ISTANBUL TECHNICAL UNIVERSITY ★ INSTITUTE OF SCIENCE AND TECHNOLOGY

EVOLUTIONARY ENGINEERING OF CHROMIUM-RESISTANT *Saccharomyces cerevisiae* FOR BIOMIMETICS

M. Sc. Thesis by

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EVRİMSEL MÜHENDİSLİK YÖNTEMİ İLE KROMA -DİRENÇLİ *Saccharomyces cerevisiae* SUŞLARININ BİYOBENZETİM AMAÇLI GELİŞTİRİLMESİ

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ABBREVIATIONS

EMS	: Ethyl methane sulphonate
YPD	: Yeast complex medium
YMM	: Yeast minimal medium
FAAS	: Flame atomic absorption spectroscopy
MPN	: Most probable number
CDW	: Cellular dry weight
GSH	: Gluthathione

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EVOLUTIONARY ENGINEERING OF CHROMIUM-RESISTANT *Saccharomyces cerevisiae* FOR BIOMIMETICS

SUMMARY

The aim of this study is to obtain chromium resistant *Saccharomyces cerevisiae* strains. $CrCl_3$ was used as a stres agent. Evolutionary engineering methodology was used as an inverse metabolic engineering strategy. For this purpose ethyl methane sulphonate, a chemical mutagen, was used to obtain variant cell population "*101*" from wild type (*100*). The stress condition $CrCl_3$ was applied to the variant cell population "*101*" to obtain the first generation the survivors were then transferred to the next step to obtain the next generation. This procedure was repeated during 36 generations.

The $CrCl_3$ stress was applied by using two different strategies: continuous and pulse selection strategies were applied at increasing and constant stress levels. In continuous selection strategy the populations were exposed to continuous $CrCl_3$ stress, but in pulse selection strategy the $CrCl_3$ stress was applied to the cells only for 90 minute. At increasing stress state, gradually increasing $CrCl_3$ stress levels were applied to each successive generation. At constant stress state, the $CrCl_3$ stress levels were kept constant for all generations. After obtaining final mutant generations for each selection strategy, random selection of individual mutants from populations was performed. The chromium resistances were determined by using most probable number method (MPN). Additionally, the characterization of the mutant individuals with respect to their potential cross resistances to other stresses like cobalt, copper, ethanol, heat, oxidative and osmotic stress was also done by using MPN method. The metal contents of the mutant individuals were determined by using flame atomic absorption spectrometry (FAAS).

To summarize, highly chromium-resistant individuals, which were selected from each generation by evolutionary engineering methodology, were obtained with respect to the wild type (*100*). Additionally, some mutant individuals also gained cross-resistance to other types of stresses. Further transcriptomic and proteomic investigations might help to understand resistance mechanisms of the mutant individuals. Finally, chromium-resistant mutant individuals obtained in this study might be used in bioremediation and bionanotechnological applications.

EVRİMSEL MÜHENDİSLİK YÖNTEMİ İLE KROMA -DİRENÇLİ *Saccharomyces cerevisiae* SUŞLARININ BİYOBENZETİM AMAÇLI GELİŞTİRİLMESİ

ÖZET

Bu çalışmanın amacı kroma dirençli *Saccharomyces cerevisiae* suşlarının oluşturulmasıdır. Stres koşulu olarak $CrCl_3$ kullanılmıştır. Bir tersine metabolizma mühendisliği yöntemi olan evrimsel mühendislik stratejisi benimsenmiştir. Bunun için kimyasal bir mutajen olan etil metil sulfonat (EMS) ile muamele edilen yaban tip hücrelerden, genetik çeşitliliği artırılmış bir populasyon elde edilmiştir. Elde edilen bu populasyona stres koşulu olan $CrCl_3$ stresi her aşamada hayatta kalan bireylerin bir sonraki aşamaya aktarılmasıyla 36 nesil boyunca devam ettirilmiştir.

Krom stresi, iki farklı strateji kullanılarak uygulanmıştır. Bunlardan birincisi artan ve sabit koşul altında uygulanan sürekli stres, diğeri de artan ve sabit koşul altında uygulanan kısa süreli, şok strestir. Sürekli stres stratejisinde populasyon sürekli bir strese maruz bırakılmıştır. Şok stres stratejisinde ise stres populasyona 90 dakikalık kısa bir süre boyunca uygulanmıştır. Artan stres koşullarında $CrCl_3$ stresi her nesilde kademe kademe arttırılarak uygulanmıştır. Sabit koşullarda ise $CrCl_3$ stres seviyesi tüm nesiller boyunca arttırılmadan sabit olarak uygulanmıştır Uygulama sonunda elde edilen mutant populasyondan rastgele bireyler seçilmiştir ve bu bireylerin krom metaline olan dirençleri en muhtemel sayı (most probable number, MPN) yöntemiyle belirlenmiştir. Ayrıca bu bireylerin karakterizasyonu, herhangi farklı bir strese olabilecek çapraz dirençlerin varlığının araştırılması için farklı streslere (kobalt, bakır, etanol, sıcaklık, oksidatif, ozmotik) olan tepkileri de yine MPN yöntemi kullanılarak belirlenmiştir. Daha sonra her bir stratejiden elde edilen bireylerin krom metalini tutup tutmama özellikleri alev atomik absorbsiyon spektrometre (FAAS) cihazı kullanılarak belirlenmiştir.

Sonuç olarak, her bir stratejiden evrimsel mühendislik yöntemiyle ata suşa oranla yüksek miktarda krom metali konsantrasyonuna dirençli mutant suşlar elde edilmiştir. Krom direncinin yanı sıra başka stres koşullarına çapraz direnç gösteren mutant suşlar elde edilmiştir. Daha sonra yapılacak olan transkriptomik ve proteomik analizler direnç mekanizmalarının anlaşılmasında yardımcı olacaktır. Bu çalışmadan elde edilen kroma dirençli mutant bireylerden, uzun vadede biyolojik arıtma ve biyonanoteknoloji uygulamalarında yararlanılabilir.

1 INTRODUCTION

1.1 Brief information about *Saccharomyces cerevisiae*

Yeasts are unicellular, simple eukaryotes and their typical characteristics are highly similar to the higher eukaryotic organisms. They are the simplest eukaryotic organism and easy to manipulate. Among the yeast species; one of them is especially preferred, *Saccharomyces cerevisiae*. It is a cell-walled, small, unicellular organism with cell wall and can grow in a simple medium. The cell wall contains structural components which are proteins, lipids, pigments carboxylate, phosphate, sulphydryl, amine groups and distinct, potential metal-complexing sites. Mannan-β-glucan is the main structural polymer of the cell. *Saccharomyces cerevisiae* has a small genome consisting of about 6000 genes and cellular organelles; mitochondria and vacuole (Feldmann, 2005, Cooper et al., 2000; Zetic et al., 2001). Vacuole is the other cellular component, which is the largest compartment of the cell. It is important in the cellular physiology of the yeast cells. It has function on pH and osmo-regulation, ion transport that is important in homeostasis and other roles as protein degradation, storage of amino acids,hydrolytic enzymes, small ions, Ca^{2+} and polyphosphates (Çakar et al., 2000; Bryant et al., 1998; Gharieb et al., 1998).

The reproduction of yeast cells occurs via two ways; vegetatively and sexually. In the vegetative way, cells are proliferating by budding. Yeast cell has an asymmetric structure. Polar orientation of yeast actin cytoskeleton is the reason of this asymmetry and the budding event goes asymmetrically. Yeasts are one of the few organisms that have microtubules, these are actin cables and actin patches. At the budding site actin patches start to accumulate and the actin cables are used to organize them. It is predicted that this action is used to help the secretion of the new cell's cell wall components At the end of the proliferation process organism creates two cells ; the mother cell (big one) and the daughter cell (small one) (Alberts et al., 2002; Feldmann, 2005).

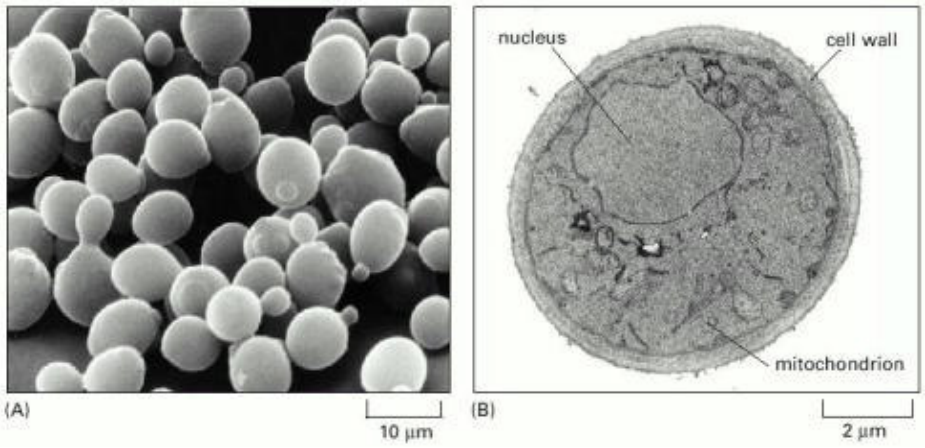


Figure 1.1 *Saccharomyces cerevisiae* (A) Scanning electron micrograph of the yeast cell clusters of *Saccharomyces cerevisiae*, (B) The transmission electron micrograph of the cellular structure of a yeast cell (Alberts, 2002).

The life cycle of *Saccharomyces cerevisiae* is seen on Figure 1.2. Yeast cells bud almost 20 times in a life period (Burke et al., 2000).

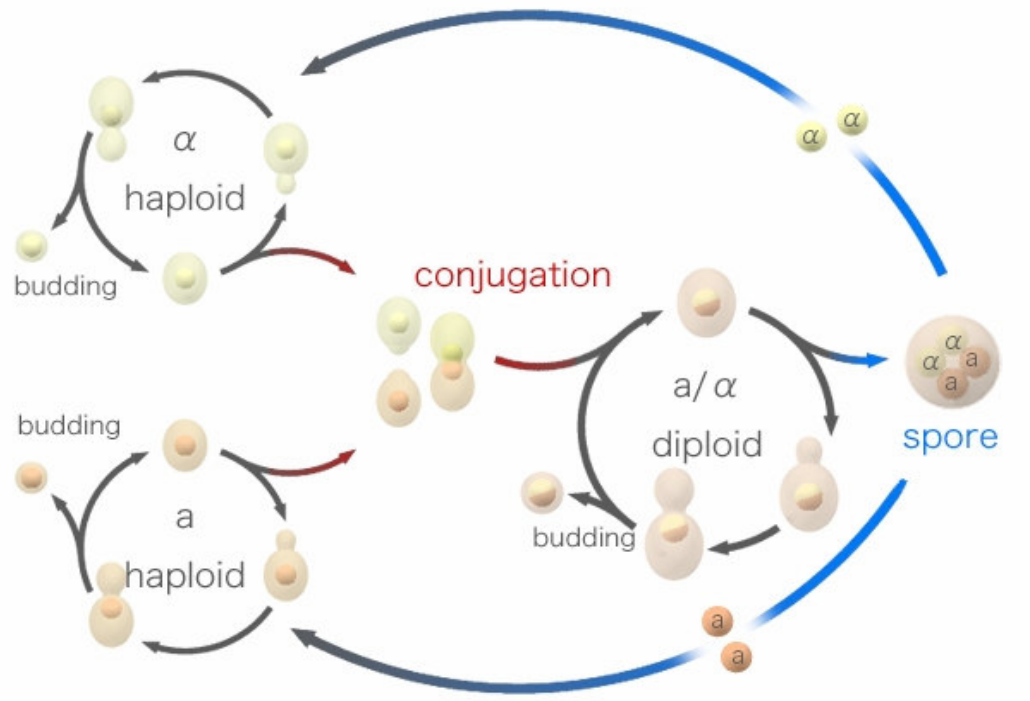


Figure 1.2 Life cycle of *Saccharomyces cerevisiae*

Another reproduction way of the yeast cells is sexual proliferation. In general yeast cells has three mating types that are MAT a (haploid cells), MAT α (haploid cells), MAT a/α (diploid cells). Sexual proliferation starts with meeting of two different

mating type cells after which budding stops. In this pattern, haploid cells MAT α and MAT a cells are coming towards each other and then fuse to generate MAT a/α cells which are diploid. MAT a/α cells have not the ability of mating, however they can undergo meiotic division to form haploid cells (Feldmann, 2005; Zetic et al., 2001).

Experimental advantages and their features make these organisms easy to study instead of complex eukaryotes. Yeasts cells are also used to study physiological and molecular mechanisms of heavy metals (Zetic et al., 2001; Kaszycki et al., 2004).

1.2 Industrial importance of *Saccharomyces cerevisiae*

Saccharomyces cerevisiae is a very special and valuable organism in industrial applications. It has a wide range of industrial uses which are indicated as follow;

- Bread making industry
- Food supplements for animal feeds and human diets as a single cell protein
- Good source of vitamin B and D production
- Ethanol, glycerol production
- Microbial culture media component as a yeast extract
- Beer and wine making industries.

Saccharomyces cerevisiae is also used for bioremediation of Cr containing waters, soils and another application it is used as a chromium-enriched biomass for nutritional usage of mammals and humans (Ksheminskaa et al., 2005; Madigan et al., 2003).

1.3 Metal stress tolerances of yeasts

Metals are the essential elements for living organisms. Some of them are cofactors of variety of enzymes and some of them have functional and structural missions in the cellular metabolism. Toxic effects appeared only at high concentrations of these elements. Toxicity indicates itself by blocking functional groups, denaturation of enzymes and other important effect is the generation of reactive oxygen species

(ROS) in the Fenton reactions, which are catalyzed by the transition metals. Reactive oxygen species causes lipid peroxidation. Metals also have the capability of inhibition of membrane-bound enzymes. This action is resulted in disruption on cellular functions, cellular metabolism and signal transductions (Gharieb et al., 1998; Pestia et al., 2000)

Antioxidant molecules such as glutathione (GSH), prevents metal ion toxicity and depletion of this molecules, causes metal ion toxicity. Recently, it was found that metal ion toxicity and glutathione depletion were hindered in *Saccharomyces cerevisiae* by alkyl hydroperoxide reductase 1 (Ahp1p, thioredoxin peroxidase) (Gharieb et al., 1998).

Uptake of heavy metals by yeasts occurs via two uptake processes. One of them is active uptake, the other one is passive uptake. In active metal uptake heavy metal can pass through the cell membrane by the help of the cells' metabolic cycle. Passive uptake or biosorption method is not related with the biological metabolic cycle. Heavy metal ions firstly entrapped in the cellular structure and then, linked with the binding sites on the cell. Briefly, active and passive uptake mechanisms are called bioaccumulation (Malik A., 2004).

Metal stres tolerance mechanisms of *Saccharomyces cerevisiae* includes;

- Impermeability or reduction of metal uptake
- Adsorption (biosorption)
- Efflux pumps (export toxic ions outside of the cell)
- Metals sequesteration by metallothioneins, polyphosphates in and/or around the cell
- Metals detoxification by chelator molecules such as GSH and subsequent transport into the vacuole

In general, yeast cells may have different important resistance mechanisms to the higher amounts of metals. These are impermeability or reduction of metal uptake, adsorption (biosorption) mechanisms, efflux pumps (export toxic ions outside of the

cell), intracellular or extracellular sequestration of metals and chelation of metal ions by using chelators. This mechanism has been shown for several metal-tolerant strains of yeasts. In metal sequestration mechanism extracellular precipitate can occur such as sulphide, binding to some specific intracellular molecules (metallothioneins, polyphosphates) or partitioning between cellular compartments (Zetic et al., 2001; Gharieb et al., 1998; Malik A., 2004).

Specific intracellular non-enzymic molecules (metallothioneins, polyphosphates and glutathiones) and antioxidative enzymes SOD (EC1.15.1.1), CAT (EC1.11.1.16), glutathione peroxidase (EC1.11.1.9) prevents the damages of heavy metal induced reactive oxygen species (ROS) which are signal molecules causes defense responses. Metallothioneins protect cells against metal ion toxicity. The synthesis of this molecule induces by the heavy metals (Liu et al., 2005).

