ISTANBUL TECHNICAL UNIVERSITY ★ INSTITUTE OF SCIENCE AND TECHNOLOGY

EVOLUTIONARY ENGINEERING OF METAL-BINDING/METAL RESISTANT YEAST FOR BIOMIMETICS

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EVRİMSEL MÜHENDİSLİK YÖNTEMİ İLE METALE-BAĞLANAN/METALE-DİRENÇLİ MAYALARIN BİYOBENZETİM AMAÇLI GELİŞTİRİLMESİ

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ABBREVIATIONS

GRAS	: Generally regarded as safe
MIC	: Minimal inhibitory concentration
EMS	: Ethyl methane sulphonate
YPD	: Yeast comlex medium
YMM	: Yeast minimal medium
HPLC	: High performance liquid chromatography
FAAS	: Flame atomic absorption spectroscopy
MPN	: Most probable number
CDW	: Cellular dry weight
MT	: Metallothionein
GSH	: Reduced gluthathione
GS-SG	: Oxidized gluthathione
ATP	: Adenosinetriphosphate
SPB	: Spindle pole body

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EVRİMSEL MÜHENDİSLİK YÖNTEMİ İLE METALE BAĞLANAN / METALE DİRENÇLİ MAYALARIN BİYOBENZETİM AMAÇLI GELİŞTİRİLMESİ

ÖZET

Bu çalışmada amaç, bir tersine metabolizma mühendisliği stratejisi olan evrimsel mühendislik yöntemi ile kobalta bağlanabilen/kobalta dirençli *S. cerevisiae* hücreleri elde etmektir. Bu amaçla, yaban tip hücreler, bir kimyasal mutajen olan etil metan sulfonat (EMS)'ye maruz bırakılmıştır. Mutasyon ile elde edilen genetik çeşitliliği artırılmış hücrelere, sabit düzeyde ve dereceli olarak artan düzeylerde olmak üzere iki ayrı stres seleksiyon stratejisi uygulanmıştır. Her aşamada hayatta kalan bireylerin bir sonraki nesile aktarılması ile seçim stratejileri nesiller boyunca devam ettirilmiştir. Hem sabit stres uygulaması hem de dereceli olarak artan stres uygulaması ile 36'şar nesil elde edilmiştir. Elde edilen son popülasyonlardan rastgele seçim ile mutant bireyler elde edilmiştir. Bireylerin karakterizasyonu için en muhtemel sayı (Most Probable Number, MPN) yöntemi ile bireylere ait stres sonrası hayatta kalma değerleri istatistiksel olarak belirlenmiştir. Herhangi bir çapraz direnç kazanımı olup olmadığını incelemek amacıyla kobalta dirençli bireyler krom, bakır ve çinko gibi metal stresleri altında ve maya biyoproseslerinde sıkça karşılaşılan oksidatif, etanol, ozmotik ve sıcaklık stresleri altında test edilmişlerdir. Bunların yanısıra, bireylerin ve popülasyonların bir stres metaboliti olan trehalozu biriktirme durumları HPLC ile incelenmiştir. Hücre içinde ve/veya hücre yüzeyine tutunmuş olarak bulunan metal varlığının incelenmesi için ise alev atomik absorpsiyon spektroskopi (FAAS) yöntemi uygulanmıştır.

Özetle, evrimsel mühendislik yöntemi başarı ile uygulanmış ve kobalta dirençli ve buna paralel olarak çapraz direnç geliştirmiş bireyler elde edilmiştir. Buna ek olarak, kobalta dirençli bireylerin hücre içinde veya hücre yüzeyine tutunmuş durumda kobaltı biriktirdikleri tesbit edilmiştir. Ancak, bakıra ve kroma dirençli bireylerin bu metalleri hücreden dışarı atarak/salgılayarak direnç gösterdikleri tahmin edilmektedir. Transkriptomik ve proteomik analizler ve araştırmalar strese karşı direnç mekanizmalarının daha iyi anlaşılmasında ve biyoarıtım ve/veya biyobenzetim uygulamalarında kullanımında faydalı olacaktır.

EVOLUTIONARY ENGINEERING OF METAL-BINDING / METAL-RESISTANT YEAST FOR BIOMIMETICS

SUMMARY

In this study, the aim was to obtain cobalt-binding/cobalt-resistant *S. cerevisiae* cells by using an inverse metabolic engineering strategy; evolutionary engineering. For this purpose, wild-type cells were firstly exposed to ethyl methane sulphonate (EMS) mutagenesis in order to increase the genetic diversity. Serial stress applications, either at constant or gradually increasing levels, were performed. Survivors of a step were passed to the following step and stress selection strategy was applied over generations of populations. The final populations were obtained as the result of both the increasing stress selection strategy and the constant stress selection strategy after 36 generations under stress conditions. The final populations were used to obtain individual mutants by random selection on solid media. The resulting individuals were characterized by determining the percent survival values of the individuals in reference to wild-type by most probable number (MPN) method statistically. Several other stress conditions were tested as well in order to test if any cross-resistances were gained by the cobalt-resistant individuals, such as resistance to other heavy metal stresses (chromium, copper, zinc) and other stress conditions frequently encountered in yeast bioprocesses such as oxidative, ethanol, osmotic and heat stresses. Moreover, accumulation of trehalose, an important stress metabolite, was investigated in the populations and individuals by HPLC. The metal contents associated with the cells were determined by flame atomic absorption spectroscopy.

To summarize, by designing and using evolutionary engineering strategies, cobalt-resistant individual cells were successfully obtained with concomitant cross-resistances towards other stress conditions. Additionally, they were found to accumulate cobalt bound to or internalized by cells. However, copper and chromium seems to be pumped out / secreted by the resistant cells as a resistance mechanism. The transcriptomic and proteomic analysis and further investigations may help to better understand the mechanism of stress resistance and ultimately exploit it for bioremediation and/or biomimetics application.

1. INTRODUCTION

1.1 *Saccharomyces cerevisiae*: Brief information

The yeast *Saccharomyces cerevisiae,* also commonly known as baker's yeast or budding yeast, is a unicellular eukaryote. The systematic classification of this single-celled fungus is given in Table 1.1.

Table 1.1 Systematic classification of *S. cerevisiae*

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

S. cerevisiae cells are oval shaped cells with dimensions around 10 μm long and 5 μm wide. They have thick, tough cell walls like other fungi. However, they are relatively immobile and involve mitochondria but not chloroplasts (Alberts, *et al*, 2002).

Culturing *S. cerevisiae* cells are considered to be easy and relatively inexpensive. They can grow in both liquid and solid media with only simple nutritional needs. A reduced carbon source (as simple as acetate), a nitrogen source (such as ammonium sulfate, urea or various amino acids), vitamin biotin, salts and trace elements are required for their growth. When the nutrients are available in growth media, they grow and reproduce as rapidly as bacteria (Alberts, *et al*, 2002).

S. cerevisiae cells can reproduce either vegetatively or sexually. Vegetative reproduction occurs by budding. During budding, a small bud emerges from the surface of the parent cell and enlarges. DNA replication occurs and distribution of chromosomes to the new cell is determined by the spindle pole body (SPB)

formation. The division of the nucleus is initiated by division signal of SPB. One set of the chromosomes are transferred to the bud from the parent cell and then the two cells are separated. At the beginning, the daughter cell is smaller than the mother cell. In spite of this biochemical inequality, both cells are genetically identical (Solomon, 1999; Lodish, *et al.*, 1995).

The number of buds formed by each parent cell is about 20-30 during the lifetime of one parent cell. Thus, the age of the parent cell can be determined by the number of bud scars formed on the cell wall. A typical budding cell and the bud scars on the parent cell are shown in Figure 1.1.

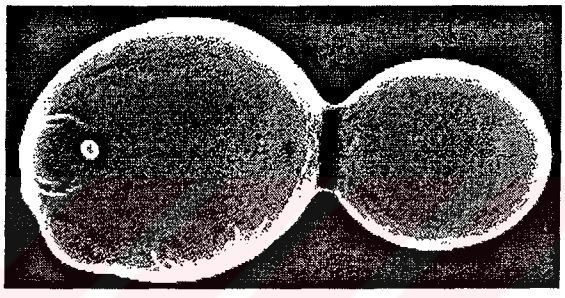


Figure 1.1 Scanning electron micrograph of budding yeast (Madigan, *et al.*, 2003)

Sexual reproduction occurs by two haploid cells fusing and forming a diploid cell. There are two mating types in sexual reproduction of yeast cells; MATa and MATα. The mating type is determined by the genetic composition of the MAT locus on chromosome III at which either gene a or gene α can be inserted. Each mating type secretes special pheromones and responds to the pheromones of the opposite type by being arrested in G1 phase of cell cycle and changing its shape, elongating and forming a pear-shaped non-dividing distinctive cell, which is called a "shmoo" (Figure 1.2). Vegetative cells can only differentiate into shmoos when the opposite mating type is present. Two shmoos are fused at their small ends and form a zygote with diploid nucleus. These stages can be observed under microscope. In contrast with higher plants and animals, *S. cerevisiae* cells can divide indefinitely in either the haploid or diploid state. Transition between these states is possible (Alberts, *et al*, 2002).

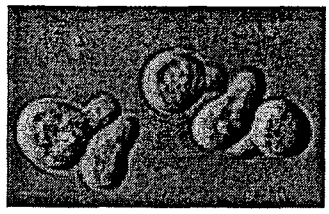


Figure 1.1 Shmoo formation in *S. cerevisiae* cells (Alberts, *et al.*, 1998)

S. cerevisiae cells can be maintained as either heterothallic or homothallic strains. Heterothallic strains can either be in diploid form or haploid form, while homothallic strains are only found in diploid form (Figure 1.3). Homothallic strains are the ones carrying an active HO gene. They are capable of switching their mating type by changing the genetic composition of the MAT locus to the opposite mating type (Madigan, *et al.*, 2003).

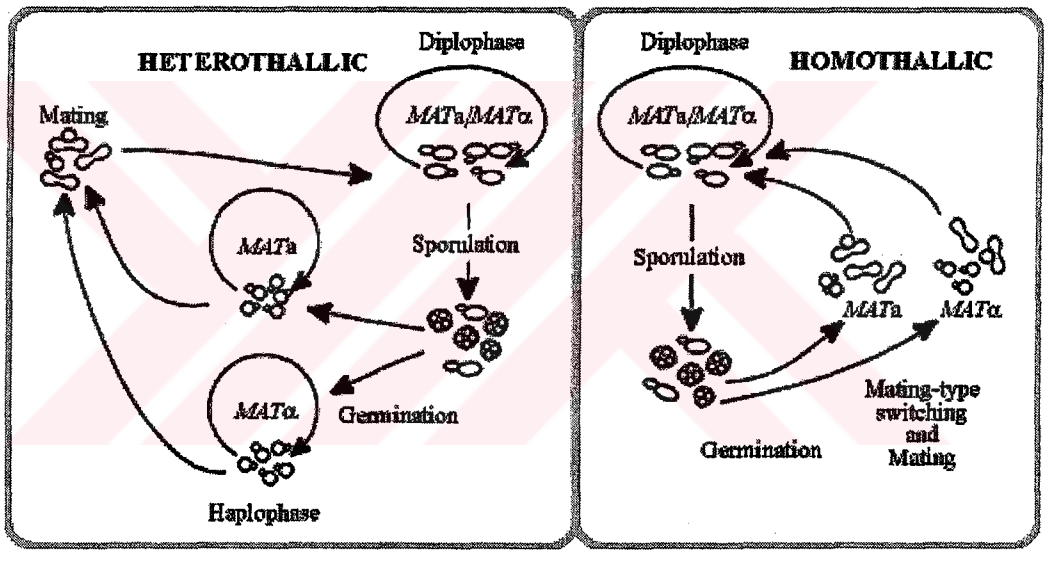


Figure 1.2 Life cycles of heterothallic and homothallic strains of *S. cerevisiae* (Sherman F., 1998)

During exponential growth, haploid cultures tend to have higher numbers of cells per cluster compared to diploid cultures. Haploid strains have a doubling time of approximately 90 minutes in complete media (YPD: 1% yeast extract, 2% peptone, and 2% glucose) and approximately 140 minutes in synthetic media at 30°C which is usually the optimum temperature for their growth (Sherman, 1998).

S. cerevisiae is the first eukaryote to have its genome sequenced. The haploid yeast genome contains 16 chromosomes. The total sequence of chromosomal DNA, constituting 13,392 kb, was released in April, 1996. *S. cerevisiae* genome size is less than 1 % that of a mammal and is 3.5 fold the genome of *E. coli*. In contrast to the

genomes of multi-cellular organisms, the yeast genome is highly compact, with 6275 genes representing 72% of the total sequence. It is estimated that it shares 23% of its genome with human. Additionally, cell control mechanisms are also similar to human cell control mechanisms (Madigan, *et al.*, 2003; Geoffrey, 2000). Thus, the baker's yeast *S. cerevisiae,* being one of the earliest domesticated organisms, is also accepted as one of the best model eukaryotic microorganisms for biological studies.

1.2 Industrial importance and advantages of *S. cerevisiae*

Yeast has economic importance in production of beer, wine, alcohol and bread since Ancient Egypt. In wine making, it is responsible for fermenting fruit juices into wine. It carries out the primary fermentation by converting glucose into ethanol. Bacteria then perform the secondary fermentation that breaks down acids in alcohol and give wine a smoother taste. *S. cerevisiae* is especially preferred as the yeast strain to be used in primary fermentation because it is less sensitive to inhibiting effect of alcohol. It is known to have the capability to produce up to 18% alcohol (Madigan, *et al.*, 2003).

Yeast is being used in bread as leavening agent which gives the bread a dough rise by producing gas. *Saccharomyces cerevisiae* cells produce alcohol and carbon dioxide during fermentation. These carbon dioxide bubbles give the bread a lighter and finer texture (Black, 2002).

Today, *S. cerevisiae* is used in a variety of commercial fermentation and biomass conversion processes. Its use is based on its ability to convert sugars and other carbon sources into ethanol in the absence of oxygen, and into CO_2 and water in presence of oxygen. Yeast is also a good food and also an uncommonly good source for vitamin B and a valuable source for low-meat / vegetarian diets. However, pure yeast is considered to be bad in taste (Ratledge, *et al.*, 2001).

It is obvious that *S. cerevisiae* is one of the most relevant organisms for mankind for its use since ancient times. However; it still draws scientists' attention for many other reasons that it is one of the most intensively studied eukaryotic model organisms in molecular and cellular biology. First of all, it can be maintained as either haploid or diploid and is commercially available. Since it is in the phylum Ascomycetes, it

produces ascospores in ascus by meiosis, and sporulate under stress. Diploid nucleus goes through meiosis producing four haploid nuclei which then incorporate into four stress-resistant ascospores, encapsulated in ascus. This packaging of meiotic products makes analysis simple. They can proliferate when they are haploid. When they are haploid they can easily be isolated due to having dispersed cells and thus, mutations can be easily studied. (Snustad, *et al.*, 2000) Another advantage is rapid growth with short generation times. Moreover, culturing *S. cerevisiae* is inexpensive and easy when compared to other microorganisms. It is known to have GRAS (generally regarded as safe) status. Thus, studying *S. cerevisiae* requires only a few precautions. Therefore, this fungus provides a highly suitable system to study basic biological processes that are relevant for many other higher eukaryotes including man (Jaafar, *et al.*, 2004).

