ISTANBUL TECHNICAL UNIVERSITY ★ INSTITUTE OF SCIENCE AND TECHNOLOGY

EVOLUTIONARY ENGINEERING OF COPPER - RESISTANT YEAST FOR BIOMIMETICS

M.Sc. Thesis by

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EVRİMSEL MÜHENDİSLİK YÖNTEMİ İLE BAKIRA -DİRENÇLİ MAYALARIN BİYOBENZETİM AMAÇLI GELİŞTİRİLMESİ

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ABBREVIATIONS

MIC	: Minimal inhibitory concentration
EMS	: Ethyl methane sulphonate
YMM	: Yeast minimal medium
YPD	: Yeast complex medium
FAAS	: Flame atomic absorption spectrometer
MPN	: Most probable number
CDW	: Cellular dry weight
MT	: Metallothionein
GSH	: Reduced gluthathione
GS-SG	: Oxidized gluthathione
OD	: Optical density
FRE1	: Ferric REductase 1
FRE2	: Ferric REductase2
CTR1	: Copper TRansport 1
CTR2	: Copper Transport 2
CTR3	: Copper Transport 3
MAC1	: Metal-binding activator 1
Ace1	: Copper-fist transcription factor
CRS5	: Copper-Resistant Suppressor
ROS	: Reactive oxygen species)
CUP1	: Metallothionein
FET4	: Low-affinity Fe(II) transport protein of the plasma membrane
ATX1	: AnTioXidant
CCS	: Copper Chaperone for SOD1
COX17	: Cytochrome c OXidase
UV	: Visible Spectrophotometer

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ÖZET

Saccharomyces cerevisiae yüzyıllardan beri endüstriyel olarak yaygın bir biçimde kullanılan önemli bir organizmadır. Hem çevresel etkenlerden dolayı hem de kullanıldığı çeşitli endüstriyel alanlara bağlı olarak, *Saccharomyces cerevisiae* çeşitli farklı streslere maruz kalmaktadır. Bu çalışmanın amacı, bir tersine metabolizma mühendisliği stratejisi olan evrimsel mühendislik yöntemi ile bakıra karşı dirençli *S. cerevisiae* suşları elde etmektir. Buna ek olarak, elde edilen suşları detaylı bir biçimde analiz ederek hem mikroorganizmadaki değişen metabolik aktiviteleri ve stress mekanizmalarini incelemek hem de uygun endüstriyel alanlarda kullanılabilirliğini arttırmaktır. Bu amaçla yabani tip hücreler öncelikle genetik çeşitliliği arttırmak için etil metan sulfonat (EMS) kimyasal mutasyonuna maruz bırakılmıştır. Genetik çeşitliliği arttırılmış hücrelere şok ve sürekli olmak üzere iki ayrı stres seleksiyon stratejisi uygulanmıştır. Her bir strateji kendi içinde sabit düzeyde ve dereceli olarak artan düzeylerde olmak üzere iki alt strateji içermektedir. Her bir stres uygulamasından sonra hayatta kalan bireyler, bir sonraki stress basamağına aktarılmış, sürekli ve şok seleksiyon stratejileri kapsamında hem sabit düzey hem de dereceli olarak artan düzeylerde uygulanan stres koşulları sonucunda bakıra karşı dirençli 56'şar nesil elde edilmiştir. Bu strese dayanıklı nesillerden rastgele seçim yöntemiyle mutant bireyler elde edilmiştir. Karakterizasyon amaçlı olarak en muhtemel sayı (Most Probable Number, MPN) yöntemi ile rastgele seçilen her bir bireyin stres sonrası hayatta kalma değerleri istatistiksel olarak belirlenmiştir. Ayrıca bakıra karşı dirençli bireylerin çapraz direnç kazanımı olup olmadığını incelemek amacıyla bu bireyler başka stress koşullarına da maruz bırakılmıştır. Bakıra dirençli bireylerin bakır, kobalt ve krom gibi farklı metal streslerine maruz bırakılarak metalleri hücre içinde ve/veya hücre yüzeyinde tutup tutmadığını ve varsa tutulan metal miktarını belirlemek için alev atomik absorpsiyon spektroskopi (FAAS) yöntemi uygulanmıştır.

Özet olarak, tersine metabolizma mühendisliği stratejisi olan evrimsel mühendislik yöntemi ile bakıra karşı dirençli nesiller elde edilmiştir. Bu bireylerden sürekli ve artan düzeyde stres uygulanarak elde edilen bireylerin bakıra daha çok direnç kazandığı belirlenmiştir. Ayrıca, dirençli bireylerin farklı bakır konsantrasyonlarında farklı bakır tutma tepkileri verdiği gözlenmiştir. Bu nedenle, elde edilen bakıra dirençli bireylerin direnç mekanizmasını aydınlatmak için daha ileri araştırma yapılması gerekmektedir. Buna ek olarak, bakıra yüksek direnç gösteren bireyler arasında krom, kobalt ve ozmotik (tuz) stres koşullarına çapraz direnç kazanımı olan bireyler olduğu belirlenmiştir. Ancak, hidrojen peroksit ile sağlanan oksidatif stres koşullarına karşı herhangi bir çapraz direnç gözlemlenmiştir. Bu çalışmada elde edilen bakıra dirençli mutantlar uzun vadede biyolojik arıtma ve biyonanoteknoloji uygulamalarında kullanılabilir.

EVOLUTIONARY ENGINEERING OF COPPER - RESISTANT YEAST FOR BIOMIMETICS

SUMMARY

Saccharomyces cerevisiae has been used for a wide variety of industrial applications for ages. It is exposed to several stress conditions due to environmental factors and industrial processing steps. The aim of this study was to obtain copper-resistant *Saccharomyces cerevisiae* strains by using evolutionary engineering technique, which is an inverse metabolic engineering strategy. Moreover, the resistant strains were analyzed in order to detect differences in metabolic activities and improved stress resistance mechanisms and thus, increase the applicability of the strains in industrial fields. For this reason, wild type cells were exposed to a chemical mutagen, ethyl methane sulphonate (EMS) to increase the genetic diversity. Two different selection strategies, which were continuous and pulse selection strategy, were applied on the genetically diversified cultures. Each strategy included two sub-strategies that were constant and increasing stress applications, The survivors of each stress step were transferred to the next stress step. By applying constant and increasing levels of continuous and pulse stress applications, 56 generations were obtained. Individual mutants were selected randomly. The survival ratios of the randomly selected individual mutants were determined statistically by most probable number (MPN) method. In addition, the cross-resistance characteristics of the copper-resistant individuals were investigated under various stress conditions. The metal contents associated with the cells were determined by flame atomic absorption spectroscopy (FAAS) analysis.

To summarize, copper-resistant individuals were obtained by using evolutionary engineering, an inverse metabolic engineering strategy. Continuous increasing stress strategy seems to be more effective in obtaining copper-resistant individuals. In addition, resistant individuals were found to have different copper holding characteristics under different copper concentrations. Thus, further analysis is required to understand illuminate the resistance mechanisms of these individuals. Copper resistant strains showed cross-resistance to chromium, cobalt and osmotic (salt) stresses. However, no cross-resistance to oxidative stress (by hydrogen peroxide) was observed. The copper-resistant mutants obtained in this study could further be exploited for applications in bioremediation and bionanotechnology.

1. INTRODUCTION

1.1 Yeast ; brief information

Yeast is a unicellular fungi and is under the phylum "ascomycetes". An important characteristic feature of this class is formation of sac like structures called "ascus" for producing and storing spores. *Saccharomyces cerevisiae,* also known as budding yeast, is the most popular member of this group. It has several application fields such as bakery, brewing, bio-fuel industries, etc. The baker's yeast *Saccharomyces cerevisiae* is a eukaryote with both a diploid and a haploid mode of existence. It has been studied as a model eukaryote for many years.

The morphology of yeast cells may be spherical, oval, or cylindrical. A single cell is about 8µm in diameter, which is a relatively large size when compared to a bacterial size (Madigan *et al.* 2003).

The baker's yeast *Saccharomyces cerevisiae,* is model microroganism for present study, needs very simple nutrients. It is not able to carry out photosynthesis and thus, requires reduced carbon sources and nitrogen source such as ammonium sulfate, vitamin such as biotin, and a variety of salts and trace elements.

Saccharomyces cerevisiae cells reproduce both sexually and asexually. This characteristic of the baker's yeast makes it attractive for geneticists. The budding is a form of asexual reproduction and contains mitotic division of the haploid parent cell. The cell division cycle starts with a single, unbudded cell. When a single cell forms a bud, a daughter cell is generated on the surface of the parent cell. When the bud reaches the size of the parent cell, it is still connected to the parent cell. Then, nuclear division occurs and then two cells separate arising two single cells.

The sexual reproduction occurs in the baker's yeast by fusion of two mating types. It has two types of sexes or mating types, which are called as a and α. The mating type of the

cell is determined by a single locus, MAT due to the differences in gene regulation. The presence of one of the two alleles at MAT locus *(MAT*α or *MAT*a*)*, results with the formation of either mating type a or *α* respectively. The phenotypic differences between a and α cells are due to a different set of genes being actively transcribed or repressed in the cells of the two mating types. Haploid cells only contain one copy of each chromosome and thus can only possess one allele of *MAT* α (either *MAT*α or *MAT*a). Diploid cells involve both mating types together and thus possess one chromosome from the *MATa* allele and another chromosome from the *MAT* allele.

The yeast strains can switch their mating types from one to other in each generation. For example, the cell with mating type a can switch to mating type α by replacing the *MATa* allele with the *MAT*α allele. This replacement is possible due to the presence of an additional silenced copy of both the *MATa* and *MAT*α alleles. *HML* (Hidden MAT *L*eft) locus typically carries a silenced copy of the *MAT*α allele, and the *HMR* (Hidden MAT Right) locus typically carries a silenced copy of the *MATa* allele. The mating type switching is a gene conversion event during which a DNA endonuclease specifically cleaves DNA at the MAT locus. Moreover, exonucleases and DNA repairing mechanisms are also included. It is a very good example for genomic rearrangements in organisms **(**Solomon, *et al* 1999**;** Lodish, *et al*, 2000**).**

A yeast cell may be both in haploid and diploid forms during its cell cycle. It includes a series of steps that involve transition between haploid phase and diploid phase**.** The transition from diploid phase to haploid phase is occurred by meiotic cell division. Four haploid nucleuses in ascospores inside an ascus were yielded by meiotic cell division of diploid cell. Ascus formation occurs under starvation conditions and enhances cell survival. On the other hand, the transition of haploid phase to diploid phase is occurred by mating of gametes. Haploid cells are capable of mating with the opposite mating type (a type can only mate with an α type, and vice versa). This is a sexual reproduction that

results with the production of a stable diploid cell. Yeast cell cycle is shown in Figure 1.1 (Madigan *et al.* 2003; Solomon *et al.* 1999).

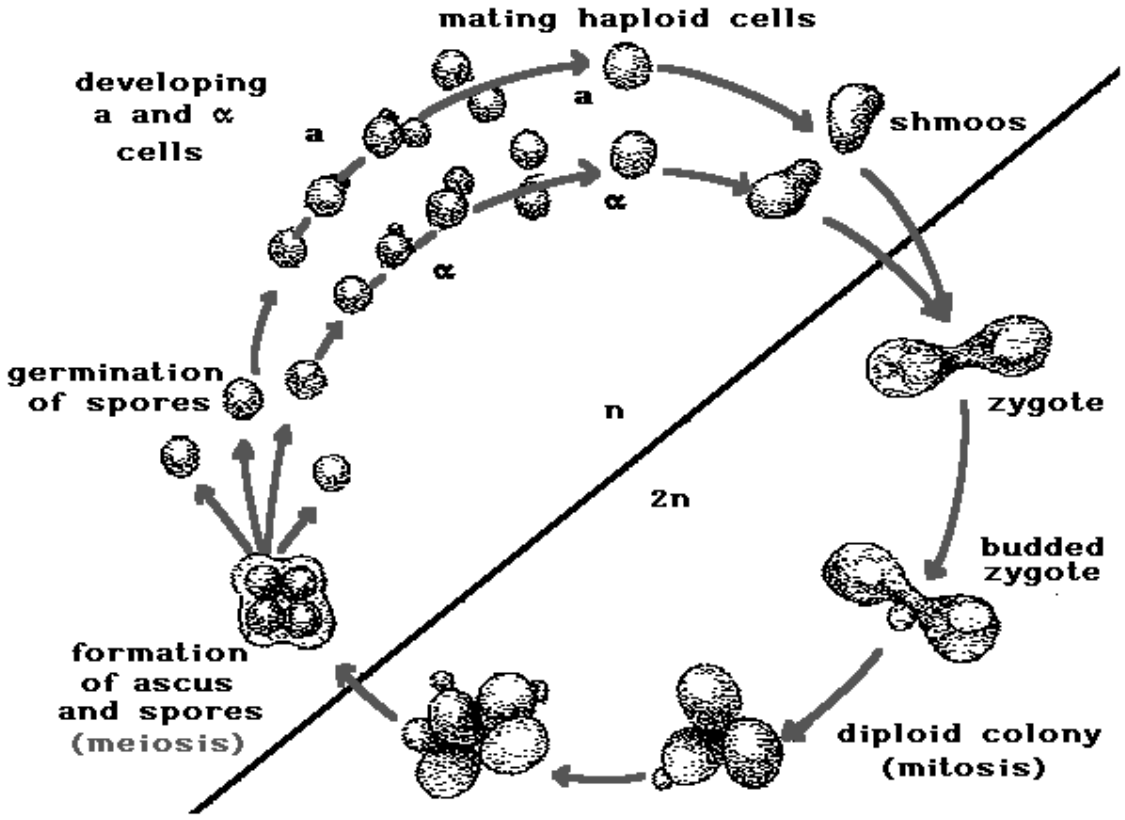

Figure 1.1 Yeast cell cycle (Ref http://www.phys.ksu.edu/gene/a2f3.html).

1.2 Industrial importance of *Saccharomyces cerevisiae*

Saccharomyces cerevisiae is an industrially important microoroganism. It has several application fields such as production of beer, wine, alcohol, and bread. It is also used as the source of Vitamin B and protein. Moreover, *Saccharomyces cerevisiae* cells are important tools for genetic research.

Yeast is able to ferment carbohydrates, during which glucose is converted into ethanol and carbon dioxide. This reaction is referred as fermentation. This feature of yeast is important in the production of beer and wine. Different yeast strains can be used for this process, but *S.cerevisiae* is the most important one among these yeast strains. These alcoholic beverages are the fermentation products of yeasts. Wine is produced from fermentation of grape juice from *Vitis vinifera* by *S. cerevisiae*. On

the other hand, beer is produced from fermentation of malt derived from the digestion of germinated barley grains by *S.cerevisiae* (Madigan *et al.* 2003; Solomon *et al.* 1999).

Saccharomyces cerevisiae has been used through ages in baking industry, due to its dough-leaving characteristics. In making bread, the baker's yeast *S. cerevisiae* cells convert carbohydrates into carbon dioxide, which causes the dough to expand or rise, this helps the formation of characteristic flavor and crumb structure of bread after which alcohol evaporates.

