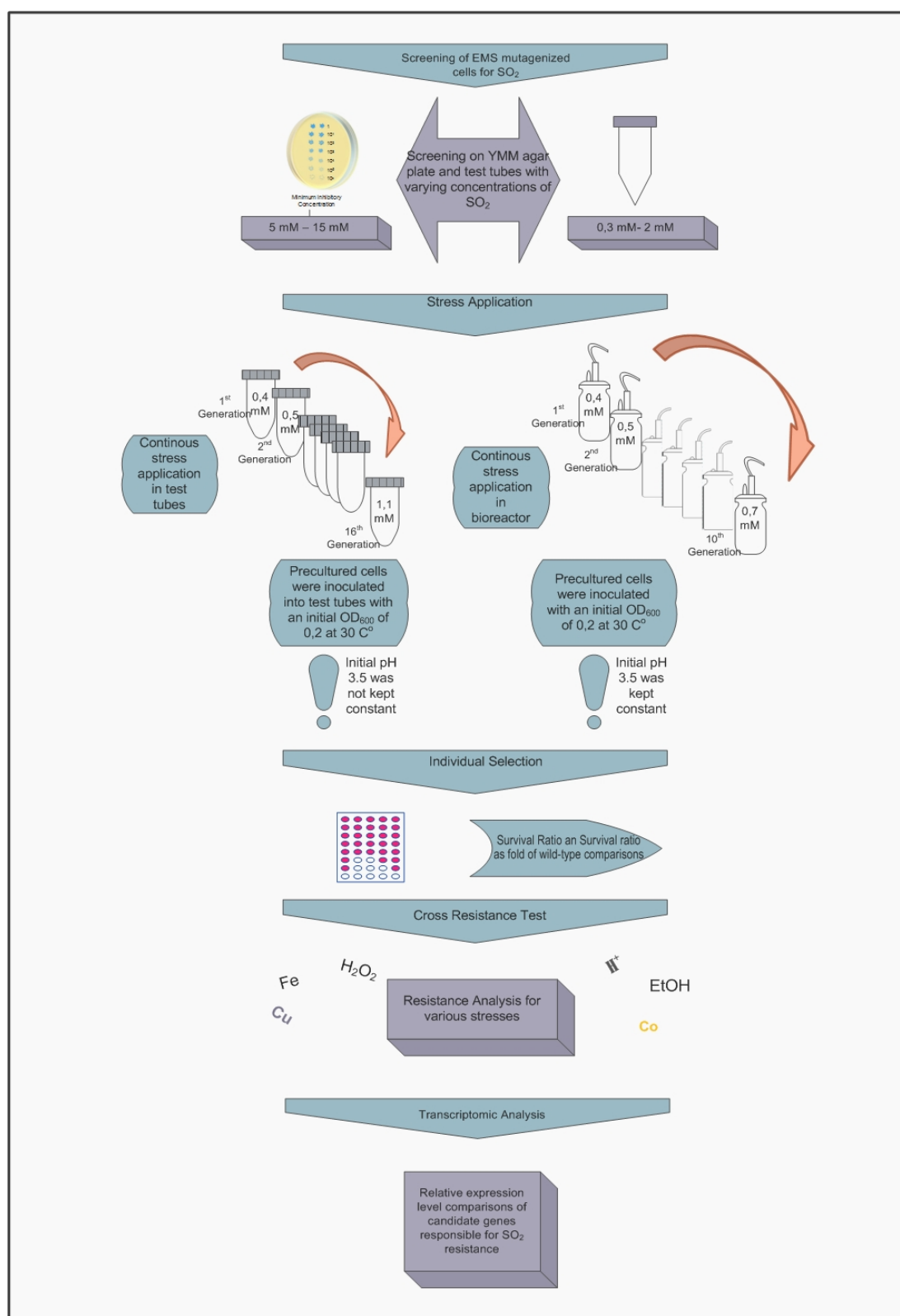


Figure 2.1: Flowchart describing the outline of the study.

2.2.1 EMS Mutagenesis

Saccharomyces cerevisiae CEN.PK 113-7D culture, approximately at a concentration of 1×10^6 cells/ml; was inoculated into 10 ml YPD, and incubated overnight at 30°C and 150 rpm in order to have the cell concentration of approximately 2×10^8 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 5×10^7 cells/ml.

Each 10 ml of cell suspension was supplemented with 300 µl of EMS in a screw-cap glass tube. The tube was vortexed and then incubated for 30 minutes at 30° C. In order to stop EMS mutagenesis, an equal volume of freshly made and filter-sterilized sodium thiosulfate solution (10%, w/v) was added into the tube. The solution was mixed well 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 stocks were prepared.

2.2.2 Initial Screening for SO₂ Resistance

EMS-mutagenized 906 strains were precultured in 10 ml YMM preadjusted to pH 3.8 in 50 ml culture tubes. Overnight grown cultures were exposed to varying concentrations of SO₂ ranging between 0.3 mM – 2 mM. Maximum concentration of SO₂ was detected at which the mutant and the wild type strains could survive. Same set of strains were also exposed to 0.5 mM SO₂ at 3 different pH values of 3.5, 4.5 and 5.5 in order to confirm the efficient pH value for selections in SO₂ solutions.

2.2.3 SO₂ Selection In Batch Cultures

Batch selection procedures were achieved in culture tubes and in a bench-top bioreactor parallelly. All the selections were accomplished by assuming a continuous approach, i.e., in every two successive cultivation cycle of 48 h, stress was applied in constant increments provided that the cells survive the previous stress concentration. Optical density measurements were done by UV-Visible Spectrophotometer at

600nm. OD₆₀₀ values were used to determine whether the that level of stress was survived.

2.2.3.1 Culture Tube Selections

Overnight cultures of 905 and 906 strains were inoculated into 50 ml culture tubes both with and without stress factor with an initial OD₆₀₀ value of 0.2 and incubated at 30 °C and 150 rpm. The initial pH was adjusted to 3.65 with 3 M. Tubes with stress factor contained the corresponding amount of stock SO₂ solution to match the desired final stress concentration beginning with 0.4 mM in the first population and ending with 1.1 mM in the last population. OD₆₀₀ values were measured at 24 h and 48 h intervals. Survival ratios were determined by calculating the ratio of the living cell number after stress to living cell number without stress. Stock cultures were prepared from cultures with favourable survival ratios and these cultures were inoculated into the next stress level.

2.2.3.2 Bioreactor Selections

Successive batch selections were also achieved in a bench-top bioreactor. 906 strain was used as the initial population. Overnight culture of 906 was inoculated into 400 ml YMM at corresponding SO₂ concentration with an initial OD₆₀₀ of 0.2 in 1 L bioreactor vessel. pH was adjusted to 3.5 ± 0.1 with 3 M HCL and maintained with 3 M KOH. The cultivation temperature was 30 °C, the stirrer speed 150 rpm, and the aeration rate was 0.50 l air min⁻¹. 500 µl of polypropylene glycol 2000 was added initially to prevent foaming. OD₆₀₀ value was measured at 24 h and 48 h. Culture with OD₆₀₀ value over 1 was inoculated into fresh medium with stress and frozen stock was prepared.

2.2.4 Stock Culture Preparation

500µl cell suspensions in YMM were mixed with 500 µl 60% (v/v) glycerol in sterile 1.5 ml microfuge tubes. The final glycerol concentration was 30%. The suspension was vortexed and transferred to deep freezer at -80°C for long-term storage.

2.2.5 Individual Selection

To obtain individual mutants from final mutant populations, final populations were plated on YMM-agar plates using dilutions to reveal individual colonies. Colonies were then picked from the plates randomly, using sterile toothpicks and transferred to culture tubes containing 10 ml YMM for overnight incubation at 30°C 200 rpm. Frozen stocks were prepared from these liquid cultures. Overnight cultures were inoculated into 96-well plates containing YMM at a final concentration of either 0.5 mM, 0.7 mM or 1 mM SO₂. Survival ratios and survival ratios normalized to wild type values were estimated by MPN method. The most promising individuals were selected and used for further analysis.

2.2.6 MPN Method

MPN is a procedure to estimate the population density of viable microorganisms in a test sample. In essence, theory of probability is applied to a standard dilution series of sample inoculums in a set of culture media tubes by taking positive growth readings into account. Positive growth response after incubation is determined by visual observation. The sample should be diluted in such a manner that higher dilutions of the sample will result in fewer positive culture tubes in the series. To this end, 10 fold serial dilutions were used in 5 tube MPN series (United States Department of Agriculture Food Safety And Inspection Service, 2008). Instead of culture tubes, 96-well plates were used. 8 dilutions were made for each five columns. After inoculation these plates were incubated at 30°C. After 24 h and 48 h of incubation last three rows for each sample were read. Positive well numbers for each rows were noted and these results were used for calculating cell numbers by using 5-tube MPN table

2.2.7 Contamination Controls

Mutant individuals obtained upon selection were checked for contamination. Contamination tests were done on Xylose-agar plates and with API ID 32C system. Mutants were precultured on YPD-agar plates. Samples were taken from these plates with micropipette tips and rubbed on Xylose-agar plate. 5 µl of samples were taken

from undiluted cultures and dropped on the plate as well. Same were done on YMM-agar plate and this plate was used as the control. The plates were then incubated at 30°C. This test is based on the inability of *Saccharomyces cerevisiae* to use xylose as a carbon source. Growth on Xylose-agar plate was monitored visually over 48 hours of incubation. Along with Xylose-agar plate testing, an identification test API ID 32C was used. Corresponding amount of sample from overnight cultures were inoculated into test strips as per manufacturer's instructions. The strips were incubated at 30°C. Positive wells on the strip, where growth was observed, were marked on the result sheets according to the strip layout. Results were evaluated by the PC software.