As a result of all these advantages, *S. cerevisiae* is an entirely sequenced model eukaryote whose genome is useful as a reference for human genome and other higher eukaryotic genes. *S. cerevisiae* is now a well-defined system with detailed genetic and biochemical knowledge. Unlike many other microorganisms, it has several markers. It is being used as cloning single or multi-copy vector in biotechnology. Several commercially available databases are present on World Wide Web pages such as; Comprehensive Yeast Genome Database (CYGD, online database), Saccharomyces Genome Database (SGD, online database) and *Saccharomyces cerevisiae* Morphological Database (SMD, online database).

1.3 Stress responses of *S. cerevisiae*

Stress response is a general property of all living organisms. Survival of cells critically depends on their ability to sense alterations in the environment and to respond to new situations through the induction of protective stress responses. Yeast is known to be one of the most suitable systems to study stress tolerance. The consequences of different types of stress can be easily observed and the cellular mechanisms can be understood in yeast. Stress conditions studied on yeast cells include heat stress, ethanol stress, hydrogen peroxide stress, rapid freezing stress, slow freezing stress, salt stress, acetic acid stress, etc (Lewis, *et al.*, 1997).

The response of yeast cells to stress conditions in the environment has been used as a model to study gene regulation networks. Moreover, understanding yeast gene regulation maintains preliminary information for studies on higher organisms. The gene expression of the yeast under stress has been studied extensively, and it has become evident that a certain group of the yeast genes is always activated during various stress treatments (Yale and Bohner, 2001).

Yeast responses towards stress conditions are not only monitored in gene expression level, but also observed directly morphologically. Most cells respond to various stress conditions (such as starvation) by elongation. Elongation helps cells to extend their cell surface and increase the chance to reach nutrients. Some of the cells get a smaller, circular shape and form a cluster that looks like grape and some cells are observed to have longer than normally grown cells. The daughter cells stay linked to the parent cells to form filaments as if searching for food (Radetsky, 1994).

Other than morphological changes cells also respond to stress conditions by changes in cellular activities. For example, they produce glycerol, which is exposed outside the cell by some cells, and stored inside the cell by others, in order to minimize effects of an undesired stress condition which is mostly a heavy metal in the growth environment. Cells change their environment by excretion of protons, by sequestration of toxic metals, production of water as a result of metabolic activity or via translocation as well. Movement of potassium and sodium between medium and cytoplasm across plasma membrane is another result of stress tolerance mechanisms in *S. cerevisiae* (Jennings, 1993).

One of the most common responses towards stress conditions is trehalose accumulation (Owobi *et al.*, 2000; Carvalheiro *et al.*, 1999). Trehalose is a nonreducing disaccharide in which two glucose molecules are linked by α,α-1,1-glycosidic linkage. This sugar is found in various organisms such as bacteria, yeast, fungi, insects, invertebrates, higher and lower plants. There are three anomers of trehalose; α,β-1,1-, β,β-1,1- and α,α-1,1. However, only α,α-trehalose has been isolated from and biosynthesized in living organisms (Figure 1.4).

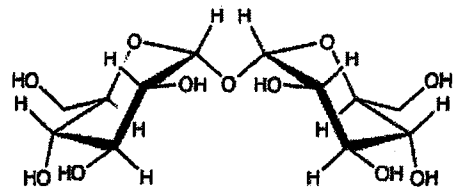


Figure 1.4 α,α-1,1-trehalose. (Elbein, *et al.*, 2003)

Levels of trehalose inside the organisms depend on the age of the cells, as well as on the stage of growth and their nutritional state. Trehalose plays a dual role as an energy and carbon reserve mainly stored in vegetative resting cells and reproductive structures and as a stress metabolite. In addition to being non-reducing, it possesses several unique physical properties, which include high hydrophilicity and chemical stability, and the absence of internal hydrogen bond formation. These properties are the main cause of trehalose for being a stress metabolite (Argüelles, 2000; Plourde-Owobi., *et al.*, 2000). It acts as a stabilizer and protectant of proteins and membranes against dehydration, damage by oxygen radicals, cold and heat. It specifically acts on plasma membranes by replacing water molecules in the polar head groups of phospholipids. It also functions as a chaperon and suppresses the formation of large aggregates of denatured proteins. Moreover, rapid trehalose degradation provides energy for correct renaturation of such proteins after stress recovery (Zaragoza, *et al.*, 2003). It functions as a sensing compound and growth regulator and as a structural component of bacterial cell wall. It serves as a stabilizer because trehalose is a non-reducing sugar. This means it cannot undergo typical browning reaction between aldehyde group on reducing sugars and amino groups on proteins. Browning reaction usually leads to denaturation of proteins (Elbein, 2003). Trehalose and some heat shock proteins are synthesized simultaneously and have a synergistic effect (Alvarez – Peral, *et al.*, 2002).

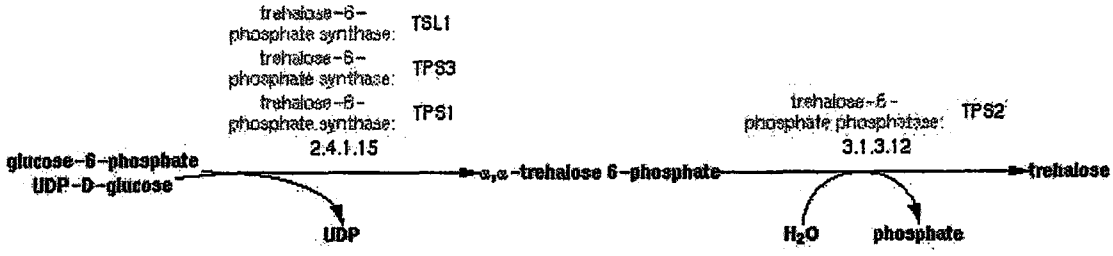


Figure 1.5 Yeast trehalose biosynthesis pathway (SGD, online database).

Trehalose synthase is a multimeric protein composed of four subunits encoded by *TPS1, TPS2, TSL1* and *TPS3*, of which only Tps1p catalyzing the formation of trehalose-6-phosphate from UDP-Glc and glucose-6-phosphate is essential for growth on rapidly fermentable carbon sources like glucose and fructose (Figure 1.5, Table 1.2). The deletion of TPS1 results in the loss of trehalose accumulation (Owobi *et al*, 1999).

Table 1.2 Genes cloned and characterized and corresponding gene products from *S. cerevisiae* involved in trehalose metabolism (Argüelles, 2000).

Gene	Product	Function	Reference
TPS1	Trehalose-6-P synthase	Required for growth on sugars	Bell *et al.* 1992
TPS2	Trehalose-6-P phosphatase	Dephosphorylation of trehalose 6-P	De Virgilio *et al.* 1993
TSL1 (TPS3)	Regulatory subunit	Stabilizer of trehalose synthase complex	Vuorio *et al.* 1993
NTH1	Neutral, cytosolic trehalase	Regulated by phosphorylation	Kopp *et al.* 1993
ATH1	Acidic, vacuolar trehalase	Regulated by catabolite repression	Destruelle *et al.* 1995

1.4 Heavy metals as pollutants

Heavy metals are metals with a density beyond 5 g/cm^3 and specific gravity greater than 4. Most heavy metals are transition elements with incompletely filled d-orbitals, which provide the formation of complex compounds. Heavy metals include cobalt, copper, zinc, nickel, lead, silver, cadmium, mercury, arsenic, chromium, and thallium. In fact, there is no precise definition for "heavy metals" in the literature. However, heavy metals are considered to be metals which are essential for growth but toxic at high concentrations. For example, arsenic is regarded as a hazardous heavy metal even though it is actually a semi-metal (Jennings, 1993).

Heavy metals are natural components of the Earth's crust and many of them fulfill essential functions in all living beings as trace elements. Heavy metal cations play important roles in biochemical reactions such as nitrogen fixation, water cleavage during oxygenic photosynthesis, respiration, re-arrangement of C-C bonds, hydrogen assimilation, cleavage of urea, transcription and development of cells. They form complexes primarily with ligands containing nitrogen and sulphur centers, rather than oxygen (Nies, 1999).

Nevertheless, a considerable number of metals are harmful to plants, animals and man in excessive concentrations. They cannot be degraded or destroyed. To a small extent they enter our bodies via food, drinking water and air. Heavy metals are dangerous because they tend to bioaccumulate, which means their concentrations increase in biological organisms over time. They are accumulated in living things any time they are taken up and stored faster than they are metabolized or excreted. For instance, cadmium is well known to remain resident for years (over decades for human) when absorbed by an organism although it is eventually excreted (Strom, 2003).

Table 1.3 Heavy metals and their industrial applications (Chua, 1998)

Metal	Industrial use
Nickel	Stainless steel, nickel electroplating, battery and accumulator manufacturing, pigments and ceramic industries
Antimony	Flame retardant, batteries, pigments, ceramics, glass
Cadmium	Batteries as rechargeable or secondary power sources, pigments, stabilizers for PVC, alloys, detergents, refined petroleum products
Chromium	Metal alloys, pigments for paints, cement, paper, rubber, air conditioning coolants
Copper	Copper pipes, additives designed to control algal growth
Lead	Petrol additives rolled and extruded products, alloys, pigments, cable sheathing, shot and ammunition.
Mercury	Batteries, non-ferrous metal production, coal combustion, crematoria.
Cobalt	Electroplating, cobalt-processing industries, radiotherapy.

Heavy metals in air, soil, and water are global problems that are a growing threat to the environment. There are multiple sources of heavy metal pollution. Heavy metals such as copper, zinc, nickel, cobalt, silver, cadmium and chromium are spread out during electroplating, metal processing industries, mining and metallurgical applications; from metal-work and metal finishing industrial effluents and corrosion of galvanized piping. These heavy metals and others, such as barium, lead, iron and mercury, are also used in the manufacture of printed circuit boards, paints, plastics, batteries, alloys, refractors, scientific instruments and paper (Chua, 1998) (Table 1.3). Heavy metals can enter a water supply by industrial and consumer waste, or even from acidic rain breaking down soils and releasing heavy metals into streams, lakes, rivers, and groundwater (Mattlock, *et al.*, 2001).

1.5 Heavy metal stress on microorganisms

Heavy metals are important for cellular mechanisms as trace elements. However, at higher concentrations, heavy metal ions form unspecific complex compounds in the cell and cause toxicity. Even certain levels of concentrations are known to affect synthesis of outer and periplasmic membrane proteins (Felicio, *et al,* 2003). Metal homeostasis must involve uptake of sufficient essential metals for optimal functioning of metabolic processes. On the other hand, protection against toxicity from excess heavy metal toxicity should be provided. This explains the importance of controlling the intracellular concentration of heavy metal ions.

There are two kinds of heavy metal ion uptake system in bacteria (Nies, 1999):

1. Fast, unspecific and constitutively expressed system: this system is used for several substrates. They are usually driven by chemiosmotic gradient across cytoplasmic membrane.
2. Slow, high substrate specificity and inducible system: it uses ATP and is produced only when required.

When the cell is exposed to high concentration of any heavy metal, the heavy metal ion is transported into the cytoplasm due to the constitutive expression of unspecific transporters. Because of this "open gate" the heavy metals are toxic for the cells. The metal tolerance can occur by mutations in these unspecific transporter genes (Nies, *et al.,* 1995). Table 1.4 shows the minimal inhibitory concentrations (MIC) of some heavy metals in *Escherichia coli* cells.

Table 1.4 Toxicity of heavy metals in *Escherichia coli* (Nies D.H., 1999).

MIC (mM)	Heavy-metal ions
0.01	Hg^{2+}
0.02	Ag^{+}, Au^{3+}
0.2	CrO_4^{2-}, Pd^{2+}
0.5	Pt^{4+}, Cd^{2+}
1.0	Co^{2+}, Ni^{2+}, Cu^{2+}, Zn^{2+}
2.0	Tl^{+}, UO_2^{2-}, La^{3+}, Y^{3+}, Sc^{3+}, Ru^{3+}, Al^{3+}
5.0	Pb^{2+}, Ir^{3+}, Os^{3+}, Sb^{3+}, Sn^{2+}, In^{3+}, Rh^{2+}, Ga^{3+}, Cr^{3+}, V^{3+}, Ti^{3+}, Bc^{2+}
10.0	Cr^{2+}
20.0	Mn^{2+}

In yeast *S. cerevisiae* uptake is also biphasic; an initial rapid phase that is independent of metabolic uptake and a slower, energy dependent phase. The initial surface binding has been observed within a few minutes in the absence of energy at low temperature and in the presence of metabolic inhibitors. This mechanism was found to be incapable of distinguishing metal ions bound by the cell wall and the ones bound to the outer membrane of the cytoplasmic membrane. The second uptake is based on the membrane transport of metals (Jennings, 1993).

In general, protection against heavy metal toxicity is performed by two mechanisms in fungi (Table 1.5): (Jennings, 1993)

1. Avoidence: This mechanism involves restricting metal entry into cell either by reduced uptake / active efflux or by formation of complexes outside the cell.

2. Sequestration: This mechanism involves reducing levels of free ions in the cytosol, either by synthesis of ligands to achieve intracellular chelation or by compartmentation into vacuoles.

Table 1.5 Mechanisms of metal tolerance (Jennings, 1993)

Cellular process	Tolerance mechanism
Secreted protein or organic molecule or organic ion	Avoidence
Fungal cell wall	Avoidence
Permeases	Avoidence
Vacuoles	Sequestration
Inorganic chelators	Sequestration
Organic chelators	Sequestration
Metal binding proteins	Sequestration

Inside the cells, heavy metal cations bind to sulfhyroxyl groups and inhibit the activity of sensitive enzymes while some others interact with physiological ions and inhibit the physiological function of that ion. For example, Ni^{2+} and Co^{2+} interact with Fe^{2+} (Nies, 1999). Similarly, competition of cobalt and magnesium for the same binding site in transport processes was demonstrated in earlier studies. It was seen that K_T for Mg^{2+} uptake was approximately equal to K_I for Mg^{2+} as an inhibitor of Co^{2+} uptake (Nelson, *et al.*, 1971). In addition, heavy metal cations may bind to

glutathione and cause formation of bisglutathionate (GSH) complexes. These complexes react with oxygen to form oxidized bisglutathione (GS-SG), the metal cation and hydrogen peroxide. GS-SG has to be reduced again by an NADPH-dependent reaction and the metal cations bind to another two glutathione molecules. This causes a considerable oxidative stress. Lastly, heavy-metal oxyanion reduction causes production of radicals (Nies, 1999).

Metal ions are primary components of all ecosystems and they cannot be degraded. Thus, all organisms must have evolved mechanisms to cope with varying levels of these elements. Many systems are being investigated for different metals. One of the most studied resistance systems is silver resistance due to its uses in several areas such as antimicrobial agents in public health hygiene, in burn, wound and ulcer treatments in medicine, coat catheters in microbial biofilm development (Silver, 2003). Moreover, *Pseudomonas putida* KT2440 genome is being studied since it encodes an unexpected capacity to tolerate heavy metals and metalloids involving several metal resistance mechanisms (Canovas, *et al.*, 2003). Metal resistance mechanisms can be classified as given below:

- Accumulation of the ion is diminished by efflux.
- Cations (especially sulfur lovers) are segregated into complex compounds by thiol-containing molecules.
- Some metal ions can be reduced to a less toxic oxidation state.
- There can be a combination of the previous two or three mechanisms (Nies, *et al.* 1995, Nies, 1999).