Yeast vacuoles has a big role in compartmentation and metal ion storage such as Li^+, Cs^+, Mg^{2+}, Ni^{2+}, Fe^{2+}, Zn^{2+} to provide homeostasis and to prevent role on metal ion toxicity of of Zn^{2+}, Mn^{2+}, Co^{2+} and Ni^{2+}. Vacuole performes its functions by the specific H^+-translocating ATPase (V-ATPase) which pumps protons into the vacuole and creates electrochemical potential across the vacuolar membrane. This action of the specific H^+-translocating ATPase (V-ATPase) activates the cation transport into the vacuole (Gharieb et al., 1998).

1.4 Information about chromium

Chromium is a transition metal it has a high boiling point that is 2676^{o}C and it is obtained from ore chromite ($FeO.Cr_2O_3$). In the earth crust chromium is the sixth most abundant element and it is found in various oxidation states that are 0, +2, +3, and +6 (Demirci et al.,2000; Gardea et al., 2005). Most common and stable oxidation states of chromium are Cr (VI) and Cr (III) at the environmental conditions, but trivalent state is the most stable form of chromium. Dominant sources of Cr (III) are tanneries and pigment producing plants. Metallurgy, mining, fossil fuel combustion, wood preservation and cooling installation effluents are the sources of Cr (VI) (Ksheminskaa et al., 2005).

It is widely used in industrial applications. Mined chromium used in different areas and purposes,

(i) metallurgy (80%),

(ii) chromium derivatives production (15%)

(iii) refractory purposes.

These are such examples of chromium application; stainless steels, leather tanning, chrome-plated metal, pigments for paints and ink (Feldmann, 2005).

Cr might be in air, water and soil. The source of chromium in air may be coming from the aerosols generated from the chemical procedures including melting, dust etc. Water origins of Cr are the natural weathering of chromium containing rocks, industrial emissions and atmospheric accumulation. Cr (III) and Cr (VI) have a different chemical activity (Gardea et al., 2005; Demirci et al., 2000).

Chromium is an essential element for mammals and exist very low amounts in tissues and biological fluids. (Vincent, 2001)

1.5 Transport and accumulation mechanisms of chromium in *Saccharomyces cerevisiae*

Living organisms shows different physiological responses to chromium stresses including the induction of stress proteins, Cr entrapment into membranous capsules, metal precipitation, chelation and active efflux (Kaszycki et al., 2004).

The toxic effects of trivalent Cr is much lower when compared with hexavalent Cr due to its characteristics of being less soluble and less suitable that are necessary for transport inside the cells. This characteristic comes from the positively charged structure of Cr (III). Cells transport the organically-bound Cr (III) derivatives by unknown mechanisms. In *Saccharomyces cerevisiae* absorption is the main mechanism of chromium accumulation (Malik, 2004).

Absorption of chromium metal accumulation process, in which biological ligands on the cell membrane have formed octahedral complexes with trivalent chromium. However, hexavalent chromium is biologically active and penetrates inside the cell membranes by anion-exchange channels (sulphate ionic channels). Cr (III) is kinetically more stable than Cr (VI) so that Cr (VI) is rapidly converted to Cr (III) ions inside the cells (Ksheminskaa et al., 2005; Zetic et al., 2001; Kaszycki et al.,

2004). Metal stress tolerance for hexavalent chromium mainly occurs by intracellular reduction to trivalent chromium. This detoxification mechanism is generally materialized with functionally active vacuole, but this is not a common mechanism for all yeast strains (Muter et al., 2001).

It is known that approximately 90% of cellular chromium is present as Cr (III). Trivalent state forms various complexes with some ligand molecules in the cell. These complexes are involved in DNA-protein cross-linking and DNA cross-linking (Ksheminskaa et al., 2005; Zetic et al., 2001; Kaszycki et al., 2004).

Another chromium uptake mechanism in yeast cells includes biosorption of which involves; adsorption, ion exchange, co-ordination and/or complexation and active accumulation mechanisms, but detailed mechanism on chromium uptake and yeast responses to chromium stress of yeasts is not known. Yeast cells have the capability of accumulating high levels of chromium (III), of which the maximum value is 30 mg/g d.w for *Saccharomyces cerevisiae* and 0.45 mg/g d.w for *Candida intermedia* (Pawel Kaszycki et al., 2004; Bingöl et al., 2004).

1.6 Toxicity of chromium

Chromium is a trace element for humans, animals, plants and microorganisms. However, it is necessary in only trace amounts and its higher concentrations has the carcinogenic and mutagenic effects on living organisms (Ksheminska et al., 2003). Over doses of inorganic chromium sources causes chromium accumulation and chromosome damage (Kingry et al., 1998; Demirci et al., 2000).

In yeast cells, chromium has toxic effects during growth phase, but the aim of its presence in yeast has not been clear. Chromium is not an essential element for yeasts. Poor absorption by yeasts makes trivalent form that is less toxic (Kro´ liczewska et al., 2004; Zetic et al., 2001; Demirci et al., 2000). The toxic effects of trivalent Cr is much lower when compared with hexavalent Cr. Trivalent chromium has no capability to pass through eukaryotic cell membranes (Ksheminskaa et al., 2005) However, trivalent chromium has an affinity to nucleic acids and sugar phosphate backbone of DNA that causes Cr-induced damages inside the cells (O'Brien et al., 2002). Chromium element is also the reason of DNA strand breaks, DNA condensation and decreasing the fidelity of DNA replication (Zetic et al., 2001).

Hexavalent chromium is the most toxic form and it is a strong oxidizing agent. It can easily penetrate inside the cells by the isostructural form with phosphate and sulphate (Kro´liczewska et al., 2004; Zetic et al., 2001; Demirci et al., 2000)

1.7 Importance of chromium tolerance

Chromium stress tolerance is important in biotechnological areas. Chromium resistant yeast strains can be used in bioremediation of Cr containing waters, soils and it is important to be used as a chromium-enriched biomass for nutritional usage of mammals and humans (Ksheminskaa et al., 2005; Madigan et al., 2003; Muter et al., 2001).

1.7.1 Bioremediation of chromium by *Saccharomyces cerevisiae*

Yeasts are useful eukaryotic organisms in biotechnological area, in chromium uptake and bioremediation of Cr containing wastes by this organism has become very important related to the increasing industrialization (effects of effluents). The consequence (results) of industrial applications chromium contamination occurs in the atmosphere, surface waters and soils. It is a serious problem for the environment, heavy metals accumulated on food chain organisms and threats human health (Ksheminskaa et al., 2005; Ferraz et al., 1999; Bingöl et al., 2004).

Chromium contamination of the effluents are, especially the results of industrial applications such as electroplating, tanning, textile dyeing (Bingöl et al., 2004). Environmental released Cr compounds have a complex chemistry and yeasts have the ability to biologically convert them into safely forms. This conversion helps to provide successful environmental control. Yeast has been applied in the management of Cr-containing waste (Ksheminskaa et al., 2005; Ferraz et al., 1999). The yeast strain, *Saccharomyces cerevisiae* has the capability of chromium and cadmium accumulation (Helena et al., 2003).

The option of biosorption is preferred instead of other bioremediation processes by the feature of its low prize, effectiveness, reusability. Biosorption is used to recover metals from the aqueous solutions by yeast, fungi, microorganisms and plants. Biosorbtion mechanisms involves; adsorption, ion exchange, co-ordination and/or complexation (Bingöl et al., 2004).

Instead of biosorption, two other general mechanisms are found for bioremediation of heavy metals by the microorganisms. These are intracellular uptake and chemical transformation of the metal ions. However, biosorption is the most rapid process between these mechanisms (Özer et al., 2003).

1.7.2 The characteristics of chromium as a nutritional supplement

Schwartz and Mertz (1959) first discovered the essentiality of trivalent chromium for mammalian normal carbohydrate, protein and lipid (improves plasma lipid levels) metabolism. It is a nutritional factor for animals and human. Deficiency of chromium causes the diabetic symptoms as seen in type II diabetes (insulin resistance) and also other diseases such as obesity, hypertension, and cardiovascular diseases (Table 1.1) (Vincent, 2000; Kro´liczewska et al., 2004).

Table 1.1 Biological functions of chromium (Volpe et al., 2001; Ding et al., 2000)

Biological Functions of Chromium Element
• Reduces fat deposition
• Cr enhances insulin binding to cell membrane receptors and improves better regulation of glucose uptake by cells by this action it controls blood glucose levels and maximizes energetic potential of the body.
• Improves amino acids entry into muscle cells
• Decreases the effects of free radicals
• Reduces blood lipids (total cholesterol, LDL cholesterol, and triglycerides)
• interacts with the thyroid metabolism
• Cr (III) binding with nucleic acids stimulates the DNA-dependant RNA synthesis
• Decreases insulin resistance

Researchers interest the effects of trace elements including chromium in the higher organisms and yeasts for the prevention of some certain diseases. Known impacts of such trace elements (Zn, Cu, Cr, Fe) have the influences on cell membranes

stabilization, synthesis of nucleic acids and the stability of the double helix of DNA while forming hydrogen bonds (Zetic et al., 2001).

Briefly, chromium is a cofactor of insulin and also picolinic acid which is a derivative of tryptophan amino acid. Chromium has a regulatory mission on the body, increases basal metabolic rate and helps to reduce body weight. The main function of insulin is about insulin sensitivity, it improves impaired glucose tolerance and this function is the reason of its regulatory character on body fat, protein and water organization (Krejpcio et al., 2001; Pittler et al., 2003).

The best sources of organically bound chromium are meats, whole-grain products and chromium-enriched yeast cells (Demirci et al., 2000).

Chromium deficiency causes these problems in the body, these are;

- decreased insulin binding
- decreased insulin binding receptor numbers
- impaired immune response
- impaired growth,
- decreased longevity,
- decreased concentrations of cholesterol and triacylglycerol,
- increased aortic plaques, brain disorder,
- decreased fertility and peripheral neuropathy

Eukaryotic cells produce TNF-α (tumor necrosis factor-alpha) against several stresses. TNF-α is a cytokine. In diabetic patients TNF-α level is high and insulin sensitivity of the cells are low. Chromium intake decreases this TNF-α levels, blood glucose and triglyceride levels in diabetes. (Jain et al., 2001; Kro´ liczewska et al., 2004; Volpe et al., 2001)

Chromium improves;

- insulin binding receptor numbers
- insulin sensitivity of the cells
- receptor enzyme's insulin sensitivity
- insulin internalization

Intake of daily chromium amount is 50-200µg for human (Ding et al., 2000).

Chromodulin, the other name is low-molecular weight chromium-binding substance (LMWCr), is a small (1500 Da) naturally-occurring oligopeptide containing cysteine, glutamate, aspartate, glycine and four chromic ions. It is discovered in rat adiposytes and is a biologically active molecule and naturally found in mammalian's livers. The mission of chromodulin is to help insulin to activate glucose metabolism. LMWCr is a cofactor of insulin receptor protein tyrosine kinase. The mechanism starts with the stimulation of insulin receptor by insulin, than LMWCr binds to the stimulated insulin receptor. LMWCr molecule increases the insulin-stimulated receptor kinase activity (nearly eight fold). Chromodulin has four equivalents of chromic ions and the level of chromium in the chromodulin oligopeptide is very important. The enhancement of insulin activity is related to chromium content of the chromodulin complexes. Chromodulin is the best naturally occuring insulin activity stimulator (Vincent, 2001; Vincent, 2000; Sumrall et al., 1997).

Yeasts have the ability to accumulate ions such as chromium which can be useful as a mineral source. Brewer's yeast has the ability to synthesize bioavailable chromium complex. This complex has a low molecular (400-600 mw) weight and includes at least six amino acids which are non-obviously leucine or isoleucine, proline, valine, alanine, serine, and glutamic acid or aspartic acid and also consists of trivalent chromium in its core. If Cr^{+3} do not exist in, GTF (glucose tolerance factor), it will not active anymore. GTF is a insulin activator obtained from yeast cells. This complex is also called as brewer's yeast GTF. Another complex from *Saccharomyces cerevisiae* is Cr(III)-glutathione-nicotinate complex (other name is glutathione) that has the similar ability with GTF in activating insulin. It is a heat and

acid stable molecule. This is the advantage of this molecule makes it stable in stomach (Vincent, 2001; Vincent, 2000; Sumrall et al., 1997).

Yeast cells uptake chromium during the cultivation with chromium containing medium and transform it into biologically available forms of chromium, which is connected with the yeast's DNA, RNA and also proteins. DNA content is much higher than RNA and proteins in Cr-rich brewer's yeast. Cultivation with chromium containing media differs the chemical content' of the cell. This cells have higher doses of glycine, alanine, lysine and glutamic acid. Cr-rich yeast cells are resistant to acidic environmental conditions which is a favourable feature for the uptake of chromium orally as a nutritional supplement (Ding et al., 2000).

In humans and mammals chromium is known as a toxic element at its high concentrations compared with the organically bound forms. Chromium chloride, chromium pinacolinate and chromium polynicotinate are the sources of dietary chromium. However, they have a very low absorptional percentages. These three forms are also inorganic sources and their bioavailibilities are insufficient (Krejpcio, 2001). Bioavailability of the organic form of chromium is higher and sufficient (Kingry et al., 1998; Demirci et al., 2000). Overdoses of inorganic chromium sources causes chromium accumulation and chromosome damages. It is necessary to intake organically bound forms of chromium to prevent these side effects (Kingry et al., 1998).

1.8 Chromium element as Biomimetic cations

Low-molecular-weight organic chromium complexes are the examples of biomimetic chromium containing cations. These molecules are preferred instead of chromium salts by their better bioavailability features. This chromium-oligopeptide complex is the biologically active form of chromium which is also called as biomimetic chromium molecule. Chromium picolinate (chromium + picolinic acid) is one of them. However, the toxic effect of chromium picolinate is the reason of losing its value as a therapeutic agent. Biological reductants in the body can be reduced picolinate ligand in the complex to form hydroxyl radicals and these radicals are the reasons of DNA mutations. Thus another chromium-oligopeptide complex is needed for supplementation. The invention of new Cr containing complexes are necessary. Up to this purpose newly chromium containing cation is synthesized by using natural

amino acid ligand (D-phenylalanine), Cr $(pa)_3$. It is a safe Cr containing oligopeptide complex and do not damage DNA under physiological conditions (Yang et al., 2005).

Another chromium-oligopeptide complex triaqua-µ3-oxo-µ-hexapropionatotri chromium (III) is a synthetic cation with the ability of insulin activation, this molecule mimics the function of naturally occuring chromodulin oligopeptide (low-molecular weight chromium-binding substance or LMWCr). Its solubility in water and acid resistant feature helps this molecule to overcome pressure in stomach and prevents its damage in the stomach, thus making this molecule orally intakable. This biomimetic cation has no oxidative effects on cells and it is a safe molecule (Figure 1.3). Low molecular weight chromium-binding biomimetic molecule has also other functions such as inhibiton of colorectal tumor formation. Therapeutic agent affects insulin sensitivity' decreases the incidence of colorectal tumors and improves the other chromium deficiency defects (Shute et al., 2002; Pickering et al., 2004).

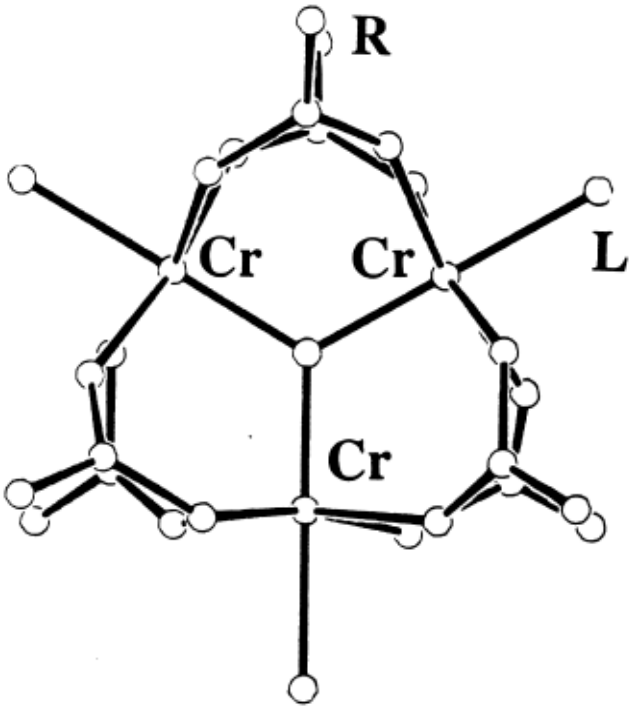


Figure 1.3 The biomimetic cation, $[Cr_3O\ (O_2\ CCH_2\ CH_3)_6\ (H_2\ O)_3]^+$. ($R=CH_2CH_3$ and $L=H_2O$) (Shute et al., 2002)

1.9 How to obtain chromium resistant *Saccharomyces cerevisiae*

Evolutionary engineering is an inverse metabolic engineering strategy, it is an effective and easy way to obtain chromium resistant yeast organism.