Yeast has about fifty percent protein of its own total biomass and the cell contains very high levels of vitamin B. Thus, yeast can be used as a nutritional supplement in a deactivated form. Its natural low-fat structure makes is a favorable product for human consumption.

Saccharomyces cerevisiae is the first eukaryotic cell, whose genomic sequence was identified with 6000 genes in 1996. Since its generation time is very short, the biochemical reactions are well known and studied very commonly, it has been used in several biotechnological applications. This unicellular eukaryote is used as a model organism in many studies to obtain information about function of higher eukaryotic systems (Peter *et al.* 2005).

1.3 General stress response mechanisms

When the microorganisms are exposed to the stress conditions such as heavy metal, heat, oxidative, they change their metabolism to tolerate against stress conditions via developing protective mechanisms such as;

1) Prevention of toxic compounds to enter into the cell
2) Reduction or removal of redox active compounds to prevent their damage in cell
3) Repairing the damaged biomolecules
4) Production of protective biomolecules that have protective functions under stress conditions

One or a combination of these mechanisms may be utilized in order to survive under stress conditions (Sebastián *et al*. 1999; Cervantes *et al*. 1994).

In this study, we are interested in heavy metal stress on *Saccharomyces cerevisiae,* and copper is used as a model heavy metal.

1.4 Heavy metals

Heavy metals are very important in biological processes. They are used as trace elements in the cell, however, when they exist at high level concentrations inside the cell, they are toxic. Heavy metals have densities above 5 g/cm^3, and they have incompletely filled d orbitals. These d orbitals determine the redox activities of heavy metal cations (Nies, 1999).

Divalent heavy metal cations are similar in structure. Their ionic diameters are between 138 and 160 pm, and are charged with double positive charge. However, the size of central ion is specific for each heavy metal. Different features of metal ions are used to distinguish the metal ions in a mixed environment. At this point, the uptake systems of the cell have important roles for selecting the desired ions. The cell has two types of uptake systems for heavy metals.

1) **First** uptake system is fast, unspecific and continuously expressed by the cell and this system is run by chemiosmotic gradient. This unspecific system leads to heavy metal ion accumulation in the cytoplasm when the cell is exposed to high heavy metal concentrations.

2) **Second** uptake system, unlike fast system, is slow, has substrate specificity, uses ATP as an energy source, and is expressed by the cell in times of need. For instance, expression of this kind of system is induced by starvation etc. (Nies D.H**,** 1999)

Heavy metal cations cause toxic effects due to high affinity for phosphate, purines, cysteinyl and histidyl side chains of proteins. The outcomes of this toxicity can occur in a variety ways:

1) Heavy metals may form complexes and clusters in the cell,

2) Heavy metals may interact with active sites of enzymes and nucleic acids.

3) Heavy metals may cause the disruption of the integrity of cellular or organelles membranes which ends up with loss of function or permeabilization of membranes.

Cells have developed some defense systems upon heavy metal stress. For instance, after an accumulation of toxic ions inside the cell, a complex is made with thiol

containing molecules, which is followed by the reduction of the metal to a less effective oxidation state (Howlett *et al*. 1997; Nies D.H**,** 1999).

Heavy metal resistance systems can be utilized in biotechnology. After making an organism gain a heavy metal resistance mechanism, that resistant characteristics of the microorganism can be used in interested biotechnological fields such as bio-mining, bioremediation of metal contaminated environments (Christopher *et al,* 1993; Nies D.H. 1999).

1.4.1 Copper is a model metal

Copper is a transition metal that is able to cycle between two redox states, oxidized Cu (II) and reduced Cu (I). The electrochemical potential of Cu^{+2} / Cu^{+} is -268 mV. It is eligible for the physiological range. Copper has high affinity to radicals. This feature makes copper a very toxic element. The minimal inhibitory concentration (MIC) of copper ions (Cu^{+2}) is 1.0 mM (Avery *et al*. 2004; Jamieson *et al*. 1998).

Copper is a redox active metal ion, which is essential for maintaining cell physiology as a co-factor for a wide variety of enzymes such as Cu-Zn superoxide dismutase, cytochrome oxidase, and ribonuclotide reductase. In addition, copper participates in generation of ROS (reactive oxygen species) formation that causes disruption of nucleic acids, proteins, and lipids by catalyzing Fenton reactions and generating hydroxyl (•OH) and superoxide (O2•-) radicals. Since, hydroxyl groups have very high damaging effects; cells have to minimize the negative effects of $OH^{\cdot}$ Radicals (Fatichenti *et al*. 2000; Avery *et al*. 2004).

If these vital metal ions are present at high concentrations in the cell, it accumulates over threshold levels in the cell; it would be dangerous and toxic for the cell. Some important results of copper toxicity are neurodegenerative disorders, autism and Alzheimer‘s disease in human (Avery *et al*. 2004).

Cells have developed balancing systems for not only maintaining the required levels of the metals but also protecting the cell against higher concentrations. These mechanisms may be listed as regulation of metal reduction, metal uptake, disruption, sequestration, and removal of the copper metal.

1.5 Copper Uptake system

The copper uptake process of *Saccharomyces cerevisiae* is initially dependent on the action of the plasma membrane reductases, Fre1p and Fre2p. The reduced copper ions are then transferred inside the cell through the cell membrane via cell surface transporters. These transporters are categorized as *high* and *low affinity transporters.* After entering the cell, copper ions are participated either to the detoxification or utilization pathways (Culotta *et al.* 2003).

Large amounts of copper ions exist in insoluble, oxidized valent states in the environment. The reduction of the cupric to the cuprous copper increases the bioavailability of these ions (Dennis *et al*. 1998).

1.5.1 Copper reductases; Fre1p and Fre2p

Some metals like Cu(II), Fe(III) require changes in their valances prior to be used. This shows that microorganisms have oxido-reduction systems for the regulation of metal uptake and bioavailability. On the other hand, this system can be a part of a resistance mechanism against high concentrations of metals in the environments (Frederick, *et al,* 1999).

Copper has to be reduced before entering into the cytoplasm. Many studies indicate that copper enteries in the cell after reducing Cu^{+2} in the environment (Thiele *et al.* 1999). This is managed with cell surface electron transport systems and enzyme reduction systems. In *Saccharomyces cerevisiae,* Fre1p and Fre2p are membranous proteins that are responsible for reducing Fe(III) to Fe(II). In addition, they are capable of reducing cupric ions, too. Environmental iron concentration regulates expression of *FRE1* and *FRE2* genes by the negative feedback mechanism (Hassett *et al.* 1995).

Fre1p, encoded by *FRE1* gene, has an important function in uptake system of copper into the cell. It has reductase activity and is located in the cell surface. *FRE1* expression requires Mac1p, since it has a Mac1p-dependent promoter element. *FRE1* is induced by copper depletion. *FRE1 and FRE2* genes are differentially affected by the Mac1p under copper depletion conditions. Fre1p reductase activity is necessary for uptake of cupric (Cu(II)) and ferric ions (Fe(III)). Kosman and co workers

showed that, FRE1 reductase activity provides 50-70 % of total copper uptake. They also identified the correlation between Mac1p and Fre1 (Hassett *et a.* 1995).

Fre2p encoded by *FRE2* gene and is an iron reductase with copper reducing activity, too. Alexandraki and co workers' studies shed light to the role of the iron reductases Fre1p and Fre2p in the copper metabolism and copper uptake. The function of Fre1p and Fre2p is to lower the valence state of copper ion to Cu(I).

In the fre1Δ strain, it was seen that copper reducing activity was achieved by Fre2p iron reductase. In addition, only Fre1p is induced by the copper starvation. Moreover, they also monitored the copper entrance into the cell and its utilization by proteins encoded from the *CUP1* and *CTR1* genes. The copper utilization pathways during which *CUP1* and *CTR1* are involved are different from each other. *CUP1* encodes a metallothionenin and induced by the Ace1p. When copper concentration increases in the cell, *CTR1* that encodes copper transporter is induced by the copper depletion and is not affected by Ace1p (Kaplan *et al*. 1999).

1.5.2 Copper membrane transporters; high and low affinity transporters

Reduced copper ions (Cu(I)) are transferred through the cell membrane with surface transporters, which may be classified as high affinity and low affinity transporters. High affinity transporters are encoded by *CTR1* and *CTR3*, low affinity transporters are *CTR2* and *FET4.*

1.5.2.1 High Affinity Copper Transporters; Ctr1p and Ctr3p

In *Saccharomyces cerevisiae, CTR1* was first identified by the Dancis and coworkers. *CTR1* was found to encode a multispanning plasma membrane transporter protein, involving 406 amino acids. It was also shown that, it was a high affinity copper transport protein in *Saccharomyces cerevisiae.* The studies demonstrated that, *CTR1* mutants were failed in both copper and ferrus iron uptake. Ctr1p exhibited a K_m value for copper of 2μM and a V_{max} of 10 pmol copper /min per 10^6 cells (Dancis *et al*. 1994; Thiele, *et al*, 1998; Hassett *et al*. 2000**).**

The other high affinity copper transporter is Ctr3p, which is encoded by *CTR3*. *CTR3* was identified by J. Thiele and coworkers (Thiele *et al*. 1999). They found that it encoded a small intracellular cysteine–rich integral membrane protein that takes role in copper uptake as a high affinity copper transporter like Ctr1p. Expression of both

CTR1 and *CTR3* are responsible for the maximization of the copper uptake under copper limiting conditions and thus promote the growth under those conditions (Thiele *et al.*1996; 1999).

High affinity uptake pathways are essentially shut down in media with copper concentrations exceeding 10μM. This behavior is very important for survival under excess metal conditions (Hassett *et al*. 2000**).**

1.5.2.2 Low Affinity Copper Transporters; Fet4p and Ctr2p

The baker's yeast *Saccharomyces cerevisiae* also exhibits low affinity uptake systems for copper. The K_m values for this process are about 40 fold greater than K_m values of high affinity uptake system.

Kosman and coworkers showed that low affinity iron transporter, Fet4p*,* also has a role as a low affinity copper transporter. They determined the role of Fet4p as a low affinity copper transporter via measuring the copper uptake with ^{65}Cu uptake assay in high affinity copper transporter deficient strains (Δctr1). They showed that the Δctr1Δctr3 strains managed copper uptake but in the Δctr1Δctr3Δfet4 strains exhibited no saturable to copper (Hassett *et al*. 2000**).**

Culotta and coworkers studied copper metallachaperones and copper transport systems. They concluded that Ctr2p encoded by *CTR2* was a low affinity copper transporter. Ctr2p has a function in delivering the copper metal ion to metallochaperones and is localized on the cell surface like Ctr1p, Ctr3p, and Fet4p. In addition, according to the Culotta and coworkers, Ctr2p has also a function for direction of copper metal ion to vacuole which functions as a store of heavy metal ions (Culotta *et al*. 2001).

1.6 Intracellular Copper Trafficking

Copper is a very toxic element, but cell requires copper for several metabolic processes. Thus, copper uptake and copper intracellular trafficking is very important for delivering it to the target location in the cell. Copper ions, which are entered into the cell are either utilized or transferred to detoxification mechanisms (Edith *et al.* 1997: Culotta *et al.* 1997).

1.6.1 Copper utilization pathway

Copper utilization pathway is including the transport of the copper ion into cell components that require the copper ions for their metabolic activities. Since, there are more than one enzymes requiring copper ions in different compartments in the cell, the transfer of the ions is important for functioning of these target enzymes.

At this point there is a question; how copper is delivered to the right site at the right time in the cell. In this process, metal ion chaperones (or metallochaperones) come into play. Metallochaperones are soluble, intracellular metal receptors, enabling the metal ion to be targeted to right location (Culotta, et al, 2003, O'Halloran, et al, 2000).

There are three different copper trafficking pathways, which are Cu transport mechanism via antioxidant Atx1, CCS, Cox17 to Golgi apparatus, cytosolic SOD, mitochondria, respectively (O'Halloranet *et al*. 2000: Edith *et al.* 1997)**.**

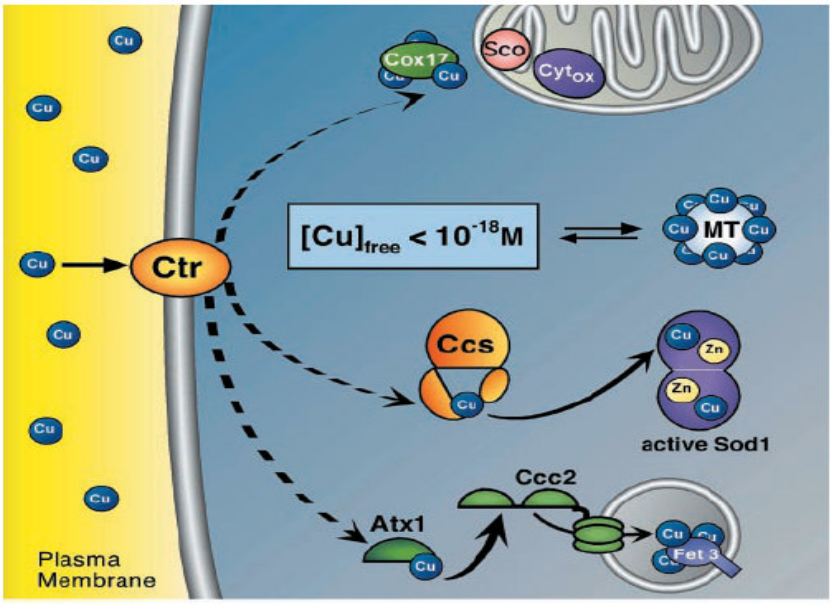

Figure 1.6.1; Copper trafficking pathways in eukaryotes.(O'Halloranet *et al*. 2000)

Atx1 encoded by *ATX1* gene, is a highly conserved intracellular copper receptor in yeast. Its homologs were identified in a variety of organism such as bacteria, plants, and human (O'Halloranet *et al*. 2000).

Atx1 was firstly identified with its protective feature against oxidative damage and it is a protein composed of a small polypeptide chain consisting of 70 amino acids. The

MXCXXC motif where M is methionine, X is any amino acid and C is cysteine, is involved in cupper binding of this protein. Yeast Atx1 copper chaperone binds only one metal ion with the two cysteine residues present in the motif (Culotta *et a.,* 2000 and O'Halloranet *et al*. 2000).

In addition, Atx1 was later found that it specifically guides copper metal to Ccc2, which is a copper transporter located in Golgi. It is also required for the activation of the Fet3 protein taking part in iron uptake. Then, this copper transporter pumps copper into Golgi lumen for its corporation with the copper enzymes. This copper transporter is a member of a large family of transporter ATPases that uses ATP as an energy source during copper transport through to membrane.