1.5.1 Roles of cell compartments in resistance mechanisms

1.5.1.1 Cell wall

Most of the metal ions are essential trace elements for cells; therefore the cell wall cannot act as a barrier to entry of such metal ions. Electron microscope studies on cell walls have showed that yeast cell walls are able to bind considerable amounts of metal ions (Jennings, 1993).

1.5.1.2 Vacuole

Data on intracellular distribution of many heavy metals demonstrates the role of vacuole in storage and detoxification of these metals. Calcium is not regarded as a heavy metal. However, studies on calcium have provided a background for a better understanding of regulation of cellular concentrations of heavy metals, especially on the role of vacuole (Jennings, 1993).

Previous studies on yeast indicated that metal ion concentration accumulated in the vacuole was significantly higher than that in the cytosol. Moreover, studies showed that, as the metal concentration increased in the vacuole, cytoplasmic metal concentrations remained constant. This result showed the role of vacuole in regulation of metal ion concentration in the cytosol (Lichko, *et al.*, 1982; Okorov, *et al.*, 1980).

Studies on *S. cerevisiae* and cobalt yielded also similar results. Repeated culturing in media containing cobalt for increasing cobalt resistance resulted with increased accumulation of cobalt in the vacuoles (White and Gadd, 1986).

1.5.2 Roles of protein families in resistance mechanisms

1.5.2.1 Transport proteins

Transport systems may use ATP hydrolysis for the transportation process. These kinds of transporters belong to the ABC-family, P-type ATPases or A-type ATPases. In bacteria a proton-influx may drive a metal cation efflux with a proton-gradient across the cytoplasmic membrane. These are the RND-transporters. In some systems, the driving force of metal transport concern the HoxN-, the Chr-, the CorA- and the CDF-family. Additional families of heavy metal ion transporters such as ZIP, ACR, FeT, OFeT, CTR1, CTR2, Nramp and MHP are also found in eukaryotes. Table 1.6 shows the important protein families for metal transport and the metal ions transported.

Table 1.6 Protein families important for heavy metal transport (Nies, 1999).

Family	Direction of transport	Energy	Metal ions	Composition
ABC	Uptake	ATP	Mn^{2+}, Zn^{2+}, Ni^{2+}, Fe^{2+}	2 membrane-integral parts + 2 ATPase parts = ABC core + periplasmic binding protein
	Efflux	ATP	-	ABC core + membrane fusion protein and outer membrane factor
P-type	Both	ATP	Mg^{2+}, Mn^{2+}, Ca^{2+}, K^{+}, Cu^{2+}, Zn^{2+}, Cd^{2+}, Pb^{2+}, Ag^{+}	1 membrane-bound protein as core
A-type	Efflux	ATP	Arsenite	1 membrane-integral protein + a dimeric ATPase subunit
RND	Efflux	Proton gradient	Co^{2+}, Zn^{2+}, Cd^{2+}, Ni^{2+}, Cu^{2+}, Ag^{+}	1 CPM proton/cation antiporter + membrane fusion protein (dimer) + outer membrane factor: CBA transport systems
HoxN	Uptake	Chemiosmotic	Co^{2+}, Ni^{2+}	Membrane-Integral protein
CHR	Antiport	Chemiosmotic	Chromate	Membrane-integral protein (ChrA)
MIT	Uptake	Chemiosmotic	Most cations	Membrane-integral protein (CorA)
CDF	Efflux	Chemiosmotic	Zn^{2+}, Cd^{2+}, Co^{2+}, Fe^{2+}	Membrane-integral protein (CzcD, ZRC1p, ZnT1)

1.5.2.2 Metallothioneins

Metallothioneins (MTs) are ubiquitous low molecular weight proteins and polypeptides of extremely high metal and sulfur content. The metallothionein gene is expressed at a basal level, but is triggered by heavy metal ions. They are thought to play roles both in the intracellular fixation of the essential trace elements zinc and copper, in controlling the concentrations of the free ions of these elements, in regulating their flow to their cellular destinations, in neutralizing the harmful influences of exposure to toxic elements such as cadmium and mercury and in the protection from of a variety of stress conditions (Kägi, *et al.*, 1988; Voet, et al, 1995).

There are three classes of metallothioneins according to an international nomenclature committee which established these classes at the Second International Meeting on Metallothionein and Other Low Molecular-Weight Binding Proteins in Zurich, 1985.

Class I: This group is found in mammalian cells. They involve 20 cysteines. There are two or more isoforms of class I metallothioneins in cells. These isoforms mostly differ in a single amino acid. Some species involve multiple subisoforms.

Class II: This group is found in yeast. They are polypeptides with locations of cysteines only distantly related to mammalian metallothioneins.

Class III: This group is found in many plants and single cell organisms. They are often called phytochelatins and cadystins. They are enzymatically synthesized metallothioneins with unusual repeating sequences of γ-glutamyl linkages of cysteines.

Class I and Class II are known to be translational products and Class III is known to be the enzymatic product since its production requires additional enzymatic steps. Class I and II metallothioneins have some similarities such as having small molecular weight, being included in enzymes in high amounts, involving cystein residues that bind metals. However, they are structurally different. On the first group MTs, two different clusters are found; whereas on the second group, various sorts of metal ions and different-length peptides are included in the metal-cysteinyl thiolase cluster (Stillman, *et al.* 1992).

1.5.3 Importance of metal tolerance

Metal-resistant simple organisms are good models for understanding tolerance mechanisms of higher organisms. Additionally, they might be used in a variety of industrial applications in the field of "biomimetics". Metal-binding proteins can be used as adapter proteins on the surfaces of metal appliances used in human body. The immunological responses are expected to be decreased significantly by the help of these adapter proteins.

Heavy metal resistant organisms can be used for bioremediation of metal-contaminated environments. A study on *Pseudomonas aeuginosa* and *Bacillus thuringiensis* demonstrated that mercury and copper appeared to be absorbed more than the other elements. These strong interactions of mercury and copper with organic matter suggested that these undesirable elements might be removed from the environment by organismal trapping (Hassen, *et al.*, 1998).

Another possible use of heavy-metal resistant microorganisms would be bio-mining applications of expensive metals. In this case, the value of the metal obtained should be more than the value of the organism used.

Moreover, addition of metal resistance to a microorganism may be useful for biotechnological processes. By this means, there would be no need for diminishing the toxic effects of heavy metals and this would be a more economical way of detoxification of efflux (Nies, 1999).

1.6 A model heavy metal: Cobalt

Cobalt is a second period heavy metal with the atomic number 27 and the atomic weight 58.9. Common oxidation states of cobalt include +2, and +3, though +1 is also seen.

The word cobalt comes from the German *kobalt* or *kobold*, meaning evil spirit. Miners gave this name to the metal, because it was poisonous and troublesome. There is a common opinion that the name may derive from the Greek word *kobalos*, which means 'mine'.

Cobalt is a brittle, hard metal, closely resembling iron and nickel in appearance. It is a hard ferromagnetic element with a metallic permeability of about two thirds that of iron. Powdered cobalt in metal form is a fire hazard. Most of the cobalt compounds should be regarded as toxic. Some cobalt compounds may be carcinogenic and should be handled with care (Material Safety Data Sheet).

Cobalt is often associated with nickel, silver, lead, copper, and iron ores, from which it is mostly obtained as a by-product. It is alloyed with iron, nickel and other metals to make an alloy called Alnico with an unusual magnetic strength, in order to be used

as magnets and magnetic recording media. Satellite alloys, containing cobalt, chromium, and tungsten, are used for high-speed, heavy-duty, corrosion-resistant, high temperature cutting tools. Cobalt is also used in other magnetic steels and stainless steels, and in super alloys used for parts in gas turbine generators and jet turbines. The metal is used in electroplating because of its appearance, hardness, and resistance to oxidation. Its salts have been used for centuries for the production of brilliant and permanent blue colors in porcelain, glass, pottery, tiles, and enamels. Cobalt has role as catalyst for the petroleum and chemical industries and as drying agents for paints, varnishes, and inks. It is the principal ingredient of Sevre's and Thenard's blue. Its chloride solution is used as a sympathetic ink. Cobalt-60, an artificial isotope, is an important gamma ray source, and is extensively used as a tracer, as a radiotherapeutic agent in medicine, in treatment of foods for cold sterilization and in industrial radiography to detect structural flaws in metal parts. Single compact sources of Cobalt-60 vary from about $1 to $10/curie, depending on quantity and specific activity (Hirata C., 2005).

Cobalt is an essential metal for the organisms in small amounts. Soils should contain 0.13 to 0.30 ppm of cobalt for proper animal nutrition. It is a central component of the cofactor B_{12} (cobalamin) that catalyses C-C, C-O, C-N rearrangements (Figure 1.6).

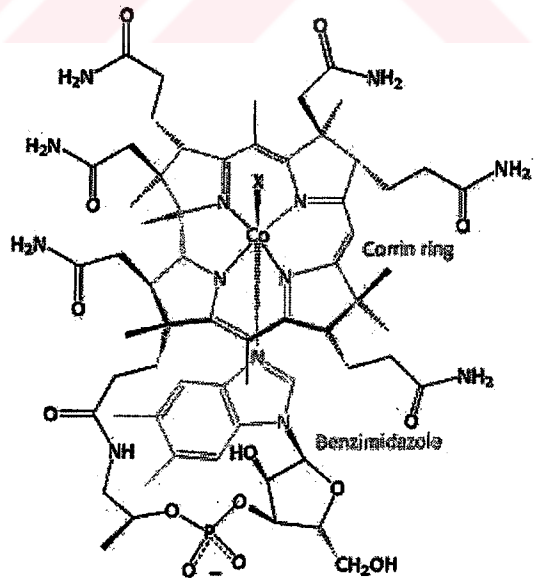


Figure 1.6 The structure of coenzyme B_{12}.

There is also a cobalt-containing enzyme. Table 1.7 shows more common cobalt-containing proteins (Kobayashi, *et al.,* 1999).

Co^{2+} is rapidly accumulated by the CorA system in most bacterial cells. First, it was thought that COT1p protein from *S. cerevisiae* transports Co^{2+} across mitochondrial membrane (Nies, 1999). However, it was later understood that transport across vacuolar membrane is present (MacDiarmid, *et al.*, 2000).

Table 1.7 Known cobalt-containing proteins (Kobayashi, *et al.*, 1999).

Enzyme or protein	Source	Cofactor content	Postulated role for cobalt
Methionine aminopeptidase	Animals, yeast, bacteria	2 Co per subunit	Hydrolysis
Prolidase	Archae	1-2 Co per subunit	Hydrolysis
Nitrile hydratase	Actinomycetes and bacteria	1 Co in each α-subunit	H_2O activation, CN-triple-bond hydration and protein folding
Glucose isomerase	Actinomycetes	1 Co per 4 subunits	Isomerization
Cobalt transporter	Actinomycetes and yeast		Cobalt uptake
Methylmalonyl-CoA carboxytransferase	Bacteria	1 Co, 1 Zn per subunit	Carboxytransferation
Aldehyde decarbonylase	Algae	1-2 Co-porphyrin per αβ subunit	Decarbonylation for aldehyde
Lysine-2,3-aminomutase[a]	Bacteria	0,5-1 Co per subunit	Mutation
Bromoperoxidase	Bacteria	0,35 Co 2 per subunits	Bromination
Cobalt-poryphrin-containing protein	Bacteria	1 Co-porphyrin per protein	Electron carrier

1.7 How to obtain cobalt resistant *Saccharomyces cerevisiae*

One of the recent attempts for obtaining genetically modified microorganisms with improved industrial properties is metabolic engineering. Metabolic engineering seems to be a suitable method for obtaining metal-resistant microorganisms.

1.7.1 Metabolic and inverse-metabolic engineering

Metabolic engineering is referred to as the directed improvement of cellular properties through the modification of specific biochemical reactions or the introduction of new ones, by recombinant DNA technology. It is a multidisciplinary method between chemical engineering, biochemistry, molecular and cell biology, and computational science. Metabolic engineering has wide range applicability both in creation of new processes and in improvement of existing processes and products (Stephanopoulos, 1999; Bailey, *et al.*, 1996).

The studies have given rise to the improvement of cellular activities by directed manipulation of enzymatic, transport, and regulatory functions of the cell. Difficulties in the selection of the desired targets due to limited background knowledge on the complex cellular systems and secondary responses to genetic manipulation accelerated the tendency to develop a new alternative technique, *inverse metabolic engineering* (Figure 1.7). The classical approach of metabolic engineering requires detailed knowledge of the enzyme kinetics and the system network, in order to have the desired phenotype at the end. However, inverse metabolic engineering starts with the identification of a desired cell behavior. Then the genetic basis for the behavior is determined and lastly development of methods for re-engineering the behavior in the organism of interest or engineering the behavior into a different organism (Yang, *et al,* 1998; Bailey, *et al.*, 1996).

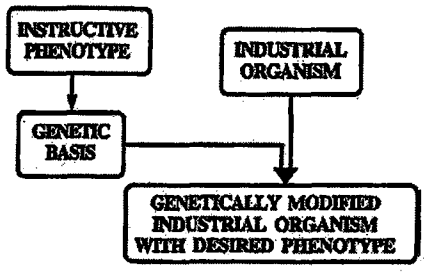


Figure 1.7 Inverse Metabolic Engineering (Bailey, *et al.*, 1996)

In order to investigate the desired phenotypes and apply them on the organisms of interest, the desired phenotypes are formed by random mutations and then selected from the heterogeneous population. If any pressure is applied in order to have desired characteristics, the strategy is called *directed evolution* (Bailey *et al.,* 1996; Gill, 2003). Use of evolutionary methods is accepted to be as a more natural procedure when compared to recombinant DNA technology. Public acceptance especially in food industry could thus be an important advantage for evolutionary techniques (Çakar, *et al.*, 2005).

1.7.1.1 An inverse metabolic engineering strategy: evolutionary engineering

Inverse metabolic engineering maintained a rapid development of new experimental methods for creating genetic diversity and for searching large populations for improved functions. Further advances in screening and selection technologies will reduce the time and cost of the experiments and will make it possible to solve more difficult problems involving multiple enzymes, multi-component enzymes, and the creation of new functional molecules (Arnold, 1999).

Directed evolution is performed first by increasing the diversity by mutagenesis. Use of non-genetic factors such as chemical or physical mutagens is a method to contribute to the generation of genetic variations. The non-genetic factors being used in this context are intrinsic instability of nucleotides, structural flexibility of biological active molecules, random encounter of interactive components, chemical and physical mutagens (Arber, 2000).

Ethyl methane sulphonate (EMS) is an alkylating agent that can modify nucleotides in DNA. Although it can modify any base, it most often modifies G nucleotides. The modified nucleotide can pair with T instead of C, a G-T pair is obtained, and the next time the DNA is replicated, the G could be replaced with an A, resulting in mutation of a G-C pair into an A-T pair. The mutation rate increases because of the significant chance of converting these mis-matched pairs into permanent changes from the original sequence (Lewin, 2000). The effect of EMS on DNA is shown in detail in Figure 1.8.

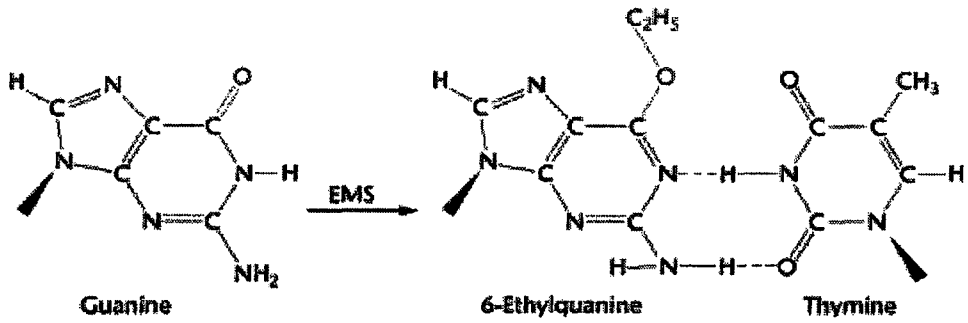


Figure 1.8 The effect of EMS on DNA (Scarr, 2002).

