ISTANBUL TECHNICAL UNIVERSITY ★ INSTITUTE OF SCIENCE AND TECHNOLOGY

EVOLUTIONARY ENGINEERING OF COBALT-RESISTANT SACCHAROMYCES CEREVISIAE FOR BIOMIMETICS

M.Sc. Thesis by

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Department : **Advanced Technologies**

Programme: **Molecular Biology – Genetics and Biotechnology**

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ABBREVIATIONS

ATP	: Adenosinetriphosphate
CDW	: Cellular Dry Weight
Cys	: Cysteine
EMS	: Ethyl Methane Sulphonate
FAAS	: Flame Atomic Absorption Spectroscopy
Glu	: Glutamic Acid
Gly	: Glycine
GRAS	: Generally Regarded as Safe
GSH	: Reduced Gluthathione
GS-SG	: Oxidized Gluthathione
h	: Hour
HPLC	: High Performance Liquid Chromatography
MIC	: Minimal Inhibitory Concentration
Min	: Minute
MPN	: Most Probable Number
MT	: Metallothionein
ROS	: Reactive Oxigen Species
YMM	: Yeast Minimal Medium
YPD	: Yeast Comlex Medium
SPB	: Spindle Pole Body

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EVOLUTIONARY ENGINEERING OF COBALT-RESISTANT SACCHAROMYCES CEREVISIAE FOR BIOMIMETICS

SUMMARY

In this study, the aim was to obtain mutant cobalt binding and/or cobalt resistant *Saccharomyces cerevisiae* cells by using evolutionary engineering methodology which is a strategy of inverse metabolic engineering. For this purpose, wild type cells were applied EMS mutagenesis in order to obtain a genetically variant population. This culture was grown until its early exponential growth phase then cobalt stress was applied for 90 min. Since cobalt stress was applied for restricted time and to early growth phase cells, this stress application was named as "pulse stress application". The cells that survived were incubated in new medium and after appropriate incubation, they were named as first generation. This new generation was used in order to obtain next generation. There were 2 different cobalt stress application strategy: "constant" and "increasing". The same $CoCl_2$ concentration was applied during obtaining constant stress generations; whereas, $CoCl_2$ content was increased gradually while obtaining increasing stress generations. 36 constant and increasing stress generations were obtained; then, individuals from these generations were randomly selected. Wild type, final constant and increasing stress generations were tested to determine their cobalt resistances by using 5 tube MPN method. There were individuals which were much more resistant to cobalt stress than wild type. The most cobalt resistant individuals, final populations and wild type were tested for various environmental stress conditions. Copper and chromium resistance were investigated and some of the individual cross-resistance to copper or chromium were demonstrated. Additionally, all the individuals and final generations showed cross tolerance to NaCl. In order to determine these individuals or final populations cobalt resistances depend on whether genetic or physiological adaptation, genetic stability test was performed and it was shown that all these cultures were gained this cobalt resistance as a result o genetic adaptation. Flame atomic absorption spectroscopy (FAAS) measurements were performed in order to determine cells associated cobalt content of wild type, final populations and individuals. Wild type associated the highest value of cobalt into its cells; whereas, the most metal resistant individual cobalt content associated with its cells had the lowest value. According to these results, it could be claimed for cobalt resistant individuals that they have a much more effective system to exclude cobalt ions that enter the cells or they do not allow the influx of cobalt as wild type do.

As a result, by applying evolutionary engineering methodology, cobalt resistant individuals were obtained. Moreover, some individuals were not only cobalt tolerant but also they were resistant to copper and chromium as well. And, all of the individuals and final generations were cross-resistant to NaCl stress. According to FAAS results the cobalt resistance mechanism were based on taking up cobalt lower than wild type cells or excluding much more cobalt out of cells than wild type.

However, in order to understand all these mechanisms previous transcriptomic and proteomic analysis are needed.

EVRİM MÜHENDİSLİĞİ YÖNTEMİ İLE KOBALTA DAYANIKLI SACCHAROMYCES CEREVİSİAE'NIN BİYOMİMETİK AMAÇLI ELDESİ

ÖZET

Bu çalışmanın amacı *Saccharomyces cerevisiae* yaban tip hücrelerinden kobalt bağlayabilen ve/veya kobalta dirençli hücrelerin, tersine metabolizma mühendisliğinin bir stratejisi olan evrim mühendisliği yöntemi ile elde edilmesidir. Bu amaçla ilk olarak, *S. cerevisiae* yaban tip hücreleri etil metan sulfonat (EMS) ile kimyasal mutasyona tabii tutularak genetik çeşitlilik sağlanmıştır. Bu hücreler, büyüme evrelerinin erken logaritmik fazında 90 dakika süreyle kobalt stresine maruz bırakılmışlardır. Kobalt stres uygulaması belli büyüme fazındaki hücrelere ve kısıtlı bir süre içinde uygulandığı için bu tip stres uygulaması *"pulse"* olarak nitelendirilmiştir. Kobalt stresi sabit ve artan olmak üzere iki farklı şekilde uygulanmıştır. Sabit kobalt stresi, hücrelere sürekli düşük konsantrasyonda kobalt stresi uygulanarak gerçekleştirilmiştir. Artan kobalt stresi uygulamasında ise, hücrelere uygulanan kobalt stres düzeyi, ortamdaki kobalt konsantrasyonunun arttırılması şeklinde sürekli arttırılmıştır. EMS uygulaması sonrası elde edilen başlangıç hücre popülasyonu, erken logaritmik evresine kadar büyütüldükten sonra, "*pulse*" kobalt stresine maruz bırakılmış ve 90 dakikalık stres sonrası taze besi yerine alınıp inkübe edilmişlerdir. Bu inkübasyon sonucunda ilk nesil elde edilmiştir. Kobalt stres uygulaması bu şekilde her yeni nesle uygulanmış, bu strese dayanabilen hücrelerin seçilimi ile devam ettirilmiş ve 36 sabit ve artan kobalt stres nesli elde dilmiştir. Son nesillerden mutant bireyler elde edilmiş ve bu bireyler, son nesiller ve yaban tip ile, istatistiksel en muhtemel sayı (Most Probable Number, MPN) yöntemi kullanılarak kobalta dirençlilikleri açısından test edilmişlerdir. Mutant bireylerin yaban tipe kıyasla kobalt stresine çok daha dirençli oldukları gözlemlenmiştir. Kobalta en dirençli bireylerin, maya hücrelerinin gerek doğada gerek endüstriyel uygulamalarda karşılaşabilecekleri stres faktörlerine karşı da olası çapraz dirençleri araştırılmıştır. Kobalta dirençli mutant bireylerden bazılarının bakır ve krom gibi diğer metallere de çapraz direnç gösterdiği anlaşılmıştır. Son nesillerin ve tüm mutant bireylerin ozmotik (NaCl) strese karşı da çapraz direnç kazandığı görülmüştür. Ayrıca, son nesillere ve bireylere, kobalt dirençlerinin kalıcı bir mutasyondan mı yoksa geçici bir adaptasyondan mı kaynaklandığını belirlemek için genetik stabilite testi yapılmıştır. Bu test sonucunda, bireylerin ve nesillerin kobalt dirençlerinin selektif olmayan (kobalt içermeyen) ortamlarda ardı ardına büyütülmelerine rağmen azalmadığı görülmüş ve bireylerin kobalt direncinin genetik stabilitesinin yüksek olduğu anlaşılmıştır. Kobalta maruz bırakılan yaban tip, son nesiller ve mutant bireyler, kobaltı hücre içinde ve hücre yüzeyinde tutma miktarlarının ölçülmesi amacıyla alev atomik absorpsiyon spektroskopisinde analiz edilmişlerdir (Flame atomic absorption spectroscopy; 'FAAS'). En çok kobalt tutan hücreler yaban tip hücreler olmuştur ve kobalt stresine en dirençli bireyin yaban tipe göre en az metal tuttuğu gözlemlenmiştir. Bu sonuçlara göre, kobalta dayanıklı