2.2.8 Sporulation Assay for Diploidization Control

Selected individuals were precultured on YPD plates. 5 µl of sample from stock cultures of individual mutants were dropped on YPD plate and left to incubate at 30°C. Samples were taken from the overnight cultures and streaked on potassium acetate (KAC) plate. Tetrad formation of the cells were observed under the light microscope.

2.2.9 Genetic Stability Tests for Mutant Individuals

Mutant individuals obtained from selections were grown for 5 successive cultivations under nonselective conditions, i.e. without being exposed to freezing stress. During each cultivation, freeze-tolerance of the culture was determined by MPN assay, as described previously. The survival rates of each successive culture were determined and compared to each other to determine genetic stability

2.2.10 Cross Resistance Analysis on Solid Medium

Prepared YMM-agar medium was melted on heater and kept at 50°C in a water bath. Necessary amount of medium was poured into seven 50 ml culture media tubes to a final volume of 15 ml. These culture tubes were separately supplemented with NaCl, CuCl₂, CoCl₂, NiCl₂, ZnCl₂, FeCl₂, Sorbitol, H₂O₂ solutions to a final concentration of 0.5 M, 0.25 mM, 2.5 mM, 0.5 mM, 10 mM, 50 mM, 2 M, 0.5 mM, respectively.

The media were then decanted onto sterile petri dishes. 5 µl of sample was dropped on agar from 10 fold dilutions of samples in 5 rows.

2.2.10 Cross-Resistance Analysis In Liquid Media

2.2.10.1 Pulse Stress Application

Cells were precultured and left for overnight incubation. From overnight cultures one ml of sample was placed into 1.5 ml microfuge tube and washed twice with glucose-free medium. Cells were then inoculated with YMM broth at a concentration of 20% (v/v) EtOH and 0.3 M H₂O₂ separately. Microfuge tubes were incubated at 30⁰C and 200 rpm 90 min for EtOH and 60 min for H₂O₂. After incubation, cells were centrifuged at 14000 rpm for 5 min in a benchtop centrifuge, and washed twice with glucose-free YMM. The cells that have been exposed to pulse stress and the control group were then transferred to 96-well plates containing YMM to determine cell viabilities according to 5-tube MPN method.

2.2.10.2 Continous Stress Application

Cells were precultured in YMM at 30⁰C and 200 rpm. From overnight cultures, 20 µl of sample was transferred to each well containing 180 µl YMM in 96-well plates supplemented with a corresponding amount of stress solution to a desired concentration. 96-well plates were incubated at 30⁰C for 72 hours. For control groups samples were inoculated into YMM without stress inoculated under the same conditions in 96-well plates. Applied stresses and final concentrations in medium is given in Table 2.6.

Table 2.6: Stresses applied and their final concentrations in the medium

Stress	Concentrations
Ethanol	6% and 8%
H ₂ O ₂	0.5 mM
CuCl ₂	0.15 mM
NaCl	0.4 M
SeL	1 mM

2.2.10.3 Cross-Resistance for Liquid Nitrogen and Cold Stresses

Cells were incubated in YMM at 30°C 200 rpm until late exponential phase of growth. After reaching desired OD₆₀₀ values (4.5-5.5), one ml culture was transferred to 1.5 ml microfuge tubes. The samples were washed twice with glucose-free YMM centrifuged at 14000 rpm for 5 min in a benchtop centrifuge to remove supernatant after each washing steps. Next, cells in microfuge tubes were submerged into a liquid nitrogen container (at - 196°C) for 25 min. After this rapid freezing step, they were left for thawing at 30°C for 10 min. Control groups were not exposed to freezing stress. Cell viability of the control group and the experimental group were determined by five-tube MPN method.

For cold stress, washed samples were placed in the deep freezer at -20°C for 90 minutes. After freezing samples were left in room temperature for defrost. Cell viabilities were determined with MPN method.

2.2.10.4 Cross-Resistance for Heat Stress

One ml of overnight cultures of mutant individuals and wild type 905 were washed with dextrose-free YMM and exposed to 60°C temperature stress for 10 minutes. Following 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°C exposure were used as control groups.

3 RESULTS

3.1 Initial Screening for SO₂ Resistance

Screening for determining the initial SO₂ tolerance of wild type and EMS-mutagenized strains was preceded by the evaluation of optimum pH level at which SO₂ was optimally effective. For this purpose, 905 and 906 strains were precultured. Overnight cultures were inoculated into 10 ml YMM with a final concentration of 0.5 mM SO₂ at pH values of 3.5, 4.5, and 5.5 separately. Initial OD₆₀₀ value was 0.2. OD₆₀₀ values were evaluated in order to determine the pH value to be used throughout the study. The results were in agreement with the literature as pH 3.5 was observed to be optimum. The results are shown in Table 3.1.

Table 3.1: OD₆₀₀ values at varying pH

SO ₂ (mM)	OD ₆₀₀ Value		pH
	905	906	
0 (Control)	4.800	4.550	3.5
0.5	0.198	0.200	
0 (Control)	4.900	4.750	4.4
0.5	0.207	0.272	
0 (Control)	5.000	4.950	5.4
0.5	4.200	4.250	

The initial populations of 905 and 906 strains were screened for the maximum stress level at which a favourable survival ratio could be observed. For this purpose, these strains were exposed to increasing concentrations of SO₂. These concentration values were decided by literature evaluation and ranged between 0.3 mM-2 mM. Overnight cultures of 905 and 906 strains were inoculated into culture media tubes containing YMM supplemented with corresponding amount of SO₂ to a final volume of 10 ml. YMM was adjusted to pH 3.5 prior to addition of SO₂. Cells were added into the YMM broth to an initial OD₆₀₀ of 0.2. The stress concentrations used were increased

by 0.2 mM increments. Culture tubes were incubated at 30 °C and 150 rpm. OD₆₀₀ values were recorded at 24 h and 48 h intervals. The survival ratio results are shown in Table 3.2.

Table 3.2: OD₆₀₀ values at the end of 48 h incubation and survival ratios for initial screening

[SO ₂]	OD ₆₀₀ Value		Survival Ratio	
	905	906	905	906
0 (Control)	1.768	1.872		
0.3 mM	0.229	0.370	0.130	0.198
0.5 mM	0.240	0.200	0.136	0.107
0.6 mM	0.194	0.203	0.110	0.108
0.7 mM	0.217	0.183	0.123	0.098
0.8 mM	0.219	0.191	0.124	0.102
0.9 mM	0.202	0.187	0.114	0.100
1.0 mM	0.222	0.196	0.126	0.105
1.5 mM	0.221	0.184	0.125	0.098
2.0 mM	0.226	0.199	0.128	0.106

Results showed that SO₂ concentrations above 0.3 mM are toxic to 905 and 906 strains. At 0.3 mM SO₂ 905 strain did not show any growth whereas 906 strain showed a very weak growth.

3.2 Stress Application and Generations Obtained

Initial populations of 905 and 906 strains were passaged in liquid medium by increasing the stress concentration after each culture wherein an OD₆₀₀ value of 0.4 was reached. Considering the initial screening results; at a SO₂ concentration of 0.3 mM, 2 successive batch cultures were carried out initially with 906 strain in order to amplify the tolerant cells in the population. Cells obtained from these cultures were then used in sequential stress application process as described in 2.1.4.

3.2.1 Culture Tube Selections

SO₂ resistant mixed cultures were obtained according to the procedure described in 2.1.4.1. Corresponding amount of SO₂ solution was introduced to the medium throughout the passages in serial batch cultures. pH was adjusted to 3.65 by 3 M HCl solution prior to inoculation and the test tubes were left to incubate for 48 hours. At

the end of the 48 h, following the OD₆₀₀ readings and stock preparation, pH check was done and pH values as low as 2.65 were observed. The cells were exposed to the increased stress concentration in every two fresh cultures. Serial batch cultures were carried out to the SO₂ concentration at which no cell growth was observed. A final concentration of 1.1 mM of SO₂ in the medium could not be exceeded and regarded as the final stress level that the mutants were able to survive. The generations obtained from culture tubes were designated as FSD and the cells obtained from every 48 h cultures were regarded as a generation. 16 generations were obtained and named accordingly. The generations obtained and the stress concentrations which they were obtained from are given in Table 3.3.

Table 3.3: Survival ratios of generations with corresponding stress concentrations

Generation	[SO ₂] (mM)	Stress (OD ₆₀₀)	Control (OD ₆₀₀)	Incubation (hours)	Survival Ratio
FSD1	0.4	4.92	5.59	48	0.88
FSD2	0.5	5.06	5.36	48	0.94
FSD3	0.6	0.30	5.49	48	0.05
FSD4	0.5	5.26	5.30	48	0.99
FSD5	0.5	5.11	5.48	48	0.93
FSD6	0.6	4.64	5.01	48	0.93
FSD7	0.6	4.97	4.85	48	1.02
FSD8	0.7	3.31	4.49	48	0.74
FSD9	0.7	4.53	4.71	48	0.96
FSD10	0.8	4.53	4.93	48	0.92
FSD11	0.8	4.60	4.90	48	0.94
FSD12	0.9	0.90	5.77	48	0.16
FSD13	0.9	5.79	5.38	48	1.08
FSD14	1.0	0.40	5.38	48	0.07
FSD15	1.0	4.95	4.82	48	1.03
FSD16	1.1	4.19	4.68	72	0.89

The survival ratios upon each generation for continuous stress application were graphed and shown in Figure 3.1

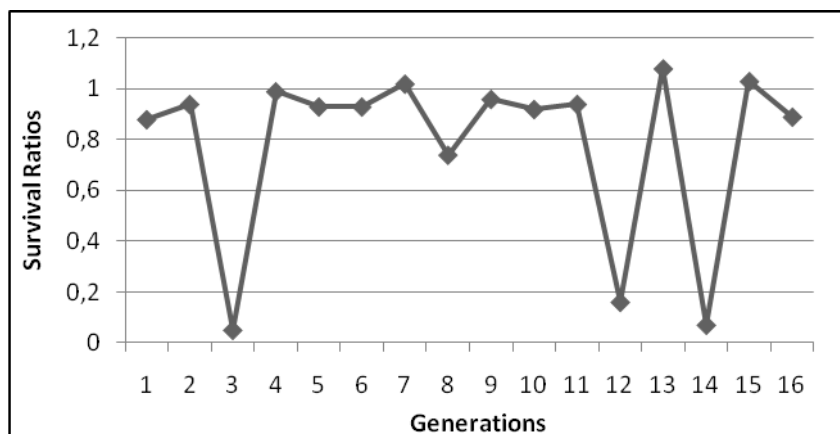


Figure 3.1: Survival ratios of culture tube generations.