1.9.1 Metabolic and inverse-metabolic engineering

Bailey et al. defined metabolic engineering as "the improvement of cellular activities by manipulation of enzymatic, transport, and regulatory functions of the cell with the use of recombinant DNA technology" (Bailey et al., 1996).

Metabolic engineering involves strain improvement by recruitment of heterologous genes and redirection of metabolite flow (Petri et al., 2004). In metabolic engineering, a wide series of manipulations and experimental approaches are performed in order to improve the productivity of a desired metabolite of an organism. Such examples of metabolic engineering include,

- improvement in productivity and/or yield
- improvement of substrate uptake,
- widening the substrate range of an organism,
- improvement of the convenience of an organism in fermentations
- analysis and modification of metabolic flux,
- elimination of metabolic overflow,
- elimination of unwanted or competing metabolic pathways,
- improvement of the organism to resist toxic conditions

(Strohl et al., 2001).

Metabolic engineering method is also very successful in strain improvement. However, it is difficult and often inefficient to apply metabolic engineering to complex or unknown, not fully investigated cellular systems. This difficulty results in new approaches including random, combinatorial approaches, such as

evolutionary engineering which is an inverse metabolic engineering strategy (Çakar et al., 2005, Sonderegger et al., 2003).

The inverse metabolic engineering strategy is an alternative approach to overcome these problems of metabolic engineering (Bailey et al., 1996).

Inverse metabolic engineering strategy is defined as a three step procedure which involves;

1. improving or identifying a desired phenotype
2. determining the genetic or the important environmental factors of that desired phenotype
3. improving or constructing that desired phenotype on another strain or organism by using directed genetic or environmental manipulation

(Gill et al., 2003).

1.9.2 An inverse metabolic engineering strategy: evolutionary engineering

Repetitive cycles of variation and selection of improved phenotypes can be used to define the term of evolutionary engineering, which mimics the idea of natural evolution. This strategy is used to create a strain with specific and desired properties.

For evolutionary engineering strategy three specific steps are necessary (Petri et al., 2004):

- synthesis of an improved strain,
- analysis of the strains' performance under desired conditions,
- designing of the next target for further optimization.

In evolutionary engineering cycle, variant cell populations are generated, the desired phenotypes are then selected after iterative cycles of selection. The design of the next target for further optimization follows these processes (Petri et al., 2004).

1.10 The aim of the present study

The aim of the present study was to obtain chromium-resistant yeast strains. Evolutionary engineering strategy was used to obtain these yeast cells. After obtaining mutant strains, cells were examined with spectrometric and most probable number methods to determine their survival according to chromium stress conditions. By using atomic absorption spectroscopy the chromium contents of the mutant cells were determined to gain insight into their chromium resistance mechanism: i,e. if they were effluxing this metal or accumulating by adsorption or intracellular accumulation ways. Further studies about chromium resistance of the mutant strains could be used in different industrial (bioremediation, biomimetics) and medical applications.

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 amino acids	6.7 g
Dextrose	20 g
Agar (for solid media)	20 g

per 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 1 liter of distilled water.

2.1.3 Yeast culture conditions

Yeast cells were cultivated at 30˚C. The agitation rates were 150 rpm for each cultivation.

2.1.4 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).

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

Chromium (III)-chloride-hexahydrate 98 % was purchased from Acros Organics (USA).

Copper (III)-chloride was purchased from Merck (Germany).

Nitric acid (65%, v/v) was purchased from Merck (Germany).

2.1.5 Buffers and solutions

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

2.1.6 Laboratory equipment

Atomic Absorption Spectrometer (ITU, Chemisty Department)	Analytik Jena Vario 6 (Germany)
Autoclaves	Tuttnauer Systec Autoclave 2540 ml (Switzerland)
	Nüve OT 4060 Steam Sterilizer (Turkey)
	Tuttnauer Systec Autoclave 3870 (Switzerland)
Balances	Precisa BJ 610C (Switzerland)
	Precisa 620C SCS (Switzerland)
Deep Freezes and Refrigerators	80˚C Heto Ultrafreeze 4410 (Denmark),
	-20˚C Arçelik (Turkey)
	+4˚C Arçelik (Turkey)

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 CT0-10AC (Japan)
Incubators	Nüve EN400 (Turkey)
	Nüve EN500 (Turkey)
Laminar Flow	Özge (Turkey)
	Faster BH-EN (Italy)

Light Microscope	Olympus CH30 (Japan)
Microfuge	Beckman®Coulter Microfuge (USA)
Micropipettes	Microfuge (Germany)
Microplate Reader	Biorad Model 3550 UV (USA)
Orbital Shaker Incubators	Certomat S II Sartorius (Germany)
pH meter	Mettler Toledo MP220 (Switzerland)
Rotor	Beckman Coulter JA-30.50i (USA)
Thermomixer	Microfuge, Thermomixer Comfort 1.5-2 ml, (Germany)
Ultracentrifuge	Beckman Coulter, AvantiJ-30I (USA)
Ultrapure Water System	USF-Elga UHQ (USA)
UV-Visible Spectrophotometer	Shimadzu UV-1700 (Japan), Perkin Elmer 25 UV/VIS (USA)
Water Bath	Memmert wb-22 (Switzerland) Nüve BS402 (Turkey)

2.2 Methods

2.2.1 EMS mutagenesis

Ethyl methane sulphonate (EMS) mutagenized *Saccharomyces cerevisiae* CEN.PK 113-7D (also named as *100* throughout this study) cells were obtained by applying the EMS mutagenesis method (Lawrence et al., 1991). *Saccharomyces cerevisiae* CEN.PK 113-7D stock culture was taken from the frozen stock (-80 ˚C) and inoculated into 50 ml test tube which contained 10ml YMM. After overnight incubation at 30 ˚C and 150 rpm, 500µl of this preculture was inoculated into 25ml YPD. This culture was incubated overnight at 30 ˚C and 150 rpm. The 2.5 ml of this culture was washed twice by using 50mM potassium phosphate buffer (pH7).Then the culture cell pellets were resuspended in 10 ml of the same solution and the final cell number was set of $5x10^7$ cells/ml. The resuspended cells were transferred into a screw-cap glass tube and 300µl EMS was added onto it and this tube was incubated at 30 ˚C for 30 min. There after, freshly made filter-sterilized sodium thiosulphate solution (10 %, w/v) was equally added to the solution to stop the mutagenesis. The solution was then entirely mixed and centrifuged at 10000 rpm for 10 min by using a Beckman Coulter JA 30.50i rotor. The supernatant was discarded and the cell pellet was washed twice by using YMM without dextrose. The mutagenized cells were inoculated into YPD and named as 101.

2.2.2 Stock culture preparation

The cultures which were kept for long-term storage at -80 ˚C and -20 ˚C, named as frozen stock. 500 µl was taken from the culture, added to the microfuge tubes and washed twice. In the washing procedure microfuge tubes were placed into the centrifuge and rotated at 12000 rpm for 5 min. Then supernatant was discarded, the cell pellet was resuspended in YMM and 500µl, 60% (v/v) glycerol solution was added into this culture suspension. The final glycerol concentrations were 30% (v/v) glycerol. At four-tube set was prepared in the same way and the solutions were then mixed well. Two of them were placed at -20 ˚C freezer and the other two were placed at -80 ˚C freezer.

2.2.3 Metal stress strategies

In order to obtain chromium resistant mutant *S. cerevisiae*, two different stress strategies were applied: pulse and continuous stress selection strategies.

2.2.3.1 Pulse stress selection strategy

Increasing and constant stress selections were used for pulse stress strategy. In the pulse stress strategy, the cultures were exposed to metal stress only for 90 min. Exposure to metal stress was limited. A frozen stock aliquot of *101* culture was inoculated into 10 ml YMM and vortexed. Incubation temperature of this pre-culture was 30 ˚C and it was agitated at 150 rpm. After overnight incubation, spectrophotometric measurements were done and the cells were inoculated into 10 ml YMM. Inoculation was performed according to OD_{600} results of the preculture, the cells OD_{600} upon inoculation result was adjusted to 0.2. When the culture OD_{600} values reached between 0.5-0.6, one ml of the culture was transferred into a 1.5 ml microfuge tube. Four tubes were prepared; two of them were used as control, one of them was used for increasing stress application, and the last one was used for constant stress application. They were then exposed to metal stress, except for the control tubes. The steps of the experimental procedure are shown in the Figure 2.1. The difference between constant and increasing stress applications was the applied stress level. In constant stress application, cells were exposed to 0.2 mM $CrCl_3$ stress throughout the study; whereas, in increasing stress application, the applied stress first level was 0.2 mM $CrCl_3$ and this level was increased gradually until the last generation was obtained.

After 90 min stress exposure, the cells were washed twice with YMM and inoculated into 5 ml YMM (without metal). OD_{600} measurements and hemocytometric cell counts were performed after 15-18h of incubation and the survival ratios were determined for optical density results by dividing the OD_{600} values of stress applied samples to the OD_{600} result of its control group for optical density result. For hemacytometric counts the survival ratios were determined similarly. As a result, the first generation of constant and increasing stress generations were obtained. The next generations were obtained by the same metal stress application procedures written above. Frozen stock cultures were prepared after each mutant generation was

obtained. The increasing stress selection experiments were performed according to the survival ratios of generations. When the lowest survival ratio was obtained for the increasing stress generations, it was stopped to obtain the next generation. Constant stress generation number was obtained as the same number of increasing stress generation. The pulse stress strategy constant and increasing stress levels, which were applied in order to obtain generations shown on the Table 3.3 and Table 3.4.

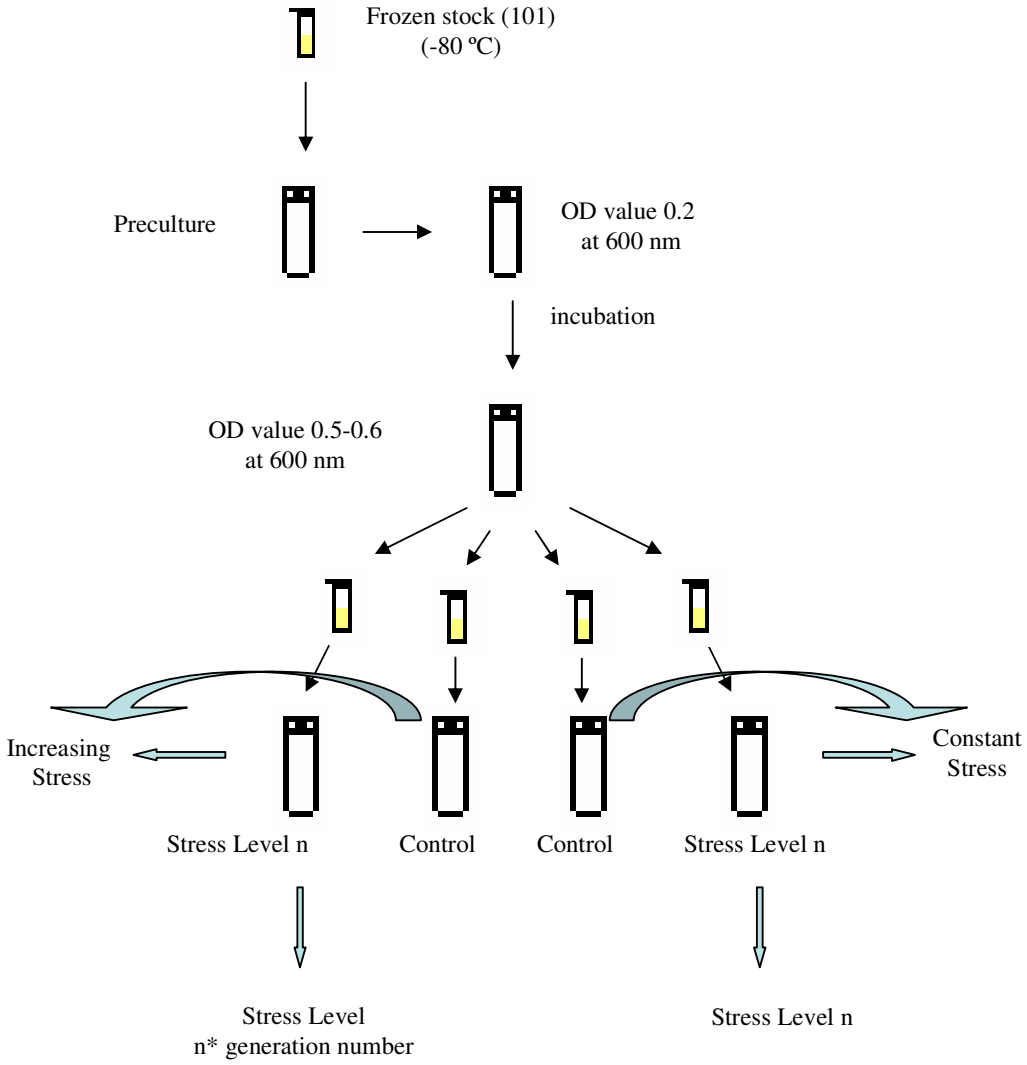


Figure 2.1 The schematic representation of the process of pulse stress application.

2.2.3.2 Continuous stress selection strategy

In continuous stress selection strategy, the cultures were exposed to chromium stress from the beginning of the inoculation to the end of the incubation. The incubation time could vary between 24, 48 and 72h according to the level of metal stress and survival ratios of the cultures. Firstly, frozen stock culture of 101 was inoculated into 10 ml YMM. Overnight incubation was performed at 30 ˚C and 150 rpm. Two tubes that contained only YMM were used as controls, and two other tubes that contained $CrCl_3$, were used for stress application. According to the optical density of the pre-culture, same levels of cultures were transferred into these tubes, which contained 10 ml YMM and $CrCl_3$ containing YMM.

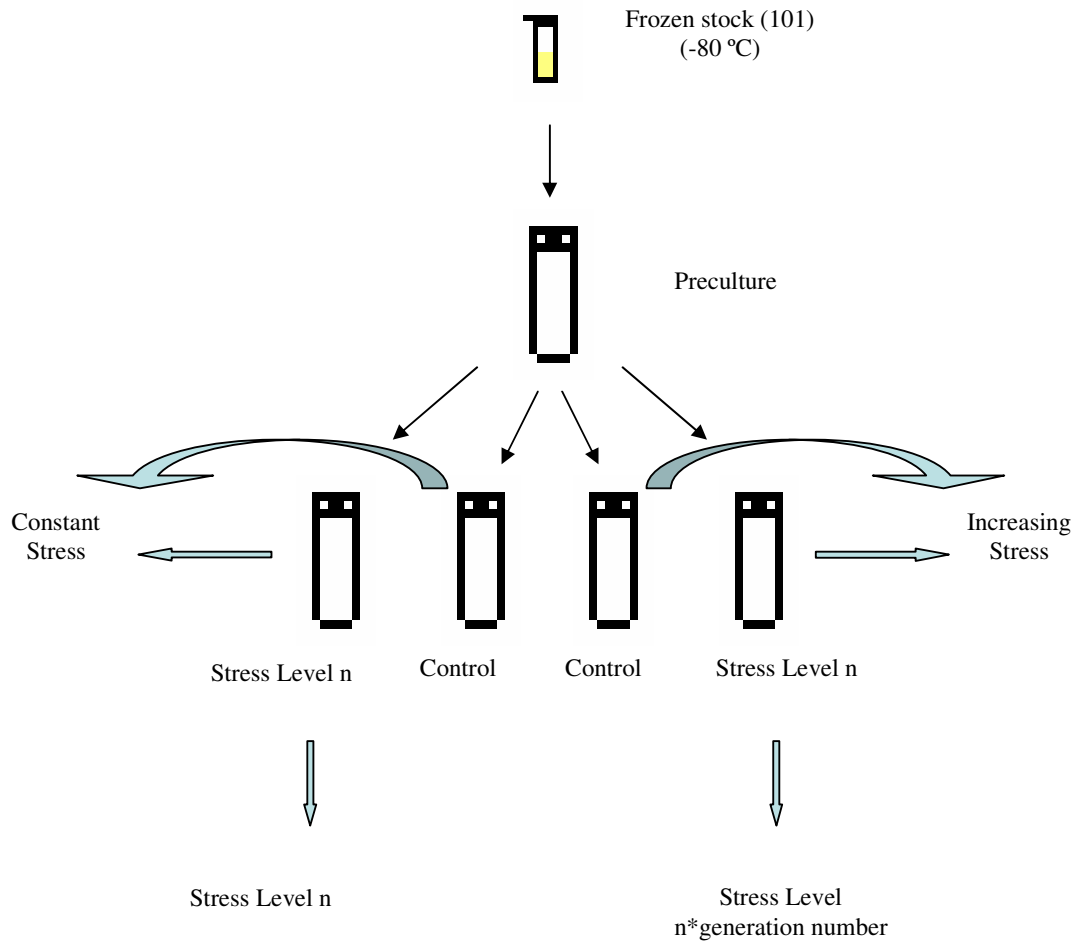


Figure 2.2 The schematic representation of the process of continuous stress application.