bireyler, kobaltı yaban bireyden daha az içeri alabiliyor ya da içeri girmiş kobaltı yaban tipten daha fazla dışarı atabiliyor olabilirler.
Sonuç olarak, evrim mühendisliği yöntemi ile kobalta dayanıklı mutant bireyler elde edilmiştir. Bu mutant bireyler arasından diğer metallere de çapraz dirençli olanları seçilmiştir. Tüm mutant bireyler içinse tuz stresine çapraz direnç gözlemlenmiştir. Alev atomik absorpsiyon spektroskopi kullanılarak, yaban tipin kobaltı en fazla miktarda hücre içi ve hücre yüzeyinde tutan tür olduğu anlaşılmıştır. Buna karşılık kobalta en dayanıklı mutant bireyin kobaltı en az tuttuğu görülmüştür. Bu nedenle, kobalta dirençli mutant bireylerin kobaltı hücre içinden uzaklaştırma mekanizmaları daha gelişmiş olan hücrelerin seçilimi sonucunda elde edildikleri ileri sürülebilir. Aynı zamanda bu bireylerin kobaltı hücre içine daha az alma eğiliminde oldukları da önerilebilir. Bu direnç mekanizmasının tam anlaşılabilmesi için transkriptomik ve proteomik analizlerin yapılması gerekmektedir.

1. INTRODUCTION

1.1 *Saccharomyces cerevisiae*

The yeast *Saccharomyces cerevisiae* is a minimal model eukaryote for scientists. It is a single-celled fungus and has other common characteristics common with other fungi. It has mitochondria and a rigid cell wall but does not have chloroplast. It is accepted as relatively immobile. It can reproduce as rapid as a bacterium if abundant supply of nutrients exist. Yeast DNA is 2.5 fold of *Escherichia coli* DNA; for this reason, it is preferentially used in genetic analysis. It has already been used to understand crucial genetic and/or biochemical mechanisms, such as cell-division cycle and it would further be used for these purposes (Alberts *et al*. 1998).

S. cerevisiae forms bud in order to grow and this process is called as "budding". The newly formed bud is named as "daughter cell" and the cell from which a bud originated is referred as "mother cell". Mother cell gives rise to a new bud near the commencement of the cell cycle. The separation of bud from mother cells occurs when the cell cycle finishes (Burke *et al*., 2000).

Yeast cells have haploid and diploid forms which differ in a wide variety of characteristics. The size of the diploid cells is larger than that of the haploid cells and diameter of diploid cells is 1.3 times of haploid cells' diameters. Moreover, diploid cells have ovoid shaped cells; whereas, haploids cells are nearly round. Another difference lies in the budding pattern of haploid and diploid cells. The emergence of new bud happens adjacent to the previous bud in an axial pattern in haploid cells, and in diploid cell, polar pattern of bud emergence occurs. Buds can be sorted where the mother cells elongate in the ends in diploid cells. Twenty buds can be formed for each yeast cells before the cells' senescence. Bud scars are the chitin rings where old bud emerged from. There are 3 mating type of yeast cells: *MAT*a, *MAT*α, and *MAT*a/*MAT*α. *MATa and MAT*α mate and yield *MAT*a/*MAT*α. *MAT*a and *MAT*α can mate but *MAT*a/*MAT*α can not mate any of these 3 different mating types. The

pheromones of different mating types affect each other and cause to stop cells as unbudded throughout the cell cycle. At this time, genes encoding the proteins needed in mating process are induced. A projection forms owing to pheromones toward the mating partner in order to realize cells fusion. The shape of the yeast cells are called as "*shmoos*". The tips from the *shmoos*' projections are combined. Then, the fusion of cytoplasm and nuclei occur respectively in order to provide the formation of diploid *MAT*a/α nucleus. This new diploid cell is called as zygote (Burke *et al*., 2000).

1.2 Cells in Various Environmental Conditions

Cells' optimal growth and function take place when the specific internal conditions are provided. External environment could sometimes be rough and changeable; for this reason, organisms developed an immense number of strategies in order to continue the specific internal conditions. Autonomous mechanisms were evolved by *Saccharomyces cerevisiae* in order to adapt to various environmental conditions (Gasch *et al.* 2000).

1.2.1 Environmental changes and yeast response

There are several studies, in which gene expression of yeast in response to various environmental changes, such as heat, oxidative, osmotic stress, and extreme pH, were investigated. There are common expression changes for all types of environmental stresses; whereas there are also some special expression changes for one particular type of stress (Gasch *et al.* 2002).