3.2.2 Bioreactor Selections

Bioreactor selections were done in a 1 L bench-top bioreactor. pH values of the medium were kept constant throughout the whole process at 3.5. First three generations showed similar growth performance with culture tube generations. However, a plateau was reached at 0.6 mM and stress concentration was kept constant for 4 generations. Once the plateau was overcome same approach was assumed for 0.7 mM of SO₂. This concentration was the final concentration where the mutant generations were able to survive. The generations obtained from bioreactor were designated as BSD. Mutants showed poor general improvement compared to those obtained from culture tubes and a total of 10 generations were obtained (Table 3.4).

Table 3.4: OD₆₀₀ values of generation with corresponding stress concentrations

Mutants	[SO ₂] (mM)	Stress OD ₆₀₀	Control OD ₆₀₀	Incubation Time
BSD1	0,4	9,67	---	48
BSD2	0,5	3,83	12,8	48
BSD3	0,6	10	12,1	48
BSD4	0,6	8,76	---	48
BSD5	0,6	8,45	---	48
BSD6	0,6	4,78	---	48
BSD7	0,7	9	---	48
BSD8	0,7	6,79	---	48
BSD9	0,7	8,42	---	72
BSD10	0.7	6.63	---	48

3.3 Mutant Individual Selection by MPN Method

Serial dilutions of samples in the range of 10^{-3} - 10^{-5} were used on YMM-agar plates to reveal single colonies. Seven colonies were picked for each final population obtained from bioreactor and culture tubes. YMM media with a final concentration of 0.5 mM, 0.7 mM and 1 mM SO₂ were used for the procedure. Mutant individual selection was done as described in 2.2.5. Mutant survival ratios and survival ratios as fold of wild type values were evaluated to decide the final mutant individuals (Table 3.5).

Table 3.5: Survival ratios of mutant individuals after 24 h and 48 h of incubation.

Mutant individual	Survival Ratio			Incubation Time (h)
	0.5 mM [SO ₂]	0.7 mM [SO ₂]	1 mM [SO ₂]	
F1	0.500	0.657	1.143	24
F2	0.100	0.225	0.000	
F3	0.444	0.100	0.000	
F4	0.296	0.001	0.000	
F5	0.007	0.001	0.000	
F6	0.058	0.022	0.000	
F7	0.444	0.100	0.000	
B1	0.444	0.444	0.000	48
B2	0.150	0.002	0.000	
B3	0.261	0.003	0.000	
B4	1.000	0.000	0.000	
B5	0.041	0.003	0.000	
B6	1.000	0.000	0.000	
B7	0.708	0.010	0.000	
905	0.017	0.000	0.000	
F1	0.500	0.786	0.671	
F2	0.458	0.917	0.000	
F3	2.037	1.704	0.017	
F4	1.915	0.057	0.001	
F5	0.010	0.010	0.001	
F6	0.588	0.100	0.002	
F7	0.648	0.333	0.030	
B1	0.444	0.444	0.030	
B2	0.857	0.081	0.001	
B3	0.587	0.380	0.030	
B4	3.143	0.000	0.002	
B5	0.077	0.131	0.000	
B6	1.296	0.010	0.010	
B7	0.743	0.263	0.069	
905	0.018	0.023	0.000	

Survival ratios as fold of wild type for initial selections in 0.7 mM and 1mM SO₂ are given in Figure 3.2 and Figure 3.3 respectively.

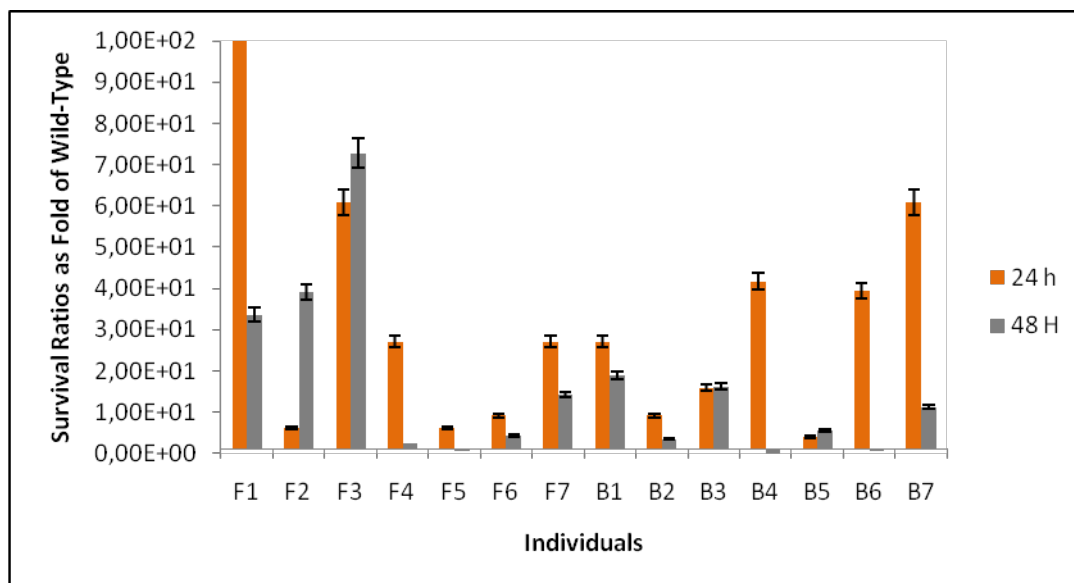


Figure 3.2: Survival ratios as fold of wild type in 0.7 mM SO₂ after 24 h and 48 h of incubation.

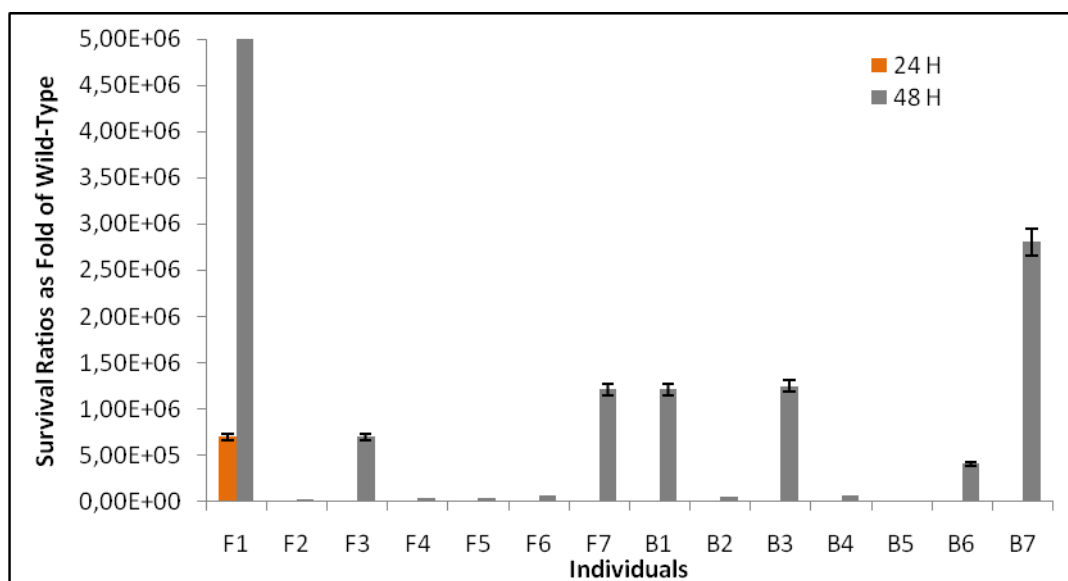


Figure 3.3: Survival ratios as fold of wild type in 1 mM SO₂ after 24 h and 48 h of incubation.

In order to select the preliminary mutant individual candidates the survival ratio and survival ratio as fold of wild type values were taken into consideration. Mutants were coded according to their source, i.e., individuals from culture tubes were designated as F while individuals from bioreactor were designated as B and a random number. More selective results were achieved in YMM media with a final concentration of

0.7 mM and 1 mM SO₂. F1 mutant showed 2.7E+07 fold increase in 1 mM SO₂, however this result could not be reproduced and F1 mutant was discarded. Individuals F3, F7, B1, B6 and B7 were selected for the secondary screening. 0.7 mM SO₂ was preferred for the procedure. The results for the secondary screening is given in Table 3.6, Figure 3.4 and Figure 3.5.

Table 3.6: Cell numbers and survival ratios of mutant individuals at 0.7 mM SO₂ after 24 h and 48 h of incubation.