COX17 and SCO1 are involved in the delivering mechanism of copper metal to cytochrome oxidase, which is a mitochondrial enzyme requiring three copper ions as cofactor. COX 17 is a shuttle protein (metallochaperone). Yeast COX17 can be located both in cytosol and in inner membrane space of the mitochondria. It is an 8.0-kDa protein whose 6 cysteines are conserved in both yeast and human proteins. It delivers copper ion to mitochondria. It is thought that COX17 may take role as a shuttle protein responsible for delivery of copper to SCO1/SCO2. SCO1 is a mitochondrial inner membrane protein. It plays an important role during the copper and cytochrome oxidase complex formation. There is a homology between SCO1 and subunit 2 of cytochrome oxidase and this homology region includes two conserved copper–binding cysteinyl ligands. SCO2 is a homolog of SOC1 and is thought to have a role in this system (Glerum *et al*. 1996; Thiele *et al*. 2002).

Another identified copper chaperone is CCS**,** which is responsible for the delivery of copper ion to cytosolic superoxide dismutase (SOD1) enzyme. CCS has three functionally distinct protein domains. The N terminal domain shows homology to Atx1 protein whereas, the central domain of CCS (Domain II) is homologous to SOD1. Copper and zinc containing superoxide dismutase (SOD1) is a homodimeric enzyme which is a very important cytosolic enzyme protecting the cell against oxidative damage. Its role is to scavenge the toxic superoxide anion radicals (Thiele *et al*. 2002: O'Halloranet *et al*. 2000). SOD1 needs only one CCS protein for its enzymatic activity. CCS directly delivers copper ion into the SOD1 enzyme. However, SOD1 can take copper ion independently from CCS when the cell is

exposed to toxic doses of copper (Glerum *et al*. 2002**;** Culotta *et al*. 2003: Thiele *et al.* 2002).

1.6.2 Copper detoxification as a defense system

The baker's yeast *Saccharomyces cerevisiae* has some defense mechanisms based on detoxification of heavy metal ions. These mechanisms can be sub-grouped into two, which are enzymatic and non enzymatic defense systems to maintain the vitality of the cell. Enzymatic system includes catalase enzyme and SOD; non enzymatic system involves metallothioneins (MT) and glutathione (GSH).

1.6.2.1 Non-enzymatic defense system

Non-enzymatic system has two important molecules: metallothioneins and glutathione. The molecules participate to metal defense system in the cell.

1.6.2.1.1 Metallothioneins

Metallothioneins (MT) were firstly identified in 1957 by Margoshes and Valle as a response component against natural cadmium accumulation in mammalian kidney. An important feature of MTs is their inducibility with spesific agents and conditions.

MTs are a group of proteins having low molecular weight (> 10 kDa). These proteins are rich in cysteine, which is a naturally occurring, sulfur-containing (sulfhydryl) amino acid with a thiol group. Their structures do not include aromatic amino acids and disulfide bridges. The high sulphydryl content of these proteins makes it easy for them to bind numerous metal ions in the form of discrete metal–thiolate clusters (Avery, *et al*, 2001; Anthony *et al***.** 1997).

The baker's yeast *S.cerevisiae* has a response system against high level of copper ions by the activation of MT genes; *CUP1* and *CRS5* encoding copper binding/detoxification proteins. Induction of metallothionein genes are regulated transcriptionally by *ACE1* gene encoding a copper dependent transcription factor (Thiele *et al*. 1997 and Kaplan *et al.* 1999**).** *CRS5* gene product is less effective than *CUP1* gene product in copper detoxification *(*Avery *et al.* 200l; Culotta 1994).

1.6.2.1.2 *Glutathione*

In addition to metallothioneins, there is another antioxidant molecule having an important role in metal detoxification mechanism in *S. cerevisiae*: glutathione. Glutathione (GSH), a tripeptide γ- L- cystinylglycine, is an antioxidant molecule, which is a cysteine rich protein like Metallothioneins.

GSH is a radical scavenging protein with the aid of sulphydryl groups in its structure. When sulphydryl group of GSH reacts with an oxidant, reduced gluthationein is produced (GSSG). The genes responsible for GSH synthesis in *S.cerevisiae* are *GSH1* and *GSH2*, encoding respectively γ-glutamylcysteine synthetase and glutathione synthetase. On the other hand, *S. cerevisiae* can also use GSH as a nitrogen and sulphur source under starvation conditions. It participates in different cellular activities such as detoxification of metal ions, removal of hydroperoxides, and protection of the normal sulfhydryl structure of proteins *(*Avery *et al. 2001;* Theodorus *et al*. 1981).

1.6.2.2 Enzymatic defense system:

Enzymatic defense system is based on the enzymatic activity of enzymes such as catalase and superoxide dismutase in the cell. The enzymes catalyze heavy metal defense system reactions.

1.6.2.2.1 *Catalase*

Catalase has a role in breaking down of H_2O_2 to O_2 and H_2O. This enzyme is important for resistance against toxic effects of H_2O_2. When copper concentration levels reach to a toxic level for a cell, the cell generates free radicals that cause damages to the cell content. At this point, the free radicals are replaced with physiologically important metal ions located on metalloproteins **(**Avery *et al*. 2004**).**

When the baker‘s yeast *Saccharomyces cerevisiae* is exposed to copper stress, this stress causes an increase in generation of reactive oxygen species (ROS), induces oxidative stress, and affects some key enzymes related to fermentative catabolism of glucose **(**Avery, *et al*, 2004**).** Hydrogen peroxide (H_2O_2), superoxide anion (O^{2-}), and hydroxyl radicals (OH^-) are reactive oxygen species. ROS are normally generated by a cell as a yield of respiration, thus the cell has to develop a protecting defense

mechanism against basal level of ROS and their potential damage on proteins, lipids and nucleic acids.

$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + OH^{\cdot} + OH^{-}$ (Fenton reaction).

Redox active metals like copper and iron participate in the Fenton reactions and promote the generation of ROS in the cell as result of a univalent reduction of H_2O_2 generating $OH^{\cdot}$ radical, which is a very highly reactive oxidant (Avery *et al*. 2001; Fatichenti *et al*. 2000; Sthohs *et al.* 1995) .

1.6.2.2.2 *Superoxide dismutase*

Super oxide dismutase (SOD) is a homodimeric zinc and copper containing enzyme catalyzing the decomposition of superoxide (O^{-2}) to hydrogen peroxide (H_2O_2) and oxygen by the help of utilization of redox properties of copper ions. Yeast has two superoxide dismutases: Cu/Zn SOD and Mn SOD. The Cu/Zn SOD is encoded by the *SOD1* gene and is located in cytoplasm, whereas the Mn SOD is encoded by *SOD2* gene located in mitochondria in cell. SOD1 protects the cell from oxidative stress via scavenging toxic superoxide anions via redox reactions. SOD binds copper ions with high affinity. Sod1p could bind to intracellular copper and functions as a buffering molecule for the intracellular copper concentrations. Thus, this enzyme has also a role in copper metal resistance mechanisms. Studies showed that, Sod1p increases the copper resistance in yeast cells. CCS molecules (also known as Lys7p), which are copper metallochaperones direct the copper metal ion to superoxide dismutase in the yeast cell (Thiele *et al.* 2002: Avery *et al. 2001* and Barry *et al*. 1992).

1.7 The role of cell contents in response to metal stress

1.7.1 The role of cell wall

Metals like copper, zinc and cobalt are essential for yeasts Thus, the cell wall would not prevent the penetration of such metals through plasma membrane under normal growth conditions. Yeast cell wall is able to bind metals and the total amount of the metal has to be at significant values. Many investigators have studied to investigate whether the metal binding on cell wall was a stress response mechanism or not. As a result of some of these studies indicate that this behavior of the cell wall may be

required for the uptake of metals by the cell, due to the this behevior the cell wall may have a role metal stress resistance mechanisms. The effect of copper ion on the plasma membrane is permeabilization. When it reaches the toxic levels, it makes changes in cellular K^+ efflux (Howlett *et al.* 1997).

1.7.2 Vacuole

Vacuoles have very vital and important physiological functions in a cell such as protein processing, pH homeostasis, osmoregulation, storage of some metabolites and degradation of some macromolecules. Yeast vacuole is acidic and contains hydrolytic enzymes. Studies showed that vacuole participates in metal ion regulation by serving as a detoxification and storage compartment in cell. Cations like Mn^{+2}, Fe^{+2}, Co^{+2}, Ca^{+2}, K^+, Li^+ are kept in vacuole, and thus the accumulation in cytosol and the possible toxic effects are prevented. The transfer of metal cations in or out of the vacuole is achieved via cation–proton exchange systems. These systems are based on vacuolar transmembrane electrochemical pH gradient, whose energy source comes from vacuolar H+ ATPase (Gadd *et al*. 1997: Gadd *et al*. 1986 and Thiele *et al.* 1997).

Information about vacuole function related to the metal storage or detoxification could be gained from Gadd and coworkers' study. They used three vacuolar deficient mutant *S. cerevisiae* strains to investigate the function of vacuole under metal stress conditions such as Cu, Cd, Co, Mn, Ni, and Zn. In this experiment, the strains were exposed to metal sulphate derivatives by incubation on agar plates to give final concentrations of 5 mM Zn, Cu, Mn, Cd, Co, Ni. The results of experiment showed that, vacuole has an important role in regulation of the toxicity of Zn, Mn and Co metals. The metal toxicity was increased in mutants lacking vacuolar functions. On the other hand, the Cu and Cd toxicity was not affected (Gadd *et al*. 1997).

1.8 Metabolic and inverse-metabolic engineering

The aim of the metabolic engineering is to improve cellular activity by using genetic technique for direction or modifying on the metabolic pathways on procaryotic and eukaryotic cells. In metabolic engineering experimental process first of all interested in genetic control of desired phenotypes. Mathematical methods have been used for identification of kinetic mechanism and structural of metabolic pathways. DNA

microarray is the improrttant genetic tool for determination of the genes charecteristic having desired improvement or identification of pathways..

Metabolic engineering involves the solution of such biotechnological problems which are identification of interested metabolic pathway and regulation of pathways. So this information about identification of bioreaction network gives the investigator for obtaining desired phenotypes on microorganism for using biotechnological process. (Stephanopoulos *et al*. 1997).

The new genetic tools have been developed and the classical approach of metabolic engineering requires detailed knowledge of the enzyme kinetics and the system network, in order to obtain the desired phenotype at the end**.** However, obtaining the desired phenotype has some difficulties due to limited background knowledge on the complex cellular and genetic systems. Thus, new alternative technique have been developed, *inverse metabolic engineering.* This type metabolic engineering support the improving of the microrganisms for an industrial process. This industrial microrganisms are then the genetic structure of the desired phenotype is defined (Bailey *et al.* 1996).

The process which are investigating the desired phenotypes and applying them on the organisms of interest, the desired phenotypes are formed by random mutations and then selected from the heterogeneous population. If any condition is applied due to have desired characteristics, the strategy is called *directed evolution* (Bailey *et al.* 1996). Use of evolutionary methods is accepted to be as a more natural procedure when compared to recombinant DNA technology. Thus evolutionary techniques give us the advantage because it is more accepted by the public for human consumption in food industry, because of the 'natural' changes or mutants in the genome, rather than insertion of a foreign or heterologous gene (Çakar *et al*. 2005).

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 Composition of yeast minimal medium (YMM)

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

per liter of distilled water.

2.1.2.2 Composition of yeast complex medium (YPD)

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

Per liter of distilled water

2.1.3 Chemicals

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

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

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

Cobalt(II)-chloride was purchased from Merck (Germany)

Chromium(VI) chloride was purchased from Merck (Germany).

Nitric acid (10M) was purchased from Merck (Germany).

2.1.4 Buffers and solutions

$CuCl_2$ solution	1 M
$CoCl_2$ solution	1 M
$CrCl_3$ solution	1 M
HNO_3 solution	6 M
H_2O_2 solution	5 M
Sodium thiosulfate solution	10 % (w/v)

2.1.5 Laboratory equipment

UV-Visible Spectrophotometer	*Shimadzu UV-1700 (Japan),*
	Perkin Elmer 25 UV/VIS (USA)
Laminar Flow	*Özge (Turkey)*
	Faster BH-EN (Italy)
Autoclaves	*Tuttnauer Stystec Autoclave 2540 ml (Switzerland)*
	NüveOT 4060 Steam Sterilizer(Turkey)
Incubators	*Nüve EN400 (Turkey)*
	Nüve EN500 (Turkey)

Atomic Absorption Spectrometer (ITU, Chemisty Department)	*Analytik Jena Vario 6 (Germany)*
Orbital Shaker Incubators	*Certomat S II Sartorius (Germany)*
Light Microscope	*Olympus CH30 (USA)*
Water Bath	*Memmert wb-22 (Switzerland)*
	Nüve BS402 (Turkey)
Microfuge	*Beckman®Coulter Microfuge (USA)*
Deep Freezes and Refrigerators	*80˚C Heto Ultrafreeze 4410 (Denmark),*
	-20˚C Arçelik(Turkey)
	+4˚C Arçelik (Turkey)
Micropipettes	*Eppendorf (Germany)*
Thermomixer	*Eppendorf, Thermomixer Comfort 1.5-2 ml, (Germany)*
pH meter	*Mettler Toledo MP220 (Switzerland)*
Ultrapure Water System	*USF-Elga UHQ (USA)*

2.2 Methods

2.2.1.EMS mutagenesis

A 500µl aliquot of *Saccharomyces cerevisiae* CEN.PK 113-7D stock culture was inoculated in YMM and incubated overnight at 30ºC and 150 rpm in a waterbath shaker. A culture volume of 2.5 ml was washed twice in 10 ml of 50 mM potassium phosphate buffer (pH =7). Wild type *S. cerevisiae* was exposed to EMS by adding

300 µl of EMS to in 10 ml of cell volume, was gently vortexed and incubated at 30ºC and 150 rpm for 30 minutes in a waterbath shaker. In order to stop EMS mutagenesis, an equal volume of filter-sterilized sodium thiosulphate solution was added and the solution was centrifuged at 10000 rpm for 10 min. in centrifuge (Beckman Coulter, JA 30.50i rotor). The pellet was washed twice with dextrose free YMM and then inoculated on YPD agar plate to transfer the mutant generation to minimal medium. Wild type was named as *100* and the chemically mutagenized calture was named as *101*.

2.2.2. Selection of copper-resistant *S. cerevisiae* populations

To obtain copper-resistant yeast cells, two types of stress strategies were applied;

1. *Continuous stress selection strategy*

2. *Pulse stress selection strategy.*

The difference between the two strategies is that the continuous stress selection strategy involves a continuous stress application throughout the cultivation, whereas the pulse selection strategy involves "pulse" of stress applied for a short period of time.

The general strategy of obtaining the generations is based on applying the initial stress application on chemically mutated culture *101* and transferring the survivors of this present stress condition to further stress applications. Each stress selection strategy includes two sub strategies; *increasing stress application* and *constant stress application,* which indicate if the copper stress is applied at increasing or constant copper concentrations.

2.2.2.1 Continuous stress selection strategy

This stress strategy was applied as two parallel approaches; *increasing stress application* and *constant stress application.* In the increasing stress application, the cultures were exposed to higher copper concentrations for each successive generation. For this purpose, $CuCl_2$ concentrations were started from 0.2 mM $CuCl_2$ and increased up stress to 20 mM in continuous increasing stress strategy. The initial stress level of 0.2 mM $CuCl_2$ was applied on *101* liquid cultures for 24 h after which the resistant cells were harvested and coded as the 1^{st} generation resistant strain (*1N*).