According to genomic expression responses under various environmental conditions, around 900 genes were found that were expressed under environmental stresses. These genes were named as "Environmental stress response (EST)" and they were classified in two groups. One of the groups contains 600 genes and transcripts of these genes decline faced on the environmental stress. The other group comprises 300 genes that had an increase in their transcripts due to stressful environmental stress conditions. It was found that 70 % of EST elements, whose transcripts decrease under stress conditions, were taking part in protein synthesis. Cells might conserve energy in order to use it when adjusting themselves to new environmental conditions. The genes that were induced fulfill various functions in the cells, such as

metabolism of carbohydrates, degradation and folding of proteins, defense against oxidative stress, DNA-damage repair etc (Gasch *et al.* 2002).

1.3 Metal Stress in *S. cerevisiae*

1.3.1 Metal uptake in *S. cerevisiae*

Metal uptake in *S. cerevisiae* is commonly biphasic; firstly, metals can rapidly enter the cell for a short period of time, and then metabolic uptake of metal begins which requires energy. It is thought that in the first phase metals are bound to the cell wall, and in the second phase metals pass through membrane channels inside the cells (Jennings, 1993). Fuhrmann *et al*. (1968) summarized Mg^{+2}, Mn^{+2}, Ca^{+2}, Sr^{+2} uptake in yeast cells. These metals are bound to cell surface anionic groups firstly, then they made interactions with a specific transport system which provides their transport into the cells. It was reported that Li+ enters the cells through K+ transport system and excluded from the cells by H^+/Li^+ antiport (Gadd et al., 1993).

1.3.2 The roles of cellular compartments in metal stress resistance of *S. cerevisiae*

1.3.2.1 Cell wall

Metals could be bound to cell walls of *S. cerevisiae*. However, the role of the cell wall in protecting cells against metal stress depends both on the metal and strain of *S. cerevisiae*. In a study by using copper-resistant and sensitive strains of *S. cerevisiae* Gadd *et al*. found that the cell wall did not significantly protect cells by blocking copper entry the cells, but it prevents cell lysis upon copper stress (Gadd et al. 1984). However, as copper, zinc, manganese, and cobalt are essential trace elements, the cell wall would not block their entry to the cells: When *S. cerevisiae* cells were exposed to mercury stress, the cell wall develops a tolerance to mercury. Cell wall in mercury-resistant mutant bound more mercury compared to mercury sensitive strain, and by this way less mercury passes to plasma membrane in mercury resistant mutant (Jennings, 1993).

1.3.2.2 Vacuole

Yeast vacuoles have very important functions inside the cell, such as they adjust pH homeostasis of cytosol, degrade macromolecules and store metabolites. Moreover, another significant role of metal ion regulation in cytosol is thought to be accomplished by vacuole for maintaining necessary metabolic functions and in order to protect cells negative effect of possible toxic metal ions. The metals that were accumulated inside the vacuole are demonstrated on Table1.1.

Table1.1 Metals sequestered inside the vacuole (Gadd *et al*. 1997)

Divalent ions	Mn^{+2} Fe^{+2} Zn^{+2} Co^{+2} Ca^{+2} Sr^{+2} Ni^{+2}
Monovalent ions	K^{+} Li^{+} Cs^{+}

Gadd *et al*. demonstrated that, when cells lack vacuole or a vacuolar functional H^{+}-ATPase, they become more sensitive to Zn, Mn, Co, and Ni and also they can not accumulate these metals inside their vacuoles. If these metals can not be stored inside the vacuole they are found in the cytosol and cause toxicity (Gadd *et al*. 1997).

1.3.3 Cellular metabolites' roles towards metal stress in *S. cerevisiae*

α,α-1,1-glycosidic linkage bounds two glucose molecules and a nonreducing disaccharide is formed what is called "trehalose". Bacteria, yeast, fungi, insects, invertebrates, lower and higher plants have this sugar and they might use it in order to supply energy. It can be used as signaling molecule in regulating metabolic pathways and even sometimes it influences growth. Moreover, in the different stress conditions, such as desiccation, dehydration, heat, cold, and oxidation, trehalose preserves cells proteins and membranes against denaturation or inactivation (Elbein et al., 2003).

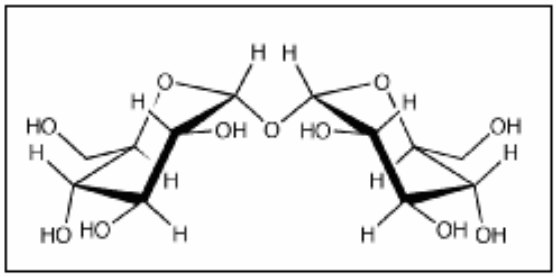


Figure 1.1 Structure of α,α1,1-trehalose (Elbein et al., 2003)

Attfield (1987) showed that trehalose can be accumulated under metal stress as well. *S. cerevisiae* cells were exposed to 5mM $CuSO_4$ stress and according to the accumulated trehalose level they concluded that $CuSO_4$ induced significant amount of trehalose accumulation.

1.4 Metal Tolerance Mechanisms

There are two types of metal tolerance mechanisms which are "avoidance" and "sequestration". The examples of these metal tolerance mechanisms are shown in Table1.2.

Table 1.2 Metal tolerance mechanisms (Jennings, 1993)

Cellular constituent/process	Result of mutation	Tolerance mechanism
Secreted protein or organic molecule or inorganic ion	Increased production resulting in increased extracellular precipitation	Avoidance
Fungal cell wall	Increased metal-binding capacity	Avoidance
Permeases	Reduced transport into the cell or increased efflux	Avoidance
Vacuoles	Increased transport into subcellular compartments	Sequestration
Inorganic chelators, e.g., sulfides	Altered metabolism	Sequestration
Organic chelators, e.g., citrates	Altered metabolism	Sequestration
Metal binding proteins	Increased synthesis	Sequestration

There exists a protein group called as "Metallothionein" that makes detoxification of metals by forming complexes. They have some common characteristics, such as they

contain significant amount of cysteine and their molecular weights are not high (Mejare et al., 2001).

Three classes of metallothionein exist: Class I, Class II, and Class III. Metallothioneins are gene products in Class I and II, but in Class III, metallothioneins are synthesized by other enzymes as polypeptides. In 1974, a Class II metallothionein was described which is a product of *CUP1* gene. Copper sensitive strains were changed to copper resistant, as result of CUP1 gene transformation (Jennings, 1993).

Ace1 is the transcription factor which becomes active in the presence of copper and silver. When Ace1 is active, it causes transcription of *CUP1* gene. It was thought that CUP1 was only activated by copper or silver; however, Tohoyama *et al*. found that cobalt also can activate CUP1 gene by using another transcription factor (Tohoyama et al., 2001).