The continuous selection strategy, with constant and increasing stress level selections is shown in Figure 2.2.

After 24 h of incubation, OD_{600} measurements and hemocytometric cell counts were done. The $CrCl_3$ stress levels were determined according to continuous stress screening results. The incubation time was increased to 48h and 72h, when the survival ratios began to decrease in the new generations**.** Frozen stock cultures were prepared for each generation.

2.2.3.2.1 Screening of mutant populations under metal stress conditions

Saccharomyces cerevisiae CEN.PK 113-7D (also named as *100*) and *101* stock cultures were taken from – 80˚C and inoculated to 10 ml YMM in 50 ml test tubes. They were incubated at overnight 30 ˚C and 150 rpm. Twelve test tubes were taken and prepared for *100* and *101*'s $CrCl_3$ stress screening applications. The tubes were labeled according to stress levels that were applied both for *100* and *101*. The tres levels were chosen as 2, 4, 6, 8, and 10 mM $CrCl_3$. One tube that contained only YMM (for control) and the other 5 tubes with different $CrCl_3$ metal concentrations were inoculated with 200 µl of *100* preculture (the same procedures were applied for *101*). All tubes were mixed well and placed into the incubator at 30 ˚C and 150 rpm. The cultures growth were controlled 24, 48, and 72h incubation, the optical densities of the cultures were then measured at 600 nm.

Pulse stress screening process was also applied for both *100* and *101*. Screening stress molarities were determined as 0, 2, 4, 6, 8, and 10 mM $CrCl_3$. The same incubation conditions were applied for pulse stress screening process as in the continuous stress screening process.

2.2.3.2.2 Determination of the metal stress molarities

The screening results were used for the determination of the metal stress molarities for further experiments. The survival ratio values of *100* and *101* were used to determine the initial increasing stress and constant stress levels for pulse and continuous stress experiments.

2.2.3.2.3 Obtaining pulse and increasing stress generations

Increasing stress and constant stress molarities were decided according to the screening results.

101 was taken from frozen stock and inoculated into 10 ml YMM in 50 ml test tube. After overnight incubation at 30 ˚C and 150 rpm, 200 µl of the pre-culture were inoculated into two 50 ml test tubes which contain 10 ml YMM (for control) and 0.2 mM $CrCl_3$ containing 10 ml YMM. Test tubes were placed to the incubator under the same conditions. Incubation was finished after 24h in order to measure cells' optical density at 600 nm wavelength (OD_{600}). The survival ratios were determined from these values. The cell counts of the cultures were also done by using a hemacytometer. The cells that were incubated in the tube with $CrCl_3$-containing YMM, were named as the first generation obtained from 0.2 mM $CrCl_3$ stress application. The other generations for increasing and constant stress were then obtained by following the same procedure. The same stress level (0.2 mM $CrCl_3$) was applied when obtaining constant stress generations and for increasing tres levels, the level was gradually increased in every generation.

Pulse stress increasing and constant stress generations were also obtained from *101* by using pulse stress strategy. According to the screening results, the starting point of constant and increasing stress level was 0.5mM $CrCl_3$. The same incubation conditions applied for continuous stress strategy were also applied in pulse stress selection strategy.

2.2.4 Selection of individual mutants

After obtaining pulse and continuous stress generations, selection of individual mutants were performed. The last mutant population generations of pulse and continuous stress strategies (both increasing and constant stress application) were inoculated into Petri-plates after serial dilution and the last dilution was 10^{-6}. The cultures were incubated at 30 ˚C for 24 h. The yeast colonies were then chosen randomly by using sterile toothpicks and transferred into 10 ml YMM. The individual stock cultures were prepared after overnight incubation.

2.2.5 Selection of the most $CrCl_3$-resistant mutant individual

Different $CrCl_3$ concentrations were applied to *100*, *101* and all the individuals which were obtained from pulse and continuous stress strategies, in order to select the most $CrCl_3$ resistant mutant individuals by using 5-tube MPN methodology (Russek and Collwell, 1983). At the beginning of the experiment, the cultures were taken from frozen stock (-80 ˚C) and they were incubated in the shaker at 30 ˚C and 150 rpm. The OD_{600} measurements were done and the cultures were reinoculated into 10 ml YMM according to their OD_{600} results. Final optical density values were adjusted to 0.2.

The individuals that were obtained by continuous and pulse stress strategies, were tested to determine their $CrCl_3$ stress resistance by using 5-tube MPN methodology. For 5-tube MPN method, 96 well plates were used and 180 µl YMM were put into the wells. The control plates were filled with only YMM and the others were filled with YMM containing different concentrations of $CrCl_3$. Then 20 µl pre-cultures were added to the first row of wells. After mixing the culture and the medium, 20 µl of it was transferred to the next row and this step was repeated until the final (8^{th}) row. Cultures were incubated at 30 ˚C and after 24, 48, 72h time period the cell number per ml values were noted and the survival ratios were determined.

2.2.5.1 Screening of individual mutants under various stress conditions

100, *101* and all the individuals which were obtained from pulse and continuous stress strategies were screened by applying different types of stress in order to determine their potential cross-resistances to corresponding stresses by using 5-tube MPN methodology.

2.2.5.1.1 Application of cobalt stress

By using continuous stress strategies, different cobalt stress levels were applied to the mutant individuals by using 5-tube MPN methodology. According to these results, a final $CoCl_2$ stress application was performed by applying 3 mM $CoCl_2$ stress continuously. After 24, 48, 72 h time periods, the mutant individual' cell number per ml values were determined and their survival ratios were calculated.

2.2.5.1.2 Application of copper stress

The same stress tests which were applied for $CoCl_2$ were performed for $CuCl_2$ and the final test was done by applying 1 mM $CuCl_2$ stress and using 5-tube MPN methodology.

2.2.5.1.3 Application of ethanol stress

Ethanol stress was studied by using continuous stress application for both pulse and continuous stress mutant individuals by using 50 ml test tubes. Three stress levels were investigated for continuous and pulse stress individuals which were 2, 5, 10% (v/v) ethanol concentrations. After 24, 48, 72 h time periods the optical density values were measured.

2.2.5.1.4 Application of heat stress

Heat stress was studied by using pulse stress strategy for both pulse and continuous stress individuals. Three heat stress levels (50 and 60 ºC) were investigated for mutant individuals with 10 min heat stress duration. These cultures were then placed to 96 well plates that contained YMM in order to apply 5-tube MPN methodology. After 72 h incubation at 30 ºC, mutant individuals' cell number per ml values were determined.

2.2.5.1.5 Application of osmotic stress

Osmotic stress was studied by using continuous stress application with 5-tube MPN method for both pulse and continuous stress mutant individuals. For continuous and pulse stress mutant individuals 10 and 15% (w/v) NaCl were applied as stress levels. After 24, 48, and 72 h time periods the results were obtained.

2.2.5.1.6 Application of oxidative stress

H_2O_2 pulse stress application was performed by applying 1 M H_2O_2 stress and using 5-tube MPN method. Mutant individuals were incubated for 1h on the medium that contained 0.3 and 1 M H_2O_2, and they were then transferred into 96 well plates that contained fresh YMM. After 24, 48, and 72 h time periods the growth results were obtained and the survival ratios were calculated.

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

Mutant individuals and wild type (*100*) precultures were inoculated into YMM and incubated overnight at 30 ˚C and 150 rpm. After overnight incubation, precultures were inoculated into 10 ml YMM with 0, 0.2, 0.5, 2.5, 5 and 14 mM $CrCl_3$, 1mM $CuCl_2$ or 3 mM $CoCl_2$ in 50 ml test tubes. Cultures were incubated up to 72 h under these stress conditions at 30 ˚C, 150 rpm. They were then centrifuged at 14000 rpm for 5 min. The supernatants were discarded and the cell pellets were washed twice by using double distilled water to separate cells from any metal residues left on the cells. In the next step, cell pellets were dried at 110 ºC for 2h and the cell dry weights were determined. The cell pellets were then hydrolysed by using 10 M HNO_3 solution for one hour at 90 ºC. At the end of this procedure, samples were measured by using a flame atomic absorption spectrometer (FAAS) an Analytik-Jena Model AAS Vario 6 (Germany), with a hollow cathode lamp. Before the measurements samples were diluted in a ratio of 1:2 in order to decrease the final acid concentration to 5 M for preventing any acid damages to the spectrometer.

Measurements were carried out with three metals which were chromium, cobalt and copper. The slit width (nm) and wavelength (nm) values for measurement of these metals are indicated in Table 2.1.

Table 2.1 Wavelength and slit width values of metals (FAAS).

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

3 RESULTS

3.1 Screening for Determination of Initial Chromium Stress Levels

After applying the screening procedure, the initial stress levels of the pulse stress and continuous stress were obtained. Optical density values at 600 nm of wild type culture under continuous stress conditions at 72 h of incubation were obtained. Pulse stress optical density measurements were done after 15-18 h incubation. Continuous stress strategy screening results showed that 2 mM $CrCl_3$ concentration could be the starting stress level for cell growth inhibition and according to the pulse stress screening results this value was higher than 20 mM $CrCl_3$ for a similar growth inhibition (Figure 3.1 and Figure 3.2).

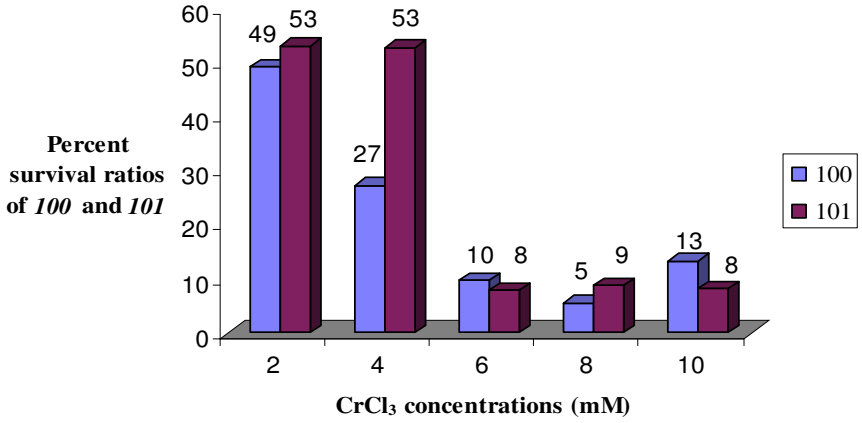


Figure 3.1 Continuous stress strategy screening results as percent survival ratios of *100* and *101* upon different $CrCl_3$ concentrations.

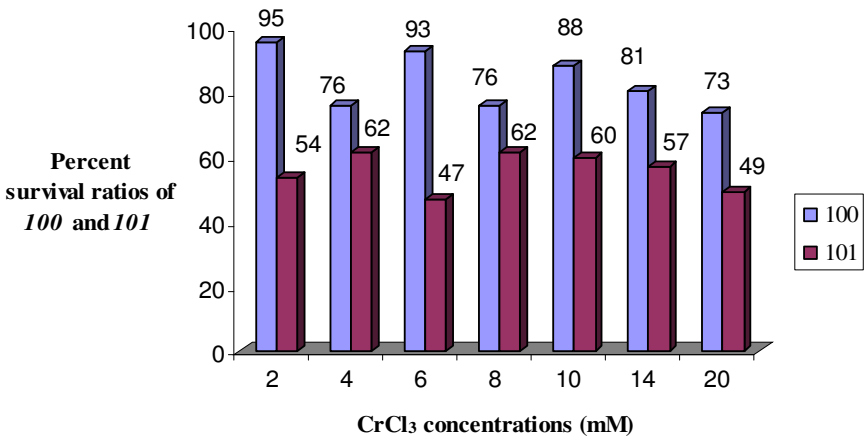


Figure 3.2 Pulse stress strategy screening results as percent survival ratios of *100* and *101* upon different $CrCl_3$ concentrations.

3.2 Obtaining the generations

To obtain the generations, two stress selection strategies were adopted: pulse stress and continuous stress. These stress strategies were also applied as two different ways which were increasing stress and constant stress (Table 3.1, Table 3.2, Table 3.3 and Table 3.4)

Table 3.1 indicates the nomenclature for the continuous increasing stress generations and their corresponding stress conditions. The level of stress condition increased at each step was adjusted according to survival ratio values of the generations. The 36^{th} generation was the final population, obtained from the application of 14 mM $CrCl_3$. This was the final population beyond which the survival levels of the mutant generations suddenly decreased.

Table 3.2 indicates the nomenclature for continuous constant stress generations and their stress conditions. For constant stress populations the stress value the generations were exposed to was always 0.2 mM $CrCl_3$. Just like to the increasing stress final generation, 36^{th} constant stress generation was accepted to be the final population of this stress selection.

Table 3.1 Nomenclature of continuous increasing stress populations.

Generation number	Code of population	Stress condition (mM $CrCl_3$)
1st increasing stress generation	1G	0.2
2nd increasing stress generation	2G	0.4
3rd increasing stress generation	3G	0.6
4th increasing stress generation	4G	0.8
5th increasing stress generation	5G	1.0
6th increasing stress generation	6G	1.1
7th increasing stress generation	7G	1.2
8th increasing stress generation	8G	1.3
9th increasing stress generation	9G	1.4
10th increasing stress generation	10G	1.5
11th increasing stress generation	11G	1.6
12th increasing stress generation	12G	2.0
13th increasing stress generation	13G	2.5
14th increasing stress generation	14G	3.0
15th increasing stress generation	15G	3.5
16th increasing stress generation	16G	4.0
17th increasing stress generation	17G	4.5
18th increasing stress generation	18G	5.0
19th increasing stress generation	19G	5.5
20th increasing stress generation	20G	6.0
21st increasing stress generation	21G	6.5
22nd increasing stress generation	22G	7.0
23rd increasing stress generation	23G	7.5
24th increasing stress generation	24G	8.0
25th increasing stress generation	25G	8.5
26th increasing stress generation	26G	9.0
27th increasing stress generation	27G	9.5
28th increasing stress generation	28G	10.0
29th increasing stress generation	29G	10.5
30th increasing stress generation	30G	11.0
31st increasing stress generation	31G	11.5
32nd increasing stress generation	32G	12.0
33rd increasing stress generation	33G	12.5
34th increasing stress generation	34G	13
35th increasing stress generation	35G	13.5
36th increasing stress generation	36G	14.0

Table 3.2 Nomenclature of continuous constant stress populations.