1.8 The aim of the present study

The aim of this study was to obtain heavy-metal-binding / heavy-metal-resistant yeast cells by developing and using evolutionary engineering strategies. For this purpose, cobalt, an important heavy metal, was chosen as a model. Different selection strategies were employed and compared with each other with respect to their efficiencies in selecting cobalt-resistant mutant yeasts. Basic characterizations of the mutants were performed by quantitatively determining their survival under various stress conditions and also by determining their cobalt content using atomic absorption spectroscopy. The mutants and the information to be obtained from their further characterization with respect to their metal-specific properties could be exploited in various industrial applications such as bioremediation and biomimetics.

2 MATERIALS AND METHODS

2.1 Materials

2.1.1 Yeast strain

Saccharomyces cerevisiae CEN.PK113-7D was kindly provided by Dr.Peter Kötter from Johann Wolfgang Goethe University, Frankfurt, Germany.

2.1.2 Yeast culture media

2.1.2.1 Yeast minimal medium (YMM)

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

in 1 liter of distilled water.

2.1.2.2 Yeast complex medium (YPD)

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

per liter of distilled water.

2.1.3 Chemicals

Ethanol (absolute) was purchased from J.T.Baker (Holland).

Hydrogen peroxide (35%, v/v) was obtained from Merck (Germany).

Sodium Thiosulphate was purchased from J.T.Baker (Holland).

D(+)-Trehalose dihydrate was obtained from Riedel-de Haën (Germany).

Kobalt (II)-chloride-hexahydrate was purchased from Merck (Germany).

2.1.4 Buffers and solutions

Potassium phosphate buffer (pH 7)	50 mM
Sodium thiosulfate solution	10 % (w/v)
H_2O_2 solution	5 M
$CoCl_2$ solution	1 M
$CrCl_3$ solution	1 M
$CuCl_2$ solution	1 M
$CuCl_2$ solution	1 M
H_2SO_4 solution	5 mM
HNO_3 solution	6 M

2.1.5 Laboratory equipment

Thermomixer	Eppendorf, Thermomixer Comfort 1.5-2 ml, (Germany)
Microfuge	Beckman®Coulter Microfuge (USA)
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)
Microplate Reader	Biorad Model 3550 UV (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	Özge (Turkey) Faster BH-EN (Italy)
Autoclaves	Tuttnauer Stystec Autoclave 2540 ml (Switzerland) 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) Nüve EN500 (Turkey)
Atomic Absorption Spectrometer (ITU, Chemisty Department)	Analytik Jena Vario 6 (Germany)
Light Microscope	Olympus CH30 (USA)

HPLC (High Performance Liquid Chromatography) System

- Refractive Index Detector	Shimadzu RID10A (Japan)
- System Controller	Shimadzu SCL10A) (Japan)
- Liquid Chromatography	Shimadzu LC-10AD (Japan)
- Degasser	Shimadzu ss DGU-14A (Japan)
- Column Oven	Shimadzu CTO-10AC (Japan)

2.2 Methods

2.2.1 EMS mutagenesis

Saccharomyces cerevisiae CEN.PK 113-7D culture, approximately at a concentration of $1X10^6$ cells/ml; was inoculated into 10 ml YPD, and incubated overnight at 30°C and 150 rpm in order to have the cell concentration of approximately $2X10^8$ cells/ml. 2.5 ml of this culture was washed twice with 50 mM potassium phosphate buffer (pH 7) and resuspended in the same buffer to obtain a final concentration of $5x10^7$ cells/ml.

300 µl of EMS was added into each 10 ml of cell suspension in a 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 this culture was named as *101*. The original wild-type cells were named as *100*.

2.2.2 Selection of Mutant Populations

Obtaining the generations is based on applying the initial stress condition to the mutated culture *101* and transferring the survivors of this present stress condition to the next stress condition. Fresh mutated culture *101* was used for the first stress application and the rest of the steps continued over the newly obtained generations. Figure 2.1 shows the algorithm for obtaining the mutant generations.

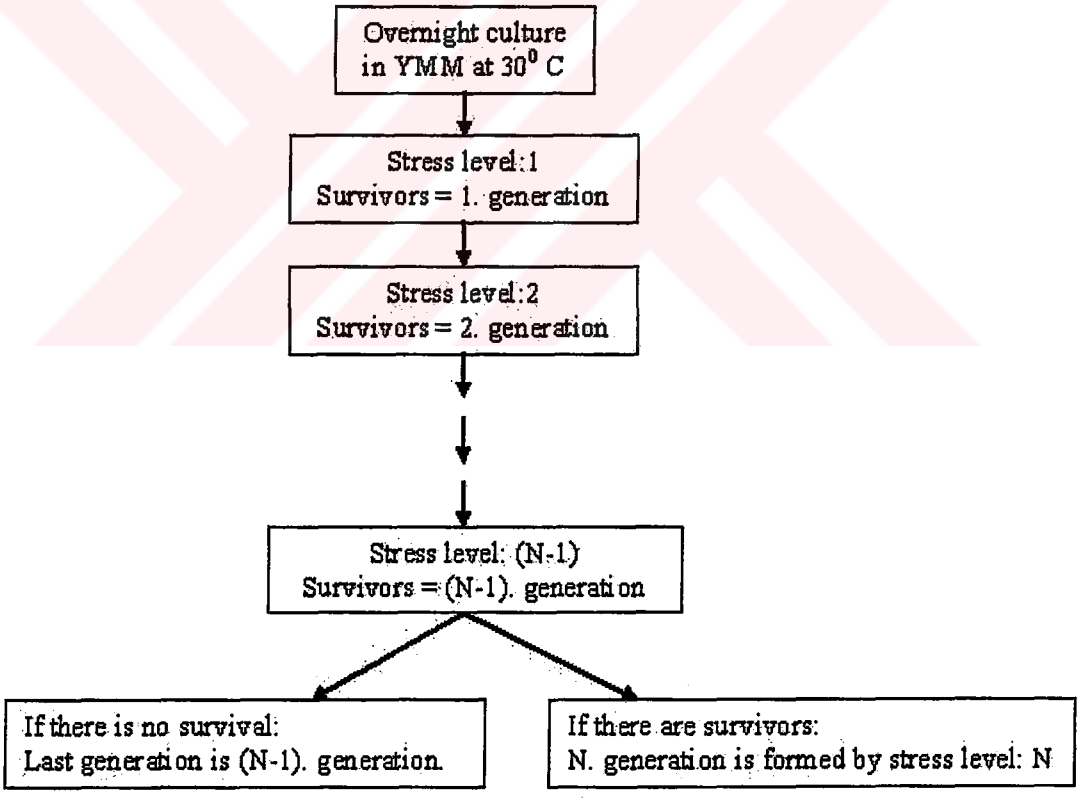


Figure 2.1 Selection algorithm for obtaining cobalt-resistant yeast mutants.

Two different strategies were adopted in parallel: increasing stress application and constant stress application. In increasing stress, cells were exposed to an increased level of metal stress at each step. The initial stress level was considered to be 0.5 mM $CoCl_2$ and this stress level increased as obtaining the following generations (0.5mM, 1mM, 1.5mM, 2mM, 2.5mM, 3mM, 3.5mM, 4mM, 4.5mM, 5mM, 5.5mM, 6mM and so on). In constant stress application, cells were exposed to a constant level of metal stress at each step. Initial stress level (0.5 mM) was applied to the cells repeatedly from 1st generation to the last generation.

Either constant or increasing, stress application was carried out continuously, which means that the stress condition was continuously present in the growth media. A stock culture of 500 µl was incubated into 10 ml of YMM. The overnight culture was used for incubation into 10 ml YMM with varying concentrations of $CoCl_2$. Optical density values and haemocytometer counting results of 24, 48 and 72. hours of cultivations were analyzed and used for survival ratio analysis.

2.2.3 Stock culture preparation

Frozen stock solutions of cultures were prepared by addition of an equal volume of 60 % (v/v) glycerol to the liquid culture to have a final ratio of 30 % (v/v) glycerol and placed at -20^0 C and -80^0 C for long-term preservation.

It is important to have all cobalt removed from the cultures before stock preparation. The cultures, which were previously grown in cobalt containing media, were washed twice by centrifuging at 10,000 rpm for 5 minutes and discarding the supernatant. The obtained pellets were resuspended by fresh sterile YMM, and after these washing steps glycerol was added.

2.2.4 Selection of individual mutants

Overnight liquid cultures of the selected highly resistant population were inoculated into agar plates. Either diluting the culture by streaking method or spreading 100 µl of the overnight culture in a dilution ratio of 1:10^6 was applied for selection of individual colonies. Figure 2.2 represents the streaking method.

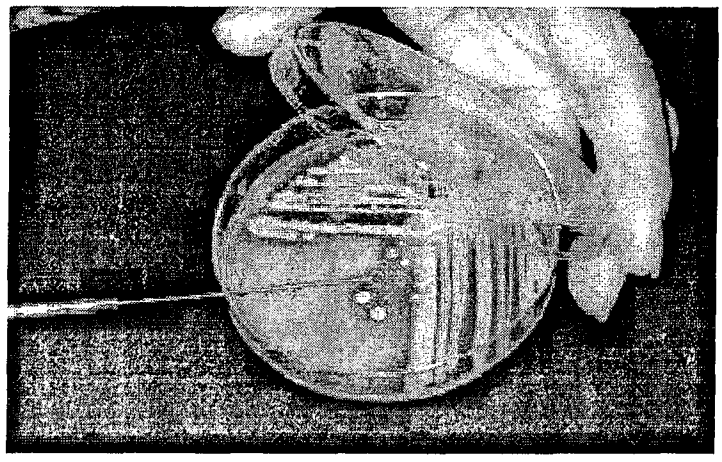


Figure 2.2 Representation of streaking method (Kaiser, 2005).

The individuals were transferred into fresh 10 ml of YMM by using sterile toothpicks and used for further investigations.

2.2.5 Characterization of individual mutants

Selected individual mutants were characterized by screening under various stress conditions such as heavy metal stress, heat stress, ethanol stress, and oxidative stress. Moreover, trehalose content in the cells was determined by HPLC (High Performance Liquid Chromatography). In addition, cobalt content associated with cells was obtained via Atomic Absorption Spectrometry.

2.2.5.1 Determination of cross-resistance to other stress conditions

2.2.5.1.1 Copper stress

Continuous stress application strategy was adopted for detecting the effect of copper on cells. Chloride salt of copper ($CuCl_2$) was applied in varying concentrations on selected cultures.

2.2.5.1.2 Chromium stress

Continuous stress application strategy was adopted for detecting the effect of chromium on cells. Chloride salt of chromium ($CrCl_3$) was applied in varying concentrations on selected cultures.

2.2.5.1.3 Zinc stress

Continuous stress application strategy was adopted for detecting the effect of zinc on cells. Chloride salt of chromium ($ZnCl_2$) was applied in varying concentrations on selected cultures.

2.2.5.1.4 Heat stress

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

2.2.5.1.5 Ethanol stress

Cells were exposed to ethanol stress for 1 hour at 30^0 C in 1.5 ml microfuge tubes. The ethanol content (v/v) showed the level of ethanol stress condition. The cells were collected by centrifugation at 14,000 rpm for 5 minutes and washed twice with YMM without dextrose. A cell suspension of 500 µl was inoculated into 10 ml of YMM and incubated at 30^0 C and 150 rpm. The growth of the cultures was checked after overnight incubation.

2.2.5.1.6 Oxidative stress

Varying amounts of hydrogen peroxide were added from 5M H_2O_2 stock solution into the liquid yeast culture of 1.5 ml. The cell pellets in microfuge tubes were resuspended in dextrose-free yeast minimal medium. The cells were incubated at 30°C for 1 hour by shaking at 30^0 C and 150 rpm. After this stress application period, they were centrifuged at 10,000 rpm for 10 minutes using a Beckman Coulter benchtop centrifuge. Following centrifugation, the supernatant was discarded and the cell pellet was resuspended in dextrose-free yeast minimal medium. A cell suspension of 500 µl was inoculated into 10 ml of liquid yeast minimal medium. The growth of the cultures was checked after overnight incubation.

2.2.5.1.7 Osmotic stress

Cells were grown in minimal media involving varying percentages of NaCl (0%, 5%, 10%, 15%). The optical density values of cells were checked after this continuous stress application strategy.

2.2.5.2 Determination of the trehalose content by HPLC

Yeast strains were inoculated into YMM and incubated overnight at 30°C and 150 rpm. Overnight cultures were used for inoculation into YMM with stress conditions. After required time of incubation for cells to grow under stress conditions, OD_{600} values were measured. The extraction of trehalose was carried out as described previously (Chuanbin et al., 1998). According to this method, 1.5 ml of the cells washed with YMM without dextrose and placed in a beaker where cells do not have a height more than 5 mm. This is important for the penetration of heat into the cells during microwave treatment. The beaker was placed into the microwave oven for 2-4 minutes at 750 W. After the microwave treatment, the content of the beaker was resuspended with an equal volume of deionized water. The extract was filtered through a 0.2 μm-filter and transferred into HPLC vials.

The analysis of trehalose was carried out using an Aminex© HPX-87H column. The method was the modified method of Ribiero et al, 1994. The retention time of trehalose in the column was determined as 6.89-7 minutes at 60°C with a flow rate of 0.6 ml/min by use of pure trehalose solution. As mobile phase, 5 mM H_2SO_4 was used. First, the standard solutions were analyzed to obtain a calibration curve. After calibration, the concentrations of trehalose in each sample were determined using the calibration curve. Cellular trehalose content was calculated as the percentage of trehalose in the cell (wt/wt). Cellular dry weight analysis was performed for each sample as described by Çakar (2000). Briefly, microfuge tubes were dried in an oven at 80°C for 48 hours, after which they were placed in a desiccator for 30 minutes to cool down to room temperature, and then weighed (w_1). Cells were transferred into microfuge tubes, centrifuged at 14,000 rpm for 5 minutes using a Beckman Coulter benchtop centrifuge, supernatants were discarded and cell pellets were resuspended in double distilled water to remove any residues left on the cells. The tubes were then centrifuged at 14,000 rpm

for 10 minutes using a Beckman Coulter benchtop centrifuge, and the supernatant was discarded. The tubes were dried in an oven at 80°C to evaporate the remaining liquid on the cell pellet. After 24 hours of drying, the tubes with dried cell extracts were weighed again (w_2). The cellular dry weight (CDW) was calculated by subtracting the first weight (w_1) from the second weight (w_2).