Sequestration of metals is one of the metal detoxification mechanisms and glutathione, phytochelatin and metallothionein are involved in this process. Glutathione that was formed from three amino acids (GSH; L-γ-Glu-Cys-Gly) not only binds to anions (arsenite and selenite), but also provides the synthesis of phytochelatin as a precursor. Gharieb *et al*. found that *S .cerevisiae* tolerance for Te, Zn, Co, Cu, Mn, Ni, and Cr was not dependent to gluthatione. For example, one of the tolerance mechanisms towards Co, Zn, Ni, and Mn in *S. cerevisiae* cells occurs by compartmentation of these metals in the vacuole. However, gluthatione role in accumulation and detoxification of Cd and Se is very important since Cd^{+2} toxicity can be reduced in the presence of GSH, and it can transform selenite to elemental selenium (Se^0) as a detoxification process (Gharieb *et al.*, 2004).

1.5 Cobalt Stress

1.5.1 Cobalt characteristics

Twenty nine ppm of Earth's crust is composed of cobalt which is a lostrous, silvery-blue, hard metal. It shows ferromagneticity, with Fe and Ni. It is stable in air, and it can be attempted by dilute acids in a slow manner (Pais and Jones, 2000 and Hill *et al*., 1996).

Table1.3 Characteristics of Cobalt (Pais and Jones, 2000)

Cobalt Characteristics	
Atomic number	27
Atomic weight	58.9332
Common valence state	Co^{+2}, Co^{+3}

1.5.2 Cobalt in biological systems

A substituted corrin macrocycle includes cobalt in vitamin B_{12}. Cobalt is a cofactor in vitamin-B_{12}-dependent enzyme that participate enzyme. It is not commonly involved in metallothionein like first-row transition metals. The cobalt containing proteins are listed in Table1.4 (Kobayashi *et al.,* 1999).

Table1.4 Cobalt containing protein or enzyme (Kobayashi *et al.,* 1999)

Enzyme or protein	Source	Cofactor content	Postulated role for cobalt
Methionine aminopeptidase	Animals, yeast, bacteria	2 Co per subunit	Hydrolysis
Prolidase	Archae	1–2 Co per subunit	Hydrolysis
Nitrile hydratase	Actinomycetes and bacteria	1 Co in each α-subunit	H_2O activation, CN-triple-bond hydration and protein folding
Glucose isomerase	Actinomycetes	1 Co per 4 subunits	Isomerization
Cobalt transporter	Actinomycetes and yeast		Cobalt uptake
Methylmalonyl-CoA carboxytransferase	Bacteria	1 Co, 1 Zn per subunit	Carboxytransferation
Aldehyde decarbonylase	Algae	1 Co-porphyrin per αβ subunit	Decarbonylation for aldehyde
Lysine-2,3-aminomutase[a]	Bacteria	0.5–1 Co per subunit	Mutation
Bromoperoxidase	Bacteria	≈0.35 Co per 2 subunits	Bromination
Cobalt-porphyrin-containing protein	Bacteria	1 Co-porphyrin per protein	Electron carrier

1.5.3 Proteins synthesized in cobalt stress

Conklin *et al*., stated that "The *COT1* gene was isolated as a dosage-dependent suppressor of cobalt toxicity" and "overexpression of *COT1* gene confers increased tolerance to cobalt". If *S. cerevisiae* overexpressed of *COT1* gene, it could even grow in 20mM $CoCl_2$-containing medium. Moreover, if *COT1* gene was deleted, *S. cerevisiae* cells became more hypersensitive and this proves that cells' high cobalt stress tolerance depends partly on this gene products. If the copy number of *COT1*, increases, cobalt uptake also increases. *COT1* resembles for this situation metallothionein; however, they are not soluble proteins (located in mitochondrial membrane), small and use probably hystidine residues (different from metallothioneins where cystein residues are involved in metal interaction (Conklin et al., 1992).

There exists another gene, *GRR1 (COT2,* and the mutations in this gene results from resistant yeast cells to metal ions cobalt, zinc, manganese, nickel whose ionic radii was approximately 0.075 nm. These metals were accumulated by the divalent cation transport system. *GRR1* roles might be to control metal permeability of membranes or to provide uptake energy for metal ions. If this gene does not function, the permeability of membranes to metals can cease, and yeast cells become resistant to these divalent ions. Moreover, one of its possible roles is to activate glucose for the plasma membrane ATPase (Conklin *et al.,* 1993).

Stadler *et al*. (2002) found that when cobalt stress was applied to yeast cells, iron regulon was induced and proteins involved in iron uptake were synthesized. Moreover, they demonstrated that these cells tolerate cobalt stress by taking up iron ions inside the cells. Another interesting result was that when a subtoxic cobalt stress was applied, the only iron regulon was induced instead of stress mechanism genes.

In another study, *Neurospora crassa* 20 times more resistant to cobalt than the wild type was obtained. Cell-free extracts of cobalt-resistant and wild type strains were taken in order to see the proportion of cobalt and cobalt bounded protein. 80 % of the cobalt that was found in cell-free extracts in cobalt-resistant strain was protein-bound and the corresponding value for the wild type was 25 %. A cobaltoprotein purification was done which was overproduced in cobalt resistant strain. This protein is a small molecular weight, brown glycoprotein, and it has absorption peaks at 275, 350 and 440 nm. The protein consisted of upto 28-30 % of mannose and the major amino acids found in this protein were cysteine, glycine, glutamic acid and aspartic acid (Sajani *et al*., 1998).

1.6 Obtaining Cobalt-Resistant *S. cerevisiae*

1.6.1 The method of obtaining cobalt-resistant *S. cerevisiae*

Random mutagenesis or classical breeding and genetic crossing are used traditionally in order to obtain desired phenotype of yeasts. However, thanks to improvements in recombinant DNA technology, scientists can now handle and intervene directly to the desired pathway. It was stated that “Directed improvements of the cellular properties achieved from the interplay of theoretical analysis, relying on biochemical

information, and the application of genetic engineering has been referred to as metabolic engineering" (Ostergaard *et al*., 2000).