Individuals	Cells/ml		Survival	Incubation Time
	0.7 mM SO ₂	Control		
F3	2.4E+02	3.5E+06	0.00007	24 h
F7	2.4E+02	2.4E+06	0.0001	
B1	2.4E+02	5.4E+06	0.00004	
B6	2.4E+02	2.4E+06	0.0001	
B7	2.3E+01	2.4E+06	0.00001	
905	2.3E+01	5.4E+06	0	
F3	1.6E+05	5.4E+06	0.03	48 h
F7	3.5E+04	7.0E+06	0.005	
B1	1.1E+05	5.4E+06	0.02	
B6	5.4E+05	3.5E+06	0.154	
B7	9.2E+03	3.5E+06	0.003	
905	2.3E+01	9.2E+06	0	

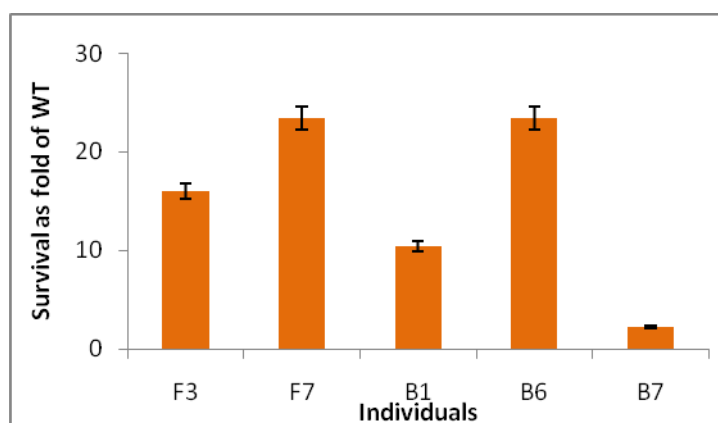


Figure 3.4: Survival as fold of wild type values after 24 h of incubation.

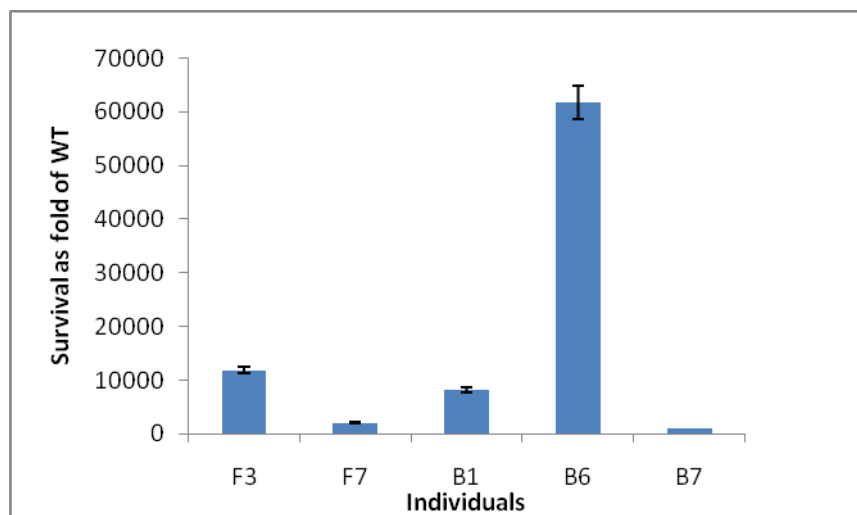


Figure 3.5: Survival as fold of wild type values after 48 h of incubation.

At the end of the screening process, mutant individuals F3, B1 and B6 showed the best growth performance under screening conditions. F3 represented the mutants obtained from culture tubes whereas B1 and B6 were selected among bioreactor mutants. Although survival ratios and survival as fold of wild type values of bioreactor individuals were higher than F3, this individual produced higher cell numbers if considered along with the first screening process under screening conditions. F3 also stood out as the most improved mutant as it could survive under higher stress levels.

3.4 Genetic Stability Determination

In order to determine the permanency of tolerance to SO_2 over generations, a genetic stability test was carried out. For this purpose, mutant individuals were grown in YMM medium without stress for 5 consecutive cultures. At the end of each 24 hours of incubation period, samples were taken and tested for their ability to survive in YMM medium with a final SO_2 concentration of 0.6 mM via MPN method. Survival ratios of mutants obtained at the end of each cultivation are given in Figure 3.6 and Table 3.7.

Table 3.7: Survival ratios of mutant individuals over 5 cycles of cultivation at 0.6 mM SO₂.

Cycle	Survival Ratio					
	24 h			48 h		
	F3	B1	B6	F3	B1	B6
1	0.154	0.026	0.100	1.314	0.120	0.146
2	0.100	0.263	0.100	0.907	0.400	0.444
3	0.044	0.011	0.004	0.296	0.246	0.017
4	0.146	0.000	0.001	0.250	0.007	0.007
5	0.015	0.000	0.000	0.462	0.017	0.010

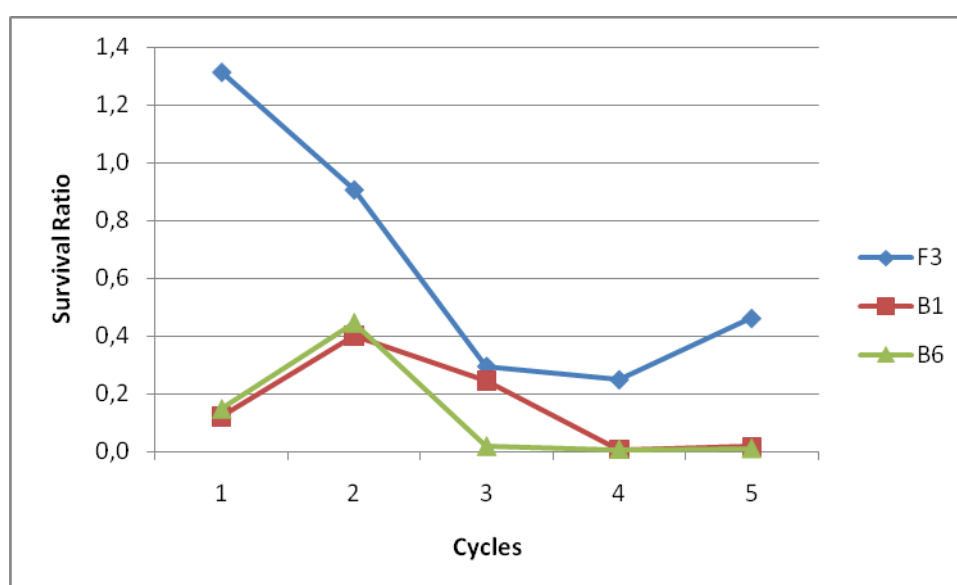


Figure 3.6: Graph of survival ratios after 48 h of incubation through 5 generations.

Mutant individuals B1 and B6 were genetically less stable and almost lost resistance to SO₂ at the end of 5 consecutive stress-free cultures. F3 individual showed rather better performance compared to bioreactor individuals B1 and B6 as the survival ratio dropped down under 60% and fluctuated between 20% - 60%.

3.5 Contamination Controls

F3, B1, B6 and 905 stocks to be used in characterization assays were checked for any contamination. Controls on Xylose-agar plate (Figure 3.7) and API ID 32C system were done as described in methods.

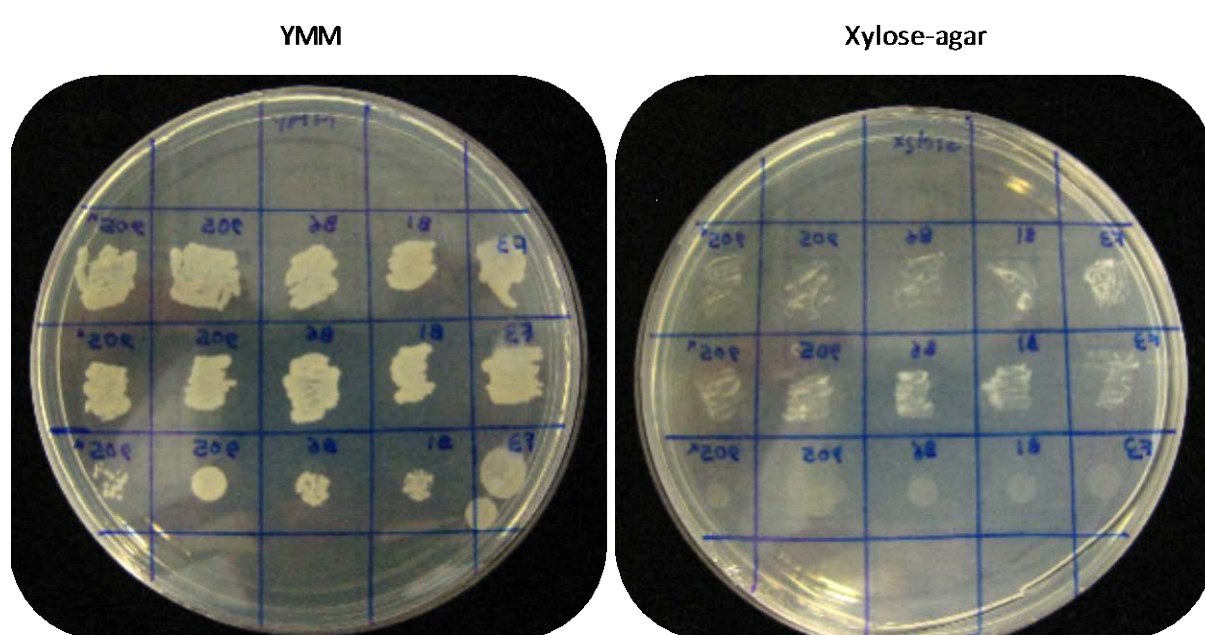


Figure 3.7: Images of samples from mutant and wild type overnight cultures grown on YMM and Xylose agar plates after 48 hours of incubation.