This step was repeated until the final resistant generation named as *56N,* which is resistant to 20mM $CuCl_2$, was obtained.

In constant stress application, starting with *101* new generations were obtained by applying 0,2 mM $CuCl_2$ stress repetitively from the first generation to the 56^{th} generation. Stock cultures were prepared for each of the generation obtained.

The overnight culture was used for incubation into 10 ml YMM with varying concentrations of $CuCl_2$ for each of increasing and constant stress generations. Optical density values at 600 nm wavelength OD and haemacytometer counting results of 72 h of incubation were analyzed and used for survival ratio analysis.

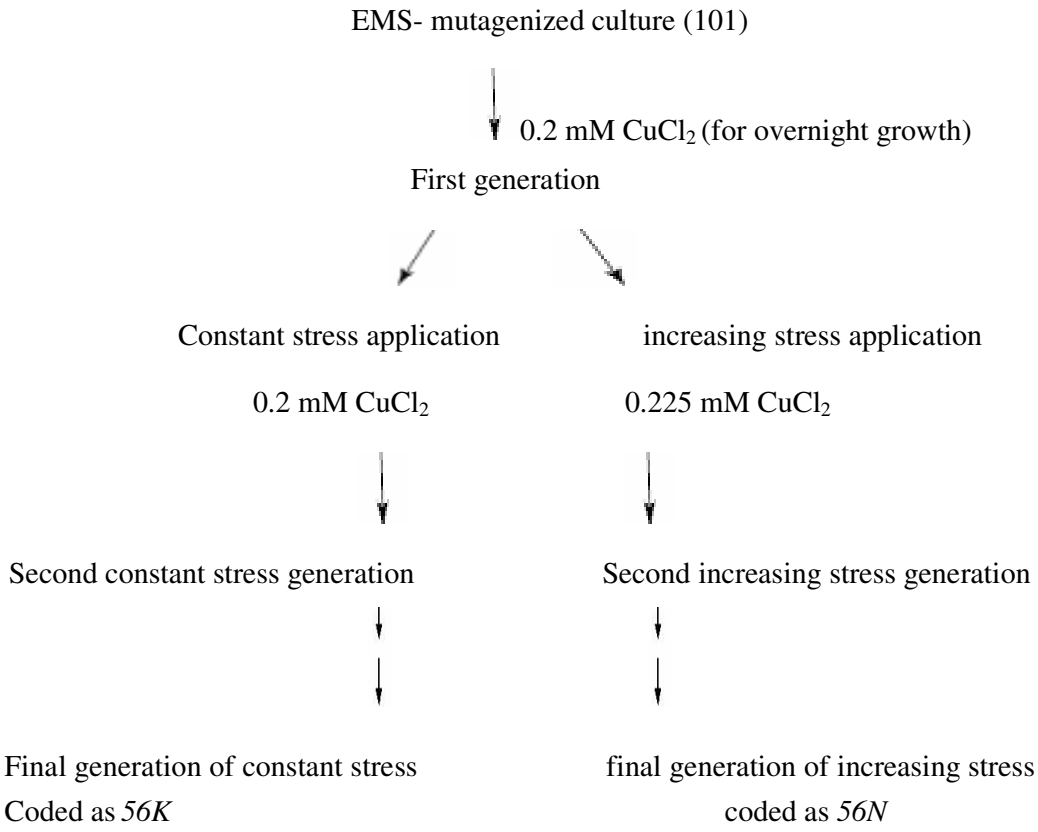

Figure 2.2.2.1 The general process of obtaining generations with continuous stress selection strategy

2.2.2.2 Pulse stress selection strategy

Similar to continuous stress selection strategy increasing stress and constant stress applications were also used in pulse stress selection strategy.

During increasing stress application, overnight incubated cultures were inoculated into fresh YMM to obtain an initial OD_{600} of about is 0.2-0.3. When the OD_{600} of culture was between 0.5-0.6, the culture was exposed to copper stress at increasing copper concentration at each successive generation. Overnight incubated pre-culture having OD_{600} between 0.5-0.6 was incubated at 30°C, 150 rpm for 1.5 h in a microfuge tube including YMM with metal stress. $CuCl_2$ concentrations ranged between 0.2 mM and to 10 mM $CuCl_2$. The initial stress level of 0.2 mM $CuCl_2$ was applied on *101 liquid* cultures for 1.5 h after which resistant cells were harvested and incubated in 10 ml YMM without metal stress for 24 h at 30°C at 150 rpm in an

orbital shaker, and then coded as the first generation resistant strain (*1N*). This step was done repeatedly until the last resistant generation named as *56N,* which is resistant to 10 mM $CuCl_2$, was obtained.

In constant stress application, starting with *101*, new generations were obtained by applying 0.2 mM $CuCl_2$ stress repetitively from 1st generation to the 56th generation. Stock cultures were prepared for each generation obtained.

Each generation's overnight culture was used for incubation into 10 ml YMM with varying concentrations of $CuCl_2$. OD_{600} data (at 600 nm wavelength) and haemacytometer counts of 24 h of incubation were analyzed and used for survival ratio analysis.

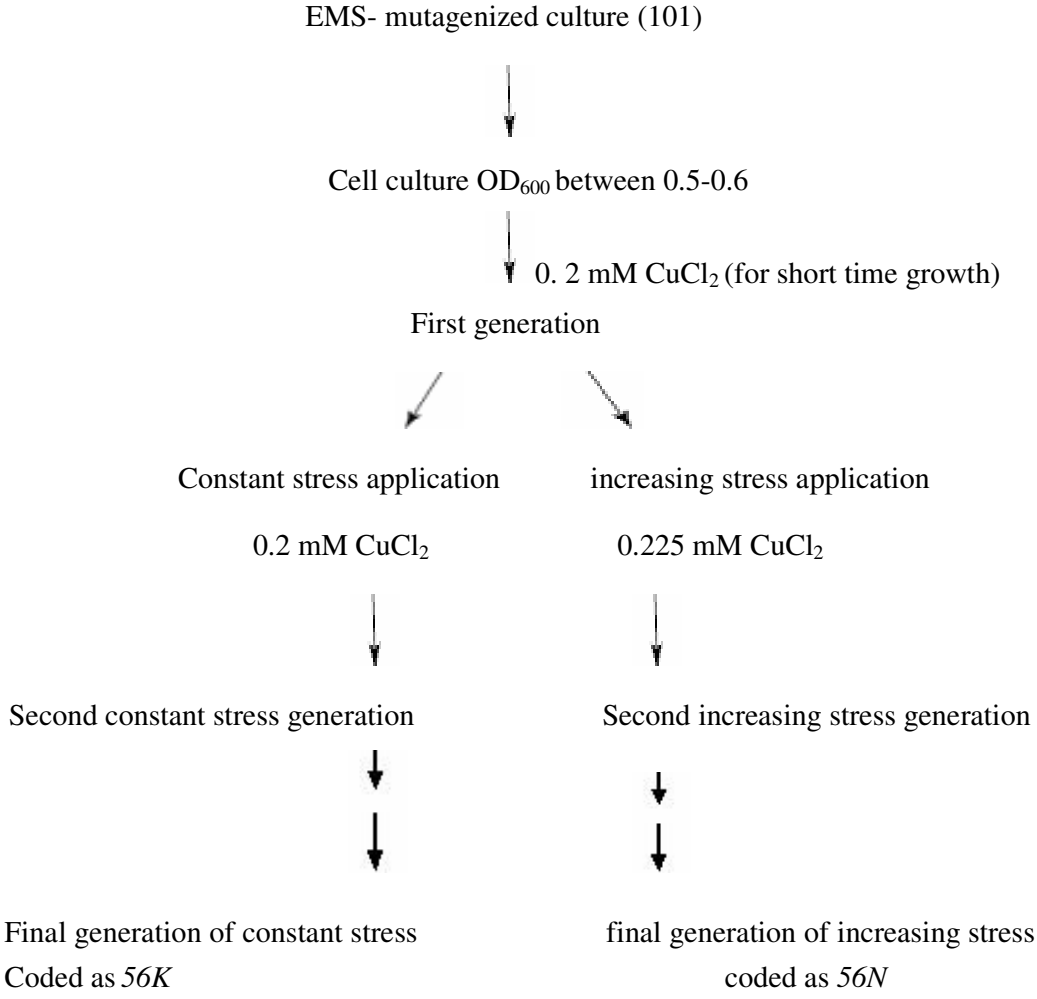

Figure 2.2.2.2 The general process of obtaining generations with pulse stress selection strategy

2.2.3 Stock culture preparation

In order to make stock cultures, heavy metal had to be removed from the culture environment. For this purpose, at the end of 72 h of incubation, 500µl culture was taken from stress-applied environment, transferred to a microfuge tube, and centrifuged at 10,000 rpm for 5 min. The supernatant was then removed and 500 µl YMM was added. After mixing gently, it was centrifuged again at 10,000 rpm for 5 minutes, and the supernatant was discarded again. Equal volumes (500 µl) of YMM and glycerol from 60 % (v/v) glycerol stock were added onto the cell pellet. After mixing gently, it was placed at -20^0 C and -80^0 C freezers for long-term preservation.

2.2.4 Selection of the $CuCl_2$ resistant individuals

Overnight final generation culture with $1:10^6$ dilutions was inoculated into YMM agar plates and incubated at 30°C. Individuals (colonies) were selected and transferred to fresh YMM. These individuals were screened by applying various concentrations of $CuCl_2$ in the 10 ml test tubes and the survival ratio was determined with respect to control tubes by spectrophotometric (OD_{600} nm) measurements. At the end of screening, highly resistant individuals were determined

2.2.5 Most probable number method (MPN)

MPN is a serial dilution test which measures the concentration of the viable cells in the culture. MPN Table is consulted to determine the most probable number of organisms (causing the positive results) per unit volume of the original sample. For this purpose, 96-well plates were used

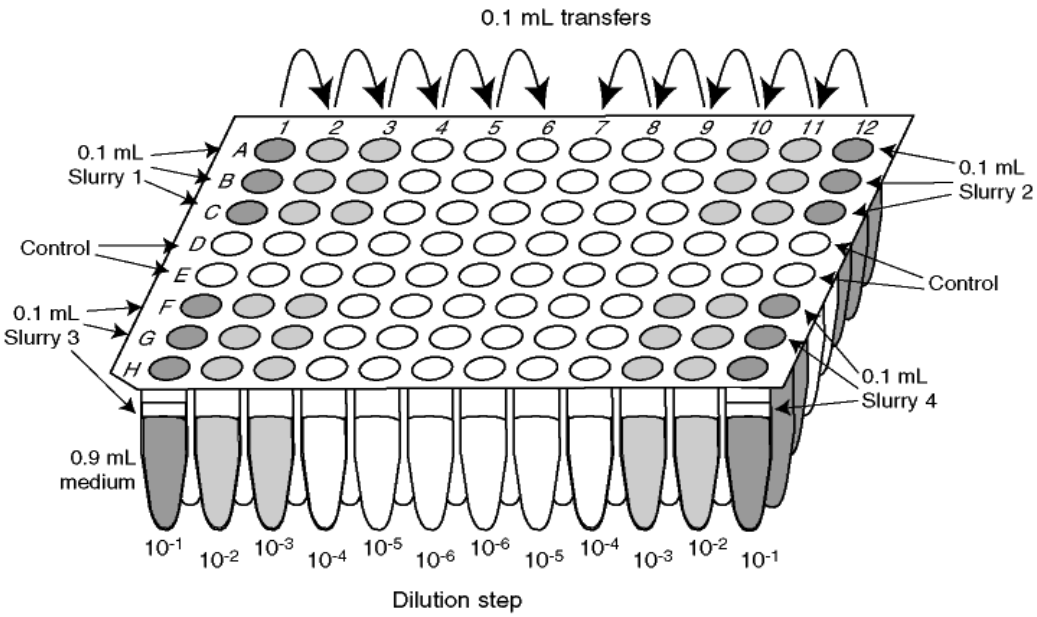

Figure 2.2.5.1 A 96 well plate for MPN (reference; http://www odp.tamu.edu/publications/201_IR/chap_05/images/05_f10.gif).

Each well contained 20 µl of cell culture and 180 µl of either YMM or metal containing YMM. Firstly, 20 µl of culture were inoculated into 5 well on the A line (figure 2.2.5.1) which contained 180 µl YMM with a specific metal concentration. After the first inoculation, using a multipipette the newly inoculated cultures in the first 5 well were serially diluted to the 5 wells on the next line (line B) and so on. After inoculation, each 96 well plate was incubated at 30°C, for 72 h. Parallel to serial dilution in stress containing wells; the same procedure was applied also in control wells. Their percent survivals were then calculated.

2.2.6 Characterization of individual mutants

Selected individual mutants were characterized by screening under various stress conditions such as heavy metal stress (cobalt, chromium), heat stress, osmotic stress, and oxidative stress.

2.2.6.1 Analysis of cross-resistance

2.2.6.1.1 Cobalt stress application

$CoCl_2$ metal stress was applied continuously by exposing the cells to varying cobalt concentrations

2.2.6.1.2 Chromium stress

$CrCl_3$ metal stress was applied continuously by exposing the cells to varying cobalt concentrations

2.2.6.1.3 Oxidative stress application

Varying levels of oxidative stress were applied on the yeast cells. Overnight incubated pre-culture having OD_{600} between 0.5-0.6 was incubated at 30°C at 150 rpm until the OD_{600} of the cell cultures reached 1.2-1.6. One ml from each culture was centrifuged at 10.000 rpm for 5 min and the resulting cell pellet was resuspended in dextrose- free YMM. This step was repeated twice. To create stress conditions, hydrogen peroxide was added from a 5 M hydrogen peroxide stock solution. The cells were incubated for 1 h at 30˚C, 150 rpm and were then centrifuged at 10.000 rpm for 10 min. The supernatant was then discarded, and the pellet was resuspended with dextrose-free yeast minimal medium. The 500 µl dextrose-free YMM plus cell mixture was inoculated into 10 ml YMM. The OD_{600} of the culture was measured at 24, 48, and 72 h of cultivation.

2.2.6.1.4 Heat stress application

One ml of overnight culture at an OD_{600} nm of 1.2-1.6 was exposed to varying heat stress levels. 30 ˚C, 40 ˚C, 50 ˚C and 60 ˚C “pulse” heat stresses were applied for 10 min. After the heat stress application, the cultures were centrifuged at 14.000 rpm for 10 min, washed using 500 µl dextrose-free yeast minimal medium. The last resuspension of 500 µl dextrose-free YMM plus cell mixture was inoculated into 50 ml test tubes at 10 ml final culture volume in YMM. OD_{600} was measured at 24, 48, and 72 h of cultivation.

2.2.6.1.5 Osmotic stress

Overnight pre-culture was inoculated into 10 ml YMM, containing varying concentrations of NaCl such as 5%, 10% and 15% with comparable initial OD_{600} values. Those cultures were then incubated for 72 h at 30ºC, 150 rpm for 72 h a shaker. OD_{600} of the cultures were measured at 24, 48, and 72h of incubation.