However, if the genetic basis of a phenotypic property whose genetic bases is not known definitely, metabolic engineering can not be effective, because there are not enough data about all the components that have roles in the system and their interactions with the whole system. An alternative approach named as evolutionary engineering can be used to obtain desired phenotypes through continuous evolution procedures. Continuously growing cultures are treated with an appropriate selection pressure. This approach was successfully used in obtaining acetate and ethanol resistant strains, as well as multiple stress resistant yeast (Çakar *et al.*, 2005).

1.7 The Aim of the Present Study

The aim of the present study was to obtain cobalt-resistant and/or cobalt-binding yeast cells by using evolutionary engineering methodology. Cobalt-resistant mutants were selected by using different selection strategies and after determining their survival ratios quantitatively under cobalt stress, the most cobalt resistant mutants were selected. Their cobalt contents were determined by employing atomic absorption spectrometry. The mutants were also screened for any possible cross-resistance to another stress condition. These cobalt resistant yeast cells would provide us the understanding of metal tolerance or resistance mechanisms on the basis of transcriptomic and genomic analysis. These yeast cells or their metabolic products, such as proteins, can be used in biomimetics and/or bioremediation applications.

2. MATERIALS AND METHODS

2.1 Materials

2.1.1 Strain name

Saccharomyces cerevisiae CEN.PK 113.7D (MATa, MAL2-8 , SUC2) strain was used in this study that was provided by Dr. Peter Kötter from Johann Wolfgang Goethe University, Frankfurt, Germany. Throughout this study, this strain was named as 100.

2.1.2 Yeast culture media

Yeast minimal medium:

Table 2.1 Content of the yeast minimal medium

Content	Amount
D-glucose	20 g
Yeast nitrogen base without amino acids	6.7 g
Agar (for solid medium)	20 g
Distilled water	to 1 l

Yeast complex medium:

Table 2.2 Content of the yeast complex medium

Content	Amount
D-glucose	20 g
Bacto peptone	20 g
Bacto yeast extract	10 g
Agar (for solid medium)	20 g
Distilled water	to 1 l

2.1.3 Chemicals

The chemicals that were used in this study are listed in Table 2.3.

Table 2.3 Chemicals that were used in this study

Chemical	Company	Country
Cobalt (II)-chloride-hexahydrate	Merck	Germany
Chromium(III) chloride hexahydrate	Acros Organics	USA
Copper (II) chloride	Merck	Germany
Sodium chloride	Riedel-de Haen	Germany
D(+)-Trehalose dihydrate	Riedel-de Haen	Germany
Hydrogen peroxide (35%, v/v)	Merck	Germany
Ethanol (absolute)	J.T.Baker	Holland
Nitric acid 65%	Merck	Germany
Sulfuric acid 95-98%	Merck	Germany
Sodium Thiosulphate	J.T.Baker	Holland

2.1.4 Solutions and buffers

The solutions and buffers that were used in this study are listed in Table 2.4.

Table 2.4 Solutions and buffers that were used throughout this study

Solution Name	Concentration
$CoCl_2$	1M
$CrCl_3$	1M
$CuCl_2$	1M
NaCl	5 % - 10 % - 15 % (w/v)
H_2SO_4	5mM
HNO_3	6M
Ethanol	20 % (v/v)
H_2O_2	1-2-3-4-5M and 20-50mM
Sodium thiosulfate	10 % (w/v)
Potassium phosphate buffer (pH 7)	50mM

2.1.5 Laboratory equipment

Laboratory equipment used in this study is listed in Table 2.5.

Table 2.5 Laboratory equipment used in this study

Equipment	Brand/Model	Country
Laminar flow	Faster BH-EN 2003	Italy
Vortex mixer	Heidolph REAX top	Germany
Light Microscope	Olympus CH30	Japan
UV-Visible Spectrophotometer	Shimadzu UV-1700 Perkin Elmer 25 UV/VIS	Japan USA
Atomic Absorption Spectrophotometer (ITU, Chemistry Department)	Analytik Jena Vario 6	Germany
Microfuge	Beckman Coulter Microfuge®18 Centrifuge	USA
Balances	Precisa XB 620C Precisa XB 220A Precisa BJ 610C	Switzerland
Autoclave	Tuttnauer Systec Autoclave 2540 ml	Switzerland
Micropipettes	Eppendorf	Germany
Deep freezers	Heto Ultra Freeze (-80ºC) Arçelik (-20ºC)	Denmark Turkey
Refrigerator	Arçelik (+4ºC)	Turkey
Rotor	Beckman Coulter, JA 30.50i rotor	USA
Orbital shaker incubator	Sartorius Certomat®SII	Germany

2.2 Methods

2.2.1 Culture cultivation conditions

Cultures were grown in 50 ml test tubes in 10 ml volume at 30ºC and 150 rpm. YMM and YPD were used as the media throughout this study.

2.2.2 EMS mutagenesis

The EMS mutagenesis method (Lawrence *et al*.,) was adapted in order to obtain EMS mutagnesis to wild type yeast. *Saccharomyces cerevisiae* CEN.PK 113.7D precultures were inoculated into 10 ml medium in a 50 ml centrifuge tube. The culture was incubated at 30ºC and 150 rpm until the cell concentration reached $2x10^8$ cells/ml. 2.5 ml of the culture at the indicated cell concentration was washed twice in 50mM potassium phosphate buffer (pH 7). The culture was resuspended in 10 ml of the same buffer to obtain cell concentration of $5x10^7$ cells per ml. 300 µl EMS was added to this suspension. After vortexing the tube, it was incubated at 30ºC for 30 min. The EMS mutagenesis was stopped by adding the same volume of sodium thiosulphate(%10, w/v) solution into the cell suspension. After mixing the tube by vortexing, the tube was centrifuged at 10 000 rpm for 10 min (Beckman Coulter, JA 30.50i rotor) and the cells were washed twice with YMM without dextrose. The cells collected after centrifugation were inoculated into YPD and this culture was named as *101*.