No growth was observed on xylose medium. API ID 32C test resulted in perfect identification of *Saccharomyces cerevisiae*. According to the results the stocks were not contaminated.

3.6 Diploidization Screening on Sporulation Medium

The yeast strains used in this study, 905 and 906, are haploid organisms. In order to see if at any generation or in mutant individuals a diploidization took place, generations obtained from culture tubes and bioreactor selections along with the mutant individuals were screened for diploidization. The samples were left to sporulate on KAC plate as described before. After 72 hours of incubation samples were observed under light microscope and tetrad forming samples were detected. The results showed that a diploidization occurred at the 6th generation, of bioreactor populations. No diploidization was detected in culture tube generations. A light microscope image of tetrads formed in KAC plate is given in Figure 3.8.

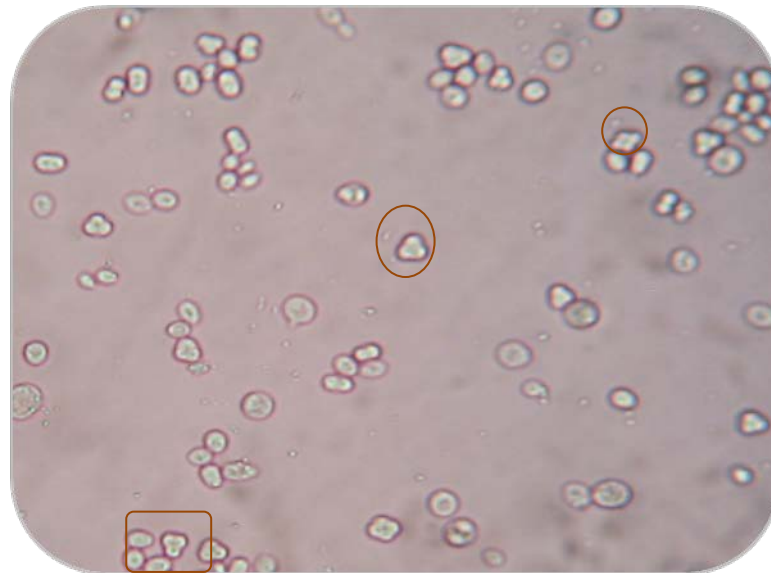


Figure 3.8: Light microscope image of tetrad formed by diploid mutants.

3.7 Characterization of Mutants

3.7.1 Cross Resistance Analysis on Solid Media

F3, B1 and B6 mutants were analyzed for their resistance against other stressors. Visual observation of growth performances under certain stress conditions on solid media was done as described in 2.2.9. Since the mutants obtained from bioreactor are diploids, a diploid wild type strain of the mutants was used. Diploid wild type was referred to as 905 (2n) while the haploid wild type was referred to as 905 (n).

Resulting plate images are given in Figure 3.9, Figure 3.10, Figure 3.11 and Figure 3.12.

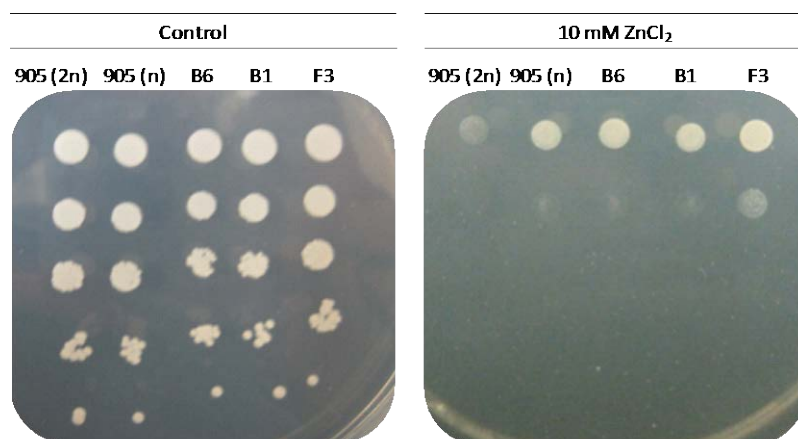


Figure 3.9: Image of the mutants and wild type strains grown on YMM plates without stress and with 10 mM ZnCl₂.

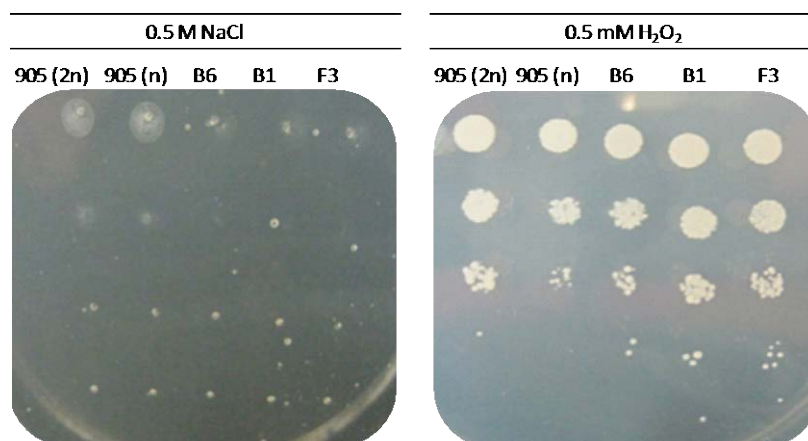


Figure 3.10: Image of the mutants and wild type strains grown on YMM plates with 0.5 M NaCl and 0.5 mM H₂O₂.

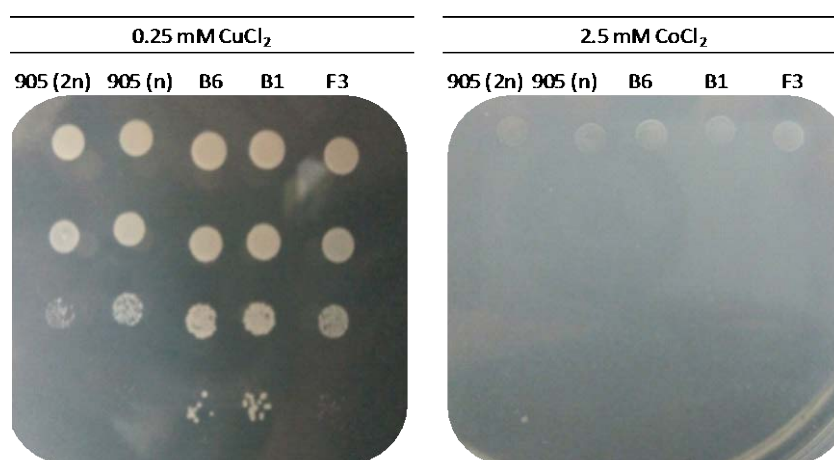


Figure 3.11: Image of the mutants and wild type strains grown on YMM plates with 0.25 mM CuCl₂ and 2.5 mM CoCl₂.

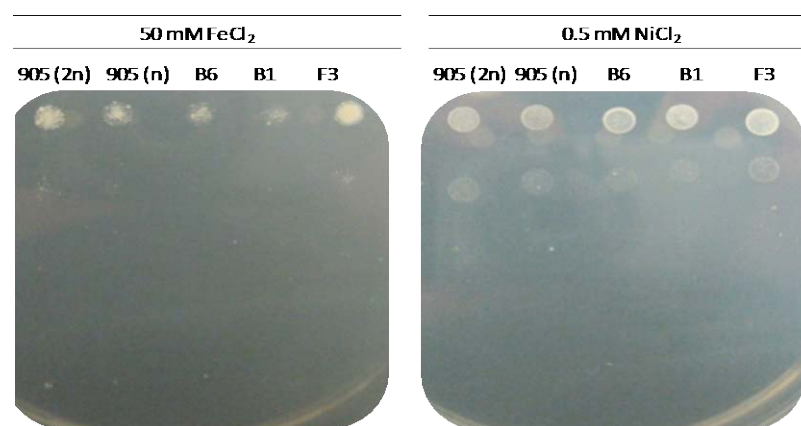


Figure 3.12: Image of the mutants and wild type strains grown on YMM plates with 50 mM FeCl₂ and 0.5 mM NiCl₂.

Judging by the growth results, it appears that none of the mutants were able to survive under minimum inhibitory levels of metal stresses except CuCl₂, compared to the wild type strains. Also no growth was observed under high osmotic stress levels, induced by 2 M Sorbitol and 0.5 M NaCl. Under oxidative stress conditions, inflicted by H₂O₂, all the mutants showed increased resistance. The mutants were scored according to the positive and negative readings. The results are summarized in Table 3.8.

Table 3.8: Estimation of resistance level as compared to wild type. “0”= No difference, “-“= sensitive, “+”= fairly resistant, “++”= noticeably resistant

	NiCl ₂ 0.5 mM	CoCl ₂ 2.5 mM	Sorbitol 2 M	NaCl 0.5 mM	CuSO ₄ 0.25 mM	H ₂ O ₂ 0.5 mM	ZnSO ₄ 10 mM	FeCl ₂ 50 mM
F3	0	0	0	-	+	++	+	0
B1	0	0	0	-	++	+	+	-
B6	0	0	0	-	++	+	+	-

3.7.2 Cross-Resistance Analysis in Liquid Media

Cross-resistance tests in liquid media were done as described in 2.2.10. For; heat, -196 °C and -20 °C tests stresses were applied as pulse. For ethanol and H₂O₂ both pulse and continuous and pulse stresses were applied. CuCl₂ and sodium selenite (SeL) stresses were applied continuously. The results and corresponding figures are below.

Cell numbers and survival ratios of mutants for continuous ethanol stress after 24 and 48 hours of incubation are given in Table 3.9. Survival ratio as fold of wild type values for 6% and 8% ethanol stress after 48 hours of incubation are given in Figure 3.13.