2.2.6.2 Flame atomic absorption spectroscopy (FAAS)

Overnight cultures were inoculated into YMM, with stress conditions which are copper, cobalt, and chromium continuous stress applications at corresponding stress levels for 72 h. The culture were harvested by centrifugation at 35.000 rpm for 5 min, the supernatant was discarded and the pellets were then resuspended with 10 ml distilled water for two times to wash out the remaining metals from the cell surface and the environment. The cells were then exposed to 6M HNO_3 for 1 h at 90^0 C for cell disruption. 11 M Nitric oxide stock solution was used to obtain the final 6 M Nitric oxide concentration. The hydrolyzed cells were used out for atomic absorption spectrometry measurements. Each sample was diluted 1:2 in order to decrease the final acid concentration to 3 M and prevent any potential acid damage to the spectrometer prior to the FAAS measurement. Copper (λ = 324.8 nm, Slith width =1.2 nm*)*, cobalt (*λ* = 242.5 nm, Slith width =0.2 nm*)* and chromium (*λ* = 357.9 nm*,* Slith width =0.2 nm*)* contents associated with cells were determined.

Additionally cell dry weight analysis was performed at the 72 h of incubation. The values of both metal content and cell dry weight were calculated for analyzing and determination of the characterization of the copper resistant mutant strains under copper, cobalt and chromium stress.

3. RESULTS

3.1 Screening for copper stress resistance to determine the initial stress application levels

The aim of this study was to obtain copper resistant strains which can survive at higher copper concentrations than the wild type. In order to determine the initial stress application level, copper stress screening test was performed. The copper stress was applied by using two different stress strategies which were "continuous" and "pulse". In continuous copper stress application, the screening technique was performed on chemically mutagenized culture (named as *101*) by continuously applying different copper stress levels (0 µM, 50µM, 100µM, 200µM, 500µM, 1000µM). The optical density value at 600 nm (OD_{600} nm) was measured at 24^{th}, $48^{th,}$ and 72^{nd} hours. It was observed that the cells could not survive at concentrations higher than 500µM $CuCl_2$. According to these results, it was decided to apply 200 µM $CuCl_2$ as the initial stress level. The results of continuous stress application screening test are shown on Table 3.1.1

Table 3.1.1 The OD_{600} results of continuous $CuCl_2$ stress aplication screening test.

Strains Name	**0 µM initial $CuCl_2$ concentr.**	**50 µM initial $CuCl_2$ concentr.**	**100 µM initial $CuCl_2$ concentr.**	**200 µM initial $CuCl_2$ concentr.**	**500 µM initial $CuCl_2$ concentr.**	**1000 µM initial $CuCl_2$ concentr.**	**2000 µM initial $CuCl_2$ concentr.**
100	5.60	5.75	6.33	5.48	6.62	0	0
101	5.91	5.88	6.14	5.64	4.44	0	0

Pulse stres was applied to *100* and *101* at varying $CuCl_2$ concentrations: 1000, 2000, 3000, 5000 µM and the optical densities were measured after 24 h of incubation at 30ºC and 150 rpm (Tabe 3.1.2).

Table 3.1.2 The OD_{600} nm results of pulse stress application screeening test.

Strain name	at 0 µM $CuCl_2$	at 1000 µM $CuCl_2$	at 2000 µM $CuCl_2$	at 3000 µM $CuCl_2$	at 5000 µM $CuCl_2$
100	6.53	6	3.79	2.31	1.00
101	5.4	5.54	2.67	1.84	0.21

3.2 Obtaining $CuCl_2$-resistant mutant generations

In order to obtain copper-resistant yeast mutants, two different stress application strategies were applied: continuous and pulse selection strategies. Both strategies were applied in two parallel sub-strategies, which are selection at increasing and constant stress levels. All of these strategies involve transferring the survivors of the previous step to the next step.

3.2.1 Continuous stress generations

3.2.1.1. Continuous increasing stress generations

Table 3.2.1. shows Nomenclature for continuous increasing stress generations including their code names and the stress levels at which they had been selected. Stress conditions were initiated by applying 200 µM $CuCl_2$ and increased by 25 µM at each step during early generations. After 21^{st} continuous increasing stress generation corresponding to the stress level of 700 µM $CuCl_2$, the increased level of $CuCl_2$ per each step was adjusted to 50 µM. The increased level of $CuCl_2$ per each step was set to 100 µM from 28^{th} to 32^{nd}, 200 µM from 32^{nd} to 35^{th}, 500 µM from 35^{st} to 43^{rd}, and 1000 µM from 43^{th} to 56^{th} continuous increasing stress generation. At the end of study, the final generation coded as *56N* was obtained, corresponding to 20 mM $CuCl_2$ stress level. All copper continuous increasing generations name and applied copper concentrations are listed in Table 3.2.1.

Table 3.2.1 Nomenclature for copper continuous increasing stress generations and the corresponding stress levels**.**

Generations	Code	$CuCl_2$ µM
1st increasing stress generation	*1N*	200
2nd increasing stress generation	*2N*	225
3rd increasing stress generation	*3N*	250
4th increasing stress generation	*4N*	275
5th increasing stress generation	*5N*	300
6th increasing stress generation	*6N*	325
7th increasing stress generation	*7N*	350
8th increasing stress generation	*8N*	375
9th increasing stress generation	*9N*	400
10th increasing stress generation	*10N*	425
11th increasing stress generation	*11N*	450
12th increasing stress generation	*12N*	475
13th increasing stress generation	*13N*	500
14th increasing stress generation	*14N*	525
15th increasing stress generation	*15N*	550
16th increasing stress generation	*16N*	575
17th increasing stress generation	*17N*	600
18th increasing stress generation	*18N*	625
19th increasing stress generation	*19N*	650
20th increasing stress generation	*20N*	675
21st increasing stress generation	*21N*	700
22nd increasing stress generation	*22N*	750
23rd increasing stress generation	*23N*	800
24th increasing stress generation	*24N*	850
25th increasing stress generation	*25N*	900
26th increasing stress generation	*26N*	950
27th increasing stress generation	*27N*	1000
28th increasing stress generation	*28N*	1100
29th increasing stress generation	*29N*	1200
30th increasing stress generation	*30N*	1300
31st increasing stress generation	*31N*	1400
32nd increasing stress generation	*32N*	1600
33rd increasing stress generation	*33N*	1800
34th increasing stress generation	*34N*	2000
35th increasing stress generation	*35N*	2500
36th increasing stress generation	*36N*	3000
37th increasing stress generation	*37N*	3500
38th increasing stress generation	*38N*	4000
39th increasing stress generation	*39N*	4500
40th increasing stress generation	*40N*	5000
41st increasing stress generation	*41N*	5500
42nd increasing stress generation	*42N*	6000
43rd increasing stress generation	*43N*	7000
44th increasing stress generation	*44N*	8000
45th increasing stress generation	*45N*	9000
46th increasing stress generation	*46N*	10000
47th increasing stress generation	*47N*	11000
48th increasing stress generation	*48N*	12000
49th increasing stress generation	*49N*	13000
50th increasing stress generation	*50N*	14000
51st increasing stress generation	*51N*	15000
52nd increasing stress generation	*52N*	16000
53rd increasing stress generation	*53N*	17000
54th increasing stress generation	*54N*	18000
55th increasing stress generation	*55N*	19000
56th increasing stress generation	*56N*	20000

The 56th continuous increasing stress generation was chosen as the final mutant generation.

3.2.1.2 Continuous constant stress generation

Continuous constant stress generations were obtained by repeating a constant level of $CuCl_2$ stress (0.2 mM) for 56 generations.

The names of copper continuous constant stress generations and their corresponding copper concentrations are shown in Table 3.2.2.

Table 3.2.2 Nomenclature for copper continuous constant stress generations and the corresponding stress levels.

Generations	Code	$CuCl_2$ µM
1st new constant stress generation	*1K*	200
2nd new constant stress generation	*2K*	200
3rd new constant stress generation	*3K*	200
4th new constant stress generation	*4K*	200
5th new constant stress generation	*5K*	200
6th new constant stress generation	*6K*	200
7th new constant stress generation	*7K*	200
8th new constant stress generation	*8K*	200
9th new constant stress generation	*9K*	200
10th new constant stress generation	*10K*	200
11th new constant stress generation	*11K*	200
12th new constant stress generation	*12K*	200
13th new constant stress generation	*13K*	200
14th new constant stress generation	*14K*	200
15th new constant stress generation	*15K*	200
16th new constant stress generation	*16K*	200
17th new constant stress generation	*17K*	200
18th new constant stress generation	*18K*	200
19th new constant stress generation	*19K*	200
20th new constant stress generation	*20K*	200
21st new constant stress generation	*21K*	200
22nd new constant stress generation	*22K*	200
23rd new constant stress generation	*23K*	200
24th new constant stress generation	*24K*	200
25th new constant stress generation	*25K*	200
26th new constant stress generation	*26K*	200
27th new constant stress generation	*27K*	200
28th new constant stress generation	*28K*	200
29th new constant stress generation	*29K*	200
30th new constant stress generation	*30K*	200
31st new constant stress generation	*31K*	200
32nd new constant stress generation	*32K*	200
33rd new constant stress generation	*33K*	200
34th new constant stress generation	*34K*	200
35th new constant stress generation	*35K*	200
36th new constant stress generation	*36K*	200
37th new constant stress generation	*37K*	200
38th new constant stress generation	*38K*	200
39th new constant stress generation	*39K*	200
40th new constant stress generation	*40K*	200
41st new constant stress generation	*41K*	200
42nd new constant stress generation	*42K*	200
43rd new constant stress generation	*43K*	200
44th new constant stress generation	*44K*	200
45th new constant stress generation	*45K*	200
46th new constant stress generation	*46K*	200
47th new constant stress generation	*47K*	200
48th new constant stress generation	*48K*	200
49th new constant stress generation	*49K*	200
50th new constant stress generation	*50K*	200
51st new constant stress generation	*51K*	200
52nd new constant stress generation	*52K*	200
53rd new constant stress generation	*53K*	200
54th new constant stress generation	*54K*	200
55th new constant stress generation	*55K*	200
56th new constant stress generation	*56K*	200

The 56th continuous constant stress generation was chosen as the final mutant generation.

3.2.2 Pulse stress generation

3.2.2.1 Pulse increasing stress generation

Table 3.2.3 shows the pulse increasing stress generations including their code names and the tress levels. Pulse stress was initiated by applying an initial stress level of 0.2 mM $CuCl_2$ to the EMS mutagenized starting population (101). The $CuCl_2$ stress level was increased throughout the study and the final applied $CuCl_2$ stress level reached 10 mM $CuCl_2$.

Table 3.2.3 Nomenclature for copper pulse increasing stress generations and their corresponding $CuCl_2$ stress levels.

Generations	Code	$CuCl_2$ µM
1st Pulse increasing stress generation	*1N*	200
2nd Pulse increasing stress generation	*2N*	300
3rd Pulse increasing stress generation	*3N*	400
4th Pulse increasing stress generation	*4N*	500
5th Pulse increasing stress generation	*5N*	600
6th Pulse increasing stress generation	*6N*	700
7th Pulse increasing stress generation	*7N*	800
8th Pulse increasing stress generation	*8N*	1000
9th Pulse increasing stress generation	*9N*	1200
10th Pulse increasing stress generation	*10N*	950
11th Pulse increasing stress generation	*11N*	1000
12th Pulse increasing stress generation	*12N*	1050
13th Pulse increasing stress generation	*13N*	1100
14th Pulse increasing stress generation	*14N*	1150
15th Pulse increasing stress generation	*15N*	1200
16th Pulse increasing stress generation	*16N*	1250
17th Pulse increasing stress generation	*17-N*	1300
18th Pulse increasing stress generation	*18-N*	1350
19th Pulse increasing stress generation	*19-N*	1400
20th Pulse increasing stress generation	*20-N*	1450
21st Pulse increasing stress generation	*21N*	1500
22nd Pulse increasing stress generation	*22N*	1550
23rd Pulse increasing stress generation	*23N*	1600
24th Pulse increasing stress generation	*24N*	1650
25th Pulse increasing stress generation	*25N*	*1700*
26th Pulse increasing stress generation	*26N*	*1750*
27th Pulse increasing stress generation	*27N*	*1800*
28th Pulse increasing stress generation	*28N*	*1850*
29th Pulse increasing stress generation	*29N*	*1900*
30th Pulse increasing stress generation	*30N*	*1950*
31st Pulse increasing stress generation	*31N*	2000
32nd Pulse increasing stress generation	*32N*	2050
33rd Pulse increasing stress generation	*33N*	2100
34th Pulse increasing stress generation	*34N*	2150
35th Pulse increasing stress generation	*35N*	2200
36th Pulse increasing stress generation	*36N*	2250
37th Pulse increasing stress generation	*37N*	2350
38th Pulse increasing stress generation	*38N*	2450
39th Pulse increasing stress generation	*39N*	2550
40th Pulse increasing stress generation	*40N*	2650
41st Pulse increasing stress generation	*41K*	2750
42nd Pulse increasing stress generation	*42N*	2950
43rd Pulse increasing stress generation	*43N*	3150
44th Pulse increasing stress generation	*44N*	3350
45th Pulse increasing stress generation	*45N*	3550
46th Pulse increasing stress generation	*46N*	3750
47th Pulse increasing stress generation	*47N*	4000
48th Pulse increasing stress generation	*48N*	4500
49th Pulse increasing stress generation	*49N*	5000
50th Pulse increasing stress generation	*50N*	5500
51st Pulse increasing stress generation	*51N*	6000
52nd Pulse increasing stress generation	*52N*	7000
53rd Pulse increasing stress generation	*53N*	8000
54th Pulse increasing stress generation	*54N*	9000
55th Pulse increasing stress generation	*55N*	9500
56th Pulse increasing stress generation	*56N*	10000

The 56th pulse increasing stress generation was chosen as the final mutant generation.

3.2.2.2 Pulse constant stress generation

Pulse constant stress generations were obtained by repeating a constant level of $CuCl_2$ pulse constant stress (0.2 mM) for 56 generations.

The names of all copper pulse constant stress generations and their corresponding copper concentrations are shown Table 3.2.4.

Table 3.2.4 Nomenclature for copper pulse constant stress generations and the applied $CuCl_2$ stress level .