2.2.3 Pulse stress selection strategies for obtaining cobalt-resistant mutants

In pulse stress selection strategy, stress was applied to a culture that was in the early exponential phase. The mutants in this study were chosen according to this methodology. 101 stock culture was taken from -80ºC and 500 µl of this culture was inoculated into 10 ml YMM. After overnight incubation in the water bath shaker at 30ºC and 150 rpm, this culture was inoculated to 10 ml YMM by adjusting the final optical density (OD_{600}) value between 0.2-0.3. The culture was then replaced into water bath shaker and the optical density was measured in certain time periods. When OD_{600} values reached between 0.5-0.6, two microfuge tubes were filled with 1 ml culture. Both of the microfuge tubes were centrifuged at 10 000 rpm for 5 min (Beckman Coulter, JA 30.50i rotor) and the supernatants were removed. One of the tubes was chosen as control tube and 1 ml YMM was added to this tube. The next tube was filled with YMM containing 0.5mM $CoCl_2$. These microfuge tubes were

incubated in the water bath shaker for 90 min. At the end of this time, the microfuge tubes were centrifuged and the supernatants were poured off. The pellets were resuspended in 1 ml fresh YMM and a second centrifugation was done. The supernatant was removed and 1 ml YMM was again added to these microfuge tubes. These 1 ml cultures were resuspended and they were added to 5 ml-YMM containing tubes. They were placed in water bath shaker and incubated for 14-16 h. At the end of this time, analysis of the optical density values of the control culture and the cultures, which were exposed to metal stress, were performed (600 nm). In addition, cell counting by using hemacytometer was done. The cultures that were exposed to metal stress were named as "first constant/increasing stress generation" and stock cultures were prepared. For this purpose, 500 µl 60% (v/v) glycerol solution was added to 500 µl culture in 1.5 ml microfuge tubes and the microfuge tubes were placed to -80ºC after vortexing.

2.2.3.1 Obtaining constant-stress generations by using pulse stress strategy

First constant/increasing stress generation stock culture was taken and inoculated into 10 ml YMM. After overnight incubation at 30^{o}C, another preculture was prepared and this preculture was inoculated into 10 ml YMM. Until its OD_{600} value reached between 0.5-0.6, it was incubated in the water bath shaker. One ml of this culture was then transferred to two microfuge tubes. After centrifugation at 10 000 rpm for 5 min (Beckman Coulter, JA 30.50i rotor), one of the tubes was filled with 1 ml YMM (control tube) and the other tube was filled with YMM containing 0.5mM $CoCl_2$. After resuspension, the microfuge tubes were incubated in the water bath shaker for 90 min. At the end of 90 min, the centrifugation was done, and the supernatant was replaced by 1 ml YMM. Second centrifugation was done after resuspension the supernatant were replaced again with 1 ml YMM. The resuspended cultures were inoculated to 5 ml YMM and these tubes were incubated for 14-16 h. At the end of this time, the OD_{600} measurements and hemacytometric counting were performed. The stock cultures of the 2nd and further constant stress generations were prepared as well. All the constant-stress generations were obtained by applying 0.5mM $CoCl_2$ metal stress to the cultures that were in the same growth phase. 36 constant-stress generations were obtained.

2.2.3.2 Obtaining increasing-stress generations by using pulse stress strategy

There was only only difference between obtaining constant and increasing stress generations. Constant-stress generations were obtained by applying always the same concentration of $CoCl_2$ (0.5mM $CoCl_2$) whereas in increasing stress application strategy, the metal concentrations were increased gradually (between 0.5mM, 5mM and 10mM).

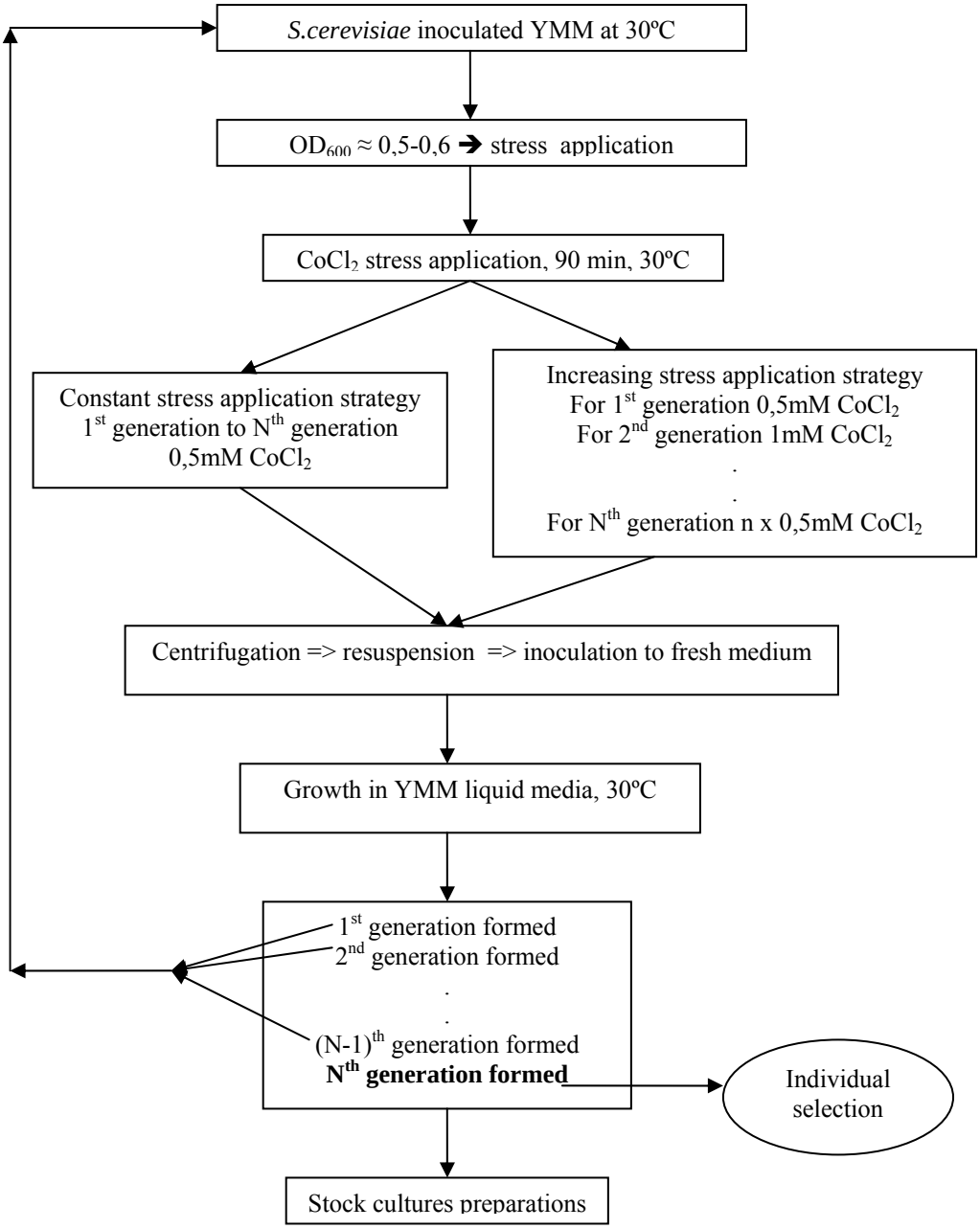


Figure 2.1 Selection algorithm of the constant and increasing stress generations

Table 2.6 $CoCl_2$ metal stress concentration that were applied to constant/increasing stress generations