Generation number	Code of population	Stress condition (mM $CrCl_3$)
1st constant stress generation	1K	0.2
2nd constant stress generation	2K	0.2
3rd constant stress generation	3K	0.2
4th constant stress generation	4K	0.2
5th constant stress generation	5K	0.2
6th constant stress generation	6K	0.2
7th constant stress generation	7K	0.2
8th constant stress generation	8K	0.2
9th constant stress generation	9K	0.2
10th constant stress generation	10K	0.2
11th constant stress generation	11K	0.2
12th constant stress generation	12K	0.2
13th constant stress generation	13K	0.2
14th constant stress generation	14K	0.2
15th constant stress generation	15K	0.2
16th constant stress generation	16K	0.2
17th constant stress generation	17K	0.2
18th constant stress generation	18K	0.2
19th constant stress generation	19K	0.2
20th constant stress generation	20K	0.2
21st constant stress generation	21K	0.2
22nd constant stress generation	22K	0.2
23rd constant stress generation	23K	0.2
24th constant stress generation	24K	0.2
25th constant stress generation	25K	0.2
26th constant stress generation	26K	0.2
27th constant stress generation	27K	0.2
28th constant stress generation	28K	0.2
29th constant stress generation	29K	0.2
30th constant stress generation	30K	0.2
31st constant stress generation	31K	0.2
32nd constant stress generation	32K	0.2
33rd constant stress generation	33K	0.2
34th constant stress generation	34K	0.2
35th constant stress generation	35K	0.2
36th constant stress generation	36K	0.2

Pulse stress process also includes the constant and increasing stress levels. Table 3.3 shows the nomenclature for pulse increasing stress generations and their corresponding stress conditions. For increasing stress populations, the initial stress value was determined to be 0.5 mM $CrCl_3$ and according to the screening results this value was increased by 0.5 mM at each step while obtaining the generations (data not shown). After 14^{th} generation, the stress level increase for each step was changed to 5mM $CrCl_3$ according to the survival ratios of the cells. The 36^{th} generation which was obtained from the application of 140mM $CrCl_3$ was accepted to be the final population.

Table 3.4 shows the nomenclature for pulse constant stress generations and their corresponding stress condition. For constant stress populations, the stress level applied to the generations was determined to be 0.5 mM $CrCl_3$. This constant stress level was applied for 36 times and the 36^{th} generation was accepted to be the final population of constant stress.

Table 3.3 Nomenclature of pulse increasing stress populations.

Generation number	Code of population	Stress condition (mM $CrCl_3$)
1st increasing stress generation	1G	0.5
2nd increasing stress generation	2G	1
3rd increasing stress generation	3G	1.5
4th increasing stress generation	4G	2
5th increasing stress generation	5G	2.5
6th increasing stress generation	6G	3
7th increasing stress generation	7G	3.5
8th increasing stress generation	8G	4
9th increasing stress generation	9G	4.5
10th increasing stress generation	10G	5
11th increasing stress generation	11G	5.5
12th increasing stress generation	12G	6
13th increasing stress generation	13G	6.5
14th increasing stress generation	14G	10
15th increasing stress generation	15G	15
16th increasing stress generation	16G	20
17th increasing stress generation	17G	25
18th increasing stress generation	18G	30
19th increasing stress generation	19G	40
20th increasing stress generation	20G	50
21st increasing stress generation	21G	60
22nd increasing stress generation	22G	70
23rd increasing stress generation	23G	75
24th increasing stress generation	24G	80
25th increasing stress generation	25G	85
26th increasing stress generation	26G	90
27th increasing stress generation	27G	95
28th increasing stress generation	28G	100
29th increasing stress generation	29G	105
30th increasing stress generation	30G	110
31st increasing stress generation	31G	115
32nd increasing stress generation	32G	120
33rd increasing stress generation	33G	125
34th increasing stress generation	34G	130
35th increasing stress generation	35G	135
36th increasing stress generation	36G	140

Table 3.4 Nomenclature of pulse constant stress populations.

Generation number	Code of population	Stress condition (mM $CrCl_3$)
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

3.3 Selection of individual mutants from final mutant populations

The final mutant populations (*U36G, U36S, U36PG, U36PS*) were used to select the individual mutants. Eight individual mutants were selected from *U36G, U36PS*, *U36PG* mutant populations and 9 individuals were selected from U36S. Selected individuals were labeled as shown in Table 3.5. The general code of all the individuals was U36.

Table 3.5 Nomenclature of mutant individuals selected from continuous and pulse increasing stress final populations (*U36G, U36S, U36PG, U36PS*).

Individuals	Code of increasing continuous application individuals	Code of increasing pulse application individuals	Code of constant continuous application individuals	Code of constant pulse application individuals
1st mutant individual	U36G1	U36P1	U36S1	U36PS1
2nd mutant individual	U36G2	U36P2	U36S2	U36PS2
3rd mutant individual	U36G3	U36P3	--	U36PS3
4th mutant individual	U36G4	U36P4	U36S4	U36PS4
5th mutant individual	U36G5	U36P5	U36S5	U36PS5
6th mutant individual	U36G6	U36P6	U36S6	U36PS6
7th mutant individual	U36G7	U36P7	U36S7	U36PS7
8th mutant individual	U36G8	U36P8	U36S8	U36PS8
9th mutant individual	--	--	U36S9	--
10th mutant individual	--	--	U36S10	--

Resistance characteristics of individual mutants were analysed with five-tube MPN method and the resistant ones were determined. Continuous strategy increasing and constant individuals were screened under three $CrCl_3$ stress levels 0.2 mM, 7 mM, 14 mM (Table 3.1).

The screening of the pulse strategy increasing and constant individuals were performed under the same $CrCl_3$ stress levels as the continuous selection strategy (Table 3.2).

3.4 Characterization of populations and individual mutants

3.4.1 Morphological analysis by microscopic techniques

Morphological analyses were performed after obtaining each generation. The morphology of the cultures were studied by light microscope and the morphological changes in the cultures after metal exposure was observed clearly under 400X total magnification. The cells of the mutant population looked like;

- Rod-like shaped cells
- Highly small structured cell

} **grouped together or dispersed one by one**

The cells had also considerably enlarged vacuoles compared to the chemically mutagenized culture (*101*) (Figure 3.3). These cells were observed to have different budding characteristics (Figure 3.3, indicated by an arrow).

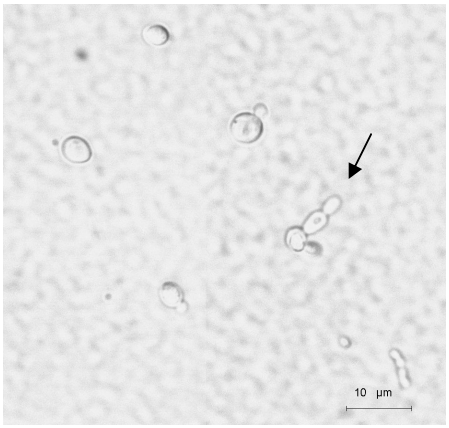
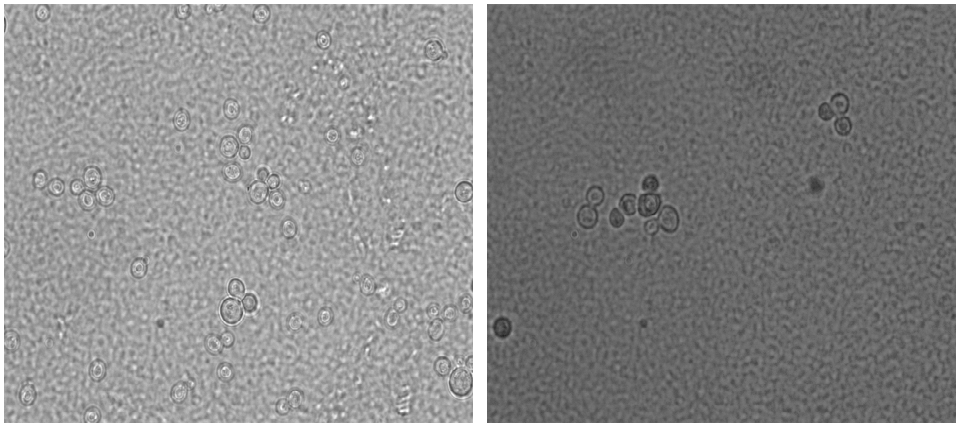


Figure 3.3 *S. cerevisiae* cells (*101*) (100X magnification with immersion oil under light microscope).

The microscopic studies showed that exposure to increasing concentrations of chromium resulted in morphological changes: the cells because longer and formed clusters.

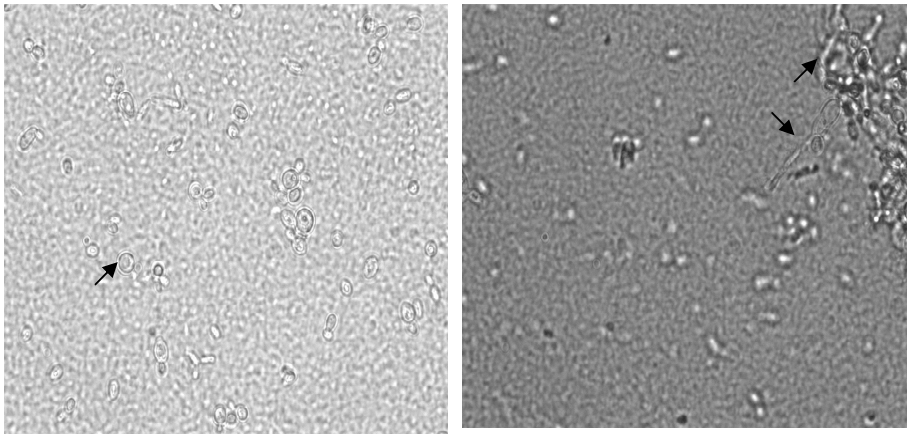
Wild type, 36G and 36P mutant populations were cultivated under non-stress and 14 mM $CrCl_3$ stress conditions and the images are shown in Figure 3.4, Figure 3.5, Figure 3.6. Chromium stress did not cause changes in the morphology of wild (Figure 3.4).



- Wild type (*100*)
- Wild type (*100*) – 14 mM $CrCl_3$

Figure 3.4 *S. cerevisiae* cells (*100*) images of cultivated cells under non-stress and 14 mM $CrCl_3$ stress conditions (Total 1000X magnification with immersion oil under light microscope).

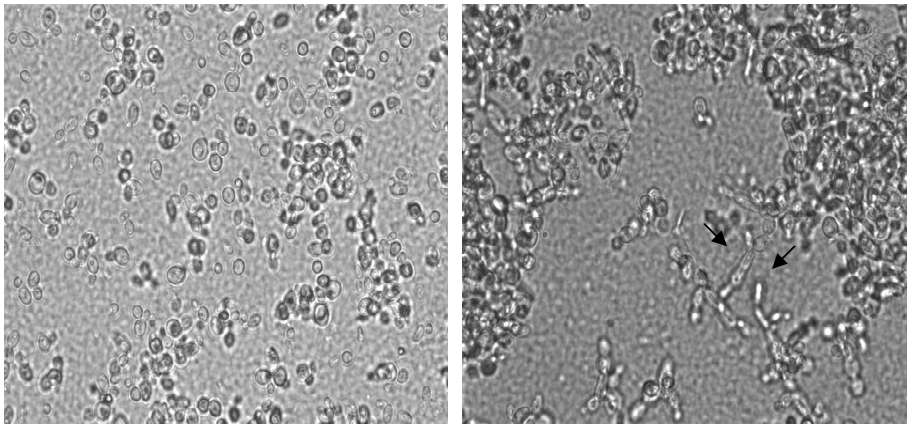
Continuous stress increasing final mutant population 36G photographs are shown in Figure 3.5. The cells with rod-like shaped morphologies are shown with arrows.



- 36G Control
- 36G – 14 mM $CrCl_3$

Figure 3.5 Mutant population 36G images of cultivated cells under non-stress and 14 mM $CrCl_3$ stress conditions (Total 1000X magnification with immersion oil under light microscope).

Pulse stress increasing final mutant population 36P images are shown in Figure 3.6. The cells with rod-like shaped morphologies and cell clusters are shown with arrows.



- 36P Control
- 36P – 14 mM $CrCl_3$

Figure 3.6 Mutant population 36P images of cultivated cells under non-stress and 14 mM $CrCl_3$ stress conditions (Total 1000X magnification with immersion oil under light microscope).

3.4.2 Determination of chromium stress resistance

3.4.2.1 Chromium stress resistances of increasing stress generations

In order to find the responses of the cells towards the metal stresses, survival ratios of each generation after metal stress exposure were calculated. The collected data of the generations were used to create a stress resistance and stress response route. The stress responses of the continuous increasing stress and pulse increasing stress generations are shown in Figure 3.7 and Figure 3.8.

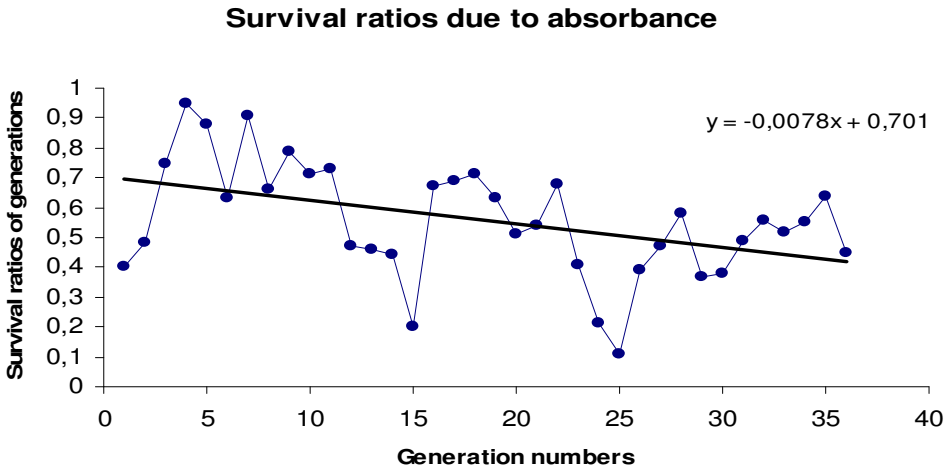


Figure 3.7 Continuous stress generations survival ratio analyses of increasing stress application.

Survival ratios were calculated according to the optical density measurements at 600 nm.

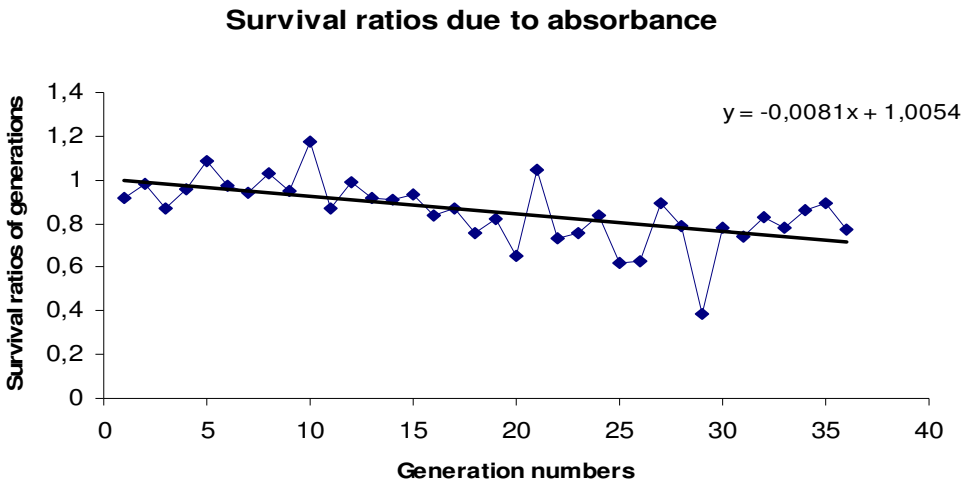


Figure 3.8 Pulse stress generations survival ratio analyses of increasing stress application.

3.4.2.2 Chromium stress resistances of continuous stress and pulse stress individual mutants

After selecting individuals from the 36th generations, the individual mutants, wild type (*100*) and chemically mutagenized culture (*101*) were tested to determine their chromium resistance using five-tube MPN method. Pulse stress strategy increasing and constant individuals, continuous stress strategy constant individuals were screened under 0.5 mM and 5 mM $CrCl_3$ stress conditions. Continuous stress strategy increasing stress individuals were screened under 0.2 mM, 7 mM and 14 mM $CrCl_3$ stress conditions. Continuous stress strategy five-tube MPN results are shown in Table 3.6, Table 3.8 and pulse stress strategy results are shown in Table 3.7, Table 3.9. All results were obtained at 72nd h of incubation.

Table 3.6 Survival ratios of continuous constant stress individuals and final populations under 0.5 mM and 5 mM $CrCl_3$ stress conditions.