Generations	Code	$CuCl_2$ µM
1st Pulse constant stress generation	1K	200
2nd Pulse constant stress generation	*2K*	200
3rd Pulse constant stress generation	*3K*	200
4th Pulse constant stress generation	*4K*	200
5th Pulse constant stress generation	*5K*	200
6th Pulse constant stress generation	*6K*	200
7th Pulse constant stress generation	*7K*	200
8th Pulse constant stress generation	*8K*	200
9th Pulse constant stress generation	*9K*	200
10th Pulse constant stress generation	*10K*	200
11th Pulse constant stress generation	*11K*	200
12th Pulse constant stress generation	*12K*	200
13th Pulse constant stress generation	*13K*	200
14th Pulse constant stress generation	*14K*	200
15th Pulse constant stress generation	*15K*	200
16th Pulse constant stress generation	*16K*	200
17th Pulse constant stress generation	*17K*	200
18th Pulse constant stress generation	*18K*	200
19th Pulse constant stress generation	*19K*	200
20th Pulse constant stress generation	*20K*	200
21st Pulse constant stress generation	*21K*	200
22nd Pulse constant stress generation	*22K*	200
23rd Pulse constant stress generation	*23K*	200
24th Pulse constant stress generation	*24K*	200
25th Pulse constant stress generation	*25K*	200
26th Pulse constant stress generation	*26K*	200
27th Pulse constant stress generation	*27K*	200
28th Pulse constant stress generation	*28K*	200
29th Pulse constant stress generation	*29K*	200
30th Pulse constant stress generation	*30K*	200
31st Pulse constant stress generation	*31K*	200
32nd Pulse constant stress generation	32K	200
33rd Pulse constant stress generation	33K	200
34th Pulse constant stress generation	*34K*	200
35th Pulse constant stress generation	*35K*	200
36th Pulse constant stress generation	*36K*	200
37th Pulse constant stress generation	*37K*	200
38th Pulse constant stress generation	*38K*	200
39th Pulse constant stress generation	*39K*	200
40th Pulse constant stress generation	*40K*	200
41st Pulse constant stress generation	*41K*	200
42nd Pulse constant stress generation	*42K*	200
43rd Pulse constant stress generation	*43K*	200
44th Pulse constant stress generation	*44K*	200
45th Pulse constant stress generation	*45K*	200
46th Pulse constant stress generation	*46K*	200
47th Pulse constant stress generation	*47K*	200
48th Pulse constant stress generation	*48K*	200
49th Pulse constant stress generation	*49K*	200
50th Pulse constant stress generation	*50K*	200
51st Pulse constant stress generation	*51K*	200
52nd Pulse constant stress generation	*52K*	200
53rd Pulse constant stress generation	*53K*	200
54th Pulse constant stress generation	*54K*	200
55th Pulse constant stress generation	*55K*	200
56th Pulse constant stress generation	*56K*	200

The *56th* generation was chosen as the final mutant generation.

3.3 Selection of individual mutants from final mutant generations

Continuous and pulse strategies were applied to obtain copper resistant strains. Finally, 56^{th} continuous increasing stress generation (the survivors of 20 mM $CuCl_2$ stress condition), 56^{th} continuous constant stress generation (obtained by repeating 0.2 mM $CuCl_2$ continuous stress application for 56 times), 56^{th} pulse increasing stress generation (the survivors of 10 mM $CuCl_2$ stress condition), 56^{th} pulse constant stress generation (obtained by repeating 0.2 mM $CuCl_2$ pulse stress application for 56 times) were obtained and defined as the final generations. In order to select the resistant mutant individuals from the final generation, the cultures were inoculated on YMM agar plates by spreading and incubated overnight at 30°C. Eight colonies were chosen randomly from these plates and they were inoculated into 10 ml YMM for overnight growth. Their frozen stocks were prepared to be used for further analysis.

The nomenclature for continuous increasing stress individuals is shown Table 3.3.1

Table 3.3.1 Continuous increasing stress mutant individuals.

Individual mutant	Code
1^{st} increasing stress individual mutant	*NB1*
2^{nd} increasing stress individual mutant	*NB2*
3^{rd} increasing stress individual mutant	*NB3*
4^{th} increasing stress individual mutant	*NB4*
5^{th} increasing stress individual mutant	*NB5*
6^{th} increasing stress individual mutant	*NB6*
7^{th} increasing stress individual mutant	*NB7*
8^{th} increasing stress individual mutant	*NB8*

The nomenclature for continuous constant stress individuals is shown Table 3.3.2.

Table 3.3.2 Nomenclature for continuous constant stress mutant individuals.

Individual mutant	Code
1st continuous constant stress individual mutant	KB1
2nd continuous constant stress individual mutant	KB2
3rd continuous constant stress individual mutant	KB3
4th continuous constant stress individual mutant	KB4
5th continuous constant stress individual mutant	KB5
6th continuous constant stress individual mutant	KB6
7th continuous constant stress individual mutant	KB7
8th continuous constant stress individual mutant	KB8

The nomenclature for pulse increasing stress individuals is shown table 3.3.3

Table 3.3.3 Pulse increasing stress mutant individuals from final corresponding stress generation .

Individual mutant	Code
1st pulse increasing stress individual mutant	PNB1
2nd pulse increasing stress individual mutant	PNB2
3rd pulse increasing stress individual mutant	PNB3
4th pulse increasing stress individual mutant	PNB4
5th pulse increasing stress individual mutant	PNB5
6th pulse increasing stress individual mutant	PNB6
7th pulse increasing stress individual mutant	PNB7
8th pulse increasing stress individual mutant	PNB8

The nomenclature for pulse constant stress individuals is shown table 3.3.4.

Table 3.3.4 Nomenclature for pulse constant stress mutant individuals.

Individual mutant	Code
1st pulse constant stress individual mutant	PKB1
2nd pulse constant stress individual mutant	PKB2
3rd pulse constant stress individual mutant	PKB3
4th pulse constant stress individual mutant	PKB4
5th pulse constant stress individual mutant	PKB5
6th pulse constant stress individual mutant	PKB6
7th pulse constant stress individual mutant	PKB7
8th pulse constant stress individual mutant	PKB8

3.4 Determination of copper stress resistance

3.4.1 Copper stress resistances of pulse and continuous increasing stress generations

In order to understand the copper resistance characteristics of all the generations, the survival ratio of each generation were calculated by taking the ratio of optical density values at 600 nm under stress condition and under non-stress condition (survival ratio determination)**.**

The survival ratios values of continuous increasing stress generations are given on Figure 3.4.1.

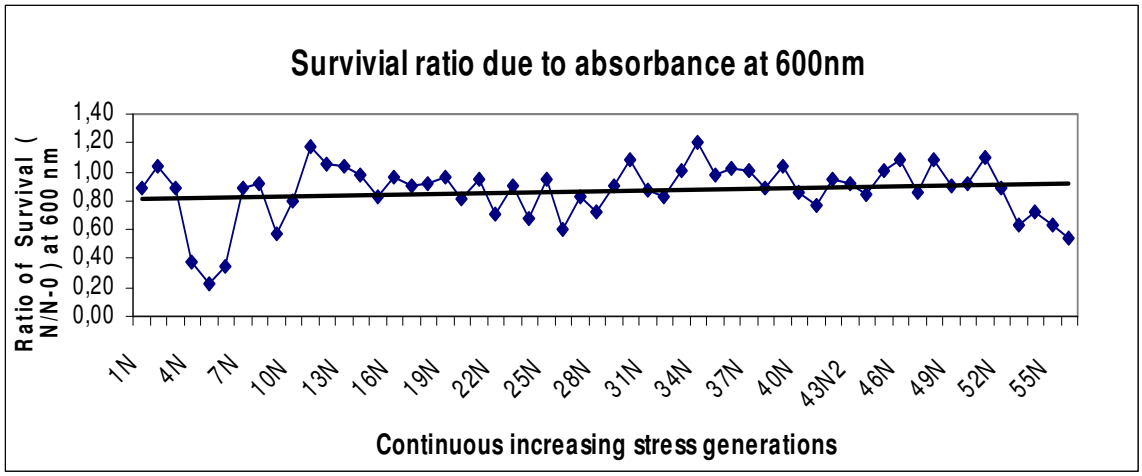

Figure 3.4.1 Survival ratio results of continuous increasing stress generations.

The survival ratios values of pulse increasing stress generations are shown in Figure 3.4.2.

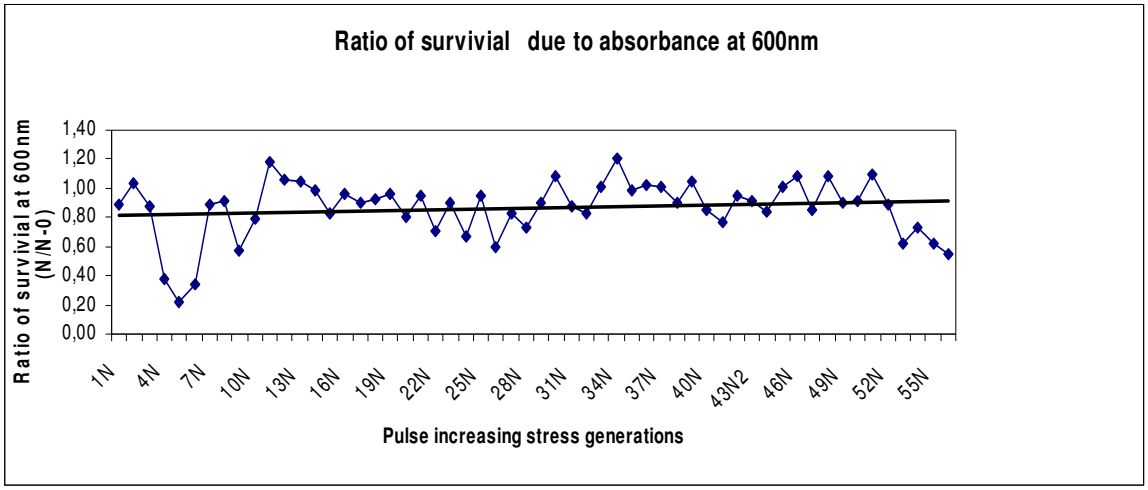

Figure 3.4.2 Survival ratio results of pulse increasing stress generations.

3.5 Characterization of individual mutants

3.5.1 Analysis of copper resistance

3.5.1.1 Copper stress resistances of continuous increasing and constant stress individual mutants

100 (wild type), *101* (EMS mutaginazed)**,** *56 K* (continuous constant stress generation, *56 N (*continuous increasing stress generation) and *8 individuals* (selected from final continuous increasing stress generation: *NB1, NB3, NB4, NB5, NB6, NB7, NB8, NB9)* were analyzed in order to determine their continuous $CuCl_2$ stress resistance characteristics by using 5-tube Most Probable Number (MPN) method. The applied $CuCl_2$ stress levels were 10 and 20 mM $CuCl_2$. The survival ratios of the individual mutants and other samples were determined by dividing stress applied samples' cell number per ml to their corresponding non-stress applied samples' cell number per ml after 96 h of incubation at 30ºC.

The survival ratios of the *100, 101, 56K, 56N, NB1, NB2, NB3, NB4, NB5, NB6, NB7, NB8, NB9* cultures are given on Table 3.5.1.

Table 3.5.1 Percent survivals of *100, 101, 56K, 56N, NB1, NB2, NB3, NB4, NB5, NB6, NB7, NB8, NB9* cultures upon 10 and 20 mM $CuCl_2$ stress.

Generation/ individual Name	% survival at 10 mM $CuCl_2$	% survival at 20 mM $CuCl_2$
100	0	0
101	0	0
56K	0	0
56N	0.70	0.45
NB1	3.37	0
NB2	2.69	0.26
NB3	0.05	0.00
NB4	0.15	0.01
NB5	0.54	0.03
NB6	0.83	8.3
NB7	0.38	0.1
NB8	0.64	0.1
NB9	0.38	0.009

According to these results *NB2, NB6, NB7,* and *NB8* individuals were selected for further analysis together with *100* (wild type), *101* (chemically mutated culture) **,** *56 K (* continuous constant stress generation), *56 N* (continuous increasing stress generation) and *7 individuals* (isolated from final continuous constant stress generation *: KB1 , KB3, KB4 , KB5 , KB7 , KB8, KB9*)**.** They were analyzed for their stress resistances upon 200 and 2000 µM $CuCl_2$ stress by using 5-tube Most Probable Number (MPN) method. The % survival of results *100, 101, 56K, 56N, KB1, KB3, KB4, KB5, KB7, KB8*, *KB9* cultures under continuous 0.2 and 2 mM $CuCl_2$ stress conditions are shown Table 3.5.2.

Table 3.5.2 Percent survival of *100, 101, 56K, 56N, KB1, KB3, KB , KB , KB , KB8, KB9* cultures under 0.2 and 2 mM $CuCl_2$ stress conditions.

Generation / individual name	% survival at 0.2 mM $CuCl_2$	% survivial at 2 mM $CuCl_2$
100	2.96	0.05
101	5.70	0.10
56K	29.60	0.06
56N	5.50	15.11
KB1	68.50	0.09
KB3	5.80	0.05
KB5	8.36	0.02
KB5	15.00	0.02
KB7	262.00	0.68
KB8	21.00	0.05
KB9	225.00	1.00

KB7 and *KB9* individuals were chosen for further analysis, according to the results of table 3.5.2.

3.5.1.2 Copper stress resistances of pulse increasing and constant stress individual mutants

100 (wild type), *101* (chemically mutagenized culture), *P56 K* (pulse constant stress generation), *P56 N* (pulse increasing stress generation) and 8 individuals (isolated from final pulse increasing stress generation: *PNB , PNB, PNB3, PNB , PNB5, PNB6, PNB7 , PNB8, PNB9*) fresh cultures were tested to determine their stress tolerance upon continuous 0.5 and 10 mM $CuCl_2$ stresses by using 5-Tube MPN method. After 96h incubation at 30ºC, % survival of these cultures was determined (Table 3.5.3).

Table 3.5.3 Percent survival of *100, 101, P56K, P56N, PNB1, PNB2, PNB3, PNB5, PNB5, PNB7, PNB8, PNB9* cultures under continuous 5 and 10 mM $CuCl_2$ stress conditions.

Generation/ individual name	% survivial at 5mM $CuCl_2$	% survivial at 10 mM $CuCl_2$
100	0	0
101	0	0
P56K	0	0
P56N	10	0.01
PNB1	0	0
PNB2	0.00	0
PNB3	0.1	0.04
PNB4	0.15	0.05
PNB5	1	0.64
PNB6	4.44	0.64
PNB7	2.25	0.22
PNB8	1	0.02

Table 3.5.4 Percent survival of *100, 101, P56K, P56N, PKNB1, PKNB2, PKNB3, PKNB4, PKNB5 , PKNB6, PNKB7, PKN8,* cultures upon continuous 5 and 10 mM $CuCl_2$ stress conditions.

Generation / individual name	% survival 0,2 mM $CuCl_2$
100	9.41
101	31.76
P56K	10.00
P56N	54.12
PKB1	31.43
PKB2	337.50
PKB3	49.09
PKB4	3.80
PKB5	6.48
PKB6	29.63
PKB7	31.76
PKB8	45.71

3.5.2 Analysis of Cross-resistance

3.5.2.1 Other Metal Stresses

3.5.2.1.1 Cobalt stress resistance of continuous increasing and constant stress individual mutants

56K (continuous stress constant generation), *56N* (continuous stress increasing generation), *NB2, NB6, NB7, NB8* (continuous increasing stress individuals mutants) fresh cultures were inoculated to YMM, and 2.5 and 5 mM $CoCl_2$- containing YMM, in 50 ml test tubes at 10 ml final culture volume. These cultures were incubated at 30ºC and 150 rpm continuously. OD_{600} values were taken at 72^{nd} h and % survival values were determined (Table 3.5.5).

Table 3.5.5 Perccent survivals of *100, 101, 56K, 56N, NB2, NB6, NB7, NB8* cultures under continuous 2.5 mM and 5 mM $CoCl_2$ stress conditions.