Table 3.9: Continuous ethanol stress results.

Mutant	24 h of Incubation			48 h of Incubation			EtOH (%)
	Cells/ml		Survival Ratio	Cells/ml		Survival Ratio	
	Cont	Stress		Control	Stress		
F3	5.4E+06	5.4E+04	0.01	5.4E+06	2.2E+06	0.41	6
B1	2.4E+06	2.4E+05	0.1	2.4E+06	2.4E+06	1.00	
B6	3.5E+06	2.4E+05	0.07	3.5E+06	5.4E+06	1.54	
905 (n)	4.6E+06	5.4E+04	0.01	7.0E+06	1.7E+06	0.24	
905 (2n)	3.5E+06	2.4E+05	0.07	3.5E+06	5.4E+06	1.54	
F3	9.2E+06	2.4E+02	0	9.2E+06	9.2E+05	0.10	8
B1	5.4E+06	2.4E+02	0	5.4E+06	9.2E+05	0.17	
B6	2.4E+06	2.4E+02	0	2.4E+06	3.5E+05	0.15	
905 (n)	2.4E+06	2.4E+02	0	9.2E+06	3.5E+05	0.04	
905 (2n)	3.5E+06	2.4E+02	0	3.5E+06	9.2E+05	0.26	

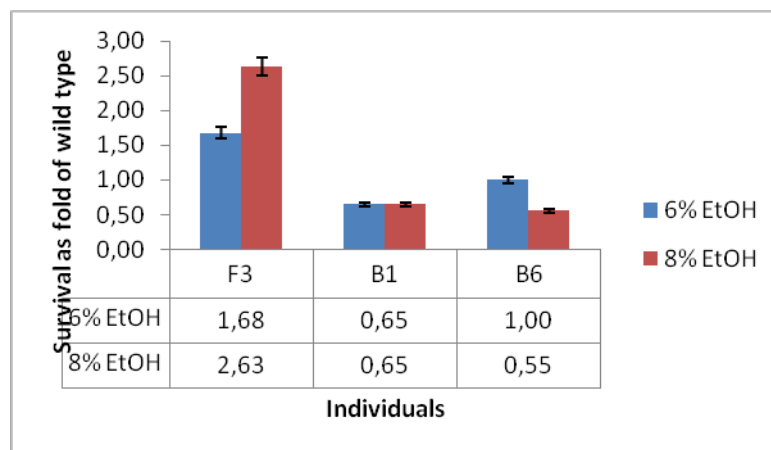


Figure 3.13: Survival ratio as fold of wild type values after 48 h of incubation.

Cell numbers and survival ratios under 20% pulse ethanol stress is given in Table 3.10. Survival ratio as fold of wild type values for %20 pulse ethanol stress are shown in Figure 3.14.

Table 3.10: Cell numbers and survival ratios for pulse ethanol stress.

Mutant	24 h of Incubation			48 h of Incubation		
	Cells/ml		Survival Ratio	Cells/ml		Survival Ratio
	Control	Stress		Control	Stress	
F3	9.2E+06	2.3E+01	0.000003	1.7E+07	1.7E+02	0.000010
B1	1.7E+06	2.3E+01	0.000014	2.6E+06	2.3E+01	0.000009
B6	2.4E+06	2.3E+01	0.000010	4.6E+06	2.3E+01	0.000005
905 (n)	5.4E+06	2.3E+01	0.000004	5.4E+06	2.3E+01	0.000004
905 (2n)	2.4E+06	2.3E+01	0.000010	4.6E+06	2.3E+01	0.000005

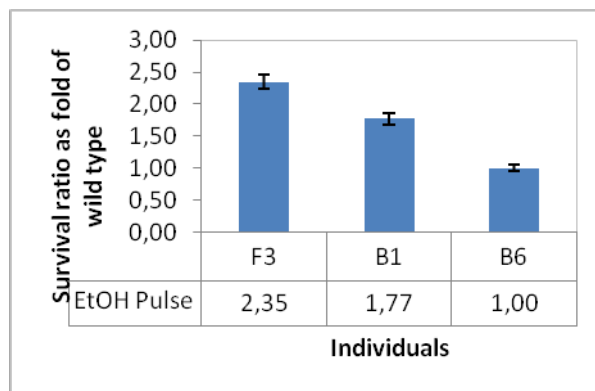


Figure 3.14: Survival ratio as fold of wild type values under %20 pulse ethanol stress after 48 h of incubation.

Cell numbers and survival ratios of the mutants after 24 and 48 hours of incubation under 0.5 mM continuous H_2O_2 stress are given in Table 3.11.

Table 3.11: Cell numbers and survival ratios for continuous H_2O_2 stress.

Mutant	24 h			48 h		
	Cells/ml		Survival Ratio	Cells/ml		Survival Ratio
	Control	Stress		Control	Stress	
F3	5.4E+06	9.2E+05	0.17	5.4E+06	2.2E+06	0.41
B1	2.4E+06	2.4E+06	1.00	2.4E+06	9.2E+06	3.83
B6	3.5E+06	1.1E+07	3.14	3.5E+06	1.1E+07	3.14
905 (n)	4.6E+06	2.4E+06	0.52	7.0E+06	2.4E+06	0.34
905 (2n)	3.5E+06	3.5E+06	1.00	3.5E+06	3.5E+06	1.00

Survival as fold of wild type values at 0.5 mM H₂O₂ continuous stress after 48 hours of incubation are shown in Figure 3.15.

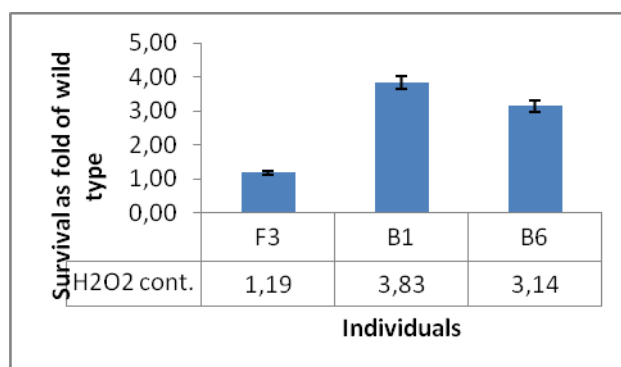


Figure 3.15: Survival as fold of wild type values after 48 h of incubation under continuous H₂O₂ stress.

No growth was observed after 72 hours of incubation for 0.3 mM H₂O₂ pulse stress application.

Cell numbers and survival ratios of mutants after 24 and 48 hours of incubation for heat stress exposure are given in Table 3.12.

Table 3.12: Cell numbers and survival ratios after heat stress exposure

Mutant	24 h			48 h		
	Cells/ml		Survival Ratio	Cells/ml		Survival Ratio
	Control	Stress		Control	Stress	
F3	9.2E+06	1.6E+04	0.0017	9.20E+06	1.70E+04	0.0018
B1	5.4E+06	2.3E+01	0.0000	5.40E+06	2.30E+01	0.0000
B6	2.4E+06	3.1E+02	0.0001	2.40E+06	4.60E+02	0.0002
905 (n)	2.4E+06	2.3E+01	0.0000	9.20E+06	2.30E+01	0.0000
905 (2n)	3.5E+06	2.3E+01	0.0000	3.50E+06	2.30E+01	0.0000

Survival ratio as fold of wild type values upon heat stress application after 48 hours of incubation is given in Figure 3.16.

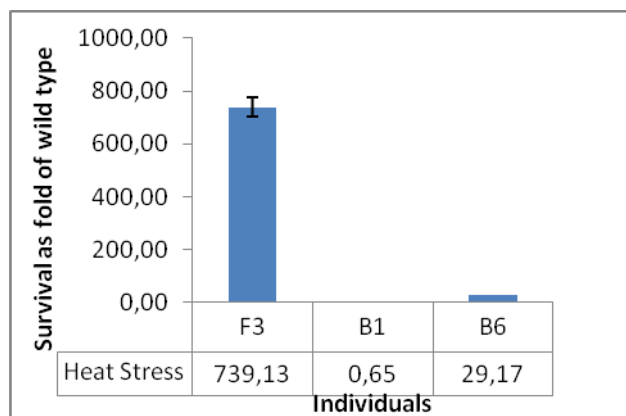


Figure 3.16: Survival ratio as fold of wild type values after 48 h of incubation for heat stress.

Cell numbers and survival ratios under 0.15 mM CuCl₂ after 24 and 48 hours of incubation are given in Table 3.13.

Table 3.13: Cell numbers and survival ratio values for 0.15 mM CuCl₂ stress.

Mutant	24 h			48 h		
	Cells/ml		Survival Ratio	Cells/ml		Survival Ratio
	Control	Stress		Control	Stress	
F3	9.2E+06	2.4E+02	0.0000	9.2E+06	2.4E+03	0.0003
B1	1.6E+06	2.4E+03	0.0015	1.6E+06	2.4E+05	0.1500
B6	3.5E+06	2.4E+03	0.0007	3.5E+06	5.4E+03	0.0015
905 (n)	1.6E+07	2.4E+02	0.0000	1.6E+07	1.6E+03	0.0001
905 (2n)	5.4E+06	2.4E+02	0.0000	5.4E+06	2.4E+02	0.0000

Survival ratio as fold of wild type values of the mutants after 48 hours of incubation with 0.15 mM CuCl₂ is shown in Figure 3.17

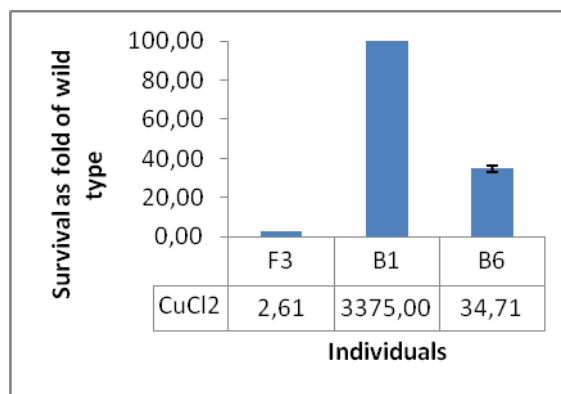


Figure 3.17: Survival ratio as fold of wild type values after 48 h of incubation under 0.15 mM CuCl₂.