2.2.5.3 Determination of metal content associated with cells via flame atomic absorption spectroscopy

Yeast strains were inoculated into YMM and incubated overnight at 30°C and 150 rpm. Overnight cultures were used for inoculation into YMM with stress conditions. After required time of incubation for cells to grow under stress conditions, cells were harvested by centrifugation at 14,000 rpm for 5 minutes. Supernatants were discarded and cell pellets were washed twice in double distilled water to remove any metal residues left on the cells. Metal content associated with cells, either inside the cytoplasm or bound to the cell wall, were extracted by acid hydrolysis. For hydrolysis, cells were exposed to 6 M nitric acid solution for one hour at 90^0 C. After acid hydrolysis, samples were ready for atomic absorption spectrometry measurements. Prior to measurement, each sample was diluted in a ratio of 1:2 in order to decrease the final acid concentration to 3 M for preventing any potential acid damages to the spectrometer. Flame Atomic Absorption Spectrometry strategy was employed as the atomization technique. An Analytik-Jena Model AAS Vario 6 (Germany) flame atomic absorption spectrometer with a hollow cathode lamp was used in this study. An air/acetylene flame was also employed. Copper, chromium and cobalt contents associated with cells were determined. The parameters for these metals were set as recommended by the manufacturers. Wavelengths and the slit width values for each metal are given in Table 2.1.

Table 2.1 Wavelength and slit width values of cobalt, copper and chromium for Flame Atomic Absorption Spectrometry (FAAS).

	Cobalt	Cupper	Chromium
Wavelength (nm)	242.5	324.8	357.9
Slit width (nm)	0.2	1.2	0.2

Cell dry weight analysis was performed at the 72. hour of incubation. FAAS results showing the metal content associated with the cells were evaluated together with dry weight analysis results.

3 RESULTS

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

Before applying the initial the stress level for obtaining the generations, the wild-type culture was screened under various concentrations of $CoCl_2$ stress (0 mM, 1 mM, 2 mM, 6 mM, 10 mM, 20 mM and 40 mM). The optical density values at 600 nm were monitored at 24^{th}, 48^{th} and 72^{nd} hours. The results showed that the growth of the cells was inhibited at $CoCl_2$ concentrations higher than 6mM (Data not shown).

3.2 Stress Application and Creation of Generations

Creation of generations was based on applying the stress conditions and transferring the survivors of the previous stress step to the next stress step. Initially, the mutated culture *101* was used as the starting culture for selection experiments. The survivors of the stress step constituted the generation and also used to prepare stock cultures at each step. Two strategies were adopted for obtaining the generations. *Increasing stress generations* were obtained by increasing the level of stress at each step (Table 3.1) and *constant stress generations* were obtained by applying a low but constant level of stress at each step (Table 3.2 and Table 3.3).

Table 3.1 is showing the increasing stress populations including their code names and stress levels in mM $CoCl_2$. Stress conditions started by applying 0.5 mM $CoCl_2$ and increased 0.5 mM at each step initially. After obtaining the 31^{st} increasing stress generation corresponding to the stress level of 15.5 mM $CoCl_2$, the increase per each step was adopted to be 5 mM. Finally, 36^{th} increasing stress generation, which constituted the survivors of 40 mM $CoCl_2$, was the final generation.

Table 3.1 Nomenclature for continuous increasing stress populations.

Generation	Code	Stress condition (mM $CoCl_2$)
1st increasing stress generation	*1G*	0.5
2nd increasing stress generation	*2G*	1.0
3rd increasing stress generation	*3G*	1.5
4th increasing stress generation	*4G*	2.0
5th increasing stress generation	*5G*	2.5
6th increasing stress generation	*6G*	3.0
7th increasing stress generation	*7G*	3.5
8th increasing stress generation	*8G*	4.0
9th increasing stress generation	*9G*	4.5
10th increasing stress generation	*10G*	5.0
11th increasing stress generation	*11G*	5.5
12th increasing stress generation	*12G*	6.0
13th increasing stress generation	*13G*	6.5
14th increasing stress generation	*14G*	7.0
15th increasing stress generation	*15G*	7.5
16th increasing stress generation	*16G*	8.0
17th increasing stress generation	*17G*	8.5
18th increasing stress generation	*18G*	9.0
19th increasing stress generation	*19G*	9.5
20th increasing stress generation	*20G*	10.0
21st increasing stress generation	*21G*	10.5
22nd increasing stress generation	*22G*	11.0
23rd increasing stress generation	*23G*	11.5
24th increasing stress generation	*24G*	12.0
25th increasing stress generation	*25G*	12.5
26th increasing stress generation	*26G*	13.0
27th increasing stress generation	*27G*	13.5
28th increasing stress generation	*28G*	14.0
29th increasing stress generation	*29G*	14.5
30th increasing stress generation	*30G*	15.0
31st increasing stress generation	*31G*	15.5
32nd increasing stress generation	*32G*	20.0
33rd increasing stress generation	*33G*	25.0
34th increasing stress generation	*34G*	30.0
35th increasing stress generation	*35G*	35.0
36th increasing stress generation	*36G*	40.0

Table 3.2 shows the constant stress populations that involve the constant stress condition of 0.5 mM. Survivors of each step were transferred to the next stress condition, where the stress condition was the same as the previous one.

Table 3.2 Nomenclature for continuous constant stress populations selected at 0.5 mM $CoCl_2$.

Generation	Code	Stress condition (mM $CoCl_2$)
1st constant stress generation	*1K*	0.5
2nd constant stress generation	*2K*	0.5
3rd constant stress generation	*3K*	0.5
4th constant stress generation	*4K*	0.5
5th constant stress generation	*5K*	0.5
6th constant stress generation	*6K*	0.5
7th constant stress generation	*7K*	0.5
8th constant stress generation	*8K*	0.5
9th constant stress generation	*9K*	0.5
10th constant stress generation	*10K*	0.5
11th constant stress generation	*11K*	0.5
12th constant stress generation	*12K*	0.5
13th constant stress generation	*13K*	0.5
14th constant stress generation	*14K*	0.5
15th constant stress generation	*15K*	0.5
16th constant stress generation	*16K*	0.5
17th constant stress generation	*17K*	0.5
18th constant stress generation	*18K*	0.5
19th constant stress generation	*19K*	0.5
20th constant stress generation	*20K*	0.5
21st constant stress generation	*21K*	0.5
22nd constant stress generation	*22K*	0.5
23rd constant stress generation	*23K*	0.5
24th constant stress generation	*24K*	0.5
25th constant stress generation	*25K*	0.5
26th constant stress generation	*26K*	0.5
27th constant stress generation	*27K*	0.5
28th constant stress generation	*28K*	0.5
29th constant stress generation	*29K*	0.5
30th constant stress generation	*30K*	0.5
31st constant stress generation	*31K*	0.5
32nd constant stress generation	*32K*	0.5
33rd constant stress generation	*33K*	0.5
34th constant stress generation	*34K*	0.5
35th constant stress generation	*35K*	0.5
36th constant stress generation	*36K*	0.5

Table 3.3 lists the generations of a second constant stress strategy with a higher constant stress level. The same constant stress application described formerly was performed at a higher constant stress level of 3 mM $CoCl_2$.

Table 3.3 Nomenclature for continuous new constant stress populations selected at 3mM $CoCl_2$.

Generation	Code	Stress condition (mM $CoCl_2$)
1st new constant stress generation	*1Ky*	3
2nd new constant stress generation	*2Ky*	3
3rd new constant stress generation	*3Ky*	3
4th new constant stress generation	*4Ky*	3
5th new constant stress generation	*5Ky*	3
6th new constant stress generation	*6Ky*	3
7th new constant stress generation	*7Ky*	3
8th new constant stress generation	*8Ky*	3
9th new constant stress generation	*9Ky*	3
10th new constant stress generation	*10Ky*	3
11th new constant stress generation	*11Ky*	3
12th new constant stress generation	*12Ky*	3
13th new constant stress generation	*13Ky*	3
14th new constant stress generation	*14Ky*	3
15th new constant stress generation	*15Ky*	3
16th new constant stress generation	*16Ky*	3
17th new constant stress generation	*17Ky*	3
18th new constant stress generation	*18Ky*	3
19th new constant stress generation	*19Ky*	3
20th new constant stress generation	*20Ky*	3
21st new constant stress generation	*21Ky*	3
22nd new constant stress generation	*22Ky*	3
23rd new constant stress generation	*23Ky*	3
24th new constant stress generation	*24Ky*	3
25th new constant stress generation	*25Ky*	3
26th new constant stress generation	*26Ky*	3
27th new constant stress generation	*27Ky*	3
28th new constant stress generation	*28Ky*	3
29th new constant stress generation	*29Ky*	3
30th new constant stress generation	*30Ky*	3
31st new constant stress generation	*31Ky*	3
32nd new constant stress generation	*32Ky*	3
33rd new constant stress generation	*33Ky*	3
34th new constant stress generation	*34Ky*	3
35th new constant stress generation	*35Ky*	3
36th new constant stress generation	*36Ky*	3

3.3 Selection of individual mutants from final mutant populations

The final generation obtained by application of continuously increasing levels of $CoCl_2$ was used for selecting the individual mutants. 36th increasing stress generation was accepted to be the final increasing stress generation, because at stress levels higher than 40mM (stress level of generation 36), almost no survival was observed (Figure 3.3). This final generation was inoculated onto YMM-agar plates by spreading and incubated overnight. Eight colonies from each plate were chosen, inoculated into liquid YMM for growth and detailed analysis and named as given in Table 3.4.

Table 3.4 Nomenclature for mutant individuals from selected increasing stress final population *(36G)*.

Individual mutant	Code
1st increasing stress individual mutant	*G1*
2nd increasing stress individual mutant	*G2*
3rd increasing stress individual mutant	*G3*
4th increasing stress individual mutant	*G4*
5th increasing stress individual mutant	*G5*
6th increasing stress individual mutant	*G6*
7th increasing stress individual mutant	*G7*
8th increasing stress individual mutant	*G8*

The final generation obtained by application of constant stress was used for selecting the individual mutants of constant stress. 36th constant stress generation was accepted to be the final constant stress generation, in order to make comparison with the individuals selected from increasing stress final generation 36G. This final generation was inoculated onto YMM-agar plates by spreading and incubated overnight. 8 colonies from each plate were chosen, inoculated into liquid media and named as given in Table 3.5.

Table 3.5 Nomenclature for mutant individuals selected from constant stress final population (*36K*).

Individual mutant	Code
1st constant stress individual mutant	*K1*
2nd constant stress individual mutant	*K2*
3rd constant stress individual mutant	*K3*
4th constant stress individual mutant	*K4*
5th constant stress individual mutant	*K5*
6th constant stress individual mutant	*K6*
7th constant stress individual mutant	*K7*
8th constant stress individual mutant	*K8*

3.4 Characterization of populations and individual mutants

3.4.1 Morphological analysis by microscopic techniques

In order to compare the morphology of the mutants with that of the wild-type, *Saccharomyces cerevisiae* cells were analyzed by use of light microscope under 100X objective with oil immersion. Chemically (EMS) mutagenized yeast cells (*101*) showed unusual budding characteristics (Figure 3.1).

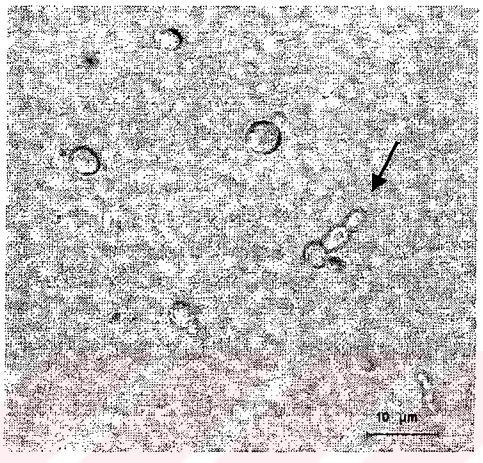


Figure 3.1 EMS mutagenized *S. cerevisiae* cells (101) under light microscope with oil immersion, 1000X total magnification.

Additionally, 22nd increasing stress generation (*22G*) yeast cells, when grown under metal stress, were observed to have significantly enlarged vacuoles, when observed under the light microscope with 100X objective and oil immersion (Figure 3.2).

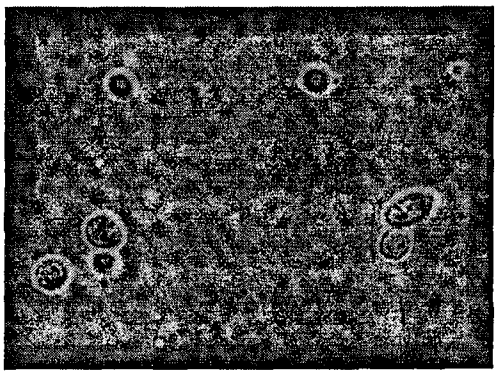


Figure 3.1 22nd increasing stress generation (*22G*) yeast cells grown overnight in the presence of 11 mM $CoCl_2$. Cells were observed at 1000X magnification (with immersion oil) under the light microscope.

3.4.2 Determination of cobalt stress resistance

3.4.2.1 Cobalt stress resistances of increasing stress generations

In order to gain insight into the cobalt-resistance characteristics of increasing stress generations, survival ratios of each generation were determined. At each step of increasing stress application, ratio of optical density values at 600 nm under stress condition and under normal condition (without stress) were used to generate the plot given in Figure 3.3.

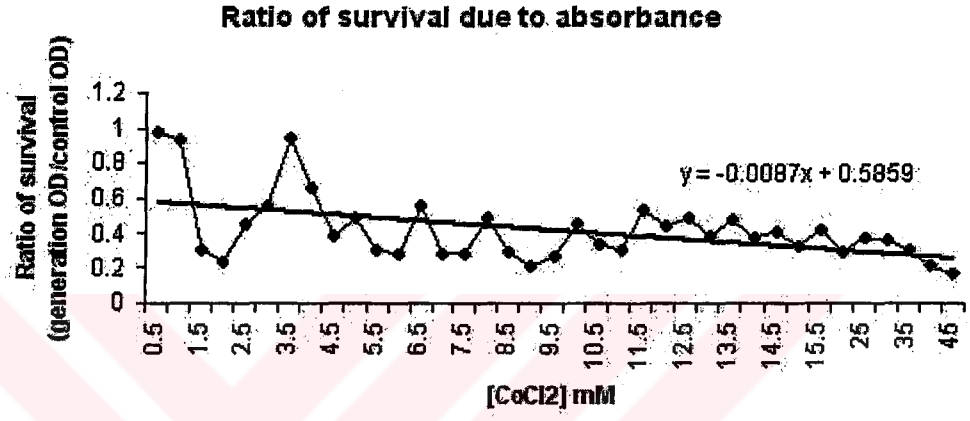


Figure 3.3 Survival analysis of increasing stress generations.

3.4.2.2 Cobalt stress resistances of final populations of increasing stress generations

In order to obtain cobalt resistance characteristics of final populations of both increasing stress selection strategy and constant stress selection strategy, fresh cultures of *100, 101, 36K* and *36G* were used for screening via 5-tube MPN method under five different $CoCl_2$ concentrations (0 mM, 0.5mM, 5 mM, 10 mM and 20 mM). The percent survival value of each condition at 48th hour in reference to growth at 0 mM is shown in Table 3.6 and Figure 3.4.

Table 3.6 Percent survival values of *100, 101, 36G, 36K* cultures under stress conditions.