Constant/Increasing stress generation number	The applied $CoCl_2$ concentration for constant stress generation (mM)	The applied $CoCl_2$ concentration for increasing stress generation (mM)
1	0.5	0.5
2	0.5	1.0
3	0.5	1.5
4	0.5	2.0
5	0.5	2.5
6	0.5	3.0
7	0.5	3.5
8	0.5	4.0
9	0.5	4.5
10	0.5	5.0
11	0.5	5.5
12	0.5	6.0
13	0.5	6.5
14	0.5	10.0
15	0.5	15.0
16	0.5	20.0
17	0.5	25.0
18	0.5	30.0
19	0.5	40.0
20	0.5	50.0
21	0.5	60.0
22	0.5	70.0
23	0.5	75.0
24	0.5	80.0
25	0.5	85.0
26	0.5	90.0
27	0.5	95.0
28	0.5	100.0
29	0.5	105.0
30	0.5	110.0
31	0.5	115.0
32	0.5	120.0
33	0.5	125.0
34	0.5	130.0
35	0.5	135.0
36	0.5	140.0

2.2.4 Stock culture preparation

Yeast cells cultures' frozen stock solutions were prepared by using 60 % (v/v) glycerol solution. Equal volume of culture and glycerol solution were mixed in order to prepare stock cultures with a final ratio of 30 % (v/v) glycerol. These stock cultures were placed at -20ºC and – 80ºC.

2.2.5 The survival ratio determination of the mutant generations

2.2.5.1 Optical density measurements

In this study, Spectrophotometric measurements were done at 600 nm. Firstly, stress applications were performed to the cultures while their OD_{600} were between 0.5-0.6. Secondly, after obtaining all the generations, their survival ratios were determined by optical density measurements as well. The survival ratio for optical density measurements was determined by dividing the optical density values of the cultures that were exposed to metal stress by these of their corresponding control groups.

2.2.5.2 Cell counting

Direct microscopic count is a method that is performed by using a microscope in order to count the cells in the samples. By this means, the measurement of population cell number could be made. If the samples are in liquid, special counting chambers must be utilized (Madigan *et al*., 2003). In these experiments a hemacytometer was used. This hemacytometer contained a grid that had 25 large squares with 16 little squares in all the 25 squares. All little squares have $25x10^{-4}$ mm^2 area and 0,01mm height, they have $25x10^{-6}$ mm^3 volume. One large square that was constituted from 16 little squares have $4x10^{-4}$ mm^3(ml) volume. In liquid culture, the Colony Forming Unit (CFU) per ml calculated as shown on the equation 2.1.

$$CFU/ml = X \times (DF) / 4x10^{-4} \text{ ml} \qquad \textbf{(2.1)}$$

In this equation, X is the average number of the cells counted on the microscope for each square that contained 16 little squares. DF is the dilution factor of the culture that was placed on the hemacytometer. $4x10^{-4}$ ml is the volume of one of the 25 squares.

2.2.6. Identification of constant and increasing stress generations' individuals

The stock cultures of final (36^{th}) constant and increasing stress generations were inoculated to 10 ml YMM for overnight growth. Then, their 10^{-6} dilutions were prepared and inoculated to YMM solid media by spread plating technique. After 48 h incubation, the single colonies appeared on both plates that contained 36^{th} constant and increasing stress generations. Twelve colonies from each Petri plate that contained both constant and increasing-stress generations were chosen randomly by

using toothpicks and they were inoculated to 10 ml YMM, and incubated at 30º C for 16-17 h. At the end of this incubation period, their stock cultures were prepared.

Table 2.7 Nomenclatures for the individuals that were obtained from the 36th constant stress generation (left); and the individuals that were obtained from the 36th increasing stress generation (right)

Constant Stress Individuals	Code
36th constant stress individual 1	*1C*
36th constant stress individual 2	*2C*
36th constant stress individual 3	*3C*
36th constant stress individual 4	*4C*
36th constant stress individual 5	*5C*
36th constant stress individual 6	*6C*
36th constant stress individual 7	*7C*
36th constant stress individual 8	*8C*
36th constant stress individual 9	*9C*
36th constant stress individual 10	*10C*
36th constant stress individual 11	*11C*
36th constant stress individual 12	*12C*

Increasing Stress Individuals	Code
36th increasing stress individual 1	I1
36th increasing stress individual 2	I2
36th increasing stress individual 3	I3
36th increasing stress individual 4	I4
36th increasing stress individual 5	I5
36th increasing stress individual 6	I6
36th increasing stress individual 7	I7
36th increasing stress individual 8	I8
36th increasing stress individual 9	I9
36th increasing stress individual 10	I10
36th increasing stress individual 11	I11
36th increasing stress individual 12	I12

33th increasing stress generation was inoculated into solid YMM containing 20mM $CoCl_2$. Three colonies were chosen from this Petri plate after incubation at 30ºC, and they were inoculated to YMM. One of these colonies had brown colour; for this reason, it was named as "Brown". The other individuals were white and were named as "White" and "Super" respectively.

Table 2.8 The individuals that were obtained from 20mM $CoCl_2$ containing solid YMM

Individuals obtained from 20mM $CoCl_2$ containing YMM
CNVR (Super)
W (White)
B (Brown)

2.2.7 Quantitative resistance determination of mutant individuals with the highest cobalt resistance for each selection strategy using a high throughput screening method (Most probable number 'MPN')

Five-tube MPN method was used in order to determine the highest cobalt resistant individuals. Five columns of a 96 well plate were filled with 180µl media. The preculture of the sample 20 µl were delivered to first row of these 5 columns and mixed. In this way, 1:10 dilution was obtained. From this first row, 20 µl of culture was then transferred to second row, after mixing, 20 µl of culture was transferred to 3rd row. The dilutions continued until 8th row and the last dilution factor was 10^8. For one sample, the same procedures were repeated with YMM and YMM that contained

different $CoCl_2$ amounts. The culture growth was observed as pellet formation on the bottom of the well or as turbidity on the well. The number of cells per ml was determined according to the 5-tube MPN table (Lindquist, J., 2001).