Generation / individual name	% survival at 2.5 mM $CoCl_2$	% survival at 5 mM $CoCl_2$
100	56	39
101	80	65
56K	37	36
56N	78	56
NB2	159	35
NB6	50	18
NB7	56	23
NB8	75	50

3.5.2.1.2 Cobalt stress resistance of pulse increasing and constant stress individual mutants

PNB3,PNB4, PNB5 (pulse increasing stress mutant individuals) and PKB1, PKB2 (pulse constant stress mutant individuals) fresh cultures were inoculated to determine their stress tolerance upon continuous 0 , 2.5 and 5 mM $CoCl_2$ stresses by using 5-Tube MPN method. After 96h incubation at 30ºC, % survival of these cultures was determined (Table 3.5.6).

Table 3.5.6 Percent survivals of *100, PNB3, PNB4, PNB5, PKB1* and *PKB2* cultures under continuous 2.5 mM and 5 mM $CoCl_2$ stress conditions

Generation / individual name	%Survival at 2.5 mM $CoCl_2$	%Survival at 5 mM $CoCl_2$
100	6.85	0.06
PNB3	34.78	0.52
PNB4	10.00	0.26
PNB5	10.00	4.44
PKB1	0.03	0.00
PKB2	0.33	0.01

3.5.2.1.3 Chromium stress resistance of continuous increasing and constant stress individual mutants

56K (continuous stress constant generation), *56N* (continuous stress increasing generation), *NB2, NB6, NB7, NB8* (continuous increasing stress mutant individuals) cultures were exposed to continuous 2.5 mM and 5 mM $CrCl_3$ stress in 50 ml test tubes with 10 ml culture volume. Optical densities of the cultures were measured at 600 nm after 72 h incubation at 30ºC. Survival ratios were determined and % survivals shown on Table 3.5.7 were calculated.

Table 3.5.7 *100, 101, 56K, 56N, NB2, NB6, NB7, NB8* cultures' percent survival upon continuous 2.5 and 5 mM $CrCl_3$ stress conditions.

Generation / individual name	% survival at 2.5 mM $CrCl_3$	% survival at 5 mM $CrCl_3$
100	77	6
101	129	15
56K	65	2
56N	97	68
NB2	11	6
NB6	5	3
NB7	5	2
NB8	53	3

3.5.2.1.4 Chromium stress resistance of pulse increasing and constant stress individual mutants

PNB3, PNB4, PNB5 (pulse increasing stress individuals mutants) and *PKB1, PKB2* (pulse constant stress individuals mutants) cultures were exposed to continuous 0 , 1 and 2.5 mM $CrCl_3$ stresses by using 5-Tube MPN method. After 96h incubation at 30ºC, % survival of these cultures was determined (Table 3.5.8).

Table 3.5.8 Percent survivals of *100, PNB3, PNB4, PNB5, PKB1* and *PKB2* cultures under continuous 1 mM and 2.5 mM $CrCl_3$ stress conditions.

Generation / individual name	% survival of 1 mM $CrCl_3$	% survival of 2.5 mM $CrCl_3$
100	0.06	1.54
PNB3	0.07	0.05
PNB4	0.10	0.00
PNB5	0.10	0.00
PKB1	3.80	10.00
PKB2	10.62	3.37

3.5.2.2 Oxidative Stress

Oxidative stress was applied to *100* (wild type), *101*(chemically mutagenized), *56K* (continuous stress constant generation), 56N (continuous stress increasing generation), *NB2, NB6, NB7, NB8* (continuous increasing stress individuals) cultures by using pulse stress application strategy. The stress levels were chosen as 1, 2, 3, 5 M H_2O_2 and the cultures were exposed to this stress for 1h. OD_{600} nm values at 72 h of incubation were used to determine % survival values (Table 3.5.9)

Table 3.5.9 Percent survival of *100, 101, 56K, 56N, NB2, NB6, NB7, NB8* upon 1, 2, 3, 5 M H_2O_2 pulse stress application.

Generation / individual name	% Survival at 1 mM H_2O_2	% Survival at 2 mM H_2O_2
100	1.85	0.00
101	16.67	0.69
56K	0.00	0.68
56N	0.00	0.96
NB2	1.83	1.22
NB6	0.98	0.76
NB7	0.81	0.81
NB8	0.00	0.00

Neither wild type nor mutant individuals survived harsher stress conditions such as 3 and 5 M H_2O_2.

Oxidative stress was applied to *100* (wild type), *PNB3, PNB4, PNB5,* (pulse increasing stress individuals) cultures and *PKB1, PKB2* (pulse constant stress individuals) by using pulse stress application strategy. The stress levels were chosen as 1, 2, 3, 5 M H_2O_2 and the cultures were exposed to this stress for 1h. OD_{600} values at 72 h of incubation were used to determine % survival values (Table 3.5.10)

Table 3.5.10 The OD_{600} results of *100, PNB3, PNB4, PNB5, PKB1, PKB2* upon 0.5 , 1,and 2 M H_2O_2 pulse stress application

Generation / individual name	0 M H_2O_2	0.5 M H_2O_2	1 M H_2O_2	2 M H_2O_2
100	22.5	0	0	0
PNB3	18.18	0	0	0
PNB4	21.36	0	0	0
PNB5	20.34	12	0.48	0
PKB1	20.04	0	0	0
PKB2	32.4	0	0	0

3.5.2.3 Heat Stress

Heat stress was applied to wild type and some of the mutant individuals by using pulse stress application strategy. One ml fresh cultures of *100* (wild type), *101* (chemically mutagenized), *56K* (continuous stress constant generation), *56N* (continuous stress increasing generation), *NB2, NB6, NB7, NB8* (continuous increasing stress individuals) and *KB7, KB9* (continuous constant stress individuals) were incubated at 30, 40, 50 and 60ºC in 1.5 ml microfuge tubes for 10 min. Following stress application, the cultures were inoculated into fresh YMM to 10 ml final volume in 50 ml test tubes. OD_{600} values at 72^{nd} h of incubation were determined, survival ratios were then calculated with respect to OD_{600} values of the corresponding cultures at 30ºC.

Percent survivals of *100, 101, 56K, 56N, NB2, NB6, NB7, NB8, KB7, KB9* upon pulse heat stress application are shown as Table 3.5.11.

Table 3.5.11 % survival results of *100,101, 56K, 56N, NB2, NB6, NB7, NB8, KB7, KB9* under pulse heat stress conditions.

Generation / individual name	% survival at 40˚C	% survival at 50˚C	% survival at 60˚C
100	110	69	98
101	112	95	106
56K	98	73	92
56N	118	107	110
NB2	115	86	115
NB6	96	88	96
NB7	67	98	99
NB8	71	105	88
KB7	105	107	100
KB9	99	82	95

All individuals showed resistance to heat stresses that had been applied.

Heat stress was applied to wild type and some of the mutant individuals by using pulse stress application strategy. One ml fresh cultures of *100* (wild type), *PNB3, PNB4, PNB5,* (pulse increasing stress individuals) cultures and *PKB1, PKB2* (pulse constant stress individuals) were incubated at 30, 40, 50 and 60ºC in 1.5 ml microfuge tubes for 10 min. Following stress application, the cultures were inoculated into fresh YMM to 10

ml final volume in 50 ml test tubes. OD_{600} values at 72nd h of incubation were determined, survival ratios were then calculated with respect to OD_{600} values of the corresponding cultures at 30ºC.

Table 3.5.12 Percent survivals of *100, PNB3, PNB4, PNB5, PKB1* and *PKB2* upon pulse heat stress application.

Generation / individual name	% Percent of 40 C	% Percent of 50 C	% Percent of 60 C
100	102	105	116
PNB3	165	153	178
PNB4	164	160	108
PNB5	100	70	98
PKB1	88	88	87
PKB2	96	106	77

3.5.2.4 Osmotic Stress

Osmotic stress was applied continuously towards *100* (wild type), *101 (*chemically mutagenized), *56K* (continuous stress constant generation), *56N* (continuous stress increasing generation), *NB2, NB6, NB7, NB8* (continuous increasing stress individuals), and *KB7, KB9* (continuous constant stress individuals) cultures. Fresh cultures were inoculated into 50 ml falcon tubes containing 10 ml of YMM with varying NaCl concentrations (0 % (w/v), 5 % (w/v), 10 % (w/v), and 15 % (w/v)). OD_{600} values at 72 h of incubation were measured and then % survivals were calculated.

Percent survivals of *100,101, 56K, 56N, NB2, NB6, NB7, NB8, KB7, KB9* upon continuous osmotic stress is shown on Table 3.5.13.

Table 3.5.13 Percent survivals of *NB6, NB7, NB8, KB7, KB9* upon continuous NaCl stress application.

Generation / individual name	% survival at 5 % (w/v) NaCl	% survival at 10 % (w/v) NaCl	% survival at 15 % (w/v) NaCl
100	*61*	52	16
101	*81*	50	21
56K	*66*	51	18
56N	*73*	55	21
NB2	*50*	31	2
NB6	*59*	30	2
NB7	*78*	50	3
NB8	*66*	26	1
KB7	*73*	55	19
KB9	*62*	38	13

Osmotic stress was applied continuously towards *100* (wild type), *PNB3, PNB4, PNB5,* (pulse increasing stress individuals) cultures and *PKB1, PKB2* (pulse constant stress individuals) cultures. Fresh cultures were inoculated inoculated to determine their stress tolerance upon continuous 0% ,5% , %10 and 15% NaCl stresses by using 5-Tube MPN method. After 72 h incubation at 30ºC, % survival of these cultures was determined Percent survivals of *100, PNB3, PNB4, PNB5, PKB1, PKB2* upon continuous osmotic stress is shown on Table 3.5.9.

Table 3.5.14 Percent survivals of *100, PNB3, PNB4, PNB5, PKB1, PKB2* upon continuous NaCl stress application

Generation / individual name	% survival of 5 % NaCl	%survival of 10 % NaCl	% survival of 15 %NaCl
100	16,364	10,606	0,000
PNB3	27,879	7,273	0,000
PNB4	7,083	66,667	0,000
PNB5	72,727	39,394	0,000
PKB1	70,769	26,923	0,000
PKB2	110,204	110,204	0,000

3.5.3 Determination of copper, cobalt and chromium metal content associated with cells using flame atomic absorption spectrometer (FAAS)

Continuous stress increasing individuals coded as *NB2, NB6, NB7, and NB8*, continuous stress constant individuals coded as *KB7 and KB9*, pulse stress increasing individuals coded as *PNB 3, PNB 4 and PNB 5*, pulse stress constant individuals coded as PKB 1 and 2 fresh culture were inoculated into 50 ml culture including 10 ml of YMM with varying metal concentrations. Each sample was incubated for 72 h at 30ºC and 150 rpm. Dried cell pellets of each culture were digested in 10 M Nitric acid (HNO_3) at 90ºC for 2h. FAAS measurements were then performed to determine the metal content hold by the cell as "*ppm values (mg/l)"*. Percentages of metal contents (% metal) associated with the cells were determined by dividing the calculated metal FAAS result to the corresponding initial metal contents exposed of the growth medium. Per cent copper weight / cell dry weight values (% metal (w/w) were determined by calculating the ratio between metal content according to culture volume (mg/ml) to cell dry weight per ml (mg/ml).

3.5.3.1 Determination of the cellular copper contents using FAAS

Determination of the copper contents with FAAS was performed at 324.8 nm wavelength with a 1.2 nm slit width. For all cultures tested, per cent copper associated with cells (% Cu) and per cent copper weight / cell dry weight values (% Cu (w/w)) results are shown in Table 3.5.15

Table 3.5.15 Wild type and mutant individuals' % Cu and % Cu (w/w) contents as determined by FAAS.

	Sample name	% Cu	% Cu (w/w)	% Cu (fold of wt)	% Cu (w/w) (fold of wt)
0 mM $CuCl_2$	100	0.00	0.00	-	0.00
	NB2	0.00	0.00	0.00	0.00
	NB6	0.00	0.00	0.00	0.00
	NB7	0.00	0.00	0.00	0.00
	NB8	0.00	0.00	0.00	0.00
	KB7	0.00	0.00	0.00	0.00
	KB9	0.00	0.00	0.00	0.00
	PNB3	0.00	0.00	0.00	0.00
	PNB4	0.00	0.00	0.00	0.00
	PNB5	0.00	0.00	0.00	0.00
	PKB1	0.00	0.00	0.00	0.00
	PKB2	0.00	0.00	0.00	0.00
0.2 mM $CuCl_2$	100	12.83	0.04	-	0
	NB2	9.84	0.03	0.77	0.73
	NB6	11.89	0.05	0.93	1.20
	NB7	12.52	0.03	0.98	0.87
	NB8	18.27	0.10	1.42	2.56
	KB7	13.94	0.05	1.09	1.29
	KB9	11.65	0.05	0.91	1.35
	PNB3	13.31	0.07	1.04	1.76
	PNB4	16.69	0.06	1.30	1.53
	PNB5	16.46	0.06	1.28	1.50
	PKB1	11.10	0.04	0.87	1.12
	PKB2	7.95	0.02	0.62	0.54
2 mM $CuCl_2$	100	2.69	5.68	0	0
	NB2	8.58	0.24	3.20	0.04
	NB6	9.98	0.26	3.72	0.05
	NB7	9.18	0.22	3.42	0.04
	NB8	13.10	0.48	4.88	0.08
	KB7	0.69	0.79	0.26	0.14
	KB9	1.59	1.44	0.59	0.25
	PNB3	12.31	0.88	4.59	0.16
	PNB4	13.61	0.44	5.07	0.08
	PNB5	10.30	0.37	3.84	0.07
	PKB1	4.49	0.35	1.67	0.06
	PKB2	7.91	0.45	2.94	0.08

	Sample name	% Cu	% Cu (w/w)	% Cu (fold of wt)	% Cu (w/w) (fold of wt)
5 mM $CuCl_2$	100	3.70	13.07	-	-
	NB2	1.91	0.18	0.52	0.01
	NB6	2.15	0.22	0.58	0.02
	NB7	2.33	0.19	0.63	0.01
	NB8	1.92	0.19	0.52	0.01
	KB7	2.99	6.33	0.81	0.48
	KB9	1.03	2.73	0.28	0.21
	PNB3	5.78	0.72	1.56	0.06
	PNB4	3.58	1.07	0.97	0.08
	PNB5	5.48	0.56	1.48	0.04
	PKB1	2.83	0.55	0.76	0.04
	PKB2	1.15	2.14	0.31	0.16
10 mM $CuCl_2$	100	1.74	27.60	-	-
	NB2	1.13	0.23	0.65	0.01
	NB6	0.99	0.15	0.57	0.01
	NB7	0.93	0.29	0.53	0.01
	NB8	0.85	0.25	0.49	0.01
	KB7	1.41	6.89	0.81	0.25
	KB9	1.29	11.69	0.74	0.42
	PNB3	1.76	0.46	1.01	0.02
	PNB4	1.28	0.63	0.74	0.02
	PNB5	1.37	6.71	0.79	0.24
	PKB1	1.60	9.24	0.92	0.33
	PKB2	1.31	0.00	0.76	0.00

NB2 seems to have increased its copper holding at higher levels of copper stress. NB2 seems to behave differently in holding copper among other continuous mutant individuals.