Generation name	% survival at 0.5 mM	% survival at 5 mM	% survival at 10 mM	% survival at 20 mM
100	0.6000	0.0005	0.0005	0.0005
101	16.6667	0.0077	0.0077	0.0077
36K	44.0000	0.0005	0.0005	0.0005
36G	17.1429	1.7143	0.1714	0.0017

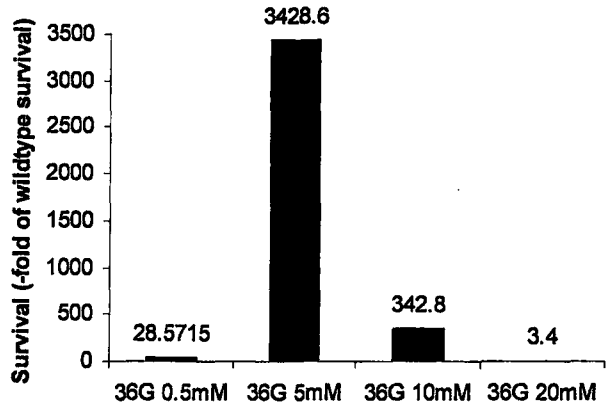


Figure 3.4 The survival of *36G* (as –fold of wild-type survival under the same conditions) at various $CoCl_2$ concentrations between 0.5 mM and 20 mM.

The final population of the increasing stress selection strategy (*36G*) survived up to 3428-fold of wild-type under stress conditions (Figure 3.4). Additionally, the final population of the constant stress selection strategy (*36K*) survived 44% under the stress condition applied for 36 generations (Table 3.6). However, the percent survival values of *36K* at higher stress levels were not different than those of the wild-type.

3.4.2.3 Cobalt stress resistances of increasing stress and constant stress individual mutants

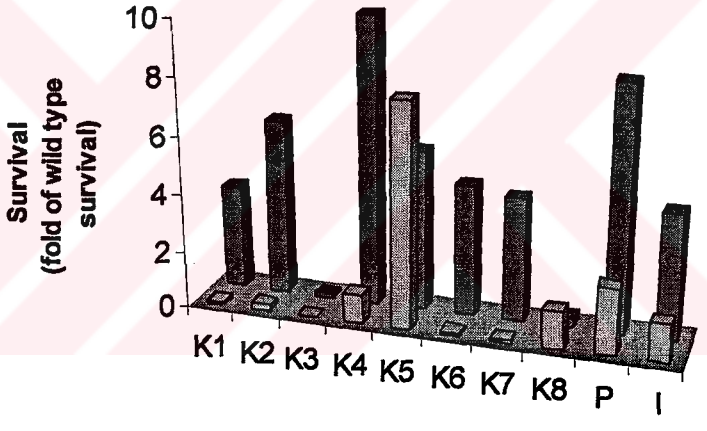
100, 101, 36K, 36G, and 8 constant stress individual mutants (*K1, K2, K3, K4, K5, K6, K7, K8*) were screened via 5-tube MPN strategy under stress conditions involving 0.5 mM and 5 mM $CoCl_2$. Percent survival values of each culture were detected by analyzing growth at stress conditions in reference to growth at 0 mM $CoCl_2$. Table 3.7 shows the percent survival ratios after 48^{th} hour of incubation.

Table 3.7 Percent survival ratios of constant stress individual mutants and final populations after exposure to 0.5 mM and 5 mM $CoCl_2$ stress conditions.

Population / individual name	0.5 mM	5 mM
100	10.0	0.08
101	10.0	0.05
36K	84.6	0.18
36G	44.0	0.26
K1	36.4	0.01
K2	61.5	0.02
K3	1.1	0.00

K4	100.0	0.08
K5	56.0	0.60
K6	45.8	0.01
K7	43.3	0.01
K8	3.4	0.10

Table 3.7 shows that percent survival ratios of the individuals selected from the final constant stress population (*36K*) are in a varying range when compared to *36K*. Figure 3.5 points out the differences in survivals between the individuals and the final population as folds of wild-type survivals under 0.5 mM and 5 mM $CoCl_2$ stress conditions. "P" represents the final constant stress population and "I" represents the arithmetic average value of eight individuals' survivals selected randomly from the final population.



	K1	K2	K3	K4	K5	K6	K7	K8	P	I
5 mM CoCl2	0.14	0.23	0.00	1.04	7.82	0.12	0.10	1.30	2.31	1.35
0.5 mM CoCl2	3.64	6.15	0.11	10.00	5.60	4.58	4.33	0.34	8.46	4.35

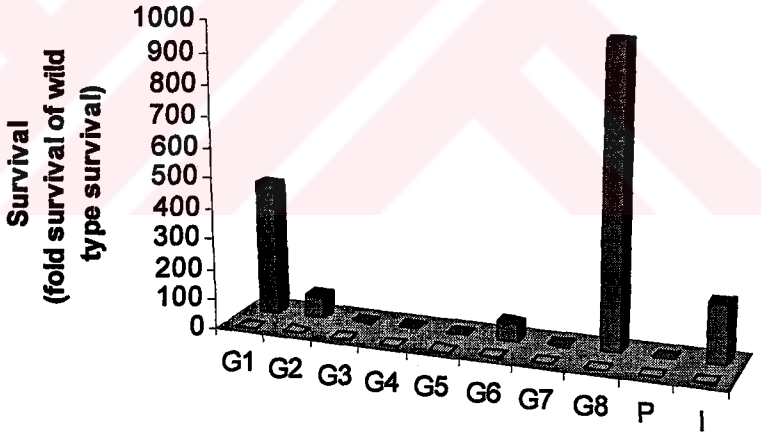
Figure 3.5 Cobalt stress resistances as folds of (100) survival ratio under 0.5 mM and 5 mM $CoCl_2$ stress conditions.

100, 101, 36K, 36G, and 8 increasing stress individual mutants (*G1, G2, G3, G4, G5, G6, G7, G8*) were screened via 5-tube MPN strategy under two different stress conditions (5mM and 30 mM $CoCl_2$. Percent survival values of each culture were determined by analyzing growth at stress conditions in reference to growth at 0 mM $CoCl_2$. Table 3.8 shows the percent survival ratios after 48. hour of incubation.

Table 3.8 Percent survival ratios of increasing stress individual mutants.

Population / individual name	% survival at 5 mM $CoCl_2$	% survival at 30 mM $CoCl_2$
100	0.0018	0.000135
101	0.0311	0.000003
36K	0.0010	0.000000
36G	0.0063	0.000007
G1	**0.7857**	0.000082
G2	**0.1364**	0.000011
G3	0.0050	0.000011
G4	0.0048	0.000046
G5	0.0005	0.000046
G6	**0.1000**	0.000096
G7	0.0009	0.000026
G8	**1.7647**	0.000135

In Figure 3.6, "P" represents the final increasing stress population and "I" represents the average value of eight increasing stress individuals selected from the final population.



	G1	G2	G3	G4	G5	G6	G7	G8	P	I
30 mM CoCl2	0.61	0.08	0.08	0.34	0.34	0.71	0.19	1.00	0.05	0.42
5 mM CoCl2	436.	75.7	2.78	2.67	0.27	55.5	0.49	980.	3.56	194.

Figure 3.6 Cobalt stress resistances as folds of wild-type (*100*) survival ratio under 5 mM and 30 mM $CoCl_2$ stress conditions.

The results show that individuals selected from the final increasing stress population survived up to 980-fold of wild-type under 5 mM $CoCl_2$ stress condition. Under 30 mM stress condition, the percent survival values of the individuals were not higher than the wild-type.

3.4.3 Determination of various other stress resistances

3.4.3.1 Chromium stress

Cultures *100, 101, 36K, 36G* and three increasing stress individuals (*G1, G6, G8*) were screened under continuous $CrCl_3$ stress levels (0 mM, 3mM, 5 mM). OD_{600} values were taken at 72nd hour. Survival ratio values with respect to growth of each culture at 0 mM $CrCl_3$ are given in Table 3.9.

Table 3.9 Survival ratio values of cultures after 72 hours of incubation at YMM containing 3 mM and 5 mM $CrCl_3$.

Population / individual name	Survival ratio at 3 mM $CrCl_3$	Survival ratio at 5 mM $CrCl_3$
100	0.80	0.25
101	0.79	0.32
36K	0.87	0.72
36G	0.52	0.30
G1	**0.92**	**0.66**
G6	0.55	0.16
G8	0.88	0.64

Cultures *100, 101, 36K, 36G,* four increasing stress individual mutants (*G1, G5, G6, G8*) and three constant stress individual mutants (*K2, K4, K5*) were used for estimation of number of survivors of continuous chromium (3 mM $CrCl_3$) stress application. 5-tube MPN method was used for statistical cell number estimation. Percent survival values are given in Table 3.10.

Table 3.10 Percent survival values of cultures determined by 5-tube MPN method, after 96 hour of incubation. Cells were exposed to 3 mM $CrCl_3$ as a continuous stress.

Population / individual name	% survival at 3 mM $CrCl_3$
100	1,546
101	0,189
36K	0,001
36G	0,002
G1	**5,556**
G5	0,000
G6	1,875
G8	0,294
K2	0,014
K4	0,001
K5	0,002

The results show that increasing stress individual *"G1"* gained cross-resistance to chromium stress when compared to wild-type.

3.4.3.2 Copper stress

Cultures *100, 101, 36K, 36G,* four increasing stress individuals and three constant stress individuals were used for estimation of number of survivors of continuous copper (1 mM) stress application Estimated cell numbers at 96th hour are given in Table 3.11.

Table 3.11 Percent survival values of cultures as a result of 5-tube MPN method, after 96 hour of incubation. Cells were exposed to 1 mM $CuCl_2$ as a continuous stress.

Population / individual name	% survival at 1 mM $CuCl_2$
100	0.0064
101	0.0000
36K	0.0000
36G	0.0000
G1	0.0002
G5	**0.0135**
G6	0.0000
G8	0.0018
K2	0.0013
K4	0.0027
K5	0.0001

The results show that increasing stress individual *"G5"* gained cross-resistance to copper stress when compared to wild-type.

3.4.3.3 Zinc stress

Fresh cultures of *100, 101, 36G, Ky, G1, G5, G6* were inoculated into 10 ml of YMM containing different concentrations of $ZnCl_2$ (0mM, 5mM, 10mM) in 50 ml tubes. The cultures were incubated at 30^0 C, 150 rpm for 72 hours. Data showing the content of media with each percentage is given in Table 3.12.

Table 3.12 YMM and $ZnCl_2$ contents in YMM with varying metal concentrations.

Metal concentration (mM)	YMM (µl)	$ZnCl_2$ (µl from 1M stock solution)
0	10000	0
5	9950	50
10	9900	100

Optical density values at 72nd hour of incubation were determined and used for obtaining the survival ratios (Table 3.13).

Table 3.13 Survival ratios of different cultures grown at 5 and 10 mM $ZnCl_2$ with respect to growth at 0 mM $ZnCl_2$.

Population / individual name	Survival ratio at 5 mM	Survival ratio at 10 mM
100	0.98	0.72
101	0.90	0.63
36G	0.95	0.69
36Ky	0.90	0.49
G1	0.98	**0.88**
G5	0.94	0.76
G6	0.82	0.71

The results show that increasing stress individual *"G1"* gained a slight cross-resistance to zinc stress when compared to wild-type.

3.4.3.4 Heat stress

30^0 C, 40^0 C, 50^0 C and 60^0 C stress were applied as pulse stress (10 minutes of exposure) on cultures *100, 101, 36K, 36G, G5* and *G6* during late exponential phase of growth. OD_{600} values on 48^{th} hour of growth after inoculation into fresh media following stress were analyzed to obtain survival ratios with respect to growth at 30^0 C, the optimal growth temperature (Table 3.14).

Table 3.14 Survival ratio values of different cultures tested after pulse exposure to heat stress (30^0 C, 40^0 C, 50^0 C, 60^0 C).

Population / individual name	Survival ratio at 40^0 C	Survival ratio at 50^0 C	Survival ratio at 60^0 C
100	0.96	0.88	0.41
101	0.99	0.76	0.68
36K	0.92	0.88	0.82
36G	0.92	0.70	0.44
G5	0.91	0.71	**0.69**
G6	0.93	0.89	**0.73**

The results may imply that increasing stress individuals *"G5"* and *"G6"* gained cross-resistance to heat stress when compared to wild-type.

3.4.3.5 Ethanol stress

Fresh cultures of *100, 101,* increasing stress population *36G,* constant stress population *36Ky,* increasing stress individual mutants *G1, G5, G6* were inoculated into 10 ml of YMM containing different percentages of ethanol (0%, 2%, 5%, 10%, 15%). Data showing the contents of media with varying ethanol percentages are given in Table 3.15.

Table 3.15 YMM and pure ethanol content in YMM with varying ethanol percentages.

Ethanol (%)	YMM (µl)	Ethanol (µl)
0	10000	0
2	9800	200
5	9500	500
10	9000	1000
15	8500	1500

Spectrophotometric measurements were done at 72nd hour of incubation and data were used for determining the survival ratios (Table 3.16).

Table 3.16 Survival ratios of different cultures grown at varying ethanol stress conditions, with respect to groth at 0% ethanol.

Population / individual name	Survival at 2% ethanol	Survival at 5% ethanol	Survival at 10% ethanol	Survival at 15% ethanol
100	0.552	0.358	0.011	0.010
101	0.642	0.348	0.010	0.010
36G	0.112	0.006	0.005	0.004
36Ky	0.572	0.387	0.004	0.002
G1	0.818	0.154	0.006	0.004
G5	0.810	0.006	0.005	0.004
G6	0.102	0.005	0.005	0.004

The results show that the cobalt- resistant mutant populations and individuals did not have a cross-resistance to ethanol stress.

3.4.3.6 Oxidative stress

One ml fresh cultures of *100, 101, 36G, 36Ky, G1, G5, G6* were centrifuged at 10,000 rpm for 10 minutes. Supernatants were discarded and cell pellets were resususpended in microfuge tubes involving YMM with varying hydrogen peroxide (H_2O_2) concentrations (Table 3.17). The cells were incubated at 30°C for 1 hour by shaking at 30^0 C and 150 rpm. After this pulse stress application, the cells were washed by centrifugation at 10,000 rpm for 10 minutes and cell pellets were inoculated into 10 ml of YMM.

Table 3.17 YMM and pure H_2O_2 contents of various media (0-4 M H_2O_2) for oxidative stress application in 1 ml microfuge tubes.

H_2O_2 final concentration (M)	YMM (µl)	H_2O_2 (µl) (from 5M stock solution)
0	1000	0
1	800	200
2	600	400
3	400	600
4	200	800

Optical density values at 72nd hour of incubation and used for determining the survival ratios (Table 3.18).

Table 3.18 Survival ratios of different cultures exposed to pulse oxidative stress, with respect to growth at 0 M H_2O_2.

Population / individual name	Survival ratio at 1 M H_2O_2	Survival ratio at 2 M H_2O_2	Survival ratio at 3 M H_2O_2	Survival ratio at 4 M H_2O_2
100	0.84	0.83	0.82	0.81
101	0.95	0.92	0.03	0.02
36G	0.79	0.83	0.78	0.02
36Ky	0.99	0.80	0.05	0.05
G1	0.65	0.10	0.09	0.06
G5	**0.99**	**0.98**	**0.97**	0.81
G6	0.90	0.35	0.07	0.04

The results show that *G5* was more resistant to oxidative stress conditions up to 3 M H_2O_2 when compared to wild-type.

3.4.3.7 Osmotic stress

Fresh cultures of *100, 36G, 36Ky, G1, G5, G6* were inoculated into 50 ml tubes involving 10 ml of YMM with varying NaCl concentrations (0%, 5%, 10%, 15%). Optical density values at 72nd hour of incubation were used for determining the survival ratios (Table 3.19).

Table 3.19 Survival ratios of different cultures exposed to osmotic stress (5%, 10%, 15% NaCl), with respect to growth at 0% NaCl.