Survival ratios for mutants and wild type cells grown under 1 mM of sodium selenite (SeL) after 24 and 48 hours of incubation are given in Table 3.14.

Table 3.14: Cell numbers and survival ratios of cells grown under 1 mM SeL after 24 hours of incubation.

Mutant	24 h		
	Cells/ml		Survival Ratio
	Control	Stress	
F3	9.2E+06	1.6E+07	1.7391
B1	1.6E+06	3.5E+06	2.1875
B6	3.5E+06	5.4E+06	1.5429
905 (n)	1.6E+07	2.4E+06	0.1500
905 (2n)	5.4E+06	2.4E+06	0.4444

Survival as fold of wild type values for 1 mM SeL stress after 24 hours of incubation are shown in Figure 3.18.

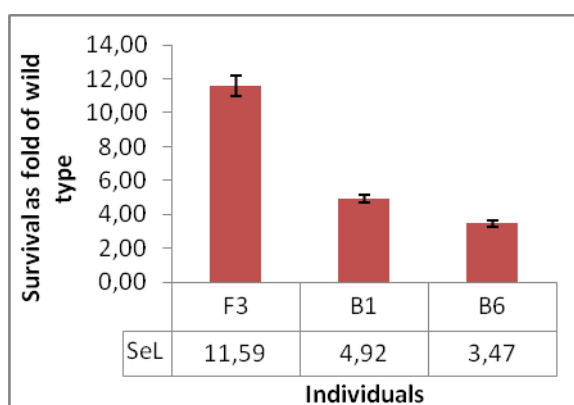


Figure 3.18: Survival as fold of wild type values after 24 h of incubation for 1 mM SeL stress.

Cell numbers and survival ratio values of mutants following -196 °C stress application after 48 and 72 hours of incubation are given in Table 3.15.

Table 3.15: Cell numbers and survival ratio values for -196 °C stress after 48 and 72 h of incubation.

Mutant	48 h			72 h		
	Cells/ml		Survival Ratio	Cells/ml		Survival Ratio
	Control	Stress		Control	Stress	
F3	1.7E+07	3.1E+04	0.0018	1.7E+07	3.1E+04	0.0018
B1	2.6E+06	1.3E+03	0.0005	2.6E+06	2.4E+03	0.0009
B6	4.6E+06	1.1E+02	0.0000	4.6E+06	1.7E+02	0.0000
905 (n)	5.4E+06	4.6E+03	0.0009	5.4E+06	4.6E+03	0.0009
905 (2n)	4.6E+06	5.4E+02	0.0001	4.6E+06	5.4E+02	0.0001

Survival ratio as fold of wild type comparison of mutants after 48 and 72 hours of incubation following -196 °C stress application is shown in Figure 3.19.

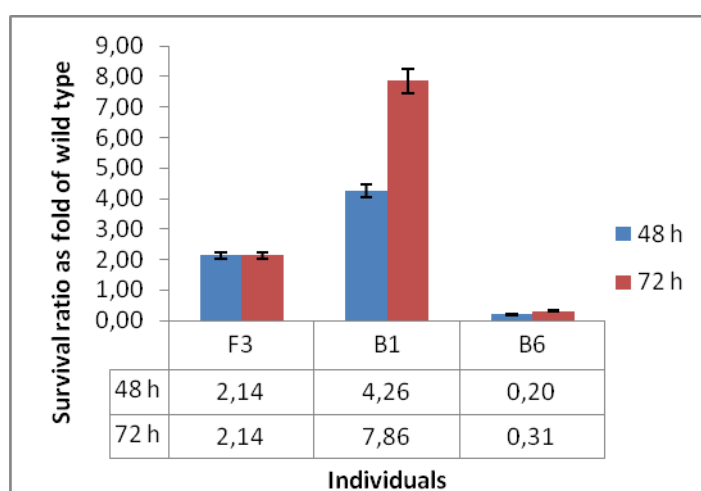


Figure 3.19: Comparison of survival ratio as fold of wild type values of mutants for -196°C stress.

Survival ratios and cell numbers after exposure to cold stress (-20°C) at 24 hours and 48 hours are given in Table 3.16

Table 3.16: Survival ratio and cell number values after 24 h and 48 h of incubation for cold stress.

Mutant	24 h			48 h		
	Cells/ml		Survival Ratio	Cells/ml		Survival Ratio
	Control	Stress		Control	Stress	
F3	9.2E+06	3.5E+06	0.38	1.7E+07	2.8E+06	0.16
B1	1.7E+06	2.4E+06	1.41	2.6E+06	2.4E+06	0.92
B6	2.4E+06	2.4E+06	1.00	4.6E+06	5.4E+06	1.17
905 (n)	5.4E+06	3.5E+06	0.65	5.4E+06	3.5E+06	0.65
905 (2n)	2.4E+06	3.4E+05	0.14	4.6E+06	1.6E+06	0.35

Survival as fold of wild type values after 48 hours of incubation for cold stress are shown in Figure 3.20.

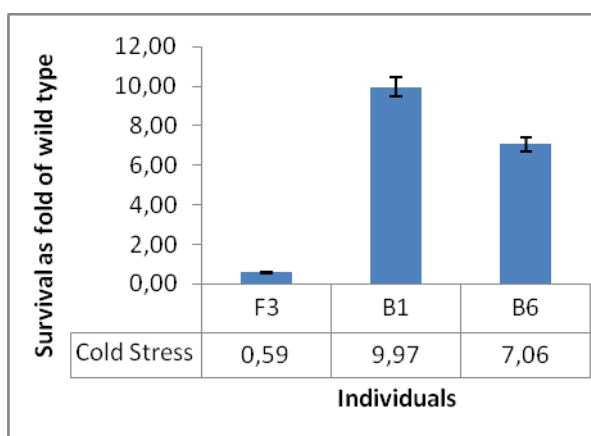


Figure 3.20: Survival as fold of wild type values for cold stress after 48 h of incubation.

Survival ratios and cell numbers recorded for mutants grown under 0.4 M NaCl stress after 48 and 72 hours of incubation are given in Table 3.17.

Table 3.17: Cell numbers and survival ratio values of mutants under NaCl stress after 48 and 72 h of incubation.

Mutant	48 h			72 h		
	Cells/ml		Survival Ratio	Cells/ml		Survival Ratio
	Control	Stress		Control	Stress	
F3	5.4E+06	2.4E+02	0.000	5.4E+06	2.4E+03	0.000
B1	2.4E+06	2.4E+02	0.000	2.4E+06	2.4E+04	0.010
B6	3.5E+06	2.4E+02	0.000	3.5E+06	2.4E+04	0.007
905 (n)	4.6E+06	2.4E+02	0.000	9.4E+06	1.6E+05	0.017
905 (2n)	3.5E+06	2.4E+02	0.000	3.5E+06	2.4E+04	0.007

Survival as fold of wild type values at the end of 72 hours of incubation under NaCl stress are graphed in Figure 3.21.

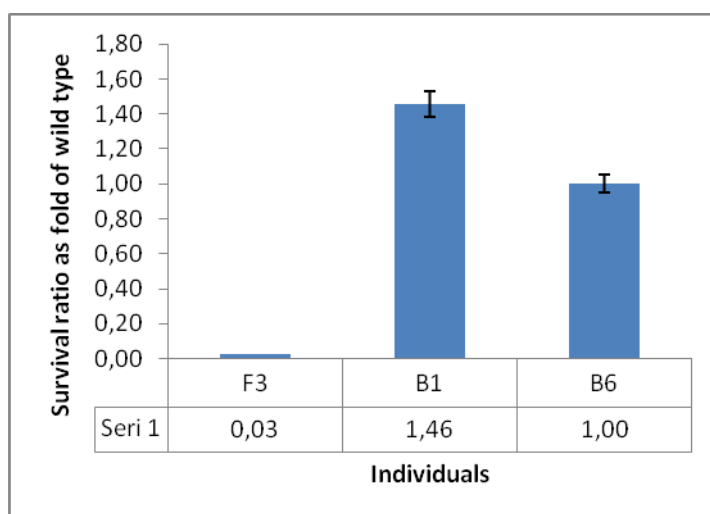


Figure 3.21: Survival ratio as fold of wild type values for NaCl stress after 72 h of incubation.

4. DISCUSSION AND CONCLUDING REMARKS

In this study, an experimental set-up established in previous studies (Cakar et al., 2005) was redesigned and optimized for obtaining sulphur dioxide resistant *Saccharomyces cerevisiae* cells. Inverse metabolic engineering strategy formed the basis of the study where a set of evolutionary engineered mutants were used to gain insight into the mechanism underlying the stress response. In this context, a genetically diverse initial population was generated by chemical mutagenesis of a haploid laboratory strain of *Saccharomyces cerevisiae*. Ethyl methane sulphonate (EMS) was used as the mutagenizing agent.