Table 3.5.16 *NB2, NB6, NB7, NB8, PNB3, PNB4*, and *PNB5* CDW/ml and AAS results.

Name of the sample	Calc.ed (mg/ml) At 2 mM $CuCl_2$	CDW/ ml at 2 mM $CuCl_2$	% Cu At 2 mM $CuCl_2$	Calc.ed (mg/ml) At 5 mM $CuCl_2$	CDW/ ml at 5 mM $CuCl_2$	% Cu At 5 mM $CuCl_2$	Calc.ed (mg/ml) At 10 mM $CuCl_2$	CDW/ ml at 10 mM $CuCl_2$	% Cu At 10 mM $CuCl_2$
NB2	10.9	4.58	8.58	6.08	3.38	1.91	7.20	3.14	1.13
NB6	12.68	4.85	9.98	6.84	3.11	2.15	6.27	4.09	0.98
NB7	11.66	5.30	9.18	7.40	3.99	2.33	5.89	2.04	0.92
NB8	16.64	3.48	13.1	6.10	3.25	1.92	5.40	2.17	0.85
PNB3	15.64	1.77	12.3	18.36	2.55	5.78	11.20	2.41	1.76
PNB4	17.28	3.90	13.6	11.38	1.06	3.8	8.14	1.29	1.28
PNB5	13.08	3.54	10.29	17.40	3.13	5.48	8.72	0.13	1.37

The data does not provide information in the exact location of copper in the cell, i.e. it is not known if copper is held or on the cell.

Constant stress mutant individuals do not seem to be more resistant than wild type upon 2 mM $CuCl_2$ stress application.

Table 3.5.17 CDW/ml, % Cu, % Cu (w/w) results for *NB7, NB8, PNB3, PNB4, PNB5*, and *PKB1* mutant individuals.

Name of the sample	At 2mM $CuCl_2$ CDW mg/ml	% Cu	% Cu (w/w)	At 5 mM $CuCl_2$ CDW mg/ml	% Cu	% Cu (w/w)	At 10 mM $CuCl_2$ CDW mg/m	% Cu	% Cu (w/w)
NB2	4.58	8.58	0.23	3.38	1.91	0.17	3.14	1.30	0.22
NB6	4.85	9.98	0.26	3.11	2.15	0.20	4.09	0.98	0.15
NB7	5.3	9.18	0.22	3.99	2.33	0.19	2.04	0.93	0.29
NB8	3.48	13.1	0.22	3.25	1.92	0.19	2.17	0.85	0.25
PNB3	1.77	12.31	0.88	2.55	5.78	0.72	2.41	1.76	0.46
PNB4	3.9	13.6	0.44	1.06	3.58	1.07	1.29	1.28	0.63
PNB5	3.54	10.3	0.37	3.13	5.48	0.56	-	-	-
PKB1	1.61	4.49	0.35	1.62	2.83	0.55	-	-	-

It seems like there may be a difference in copper resistances of continuous increasing mutant individuals and pulse increasing and constant mutant individuals upon 5 mM $CuCl_2$ stress application. The continuous increasing mutant individuals had generally lower resistances than pulse increasing and pulse constant mutant individual.

Constant stress mutant individuals were usually less resistant than the wild type upon 2 mM $CuCl_2$ stress application.

3.5.3.2 Determination of the cellular cobalt contents using FAAS

FAAS was used at 252.5 nm wavelength with a 0.2 nm slit width in order to determine wild type and individual mutants % Co and % Co (w/w). The corresponding values for 2.5 mM $CoCl_2$ stress application are shown on Table 3.5.18.

Table 3.5.18 Percent cobalt hold various individual mutants and the wild type, as determined by FAAS.

	Sample name	% Co	% Co (w/w)	% Co (fold of wt)	% Co (w/w) (fold of wt)
0 mM $CoCl_2$	100	0.00	0.02	-	-
	NB2	0.00	0.01	0.00	0.00
	NB6	0.00	0.02	0.00	0.00
	NB7	0.00	0.03	0.00	0.00
	NB8	0.00	0.02	0.00	0.00
	KB7	0.00	0.02	0.00	0.00
	KB9	0.00	0.03	0.00	0.00
	PNB3	0.00	0.01	0.00	0.00
	PNB4	0.00	0.02	0.00	0.00
	PNB5	0.00	0.02	0.00	0.00
	PKB1	0.00	0.03	0.00	0.00
	PKB2	0.00	0.02	0.00	0.00
2.5 mM $CoCl_2$	100	1.61	0.12	-	-
	NB2	2.49	0.16	1.54	1.34
	NB6	1.95	0.14	1.21	1.17
	NB7	2.25	0.15	1.39	1.31
	NB8	1.63	0.18	1.01	1.51
	KB7	1.73	0.16	1.07	1.32
	KB9	1.74	0.24	1.08	2.06
	PNB3	1.68	0.28	1.04	2.37
	PNB4	1.50	0.22	0.93	1.87
	PNB5	1.93	0.20	1.20	1.69
	PKB1	1.72	0.32	1.07	2.68
	PKB2	1.92	0.32	1.19	2.67
5 mM $CoCl_2$	100	1.03	0.35	-	-
	NB2	0.87	0.44	0.84	1.25
	NB6	0.75	0.47	0.73	1.36
	NB7	0.92	0.33	0.89	0.94
	NB8	0.74	0.40	0.72	1.15
	KB7	0.99	0.61	0.95	1.74
	KB9	1.14	0.64	1.11	1.83
	PNB3	0.91	0.75	0.88	2.15
	PNB4	1.09	0.73	1.05	2.11
	PNB5	0.99	0.45	0.95	1.28
	PKB1	0.73	0.50	0.71	1.42
	PKB2	0.78	0.58	0.76	1.67

NB2, NB6 and NB7 CDW/ml did not decrease as significantly as the other individual mutants and also NB7 had the same CDW/ml for control and 2.5 mM $CoCl_2$ stress applied sample.

Table 3.5.19 AAS results for wild type*, NB2, NB6*, and *NB7.*

Culture Name	**At 0 mM $CoCl_2$ CDW (mg/ml)**	**At 2.5 mM $CoCl_2$ CDW (mg/ml)**	**At 2.5 mM $CoCl_2$ % Co**	**% Co (w/w)**
100	3.35	1.98	1.61	0.12
NB2	3.83	2.28	2.49	0.16
NB6	2.40	2.04	1.95	0.14
NB7	2.11	2.11	2.25	0.15

% Co values of NB2 and NB7 were higher than the wild type although their CDW/ml were similar.

3.5.3.3 Determination of the cellular chromium content using FAAS

By using FAAS at 357.9 nm wavelength with, and a 0.2 nm slit width, % Cr and % Cr (w/w) were determined upon continuous 1 and 2.5 mM $CrCl_3$ stress application. The results are shown on Table 3.5.20.

Table 3.5.20 Percent chromium hold by various individual mutants and the wild type, as determined by FAAS

	Sample name	% Cr	%Cr (w/w)	% Cr (fold of wt)	% Cr (w/w) (fold of wt)
0mM $CrCl_3$	100	0	0.00	-	-
	NB2	0.00	0.00	0.00	0.00
	NB6	0.00	0.02	0.00	0.00
	NB7	0.00	0.01	0.00	0.00
	NB8	0.00	0.02	0.00	0.00
	KB7	0.00	0.02	0.00	0.00
	KB9	0.00	0.01	0.00	0.00
	PNB3	0.00	0.05	0.00	0.00
	PNB4	0.00	0.40	0.00	0.00
	PNB5	0.00	0.01	0.00	0.00
	PKB1	0.00	0.00	0.00	0.00
	PKB2	0.00	0.00	0.00	0.00
1 mM $CrCl_3$	100	4.17	0.06	-	-
	NB2	5.13	0.10	1.23	1.64
	NB6	6.69	0.10	1.60	1.70
	NB7	6.62	0.12	1.59	2.09
	NB8	17.73	0.29	4.25	4.95
	KB7	14.81	0.23	3.55	3.95
	KB9	7.98	0.11	1.91	1.91
	PNB3	10.48	0.15	2.51	2.55
	PNB4	15.58	0.18	3.73	3.08
	PNB5	25.92	0.33	6.21	5.68
	PKB1	19.31	0.23	4.63	4.03
	PKB2	18.19	0.23	4.36	3.95

Pulse increasing and constant mutant individuals could grow well and they could also hold high amount of chromium. Moreover, their % Cr results were much higher than those of the continuous stress mutant individuals (except NB8).

4. DISCUSSION AND CONCLUSION

The aim of this study is to obtain copper resistance strains that can survive at higher copper concentrations than wild type. Evolutionary engineering, which is an inverse metabolic engineering approach was adopted throughout the study.

First of all, wild type cells were exposed to ethyl methane sulphonate (EMS) mutagenesis for obtaining a culture with an increased genetic diversity. In order to determine the first stress application level, copper stress screening was performed. After determining the initial stress level, copper stress was applied on chemically mutagenized culture (named as *101*) by using two different stress selection strategies, which were "continuous" and "pulse" stresses. Both continuous and pulse stress selection strategies were applied in two different sub-strategies, which were increasing stress and constant stress applications.

In the continuous stress selection strategy, wild-type and the chemically mutagenized culture were resistant under the stress conditions lower than 1 mM of $CuCl_2$, but could not tolerate higher concentrations. In the pulse stress selection strategy, wild-type and the chemically-mutagenized culture were resistant under the stress conditions lower than 5 mM of $CuCl_2$. According to these results, in both the constant and the increasing levels of continuous and pulse stress applications, 0.2 mM $CuCl_2$ was determined to be the initial copper stress level. At the end of applying pulse and continuous of copper stresses, 56 generations were obtained. 56^{th} increasing pulse and increasing continuous stress generations, which were the survivors of 10 mM and 20 mM $CuCl_2$ stress application, respectively, and 56^{th} constant pulse and constant continuous stress generations, which were obtained by repeating 0.2 mM $CuCl_2$ for 56 times were determined to be the final generations for each strategy. The 56^{th} generations were determined to be the final generation for each stress selection strategy, due to decreased survival ratio values.

The final generations obtained by continuous constant, continuous increasing, pulse constant and pulse increasing stress selection strategies were used to select mutant individuals randomly. The survival ratio values of the randomly selected individual mutants were analyzed for characterization by most probable number (MPN) method statistically.

In addition, the cross-resistance characteristics of the copper-resistant individuals were investigated under various stress conditions. The metal contents associated with the cells were determined by flame atomic absorbtion spectroscopy (FAAS) analysis.

Preliminary expriments showed that wild type cells were not capable of surviving under copper stress conditions higher than 1 mM $CuCl_2$. However, the continuous increasing stress generations were shown to survive under 20 mM $CuCl_2$ concentration. Thus, it is obvious that evolutionary engineering is an effective technique to obtain copper resistant yeast cells.

The percent survival values of continuous increasing and pulse increasing stress mutant individuals were analyzed under low copper concentrations (Table 3.5.1 and Table 3.5.3). According to the results, all of the selected continuous increasing stress mutant individuals had higher percent survival values than wild type. Some of the mutant individuals had significantly high resistance characteristics. Moreover, they were resistant under high copper concentrations (10 mM $CuCl_2$). However, pulse increasing stress mutant individuals could not tolerate high copper concentrations. They were more resistant than wild type under mild copper stress conditions were (5 mM $CuCl_2$).

All of the continuous constant stress mutant individuals found to have significantly high percent survival values than wild type under the stress condition (0.2 mM $CuCl_2$) applied during obtaining the final generation that these individulas were selected from (Table 3.5.2 and Table 3.5.4). In addition, some mutant individuals had very high percent survival values than other constant mutant individuals.

Four continuous increasing stress mutant individuals were selected and coded as *NB2, NB6, NB7, NB8* and two continuous constant stress mutant individuals were selected and coded as *KB7* and *KB9*. The individuals were exposed to other stress conditions such as other metal streses (cobalt and chromium), osmotic stress, oxidative stress and heat stress and survival ratio values were analyzed to investigate cross- resistance characteristics.

It was shown that, continuous increasing stress mutant individuals were able to survive under 5 mM $CoCl_2$ and 2.5 mM $CrCl_3$. However, percent survival values of the mutant individuals at the corresponding stress conditions were not more resistant than wild type. They seem not to have cross-resistances under cobalt and chromium stress conditions.

The mutant individuals found to have no cross-resistance under oxidative stress conditions. However, *NB2,* which is a continuous increasing mutant individual, had significantly high resistance under 2 mM H_2O_2 stress condition (Table 3.5.7).

According to cross-resistance characteristics results of continuous increasing and continuous constant mutant individuals under heat stress conditions, the percent survival values are similar to wild type. There seems to be no significantly resistant mutant individuals.

Table 3.5.9 showing the percent survival results of continuous increasing and continuous constant mutant individuals under osmotic stress conditions, indicates that continuous increasing and continuous constant mutant individuals were not able to resist under high osmotic stress levels (10 % and 15 % NaCl). However, they had slightly higher survival under low osmotic stress level (5 % NaCl). KB7, which is an increasing constant individual mutant, had a slightly higher resistance than other individuals.

Flame atomic absorption spectroscopy analysis was performed to characterize the selected mutant individuals for metal holding proporties. As shown on Table 3.5.10, pulse increasing stress mutant individuals seem to hold the copper content associated with the cells slightly more than continuous increasing stress mutant individuals.

As shown on Table 3.5.10, pulse constant stress mutant individuals seem to hold the copper content associated with the cells slightly more than continuous constant stress mutant individuals under 2 mM copper stress condition

It is obvious that under 2 mM $CuCl_2$ stress condition, continuous increasing stress, pulse increasing stress and pulse constant stress mutant individuals hold the copper content associated with the cell. However, continuous constant mutant individuals seem to take the metal content out of the cells.

As shown in Table 3.5.13, continuous and pulse stress individuals decreased the cobalt ion hold associated with the cell, when cobalt concentrations increased from 2 to 5 mM. In addition, as given in Table 3.5.14, percent cobalt values of *NB2* and *NB7* were higher than the wild type, although their CDW/ml values were similar. Thus, it can be said that these individuals have more cobalt holding capacity than wild type.

According to the results showing the chromim holding characteristics of the copper resistant individuals in Table 3.5.15, pulse increasing and pulse constant mutant individuals could resist chromium stesses and they could also hold high amount of chromium. Moreover, results showing the percent chromium hold by pulse stress individual mutants seem to have much higher than those of the continuous stress mutant individuals (except *NB8* and *KB7*).

In conclusion, our results demonstrate that evolutionary engineering, which is an inverse metabolic engineering stratagy, is a powerful method for obtaining resistant strains under

stress conditions. Similarly, in our study copper resistant individual mutants were obtained by this method. When the four different approaches are compared in terms of copper resistance, it can be said that continuous increasing stress application is succesful in obtaining resistance under high copper stress levels. On the other hand, pulse stress application strategy is effective for generating resistant strains under low stress conditions. In order to determine the specific copper resisitance mechanisms of the selected individual mutants, furher analysis are required.

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