Population / individual name	Survival ratio at 5% NaCl	Survival ratio at 10% NaCl	Survival ratio at 15% NaCl
100	0.57	0.36	0.15
101	0.65	0.37	0.15
36G	0.63	0.37	0.14
36Ky	0.76	0.43	0.14
G1	0.65	0.40	0.15
G5	0.63	0.39	0.16
G6	0.73	0.36	0.13

The results show that none of the mutant populations and individuals had an improved cross-resistance to osmotic stress.

3.5 Determination of metal content associated with cells using flame atomic absorption spectrometer (FAAS)

Fresh cultures of *100, 36G, 36Ky, G1, G5, G6* were inoculated into 50 ml falcon tubes involving 10 ml of YMM with varying metal concentrations (Table 3.20).

Table 3.20 Stress levels applied to the cultures prior to determination of metal contents with atomic absorption spectrometry.

Metal	Stress level	Corresponding "ppm" value
Cobalt	10 mM	580 ppm
	20 mM	1160 ppm
Copper	10 mM	635 ppm
Chromium	5 mM	260 ppm

After necessary time of incubation for growth, cell pellets were exposed to acid hydrolysis and FAAS measurements were performed. The results represent the metal content emerged after acid hydrolysis of the cell pellets as "ppm values (mg/l)". Percentages of metal contents hold by cells were obtained by analyzing the initial metal stress exposed to the cells prior to FAAS analysis. Cell dry weight analysis was performed for the cultures at 72nd hour of incubation and percent metal contents associated with cells were determined as " (metal weight / cell dry weight) X 100".

3.5.1 Cobalt content associated with cells

Cobalt detection was performed at 242.5 nm wavelength with a 0.2 nm slit width. Percent cobalt hold by the cells (the cobalt content associated with cells in reference to cobalt content previously added to the growth media) is shown in Table 3.21.

Table 3.21 Percent cobalt hold by the cell pellets of different cultures tested.

Individuals and populations	% cobalt hold by the cells
100 - 0 mM	0
36G - 0 mM	0
36Ky - 0 mM	0
G1 - 0 mM	0
G5 - 0 mM	0
G6 - 0 mM	0
100 - 10 mM	0.25
36G - 10 mM	0.74
36Ky - 10 mM	0.52
G1 - 10 mM	0.65
G5 - 10 mM	0.46
G6 -10 mM	0.61
100 - 20 mM	0.05
36G - 20mM	0.24
36Ky - 20mM	0.32
G1 - 20mM	0.05
G5 - 20mM	0.04
G6 - 20mM	0.31

Percent cobalt hold by the cells (the cobalt content associated with cells in reference to cobalt content previously added to the growth media) in folds of percent cobalt hold by wild-type at the same conditions is shown in Figure 3.7.

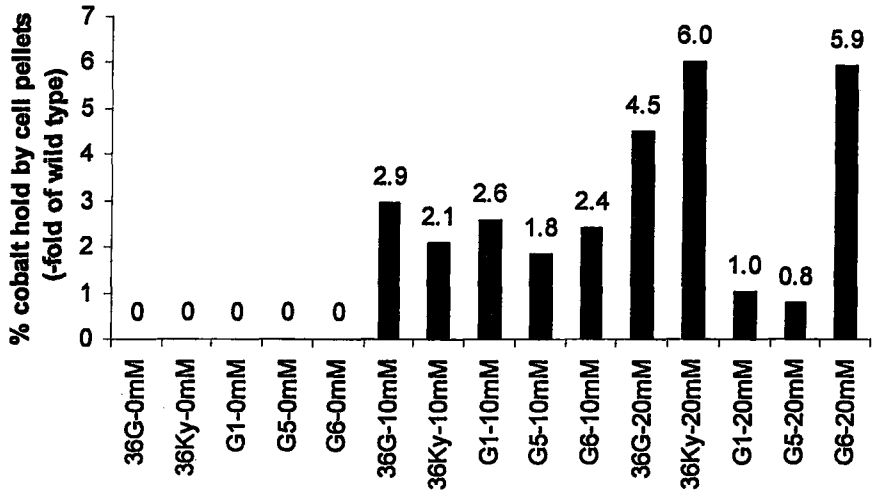


Figure 3.7 Percent cobalt hold by the cell pellets of different cultues tested in folds of wild-type at the same conditions.

Percent cobalt weight / cell dry weight values for each culture are shown in Figure 3.8.

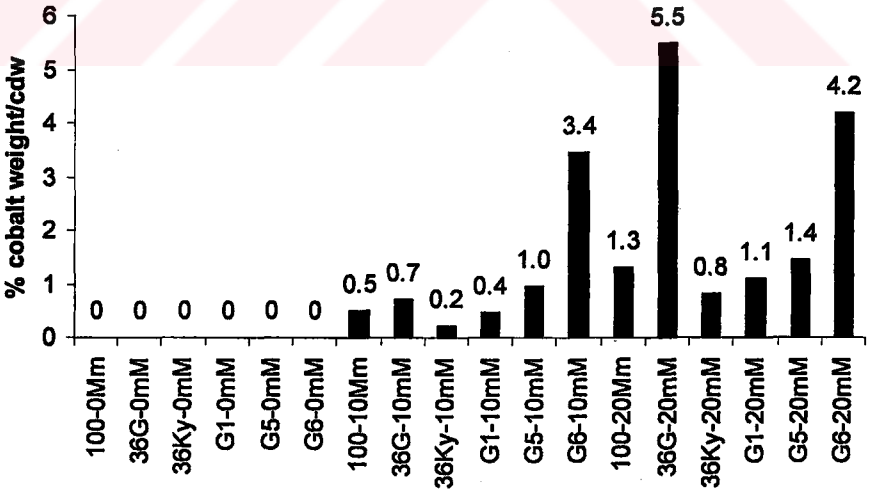


Figure 3.8 Percent cobalt weight / cell dry weight values of different cultures tested.

The results show that the cobalt content bound to cell pellets under $CoCl_2$ stress conditions were approximately 3-5 folds that of the cell pellets under non-stress conditions.

3.5.2 Chromium content associated with cells

Chromium detection was performed at 357.9 nm wavelength with a 0.2 nm slit width. Percent chromium hold by the cells (the chromium content associated with cells in reference to chromium content previously added to the growth media) is shown in Table 3.22.

Table 3.22 Percent chromium hold by the cell pellets of different cultures tested.

Individuals and populations	% chromium hold by the cells
100 - 0mM	0
36G - 0 mM	0
36Ky - 0 mM	0
G1 - 0 mM	0
G5 - 0 mM	0
G6 - 0 mM	0
100 - 5 mM	0.80
36G - 5 mM	0.32
36Ky - 5 mM	0
G1 - 5 mM	0
G5 - 5 mM	0
G6 - 5 mM	0.61

Percent chromium hold by the cells (the chromium content associated with cells in reference to chromium content previously added to the growth media) in folds of percent chromium hold by wild-type at the same conditions is shown in Figure 3.9.

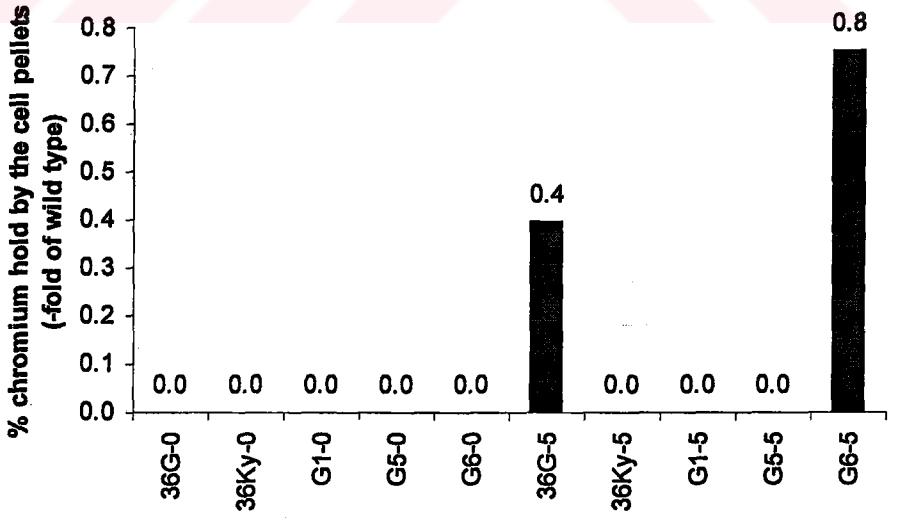


Figure 3.9 Percent chromium hold by the cell pellets of different cultues tested in folds of wild-type at the same conditions.

Percent chromium weight / cell dry weight values for each culture are shown in Figure 3.10.

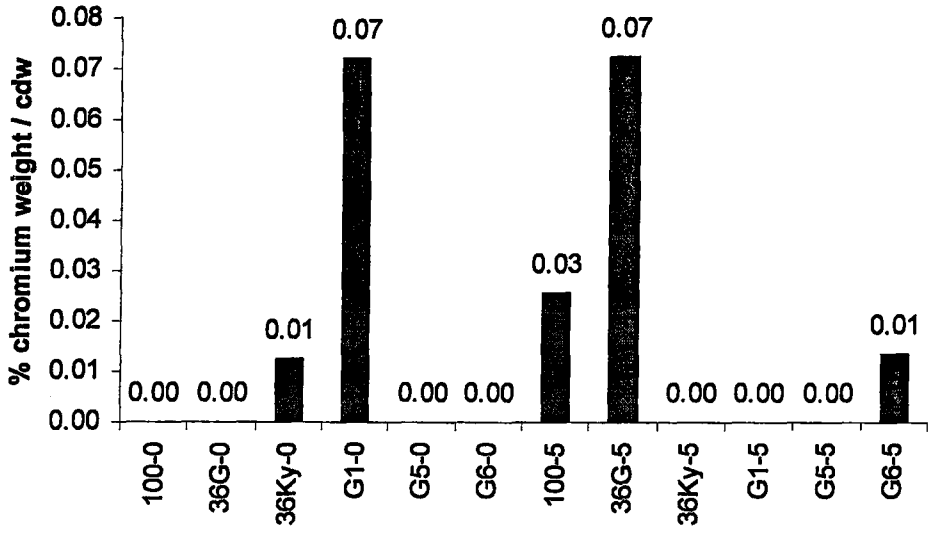


Figure 3.10 Percent chromium weight / cell dry weight values of different cultures tested.

The results show that chromium content associated with the individual mutant cells was lower when compared to wild-type.

3.5.3 Copper content associated with cells

Copper detection was performed at 324.8 nm wavelength with a 1.2nm slit width. Percent copper hold by the cells (the copper content associated with cells in reference to copper content previously added to the growth media) is shown in Table 3.23.

Table 3.23 Percent chromium hold by the cell pellets of different cultures tested.

Individuals and populations	% copper hold by the cells
100 - 0 mM	0
36G - 0 mM	0
36Ky - 0 mM	0
G1 - 0 mM	0
G5 - 0 mM	0
G6 - 0 mM	0
100 - 10 mM	0.70
36G - 10 mM	0
36Ky - 0 mM	0.89
G1 - 10 mM	0
G5 - 10 mM	0
G6 - 10 mM	1.49

Percent copper hold by the cells (the copper content associated with cells in reference to copper content previously added to the growth media) in folds of percent copper hold by wild-type at the same conditions is shown in Figure 3.11.

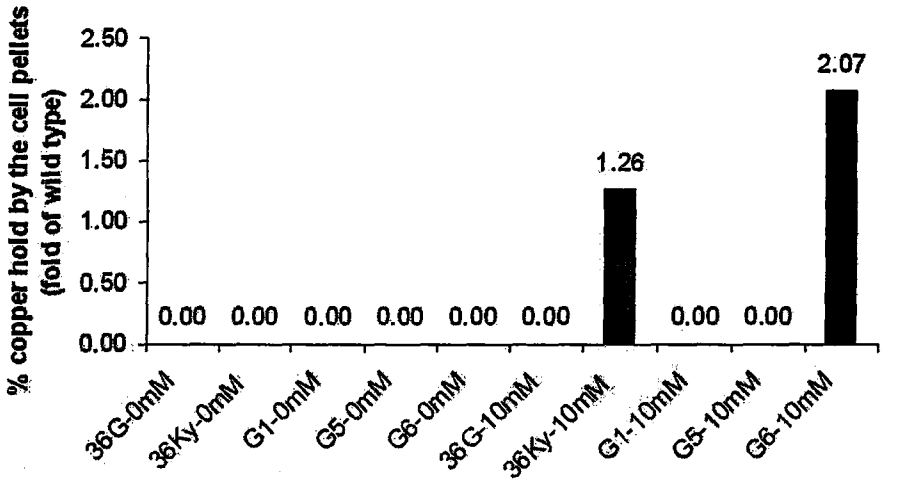


Figure 3.11 Percent copper hold by the cell pellets of different cultues tested in folds of wild-type at the same conditions.

Percent copper weight / cell dry weight values for each culture are shown in Figure 3.12.

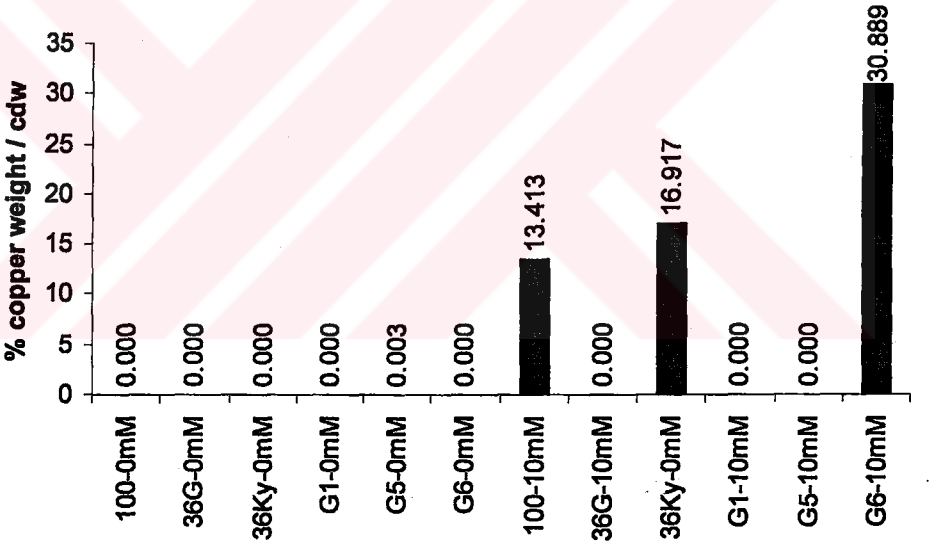


Figure 3.12 Percent copper weight / cell dry weight values of different cultures tested.

The results show that copper contents associated with the individual mutants *G1* and *G5* were lower when compared to wild-type. On the other hand, *G6,* which was sensitive under copper stress conditions, had 30-fold copper content in the cell pellets.

3.6 Determination of trehalose content of populations and individual mutants

As trehalose is an important carbohydrate associated with stress in yeasts, the trehalose contents of the cultures were determined by High Performance Liquid Chromatography (HPLC) under both stress and non-stress conditions. The cultures analyzed by HPLC were as follows:

- "*100*" non-mutagenized original culture
- "*101*" initially mutagenized culture
- "*36G*" final increasing stress population
- "*36Ky*" final constant stress population
- "*G1*" increasing individual number 1
- "*G5*" increasing individual number 5
- "*G6*" increasing individual number 6
- "*G8*" increasing individual number 8

Extraction of trehalose was performed by a previously explained method by Chuanbin *et. al.*, which is briefly based on microwave treatment on cells (Chuanbin et. al.,1998).