Population / individual name	0.5 mM	5 mM
100	0,20	0.00
101	1,00	3.09
U36G	3,18	2.00
U36S	1,00	0.04
U36S1	1,74	0.10
U36S2	2,04	0.06
U36S3	0,58	0.00
U36S4	0,54	0.00
U36S5	0,23	0.00
U36S6	4,58	0.02
U36S7	1,00	0.00
U36S8	1,50	0.00

Table 3.7 Survival ratios of pulse constant stress individuals and final populations under 0.5 mM and 5 mM $CrCl_3$ stress conditions.

Population / individual name	0.5 mM	5 mM
100	1.80	0.00
101	1.00	0.30
36PG	1.00	0.30
36PS	0.53	0.60
U36PS1	0.37	0.00
U36PS2	1.20	0.02
U36PS3	2.40	0.01
U36PS4	1.80	0.00
U36PS5	0.12	0.00
U36PS6	0.60	0.01
U36PS7	0.20	0.00
U36PS8	0.27	0.00

In Figure 3.9, Figure 3.10, Figure 3.11 and Figure 3.12, 36^{th} generation pulse and continuous strategies are represented as *P* and symbol *I* is used to indicate arithmetic average value of randomly selected individuals of each strategies under chromium

stresses. The survival ratios of individuals as fold of wild type are shown in the Figure 3.9, Figure 3.10, Figure 3.11 and Figure 3.12.

Figure 3.9 indicates the continuous stress strategy increasing individuals' survival ratios under 0.2 mM, 7 mM, 14 mM $CrCl_3$ stress levels.

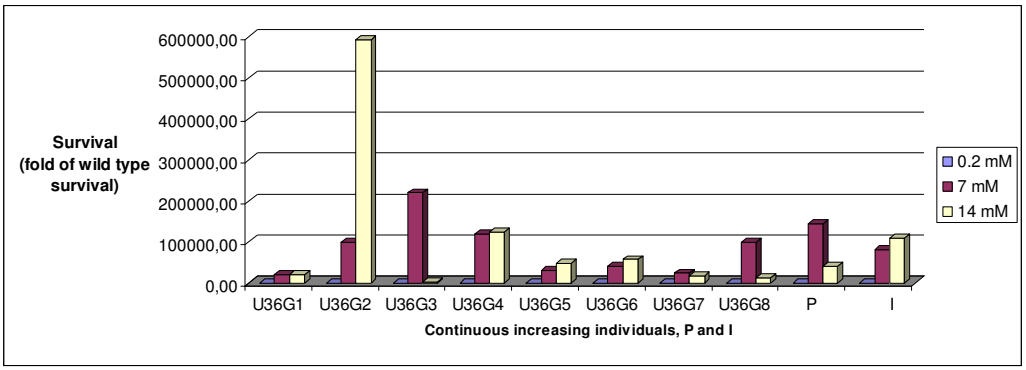


Figure 3.9 Survival ratios of continuous stress strategy increasing individuals and P, I values according to 0.2 mM, 7 mM and 14 mM $CrCl_3$ stress levels as fold of wild type.

U36G2 survived 592593 fold and U36G4 survived 124074 fold when compared to wild type which is incubated in the same stress conditions at 14 mM $CrCl_3$ concentrations (data is shown in Table 3.8).

Table 3.8 Survival ratios of 0.2 mM, 7 mM and 14 mM $CrCl_3$ stress application as fold of wild type.

Populations / individuals	0.2mM	7mM	14mM
U36G1	3.30	21000	20370
U36G2	20.50	100000	592593
U36G3	6.80	220000	2778
U36G4	11.40	120000	124074
U36G5	1.20	31000	48148
U36G6	1.20	40000	57407
U36G7	4.50	24000	18519
U36G8	20.90	100000	12778
P	6.60	145000	40741
I	8.70	82000	109574

Figure 3.10 indicates the continuous stress strategy constant individuals survival ratios under 0.5 mM, 5 mM $CrCl_3$ stress levels.

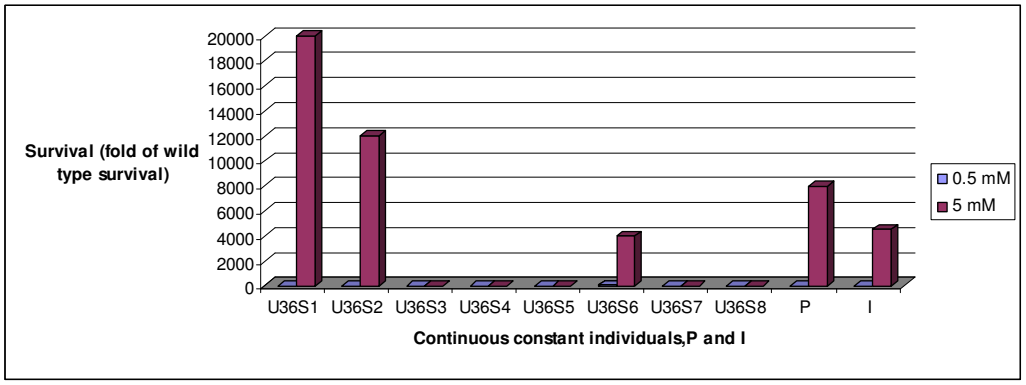


Figure 3.10 Survival ratios of continuous stress constant individuals and P, I values according to 0.5 mM and 5 mM $CrCl_3$ stress levels as fold of wild type.

U36S1 survived 20000 fold and U36S2survived 12000 fold when compared to wild type of which is incubated in the same conditions at 5 mM stress level (data is shown in Table 3.9).

Table 3.9 Survival ratios of individuals according to 0.5 mM and 5 mM $CrCl_3$ stress application as fold of wild type.

Population / individual name	0.5 mM	5 mM
U36S1	8.70	20000
U36S2	10.20	12000
U36S3	2.90	0.60
U36S4	2.70	0.56
U36S5	1.15	0.24
U36S6	22.90	4000
U36S7	5.00	6.00
U36S8	7.50	8.72
P	5.00	8000
I	7.63	4500

Figure 3.11 indicates the pulse stress strategy increasing individuals' survival ratios under 0.2 mM, 7 mM and 14 mM $CrCl_3$ stress levels.

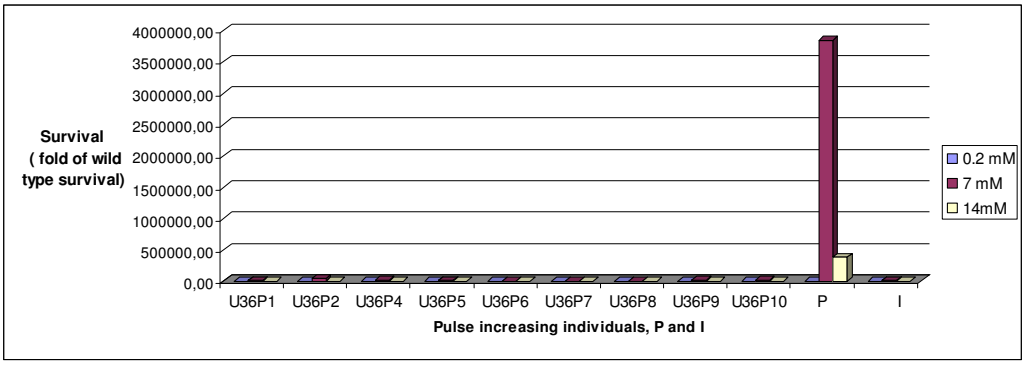


Figure 3.11 Survival ratios of pulse stress strategy increasing individuals and P, I values according to 0.2 mM, 7 mM and 14 mM $CrCl_3$ stress levels as fold of wild type.

Pulse stress strategy increasing individuals survived at high rates up to wild type which is incubated in the same conditions at 7 mM and 14 mM $CrCl_3$ stress level (data is shown in Table 3.10).

Table 3.10 Survival ratios of individuals according to 0.2 mM and 7 mM and 14 mM $CrCl_3$ stress application as fold of wild type.

Populations / individuals	0.2 mM	7 mM	14 mM
U36P1	0.53	8385	0.84
U36P2	4.18	41923	41.96
U36P4	1.08	16923	16.92
U36P5	1.66	5923	26.38
U36P6	2.97	2962	2.98
U36P7	0.53	5385	54.31
U36P8	0.07	1000	1.00
U36P9	1.71	16923	16.92
U36P10	1.16	16923	16.92
P	6.92	3846153	384615
I	1.54	12962	19.85

Figure 3.12 indicates the pulse stress strategy increasing individuals' survival ratios under 0.2 mM, 7 mM and 14 mM $CrCl_3$ stress levels.

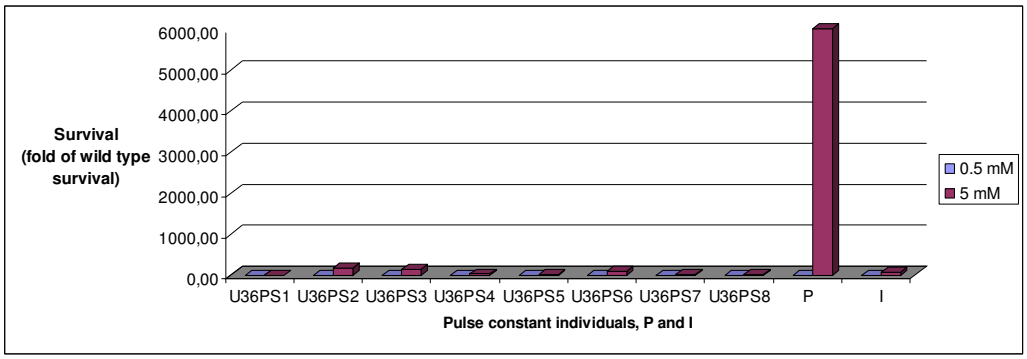


Figure 3.12 Survival ratios of pulse stress constant individuals and P, I values according to 0.5 mM and 5 mM $CrCl_3$ stress levels as fold of wild type.

Pulse stress strategy increasing individuals survived at high rates up to wild type which is incubated in the same conditions at 70 mM and 140 mM $CrCl_3$ stress level at pulse application (data is shown in Table 3.11).

Pulse stress strategy increasing individuals *U36P5, U36P9* and *U36P10* survived at 140 mM $CrCl_3$ stress level at pulse application when compared to wild type.

Table 3.11 Survival ratios of individuals according to 0.5 mM and 70 mM and 140 mM $CrCl_3$ stress pulse application as fold of wild type.

Populations / individuals	0.5 mM	70 mM	140 mM
U36P1	1.76	2.18	0.53
U36P2	2.63	0.06	0.22
U36P4	2.63	0.57	0.06
U36P5	0.61	2.87	2.18
U36P6	1.00	0.08	0.13
U36P7	1.55	0.57	0.34
U36P8	1.82	0.57	0.26
U36P9	1.16	1.49	1.15
U36P10	2.87	2.87	2.82
P	2.63	2.18	2.18
I	1.78	1.25	0.85

Pulse stress strategy constant individuals survived at high rates up to wild type which is incubated in the same conditions at 0.5 mM and 5 mM $CrCl_3$ stress level. U36PS2 survived 180 fold and U36PS3 survived 140 fold when compared to wild type of which is incubated in the same conditions at 5mM stress level (data is shown in Table 3.12).

Table 3.12 Survival ratios of individuals according to 0.5mM and 5mM $CrCl_3$ stress application as fold of wild type.

Population / individual name	0.5 mM	5 mM
U36PS1	0.21	0.40
U36PS2	0.67	180
U36PS3	1.33	140
U36PS4	1.00	32.00
U36PS5	0.07	21.00
U36PS6	0.33	100
U36PS7	0.11	18.00
U36PS8	0.15	27.00
P	0.29	6000
I	0.48	64.80

3.4.3 Determination of cross-resistances to other stresses

Cultures of wild type (*100*) and chromium resistant selected individuals were tested to determine if they have gained any cross-resistance against other stresses. The purpose of using wild type is to make comparison between mutant individuals and the wild type.

3.4.3.1 Cobalt stress

Selected individuals and *100* were tested under 3 mM $CoCl_2$ stress by using five-tube MPN method. Cells were exposed to continuous stress and the results belonged to 72^{nd} h of incubation at 30 ºC, 150 rpm. Results are shown in Table 3.13. Individual *U36G6* seems to be 9.4 fold resistant towards 3 mM $CoCl_2$ stress when compared to wild type. As shown in Table 3.13, only one of the 12 selected individual mutants gained cross-resistance to cobalt stress.

Table 3.13 Survival ratios of the individuals as fold of wild type, upon 3 mM $CoCl_2$ stress.

Populations / individuals	3 mM $CoCl_2$
U36S1	0.0026
U36S2	0.0022
U36G2	0.0026
U36G3	**1.5000**
U36G4	0.1500
U36G5	0.1400
U36G6	**9.4000**
U36PS2	0.6900
U36PS3	0.4400
U36P5	0.1000
U36P9	0.1700
U36P10	0.0150

3.4.3.2 Copper stress

Selected individuals and *100* were used to study copper stress under 1 mM $CuCl_2$ by using five-tube MPN method. Copper stress was applied to the cells continuously and the results belonged to 72^{nd} h incubation at 30 ºC, 150 rpm. The results are shown in Table 3.14.

Table 3.14 Survival ratios of the individuals as fold of wild type, upon 1 mM $CuCl_2$ stress.

Populations / individuals	1 mM $CuCl_2$
U36S1	0.14
U36S2	0.12
U36G2	0.14
U36G3	0.02
U36G4	0.00
U36G5	0.00
U36G6	0.00
U36PS2	0.38
U36PS3	**2.17**
U36P5	0.00
U36P9	0.14
U36P10	**2.17**

3.4.3.3 Heat stress

Wild type (*100*) and selected individuals were investigated under two different heat stress levels which were 50 ºC, 60 ºC. Heat stress was applied as a 1 h-pulse treatment. The results showed that none of the mutants had a cross-resistance to heat stress when compared to wild type. Table 3.15 indicates the survival ratios of the continuous increasing stress individuals, as determined by MPN methodology.

Table 3.15 Survival ratios of continuous increasing stress selected individuals obtained by 5-tube MPN method exposed to 50 ºC, 60 ºC heat stress levels.

Population / individual name	Survival ratio at 50 ºC	Survival ratio at 60 ºC
100	0.100000	0.00
U36G2	0.000001	0.00
U36G3	0.000052	0.00
U36G4	0.100000	0.00
U36G5	0.000060	0.00
U36G6	0.000022	0.00

Table 3.16 shows continuous constant and pulse strategy individuals' heat stress resistance results as fold of wild type.

Table 3.16 Survival ratios of continuous constant stress and pulse strategies selected individuals obtained by 5 tube MPN method exposed to 50 ºC, 60 ºC heat stress levels.

Population / individual name	Survival ratio at 50 ºC	Survival ratio at 60 ºC
100	0.0600	0.00
U36S1	0.0012	0.00
U36S2	0.0420	0.00
U36PS2	0.0002	0.00
U36PS3	0.0042	0.00
U36P5	0.0000	0.00
U36P9	0.2400	0.00
U36P10	0.0005	0.00

3.4.3.4 Ethanol stress

Wild type and selected individuals were tested under ethanol stress with the levels 2 %, 5 %, 10 % (v/v). Stress was applied continuously to the individuals and the wild type. It was found that the individuals had no cross-resistance to ethanol stress when

compared to *100* at the same stress level. Apparently, all the individuals seem to survive 2 % ethanol stress without a significant decrease in their percent survivals. However, none of the individuals could resist ethanol concentrations higher than 2 % (v/v) (Table 3.17). All ethanol experiments were performed in test tubes.

Table 3.17 indicates the continuous stress strategy increasing individuals' results.

Table 3.17 Survival ratios of continuous increasing individuals upon ethanol stress.

Population / individual name	Survival at 2 % ethanol	Survival at 5 % ethanol	Survival at 10 % ethanol
100	0.69	0.15	0.00
U36G2	0.77	0.08	0.00
U36G3	0.99	0.06	0.00
U36G4	0.86	0.05	0.00
U36G5	0.91	0.08	0.00
U36G6	0.86	0.06	0.00

Table 3.18 indicates the continuous stress strategy constant individuals and pulse stress strategy increasing and constant individuals' results.

Table 3.18 Survival ratios of continuous, pulse constant and pulse increasing individuals up to ethanol stress.