In order to determine the preliminary stress level to be used in selection process, chemically mutagenized cells and wild type cells were screened in a range of sulphur dioxide concentrations. Since SO_2 is a pH dependent compound, cells were exposed to 0.5 mM SO_2 at varying pH values prior to screening process. The goal here was to see the pH effect on the stressor and to have a reference point for deciding the concentration range in the later stages. As expected, sulphur dioxide was the most effective at pH 3.5 and 0.5 mM SO_2 was lethal at this pH. Considering this, pH was lowered in screening application. However, the procedure did not yield favourable results as the minimum inhibitory SO_2 concentration was found to be 0.3 mM. This result was rather low when compared to the haploid strains studied in the literature (Xu et al., 1994) in fact it corresponded to the levels at which the sensitive mutants were isolated. Even in this concentration not a completely distinctive growth performance was observed for 906 strain compared to 905. The results also might indicate that mutations induced by EMS which are in favour of SO_2 tolerance are at a very low frequency or not in directly related pathways.

It is known that yeast cells grown in a glucose based medium cause a decrease in pH of the medium. In case of the strains used in this study, pH levels as low as 4.5-5 were encountered in cultures grown in YMM. Considering this and the fact that sulphur dioxide becomes more effective and available in the medium at low pH values, in a medium with a pre-adjusted acidic pH, a decrease in pH value would

result in enhanced toxicity (Pilkington & Rose, 1988) This situation applies to the cultures grown in test tubes where no interference on pH is in question. Although it is a desirable condition in terms of multiple-resistance development and could provide supplemental results, an additional acid stress might affect the efficiency of process. In connection with pH phenomena, a parallel selection procedure was adopted. According to this, selections were carried out parallelly in culture tubes under inconstant pH and in a bench-top bioreactor under constant pH. In bioreactor selections, pH value was kept constant at 3.5. On the other hand, in culture tube selections pH was initially adjusted to 3.5-3.7, however, pH values as low as 2.65 was measured after 48 hours of incubation.

Moving from the screening results, the initial 906 strain was serially cultured for 2 generations at 0.3 mM SO₂ in order to amplify the number of relatively resistant cells. Following this initial cultivations, resistant mutant generations were selected in culture tubes and bioreactor separately. Generations in both sources showed a rapid improvement for the first 2 generations; however, after this point there was a drop in survivals. Concentrations were increased in every 2 generations by 0.1 mM increments in culture tubes where 4 cultures at constant concentration were preferred in bioreactor. The selection process was ended at the concentration after which no survival was observed. From bioreactor cultures a total of 10 generations were obtained with a maximum resistance concentration of 0.7 mM SO₂. Culture tube selections yielded 16 generations under the last stress concentration of 1.1 mM SO₂. The last level of tolerance achieved in both processes still remained under the literature levels (Casalone et al., 1994; Xu et al., 1994). However, over two fold of tolerance increase in bioreactor and approximately three fold of tolerance increase in culture tube generations relative to the initial levels could not be overlooked.

Following the selections, randomly picked individuals from the last mutant populations were screened under varying amounts of SO₂ concentrations. F3, F7, B1, B6 and B7 were selected, in relation to their survival ratios under 0.7 mM and 1 mM SO₂, for a secondary screening. Among these mutant individuals F3, B1 and B6 were defined to be the most promising mutants. Since the screening results were conducted under conditions, resembling that of culture tube selections, similar survival ratios shown in screening process between F3, B1 and B3 indicates that

constant pH might not be the main obstacle against adaptation to higher levels of stress.

Prior to the further characterization, mutants were observed under light microscope for any morphological changes. A distinctive increase in cell size was observed for mutants B1 and B6. As a possible reason of cell enlargement, these mutants were tested for their sporulation ability in potassium acetate (KAC) plate in order to determine ploidy. Sporulation assay yielded positive results for both B1 and B6 whereupon all the mutant individuals and populations were screened for sporulation. No diploidization was observed among culture tube mutants and populations where all the populations and mutants were diploid after the 6th generation of bioreactor cultures. It was not surprising to see that the appearance of diploids coincided with the increase of stress concentration from 0.6 mM to 0.7 mM. This diploidization clearly conferred advantage to the cells in order to overcome the stress pressure. It also should be noted that a chromosomal rearrangement is evident in SSU1-R gene of wine yeasts (Goto-Yamamoto et al., 1998). This gene is associated with dramatically high levels of sulphite resistance. In the light of these, it may be inferred that a chromosomal rearrangement mechanism is being activated under sulphite stress with certain environmental conditions.

The clear difference of improvement between the generations of different sources could be attributed to several reasons among which a higher pH value stands out. A constant pH of 3.5 results in a decreased concentration of molecular SO₂ which is the most effective form of sulphites, against microorganisms in aqueous solutions (Stratford & Rose, 1986). Together with the pH effect, Polypropylene Glycol 2000 (PPG) is used as an anti-foam agent in bioreactor and it could affect the available concentration of molecular SO₂. Sulphite equilibrium in solutions are shown to be displaced in the direction of “HSO₃⁻” ion, which has no antimicrobial effect, with addition of non-electrolytes like ethanol and PPG. However, as mentioned above, similar survival ratios under inconstant pH conditions showed by mutant individuals might indicate a different mechanism interfering the improvement process. A possible factor could be oxidative stress; since, in bioreactor, a better O₂ distribution is possible which in turn leads to an increase in respirative metabolism of cells. As more ROS produced during higher respiratory activity they bind to the same intracellular targets with sulphites in a competitive manner. This situation could well

lead to a decrease in levels of sulphite binding molecules thus increased toxicity. Addition to those, an increased cell surface in diploid cells could provide more protein targets on membrane, thus, sulphur dioxide might inflict more severe damage to the cell (Schimz, 1980). If it was to be considered in an industrial methodological context, the last concentrations survived under different conditions obviously revealed the difference in effectiveness of these two approaches. Besides having produced a lower maximum resistance level, the difficulties encountered during the setting of bioreactor along with the other issues like spillage and mechanical problems proved this procedure to be inefficient. On the other side, from an analytical standpoint, analyzing different phenotype manifestations of two different mutants of the same stress could provide new footprints for underlying mechanisms. Cross-resistance analysis allowed further characterization on mutants. Cross-test results on solid media led to the conclusion that none of the mutants were resistant to metal stresses except copper. Mutants showed relatively better growth under 0.5 mM H₂O₂. Liquid cross-resistance analysis gave more significant results. Most remarkable improvements were observed under copper, selenite, heat and -196 °C stress conditions. According to these results, as confirmed by the solid media analysis, B1 and B6 mutants showed significant cross-resistance. In this regard, B1 showed survival values in the range of thousands-fold where B6 showed almost forty fold of wild-type. F3, on the contrary, was only improved by 2.5 fold. Another important result was the improved survivals under 1 mM sodium selenite (SeL). Selenite resistance results were parallel with SO₂ resistance of the mutants where F3 mutant showed up to 13 fold increase in survival ratio compared to the wild type. Other important resistance changes were that of F3 under heat stress by eight hundred folds and that of B1 under -196 °C stress.

When these results are evaluated together, they seem to fit in a frame of resistance mechanism mediated by oxidative response and *ssu1p*. As the elevated copper resistance in mutants B1 and B6 with putatively increased respiratory activity hints at the oxidative response mechanisms, the increases in -196 °C and cold stress (-20) resistances strengthens this hypothesis. Heat stress responses are also consistent with the possible mechanism helping these cells to cope with the stress as heat shock proteins could be aiding the cell in fixing the protein targets of SO₂ (Avram et al., 1999). Above all, selenite resistance elevations in proportion with SO₂ tolerance

capacity of the mutants support the assumption that these mutants activated different mechanisms. This assumption is based on the fact that mutants overexpressing SSU1 shows increased resistance to selenite (Park & Bakalinsky, 2000). Yet it can be inferred that FZF1, the gene coding for the transcription factor of SSU1, has different regulation pathways in mutants obtained under different conditions. Above all, it has to be born in mind that these mutants have a poor genetic stability and these cross-resistances might also be transient manifestations. If so, it is possible that sulphur dioxide induces general stress response pathways via Msn2/4 or Yap1/2 transcriptional regulators.

To sum up, sulphur dioxide resistant mutants were successfully obtained under controlled and uncontrolled growth conditions with an evolutionary engineering approach. These mutants showed different cross-resistance patterns and chromosomal make-up. These different patterns of stress responses could be used to design molecular studies for elucidating underlying mechanisms. As the study suggests a strong relation between oxidative stress response mechanisms and sulphite resistance mechanisms a transcriptomic analysis aimed at genes involved in these pathways might prove useful. To further investigate the impact of oxidative metabolism on the SO₂ tolerance, mutants could be obtained and characterized under microaerobic or anaerobic conditions. As the initial tolerance levels and the achieved tolerance levels were relatively lower compared to the literature, strains used in the study might be carrying genes causing sensitivity. In the following studies, identification and cloning of these genes might reveal novel interlinking mechanisms.

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CURRICULUM VITAE



Candidate's full name: Aziz Kaan KORKMAZ

Place and date of birth: Istanbul – 26.12.1983

Permanent Address: Istanbul Evleri Koza 19 D.24 Bahcelievler /
ISTANBUL

**Universities and
Colleges attended:**

B.Sc. Biology

Istanbul University (2003-2007)

**M.Sc. : Molecular Biology Genetics and
Biotechnology**

Istanbul Technical University (2008-2011 expected)

Presentations:

Korkmaz, Aziz Kaan , Yilmaz, Ulku, Sezgin, T., Ercan, Onur, Cakar, Z. P.
“Evolutionary Engineering of Sulphur Dioxide Resistant *Saccharomyces cerevisiae*” *The 4th International Conference on Physiology of Yeast and Filamentous Fungi (PYFF4)*, Rotterdam, The Netherlands, 1-4 June, 2010 (Poster Presentation)