3.6.1 Trehalose standard curve

Trehalose standard curve was obtained by using D(+)Trehalose solutions with concentrations from 0 mg/ml to 10 mg/ml. The standard curve was prepared by using standard D(+)Trehalose solutions (Figure 3.6.1.1).

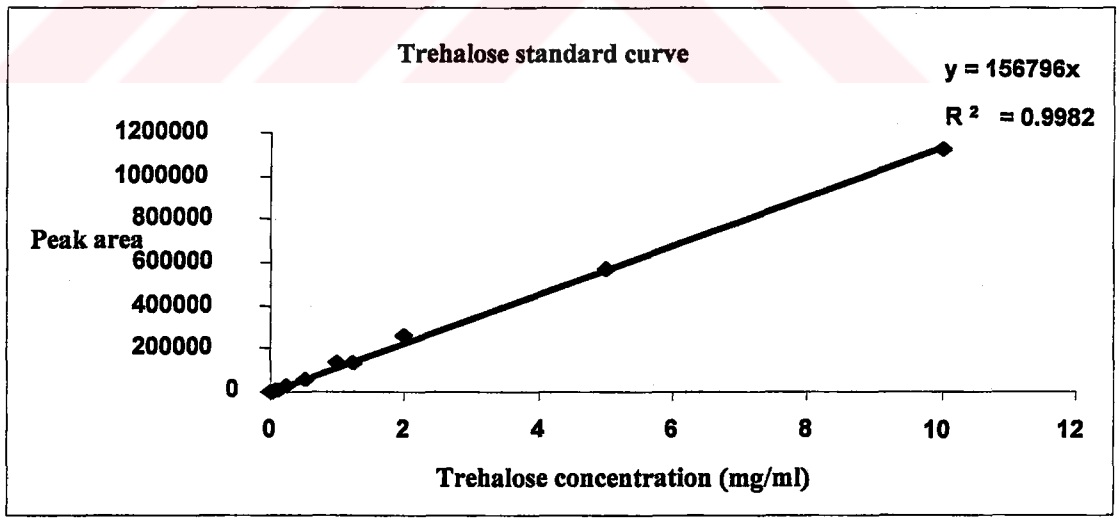


Figure 3.13 Trehalose standard curve.

The retention time of D(+)Trehalose was determined and the peak area of each sample at the determined time, which was 6.999 minutes in this experiment (data not shown), was used for obtaining the trehalose concentrations of the samples. The trehalose comncentrations were calculated by use of the peak areas according to the formula given below:

$$Trehalose\ (mg/ml) = \frac{1}{156796} Peak\ Area \qquad \textbf{Equation 3.1}$$

3.6.2 Trehalose contents of populations and individual mutants

Fresh overnight cultures of "*100*", "*101*", "*36G*", "36*Ky*" and individual mutants "*G1*", "*G5*", "*G6*", "*G8*" were used for inoculation into stress conditions. Stress resistance of cultures "*100*" and "*101*" were lower from the others. Thus, lower stress conditions were applied on these two cultures. Table 3.24 is showing the stress conditions as $CoCl_2$ concentrations.

Table 3.24 Stress conditions applied to cells prior to determination of trehalose contents.

Culture code	Stress condition
100	5 mM $CoCl_2$
101	5 mM $CoCl_2$
36G	10 mM $CoCl_2$
36Ky	10 mM $CoCl_2$
G1	10 mM $CoCl_2$
G5	10 mM $CoCl_2$
G6	10 mM $CoCl_2$
G8	10 mM $CoCl_2$

After required time of incubation, cells are used for trehalose extraction and cellular dry weight analysis. HPLC analysis and cell dry weight analysis results were expressed as percent trehalose weight / cellular dry weight (Table 3.25).

Table 3.25 Cell dry weights, trehalose concentrations and % trehalose (wt/wt) values of various cultures tested under stress and nonstress conditions.

	No stress			Under stress		
	Cell dry wt (mg/ml)	Trehalose (mg/ml)	% Trehalose (wt/wt)	Cell dry wt (mg/ml)	Trehalose (mg/ml)	% Trehalose (wt/wt)
100	0.24	0.13876	0.58	0.05	0.229183	4.58
101	0.08	0.195802	2.45	0.03	0.133588	4.45
36G	0.13	0.079447	0.61	0.23	2.239094	9.74
36Ky	0.04	0.137771	3.44	0.04	0.17311	4.33
G1	0.23	0.133855	0.58	0.03	0.137051	4.57
G5	0.2	0.080264	0.40	0.02	0.08101	4.05
G6	0.2	0.108657	0.54	0.24	0.137051	0.57
G8	0.11	0.136049	1.24	0.6	0.152989	0.25

The trehalose accumulation values of each culture were determined by examining ratio of trehalose accumulation under stress (5 mM $CoCl_2$) and without stress. The results are given in folds in reference to trehalose accumulation without stress (Figure 3.14).

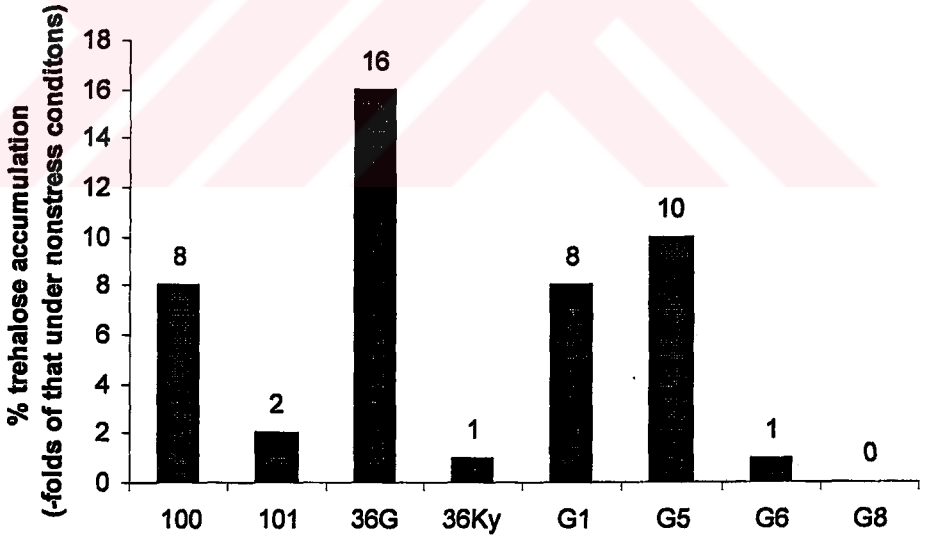


Figure 3.14 Percent trehalose accumulation values of cells under stress conditions as folds of percent trehalose accumulation under nonstress conditions.

The results indicate that similar or lower amounts of trehalose were accumulated by cobalt-resistant individuals when compared with-type under stress conditions.

4. DISCUSSION AND CONCLUSIONS

The primary aim of this study was to obtain cobalt-binding / cobalt-resistant *Saccharomyces cerevisiae* cells. In order to achieve this goal, evolutionary engineering strategies, which represent the inverse metabolic engineering approach, were designed and applied. At first, a chemical mutagen, ethyl methane sulphonate (EMS) was used for mutagenesis. By this way, the genetic diversity of the starting wild-type culture was increased.

With this genetically diverse culture and wild-type, an initial screening for stress resistance was performed. The aim was to determine the highest stress level that the wild-type and the EMS-mutagenized culture could resist. Both cultures were resistant under the stress conditions lower than 6 mM of $CoCl_2$, but could not tolerate higher concentrations. According to these results, initial stress levels to be used in selection strategies were determined.

By passing the survivors of a stress step to the following step, several generations of populations were obtained. While obtaining the generations of populations, two different selection strategies were adopted: "constant stress selection strategy" and "increasing stress selection strategy".

In the constant stress selection strategy, 36 generations of populations were obtained, each of which were exposed to a constant mild stress condition. 0.5 mM of $CoCl_2$ was chosen as the mild stress level. After obtaining the 36^{th} constant stress generation, this final population was screened under various stress levels. It was observed that the final constant stress population (*36K*) survived 44% under the stress conditions (0.5 mM) which had been applied for 36 generations. However, the same population did not behaved similarly under higher stress levels (5 mM, 10 mM and 20 mM $CoCl_2$) (Table 3.6). These results showed that applying a constant stress on the cells make them gain resistance towards the stress level applied. However, this strategy did not help the cells gain resistance to higher stress levels.

After obtaining the results of the constant stress selection strategy under 0.5 mM $CoCl_2$, another set of constant stress selection strategy was performed under 3 mM $CoCl_2$ stress level. The aim was to test if similar results would be obtained under this higher stress condition. Thirty six generations of constant (3 mM $CoCl_2$) stress application were obtained and the final population was named as *36Ky*. The screening results verified the information obtained previously. The novel constant stress population "*36Ky*" resulted with high survival values under the particular stress condition (3 mM $CoCl_2$) that had been constantly applied during selection.

While applying the increasing stress selection strategy, initial stress level applied was 0.5 mM $CoCl_2$. The stress level was gradually increased up to 40 mM in 36 generations. After obtaining 36^{th} generation, 45 mM of $CoCl_2$ was applied to cells. Even after 96 hours of incubation for growth, almost no survival was obtained (Figure 3.3). Thus, 36^{th} increasing stress generation (*36G*) was accepted as the final increasing stress generation. Screening results of *36G* showed that the increasing stress selection strategy was suitable for obtaining populations which were resistant to higher levels of stress conditions. As shown in Table 3.6, MPN results demonstrate that *36G* gained resistance towards 5 mM, 10 mM and 20 mM $CoCl_2$, when compared to both wild-type and to constant stress final population. Survival up to 3428-fold of the wild-type was observed under stress conditions (Figure 3.4).

MPN analysis on constant stress and increasing stress final populations showed that both the constant stress selection strategy and the increasing stress selection strategy were succesful in yielding cobalt-resistant *S. cerevisiae* by evolutionary engineering. However, the stress-resistance levels of the mutants obtained by increasing stress selection strategy were higher at higher stress levels, compared to those of the constant stress selection strategy.

After obtaining the final populations by constant stress and increasing stress selection strategies, individual mutants were selected randomly from the final populations. Survival behaviours of eight of the constant stress individuals (*K1, K2, K3, K4, K5, K6, K7, K8*) and eight of the increasing stress individuals (*G1, G2, G3, G4, G5, G6, G7, G8*) were analyzed by monitoring their growth under various stress conditions.

As shown in Table 3.7, eight constant stress individual mutants had varying percent survival ratios. The arithmetic average of survival values of these eight individuals (represented by "*I*") was approximately half of the survival value of the final population that these individuals were selected from (Population "*36K*" is represented by "*P*"in the plot). This result indicated that the final population was heterogeneous.

Additionally, it was clearly seen that most of the individuals selected from *36K* had survived more than 4-fold of the wild-type under 0.5 mM stress condition (Table 3.7). The screening results of the constant stress individuals under 5 mM $CoCl_2$ were approximately the same as that of the wild-type, except one of the individuals (*K5*). This result confirmed the previous results implying the constant stress strategy being capable of forming highly resistant mutants under the particular stress level applied during selection, but not being effective at higher stress conditions.

In Table 3.8, the survival values of eight increasing stress individual mutants are shown in folds of the wild-type survival. "I" representing the average survival value of these eight individuals is distantly different from "P" representing the survival of the population *36G*. This is again a result of the heterogeneity of the final population. Even though the eight individuals were the representation of a broader pool of individuals screened, they do not directly represent the population they were selected from. The survival values of the individuals were up to 980-fold of the wild-type under 5 mM $CoCl_2$ stress condition, where the survival of the population was not more than 4-folds of the wild-type (Figure 3.6).

In addition to the screening results under cobalt stress, the populations and the individuals were screened under other heavy metal stress sonditions such as; chromium, copper, zinc stress and under other stress conditions commonly encountered in yeast bioprocesses such as; heat, ethanol, oxidative and osmotic stresses. The increasing cobalt-stress individuals *G1* survived 3-fold of the wild-type under 5 mM $CrCl_3$ and *G5* survived 2-folds of the wild-type under 1 mM $CuCl_2$ stress conditions. Survival of *G1* under zinc was higher than those of the wild-type. These results demonstrate that while gaining resistance to cobalt, the individual mutants gained cross-resistances to other divalent metal ions. The resistance

mechanisms and the genes involved in these mechanisms may be similar in these metals, which remains to be investigated in future studies.

Moreover, *G5* survived under 1 M, 2M and 3 M H_2O_2 stress conditions with distinctly higher survival values than the wild-type. However, this result was not observed under higher oxidative stress levels (Figure 3.18). *G5* and *G6* were observed to be more resistant than the wild-type at 60^0 C. These individuals seemed to have gained co-resistance towards heat and oxidative stresses during evolution steps of the generations.

On the contrary, none of the individuals gained co-resistance to osmotic stress and ethanol stress. This might imply that the resistance mechanisms towards ethanol and osmotic stress conditions may not share similar pathways with metal tolerance mechanisms.

Trehalose is a critical carbohydrate to be analyzed for stress resistance studies (Owobi et al., 2000; Carvalheiro et al., 1999). For this purpose, the trehalose contents in the population and individuals were monitored by HPLC in this study. According to the results, the trehalose accumulation of the individuals were equal less than those of the wild-type (Figure 3.14). This result may imply that the individuals were adapted to the stress conditions after serial steps of exposure. So that no immediate stress response like trehalose accumulation was observed.

Flame atomic absorption spectroscopy analysis provided quantitative data about the metal contents associated with the populations and individuals comparatively. According to the results, individuals with 6-fold cobalt content of the wild-type were observed (Figure 3.7). It was generally observed that cobalt-resistant individuals contained higher values of cobalt than the wild-type. When the metal concentration applied to the cells increased, cells seem to have lower metal contents bound to cells. When the results on Figure 3.8 are taken into consideration, it is clearly seen that the low metal content was the result of decreased biomass. Thus, the first plot showing the percent cobalt hold by the cells in folds of wild-type should be used to explain the resistance mechanism. These results imply that cobalt-resistance mechanism adopted by the cells might be based on isolating the metal on the surface and/or inside the cell, rather than pumping/secreting it outside the cells. The data were not

sufficient to explain whether the metal content associated with the cells was placed inside the cells or on the surface of the cells. Further studies such as electron microscopy techniques are required in order to understand the mechanism in detail.

While selecting the cobalt-resistant individuals, some of the individual mutants were found to have cross-resistances to chromium and copper stresses. FAAS results showed that the metal contents in the cell pellets were extremely low (Figure 3.9 and Figure 3.11). This may imply that the resistance mechanisms towards these metals might work by taking the metal contents outside the cells. Further studies are required for clarification.

To summarize, cobalt-resistant populations and individuals were generated by evolutionary engineering strategy. Some individuals (*G1*, *G5*, *G6*) with cross-resistance characteristics were obtained. By further investigations of the selected individuals would explain the cobalt-resistance mechanism in *S. cerevisiae* in detail and illuminate the shared pathways in the resistance mechanisms towards other stress conditions such as heat, ethanol, oxidative, osmotic and different heavy metal stresses. Transcriptomic and proteomic analysis could also provide the necessary information for understanding the mechanisms in detail, which could ultimately be exploited in various applications in the fields of biomimetics and/or bioremediation.

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