Population / individual name	Survival at 2 % ethanol	Survival at 5 % ethanol	Survival at 10 % ethanol
100	0.75	0.21	0.00
U36S1	0.70	0.09	0.00
U36S2	0.89	0.09	0.00
U36PS2	0.82	0.05	0.00
U36PS3	0.55	0.08	0.00
U36P5	0.86	0.06	0.00
U36P9	0.72	0.07	0.00
U36P10	0.62	0.04	0.00

3.4.3.5 Oxidative stress

Wild type (*100*) and selected individuals were examined for oxidative stress resistances of 0.3 M and 1 M H_2O_2 by using five-tube MPN method. Pulse stress were applied. The continuous stress increasing individuals results are shown in Table 3.19.

Table 3.19 Survival ratios of the continuous increasing individuals upon oxidative stress by H_2O_2.

Population / individual name	Survival ratio at 1 M H_2O_2
100	0.00
U36G2	0.00
U36G3	0.00
U36G4	0.00
U36G5	0.00
U36G6	0.00

Pulse stress was also applied. The continuous stress constant individuals and the pulse stress strategy individuals' results are indicated in Table 3.20. None of the mutants had a cross resistance to H_2O_2-oxidative stress.

Table 3.20 Survival ratios of continuous and pulse strategy individuals upon oxidative stress by H_2O_2.

Population / individual name	Survival ratio at 1 M H_2O_2
100	0.00
U36PS2	0.00
U36PS3	0.00
U36S1	0.00
U36S2	0.00
U36G6	0.00
U36P5	0.00
U36P9	0.00
U36P10	0.00

3.4.3.6 Osmotic stress

100 and selected individuals were tested for continuous osmotic stress resistance by applying 10% and 15% (w/v) NaCl and by using five tube MPN method. Results are shown in Table 3.21, Table 3.22 and Table 3.23.

Table 3.21 Survival ratios of pulse stress increasing individuals as fold of wild type upon 15% (w/v) continuous NaCl stress.

Population / individual name	Survival ratio at 15 % NaCl
U36P5	0.055
U36P9	0.018
U36P10	0.028

According to the results of pulse stress resistant increasing individuals, there is no cross-resistance to osmotic stress when compared to wild type (Table 3.21).

Table 3.22 Survival ratios of continuous stress increasing individuals as fold of wild type according to 15 % NaCl stress.

Population / individual name	Survival ratio at 15 % NaCl
U36G2	6.42
U36G3	0.41
U36G4	8.93
U36G5	22.7
U36G6	2.11

According to the continuous stress results of increasing stress individuals, *U36G5* was 22.7 fold resistant to osmotic stress as fold of wild type, and the ratios were 2.11, 8.93 and 6.42 for *U36G6*, *U36G4* and *U36G2*, respectively. Also *U36G5* was scraped between others (Table 3.22).

Table 3.23 Survival ratios of pulse and continuous stress strategies constant stress individuals as fold of wild type according to 10 % and 15 % NaCl stresses.

Population / individual name	Survival ratio at 10 % NaCl	Survival ratio at 15 % NaCl
U36PS2	41800	765
U36PS3	40000	765
U36S1	3.3818	0.77
U36S2	1.9818	0.01

Osmotic stress results of the pulse constant and continuous constant stress individuals are shown in Table 3.23. *U36PS2* and *U36PS3* seemed to have gained significant cross-resistance to osmotic stress when compared to wild type.

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

The metal contents of the growth medium, as ppm (parts per million) values, before atomic absorption spectrometry are shown in Table 3.24.

Results were obtained after 72 h of incubation under the same conditions.

Table 3.24 Stress levels applied to the cultures prior to determination of metal contents with atomic absorption spectrometry.

Metals	Stress levels	Corresponding "ppm" values
Chromium	0.2mM	10.4
	0.5mM	26
	2.5mM	130
	5mM	260
	14mM	728
Copper	3mM	63.5
Cobalt	1mM	174

3.5.1 Chromium content associated with cells

The chromium contents of the cells in reference to chromium contents initially added to the growth media, and percent cobalt weight / cell dry weight results are shown in Table 3.25.

Table 3.25 Percent chromium hold by the cell pellets of the individuals and wild type.

Chromium concentrations (mM)	Individuals and wild type	% chromium hold by the cells
Controls (0 mM $CrCl_2$)	100	0.00
	U36G2	0.00
	U36G3	0.00
	U36G4	0.00
	U36G5	0.00
	U36G6	0.00
	U36S1	0.00
	U36S2	0.00
	P5	0.00
	P9	0.00
	U36P10	0.00
	PS2	0.00
	PS3	0.00
0.2 mM $CrCl_2$	100	0.00
	U36G2	0.00
	U36G3	0.00
	U36G4	0.00
	U36G5	0.00
	U36G6	0.00
	U36S1	0.00
	U36S2	0.00
	U36P5	3.47
	U36P9	0.00
	U36P10	0.00
	PS2	0.00
	PS3	0.00

0.5 mM $CrCl_2$	100	0.00
	U36G2	0.00
	U36G3	0.00
	U36G4	0.00
	U36G5	0.05
	U36G6	0.01
	U36S1	0.00
	U36S2	0.00
	U36P5	7.12
	U36P9	32.38
	U36P10	1.64
	PS2	1.75
	PS3	1.23
2.5 mM $CrCl_3$	100	0.41
	U36G2	0.01
	U36G3	0.22
	U36G4	0.02
	U36G5	0.03
	U36G6	0.02
	U36S1	0.04
	U36S2	0.36
	U36P5	0.00
	U36P9	0.17
	U36P10	1.29
	PS2	1.68
	PS3	1.05
5 mM $CrCl_3$	100	1.43
	U36G2	0.06
	U36G3	0.06
	U36G4	0.07
	U36G5	0.06
	U36G6	0.06
	U36S1	0.22
	U36S2	1.42
	U36P5	0.22
	U36P9	0.57
	U36P10	0.22
	PS2	0.94
	PS3	0.12
14 mM $CrCl_2$	100	0.07
	U36G2	0.06
	U36G3	0.31
	U36G4	0.36
	U36G5	0.32
	U36G6	0.49

U36P5 and *U36P9* seem to hold chromium at 0.5mM $CrCl_3$ stress levels when compared to the non-stress conditions. However, at increasing concentrations *U36P5* and *U36P9* seem to excrete chromium among all the other individuals. Percent chromium hold and percent chromium weight /cell dry weight values were too low (Table 3.25).

Percent chromium hold by the cells under different $CrCl_3$ concentrations analyzed as folds of percent chromium hold by wild-type at the same conditions are shown in Figure 3.13. For the controls which were incubated in YMM without $CrCl_3$, percent chromium hold were 0 and these values were not shown in the Figure.

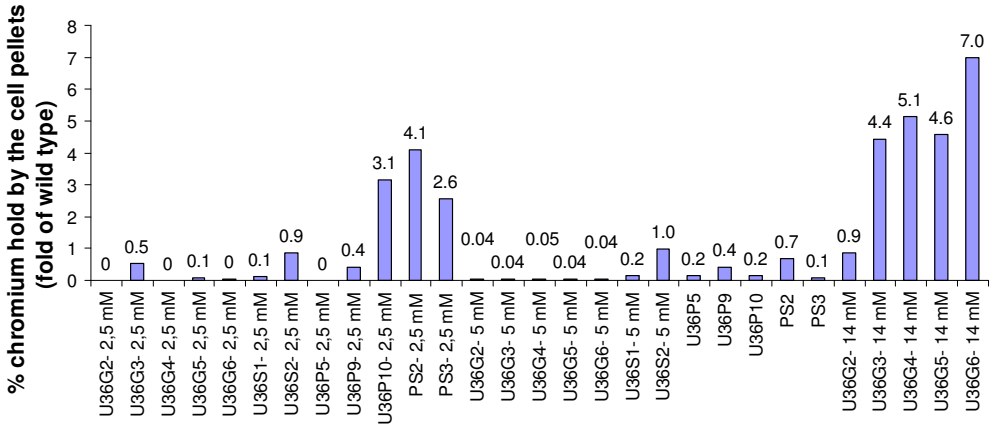


Figure 3.13 Percent chromium hold by the cells under different $CrCl_3$ concentrations analyzed as folds of wild-type at the same conditions.

Percent chromium weight / cell dry weight values for each individual and wild type are shown in Figure 3.14. For the controls which were incubated in YMM without $CrCl_3$, percent chromium weight / cell dry weight values were 0 and they were not shown in the Figure.

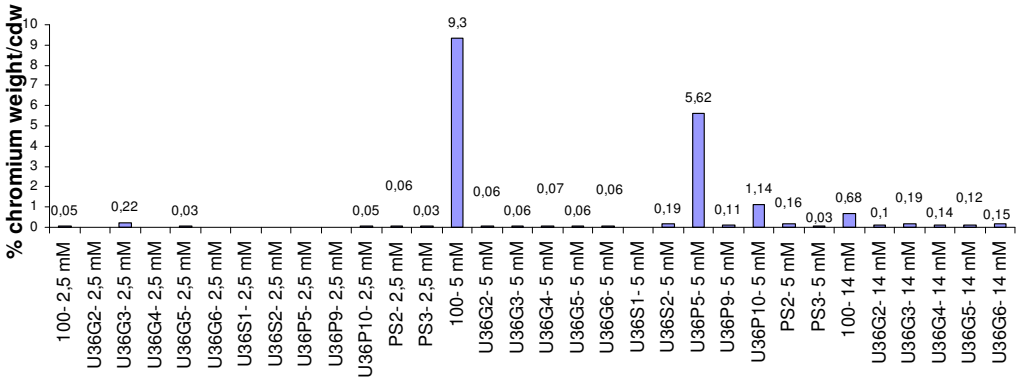


Figure 3.14 Percent chromium weight / cell dry weight values of wild type and individuals.

According to the results, chromium content of the cells under higher $CrCl_3$ stress conditions showed that up to 2.5 mM in critical concentrations, cells holds very low amounts of chromium when compared to the cells under non-stress conditions.

3.5.2 Copper content associated with cells

The copper contents of the cells in reference to copper previously added to the growth media and percent copper weight / cell dry weight results are shown in Table 3.26.

Table 3.26 Percent copper hold by the cell pellets of the mutant individuals and the wild type.

Copper concentrations (mM)	Individuals and wild type	% copper hold by the cells
Controls (0mM $CuCl_2$)	100	0.00
	U36G2	0.00
	U36G3	0.00
	U36G4	0.00
	U36G5	0.00
	U36G6	0.00
	U36S1	0.00
	U36S2	0.00
	U36P5	0.00
	U36P9	0.00
	U36P10	0.00
	U36PS2	0.00
	U36PS3	0.00
1mM $CuCl_2$	100	0.76
	U36G2	3.62
	U36G3	12.09
	U36G4	5.86
	U36G5	12.41
	U36G6	6.38
	U36S1	5.75
	U36S2	0.78
	U36P5	21.86
	U36P9	0.09
	U36P10	12.31
	U36PS2	0.36
	U36PS3	0.79

Percent copper hold by the cells under 1 mM $CuCl_2$ stress was analyzed as folds of percent cobalt hold by wild-type at the same conditions (Figure 3.15).

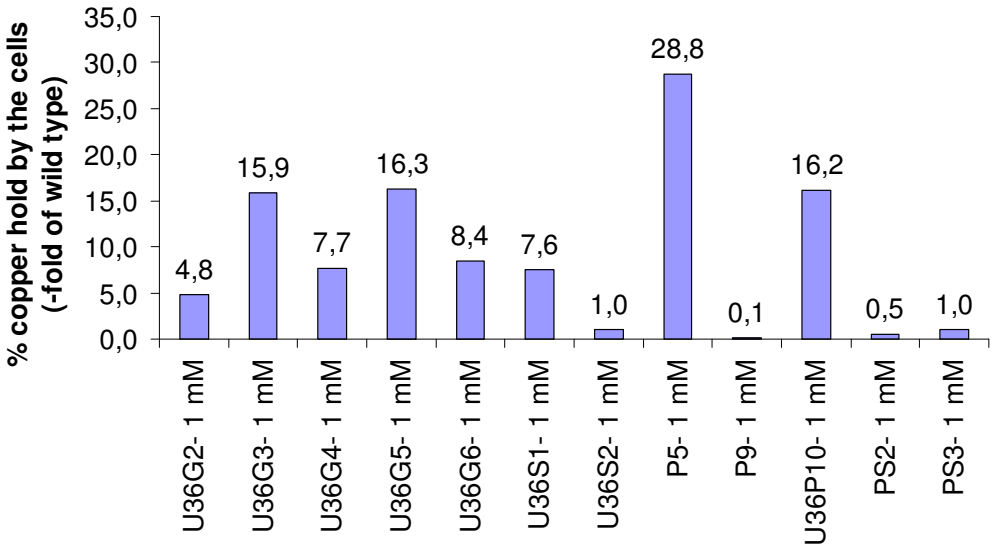


Figure 3.15 Percent copper hold by the cells under 1 mM $CuCl_2$ concentration analyzed as folds of wild-type at the same conditions.

Percent copper weight / cell dry weight values for each individual and wild type are shown in Figure 3.16. The controls which are incubated in the YMM without $CuCl_2$, percent copper weight / cell dry weight values were 0 and not shown in the Figure.

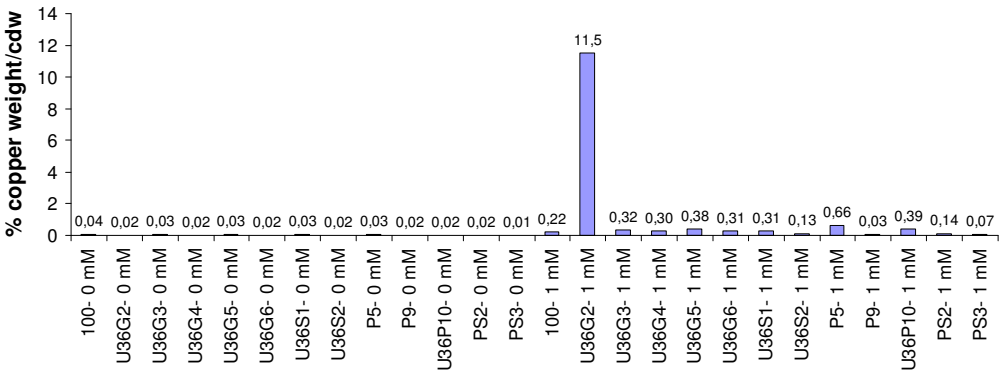


Figure 3.16 Percent copper weight / cell dry weight values of wild type and individuals.

According to the results, copper content of the cells under $CuCl_2$ stress condition showed that cells could hold low amounts of copper when compared to the cells under non-stress conditions. Copper content associated with the individual cells was higher than that of the wild type, except for *U36P9* and *U36PS2*.

3.5.3 Cobalt content associated with cells

The cobalt content of the cells in reference to cobalt contents initially present in the growth media and percent cobalt weight / cell dry weight results are shown in Table 3.27.

Table 3.27 Percent cobalt hold by the cell pellets of the individuals and wild type.

Cobalt concentrations (mM)	Individuals and wild type	% cobalt hold by the cells
Controls (0 mM $CoCl_2$)	100-0mM	0.00
	U36G2-0mM	0.00
	U36G3-0mM	0.00
	U36G4-0mM	0.00
	U36G5-0mM	0.00
	U36G6-0mM	0.00
	U36S1-0mM	0.00
	U36S2-0mM	0.00
	U36P5-0mM	0.00
	U36P9-0mM	0.00
	U36P10-0mM	0.00
	U36PS2-0mM	0.00
	U36PS3-0mM	0.00
3 mM $CoCl_2$	100-3mM	0.89
	U36G2-3mM	0.39
	U36G3-3mM	2.96
	U36G4-3mM	1.56
	U36G5-3mM	2.28
	U36G6-3mM	2.55
	U36S1-3mM	1.12
	U36S2-3mM	1.58
	U36P5-3mM	2.25
	U36P9-3mM	0.82
	U36P10-3mM	0.90
	U36PS2-3mM	2.08
	U36PS3-3mM	1.67

U36G3, *U36G5*, *U36G6*, *U36P5*, *U36PS2* individuals seem to hold low amounts of cobalt ion as shown in Table 3.26.

Percent cobalt hold by the cells under 3 mM $CoCl_2$ concentration was analyzed as folds of percent cobalt hold by wild-type at the same conditions (Figure 3.17). Cobalt contents associated with the individual cells were higher than the wild type, except for *U36G2*. The controls which were incubated in YMM without $CoCl_2$, had percent cobalt hold values 0 and they were not shown in the Figure.

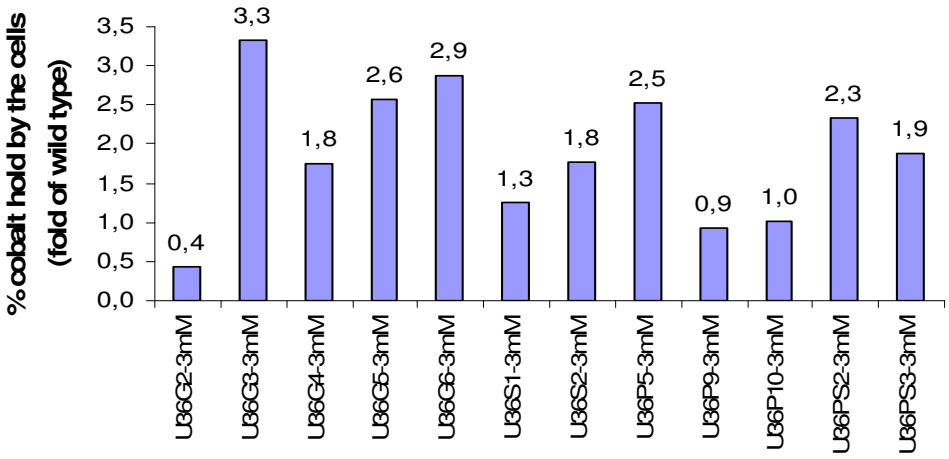


Figure 3.17 Percent cobalt hold by the cells under 3 mM $CoCl_2$ concentration analyzed as folds of wild-type at the same conditions.

Percent cobalt weight / cell dry weight values for each individual and wild type are shown in Figure 3.18.

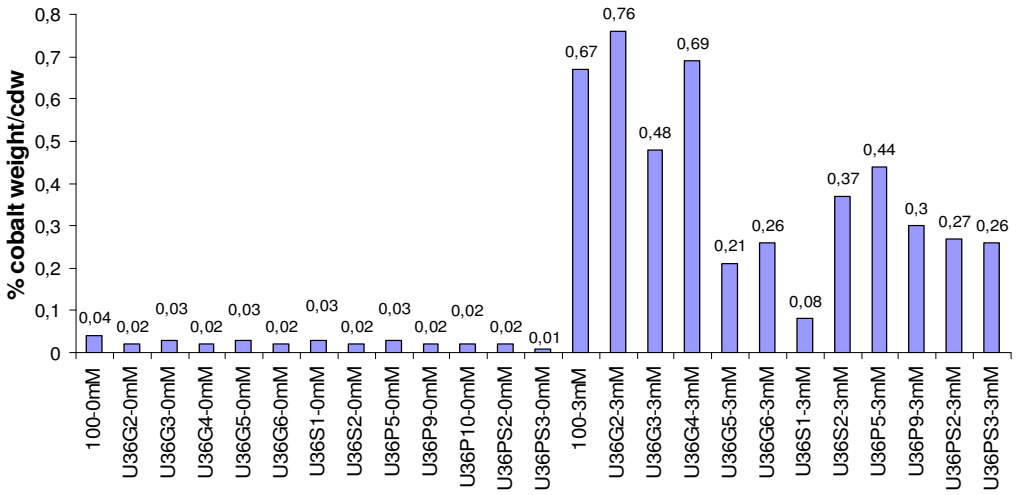


Figure 3.18 Percent cobalt weight / cell dry weight values of wild type and individuals.

According to the results shown in Figure 3.17 and Figure 3.18, the cobalt contents of the cells under $CoCl_2$ stress conditions showed that cells could hold cobalt ions as compared to the cells under non-stress conditions.

4 DISCUSSION AND CONCLUSION

In this study, Cr-resistant *Saccharomyces cerevisiae* mutant populations and individuals were obtained by using an inverse metabolic engineering approach, evolutionary engineering. Two main selection strategies which were continuous and pulse were applied in two different stress levels: constant and increasing. According to evolutionary engineering, cells were firstly mutagenized by a chemical mutagen, ethyl methane sulphonate (EMS) to obtain a variant cell population "*101*" from the wild type.

In order to determine the chromium stress levels to be applied on the generations, chemically mutagenized culture "*101*" was screened under chromium stress. Screening was performed under both continuous and pulse stress conditions. In pulse chromium stress application, both the wild type (*100*) and the chemically mutagenized culture "*101"* survived approximately 50% upon 20 mM $CrCl_3$ stress conditions with respect to the control groups. In continuous stress application, wild type survived approximately 50% with respect to control under 2 mM $CrCl_3$ and *101* survived approximately 50% under 4 mM $CrCl_3$ stress concentration (Figure 3.1 and Figure 3.2).

According to the screening results, 0.2 mM $CrCl_3$ was selected as the first continuous, increasing generations' chromium stress application level and also the continuous constant application value. The $CrCl_3$ stress levels were arranged to be 0.2 µl until the 5^{th} generation. At this step, the survival ratios decreased and thus, the stress step was determined to be 0.1 µl per each generation. After reaching the 11^{th} generation the stress steps were increased to 0.5 µl up to the final generation. The stress application value was 14 mM $CrCl_3$ at the final continuous increasing generation (36^{th}).

For pulse increasing stress populations, the initial stress value and the pulse constant application value were determined to be 0.5 mM $CrCl_3$. According to the screening

results, this value was increased by 0.5 mM at each step while obtaining the generations and the 36th generation was accepted to be the final population, which was obtained from the application of 140 mM $CrCl_3$.

Chemically mutagenized cell population "*101"* was used to obtain generations by the application of iterative cycles of chromium stress upon continuous and pulse increasing and constant strategies for selection of the chromium-resistant generations. The survival ratios of generations of increasing pulse and increasing continuous strategies were decreased as a result of increasing levels of chromium stresses. The survivors from every selection strategies after each step of stress application were used to obtain next generations and the survival ratios were obtained by using the optical density results of each generation. Thirty six generations were obtained by each selection strategies. 36th generation was named as the final generation of each strategy, but the stress level applied to obtain the corresponding final generation did not cause a high reduction in survival ratios (Figure 3.7 and Figure 3.8). The final mutant populations *U36G, U36S, U36PG, U36PS* were used to select the individual mutants. Eight individual mutants were selected from *U36G, U36PS*, *U36PG* mutant populations and 9 individuals were selected from *U36S*. These individuals were analyzed under different chromium stress conditions. In addition, cross-resistance studies of these individual mutants were performed.

It was stated that upon 15 µM chromium concentration inhibits microbial growth approximately 30 % in *Saccharomyces cerevisiae* cells (Jianlong et al., 2004). In this study, the final mutant populations from pulse and continuous selection strategies were not affected by µM levels of chromium stresses. Continuous and pulse increasing individuals could overcome at least 14 mM $CrCl_3$ stress application, except *U36P1*. Additionally, continuous and pulse constant individuals could generally overcome at least 5 mM $CrCl_3$ stress application.

Individuals' chromium resistances were analyzed by using MPN method under different $CrCl_3$ concentrations. According to the MPN analysis the survival ratios of mutant individuals and wild type cells were calculated. The survival ratios of the mutant individuals were then determined as fold of wild type.

The final generation of pulse increasing stress selection strategy resisted 140 mM $CrCl_3$ stress levels. In continuous increasing stress selection strategy, last generation resisted 14 mM $CrCl_3$ stress levels. The survival ratios did not reach lethal values at these 140 mM $CrCl_3$ pulse and 14 mM $CrCl_3$ continuous stress application. Most of the randomly selected mutant individuals from continuous and pulse increasing final generations resisted at 14 mM levels of $CrCl_3$ continuous stress application. According to the MPN results of three different chromium concentrations (0.2 mM, 7 mM and 14 mM), it is clear that the individual mutants obtained from pulse increasing selection strategy behave differently at each chromium concentration. *U36P5, U36P9* and *U36P10* had 2.18, 1.15 and 2.82 survival ratio results as folds of wild type at 140 mM $CrCl_3$ stress level, respectively (Table 3.10). However, *U36P2* had remarkable survival ratio values as fold of wild type among others for continuous application of 0.2 mM, 7 mM and 14 mM $CrCl_3$ concentrations (Table 3.9). Different individuals came forward at each chromium concentrations. These, wide range of different results of survival ratios might be originated from the different responses of defense mechanisms of mutant individuals. According to the 7 mM $CrCl_3$ results it can be said that 7 mM $CrCl_3$ is a mild stress level for all selected pulse stress increasing individual mutants. The survival ratios of each individual at this level were higher when compared to wild type. *U36P2* resisted approximately 42000 folds of the wild type (Table 3.9).

The final generations of constant pulse and constant continuous selection strategies were obtained at 0.5 mM and 0.2 mM $CrCl_3$ stress levels, respectively. To obtain the generations, the same levels of chromium stresses were applied to the cells at each step. Although low levels of chromium stresses as 0.2 mM and 0.5 mM were applied to the generations, the final generations showed high resistance levels at 5 mM $CrCl_3$. Especially, the survival ratio results of *U36PS2*, *U36PS3* and *U36PS6* as fold of wild type were above the average between all of the pulse constant stress mutant individuals. They had 100 to 180 fold of wild type' survival ratio values at 5 mM $CrCl_3$ stress application. For pulse selection strategy it was observed that the resistances of the mutant populations were significantly higher than those of the mutant individuals. Final population was genetically diverse. The individuals were selected from the population randomly and they do not completely represent the responses of the final generations. Although the mutant individuals' survival ratios

were very low compared to final generations' results, it must be emphasized that the survival ratios were significantly high when compared to that of the wild type.

U36S1, *U36S2* and *U36S6* mutant individuals from the continuous, constant selection strategy showed remarkably high resistance to 5 mM $CrCl_3$ stress, when compared to wild type. It is important that the survival ratio of *U36S1* reached 20000 fold of the wild type and *U36S2* had a survival ratio 12000 fold of the wild type. The survival value of *U36S6* was lower than *U36S1* and *U36S2* upon 5 mM $CrCl_3$ results, but among other selected mutant individuals *U36S6* had a significantly higher survival ratio (4000-fold). It must be emphasized that the resistant mutant individuals of continuous constant selection had survival ratios thousand fold of wild type survival ratio. However, *U36S3*, *U36S4* and *U36S5* mutant individuals from the continuous, constant selection strategy couldn't survive 5 mM $CrCl_3$ stress level, and 0.5 mM $CrCl_3$ survival results were lower than that of the other individuals.

Continuous increasing stress mutant individuals' survival ratios showed that, all of the individuals gained resistance to chromium stress upon 14 mM $CrCl_3$ stress level, when compared to wild type. Especially two mutant individuals, which were *U36G2* and *U36G4,* were found to be remarkably resistant to chromium under 0.2 mM, 7 mM and 14 mM $CrCl_3$ concentrations. According to the 0.2 mM results *U36G2* and *U36G8* resisted 20-21 folds of chromium stress when compared to wild type. It is obvious that, *U36G2* and *U36G8* had the highest survival ratios as fold of wild type. At 7 mM $CrCl_3$ concentrations, survival ratio values of *U36G2* and *U36G8* as fold of wild type were lower than *U36G3* and *U36G4*. However, their survival ratios with the values of 100000 and 120000 as folds of wild type were above the average value of the individuals. The survival ratio of *U36G3* reached 220000 fold of wild type. At 14 mM $CrCl_3$ concentrations, *U36G2* and *U36G4* had significantly high survival ratios as fold of wild type when compared to other mutant individuals. It is obvious that all of the continuous increasing stress mutant individuals gained resistance to chromium stress, when compared to wild type at 14 mM $CrCl_3$ concentrations.

After selecting the individuals from the different selection strategies, the characterization of the individual mutants were performed under different stress conditions which were cobalt, copper, heat, oxidative, osmotic and ethanol stresses.

The cobalt analysis (at 3 mM $CoCl_2$) showed that only two mutant individuals *U36G3* and *U36G6*, which were selected from the continuous increasing strategy, gained cross resistance to cobalt stress. Other individuals had no cross resistance to cobalt stress.

The results of the ethanol, oxidative stress and heat stress tests suggested that individuals did not gain cross resistance to these stresses.

The results of the osmotic stress indicated that, the mutant individuals selected from the continuous increasing strategy (*U36G2*, *U36G4*, *U36G5, U36G6)* and the mutant individuals selected from the pulse, constant strategy (*U36PS2* and *U36PS3)* gained cross resistance to osmotic stress at 15 % NaCl. Other individuals did not gain resistance to this stress. Especially two individuals were important *U36PS2* and *U36PS3* had both resistances 765-fold of the wild type. The pulse, constant strategy individuals (*U36PS2* and *U36PS3)* had low resistance to chromium when compared to continuous, constant strategy individuals (*U36S1* and *U36S2)* but had the highest osmotic resistance, while *U36S1* and *U36S2* had no cross resistances to osmotic stress. However, continuous increasing strategy individuals (*U36G2*, *U36G4*, *U36G5, U36G6)*, which were the most chromium resistant individuals at 14 mM $CrCl_3$ stress level, had cross resistance to osmotic stress. The responses of the individuals to various stresses were different from each other. Further analyses of the mutants at genetic level would be necessary to clarify and understand the genetic and regulatory back ground of these differences.

According to the results of the MPN analysis, some individuals were selected to investigate their chromium, cobalt and copper contents by flame atomic absorption spectroscopy analysis (FAAS). Before analyzing the chromium, cobalt and copper contents, cells were grown under those metal stresses. Flame atomic absorption spectroscopy analysis gave results of metal contents and insight about the metal holding behaviour of the mutant individuals

It is obvious that the cells seem to hold very low amounts of chromium upon chromium stress. Continuous increasing selection strategy' mutant individuals *U36G3*, *U36G4*, *U36G5* and *U36G6* had the highest resistance to chromium stress whereas they hold very low amounts of chromium even at 14 mM $CrCl_3$

concentration. Some mutant individuals, which were *U36P10*, *U36PS2* and *U36PS3,* hold very low chromium at 2.5 mM $CrCl_3$ stress level, as 3.1, 4.1, 2.6 folds of wild type, respectively. *U36PS2* and *U36PS3* pulse constant individuals were the resistant ones. However, they had the lowest resistances with respect to all selection strategy individuals. For *U36PS2* and *U36PS3*, 2.5 mM $CrCl_3$ stress level may be a critical point for the cells to show their stress response by using different mechanism. *U36P10*, *U36PS2* and *U36PS3* did not show other remarkable chromium holding patterns in another upper and lower stress application levels.

According to our results, generally two uptake mechanisms can be thought with respect to low chromium content of the cells. These mechanisms might be reduced uptake or export of the chromium ions outside the cell by using efflux pumps.

The results of the percent cobalt hold by the individual mutants as fold of wild type suggest that cells seem to hold cobalt ion at 3 mM $CoCl_2$ concentration except for *U36G2*. *U36G2* had low cobalt content with respect to wild type. However, *U36G2* had high % copper hold as fold of wild type. The individuals' responses were different according to the metal exposure levels. *U36G3* and *U36G6,* which were the cobalt resistant individuals, had the highest cobalt contents as fold of the wild type. Here some accumulation mechanisms, which includes passive or active uptake, might be used by the mutant individuals under cobalt stress. As a result of active uptake, metal sequesteration by metallothioneins and metal detoxification by chelator molecules such as glutathione (GSH), which leads to subsequent transport into the vacuole, cobalt ions might be accumulated into the cells (Zetic et al., 2001; Gharieb et al., 1998; Malik A., 2004). Now this mechanisms is unknown, but will be investigated in the future studies.

According to the percent copper content of the cells as fold of wild type results, *U36P5* seems to hold the highest level of copper and the other individuals except *U36S2, U36P9, U36PS2* and *U36PS3* seem to hold copper ion.

Further investigations ongenetic and proteomic level are necessary for the characterization of chromium resistance mechanism of the different mutants selected by these selection strategies and analysis of the relationships of the mechanisms with each other and the cross resistances.

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