2.2.8 Cobalt content determination of mutant individuals with the highest cobalt resistance using Atomic Absorption Spectroscopy (AAS)

The determination of the cobalt content of the samples after 5mM $CoCl_2$ stress application was done by using atomic absorption spectrometer. The precultures of the 100, 36^{th} constant stress generation, 36^{th} increasing stress generation and individuals were inoculated to 10 ml YMM and to YMM that contained 5mM $CoCl_2$ as well in 50 ml centrifuge tubes. They were incubated for 48 h at 30ºC and 150 rpm. The 50 ml falcon tubes were waited at 105ºC for 2 h inside the oven. At the end of this time, the tubes were waited for 15 min inside the dessicator. After weighing the tubes, the samples were transferred to these tubes. They were centrifuged and washed in double distilled water for 2 times. They were then placed to 105ºC for 2 h. They were then weighed and 6M HNO_3 was added to these samples in order to hydrolyze the cells. 6M HNO_3 might damage atomic absorption spectrophotometer that was used; for that reason, the samples were diluted by adjusting dilution factor as 2. Flame atomic absorption spectrophotometer (FAAS), Analytik-Jena Model Atomic Absorption Spectrometer (AAS) Vario 6 (Germany), was used in order to determine the cobalt content that was associated with the samples. An air/acetylene flame and hollow cathode lamp were utilized. The wavelength and slit width for cobalt are shown on Table 2.9.

Table 2.9 The wavelength and slith width that were used on FAAS

Wavelength (nm)	Slit width (nm)
242,5	0,2

2.2.9 Determination of other stress resistances

In all experiments the cultures that were indicated on Table 2.10 were used. Pulse and continuous stress applications were applied to these populations and individuals.

In pulse stress application strategy, the precultures of these populations and individuals were inoculated to YMM and they were incubated at 30ºC and 150 rpm until their early or middle exponential growth phase. At the appropriate growth

phase, 1 ml of cultures were transferred to at least 2 microfuge tubes which one of them was for the control group and the other one for stress application. The microfuge tubes were centrifuged for 10 min at 10 000 rpm (Beckman Coulter, JA 30.50i rotor) and the supernatant was removed. Cells were resuspended in 1 ml YMM without dextrose and a second centrifugation was applied. After removing YMM without dextrose the control tube was filled with 1 ml YMM and the other tubes where stress would be applied were filled with the medium that contained the stress factor. These microfuge tubes were incubated at 30º and 150 rpm for 60 or 90 min. At the end of this time, tubes were centrifuged and washed with YMM without dextrose. Their pellets were then resuspended in 1 ml YMM and inoculated to 50 ml centrifuge tubes that contained 10 ml YMM. They were incubated at 30ºC and 150 rpm and their OD_{600} measurements were done at 24, 48, and 72 h.

In order to apply continuous stress, the precultures of these populations and individuals were prepared. These precultures were inoculated to YMM and YMM with stress factor both on the 50 ml centrifuge tube with 10 ml volume and to 96 well plate in order to apply 5 tube MPN methodology. The survival ratios for inoculation to centrifuge tubes were determined by measuring OD_{600} values and for MPN method by calculating the number of cells per ml both for control and stress applied samples.

Table 2.10 The population and individuals that were used in other stress tests

Population/individual name	Code
Wild type	*100*
36^{th} constant stress generation	*36C*
36^{th} increasing stress generation	*36I*
36^{th} constant stress individual 5	*5C*
36^{th} constant stress individual 6	*6C*
36^{th} increasing stress individual 1	*I1*
36^{th} increasing stress individual 3	*I3*
36^{th} increasing stress individual 8	*I8*
36^{th} increasing stress individual 10	*I10*
Super	*Cnvr*
White	*W*
Brown	*B*

2.2.9.1 Different metal stresses (copper and chromium)

Precultures of the samples indicated on Table 2.10 were inoculated to 10 ml YMM in 50 ml falcon tubes. These precultures were used to inoculate the medium that contained different concentrations of $CuCl_2$ and $CrCl_3$ to apply these metal stresses continuously. The experiments were done in 96 well plates and also in centrifuge tubes (10 ml medium in 50 ml centrifuge tube).

2.2.9.2 Heat stress application

Populations and individuals were applied to pulse heat stress for 60 min at 52º and 80ºC. Their survival ratios were determined by measuring OD_{600} of the samples after 24, 48 and 96 h time period.

2.2.9.3 Oxidative stress application

Pulse and continuous oxidative stress applications were done to all samples. 1M H_2O_2 pulse stress was applied to these cultures. After 24 and 72 h of incubation, OD_{600} values were measured and the survival ratios of the cultures were determined.

In order to apply oxidative stress continuously, overnight precultures of the samples were inoculated to YMM and YMM that contained different concentrations of H_2O_2 (10mM, 20mM, 50mM). OD_{600} values of the cultures were measured after 24^{th}, 48^{th}, and 72^{nd} h of incubation. The experiment was applied in 96 well plates for MPN methodology as well.

2.2.9.4 Ethanol stress application

Ethanol stress was applied by using pulse stress application strategy. 20 % ethanol (v/v) was chosen as the pulse ethanol stress level. The optical density values were taken for every 24 h during 3 days.

Continuous ethanol stress was applied in 50 ml centrifuge tube with 5 and 10 % (v/v) stress levels. OD_{600} values were taken after 24, 48, and 72 h of incubation.

2.2.9.5 Osmotic stress application

Osmotic stress was applied by using both pulse and continuous stress strategy. For pulse osmotic stress application, 15 and 30 % (w/v) NaCl were applied to these cultures. After inoculations in centrifuge tubes, the survival ratios were determined by measuring OD_{600} of the samples for each 24 h during 3 days of cultivation.

Continuous stress was applied both on the 50 ml centrifuge tubes and on the 96 well plates. 5 and 10 % (w/v) NaCl stress were applied to cultures on the 50 ml centrifuge

tubes. The same stress levels were used in order to apply 5-tube MPN method to these cultures as well.

2.2.10 Genetic stability tests

100, 36^{th} constant, 36^{th} increasing stress generations and the individual precultures were prepared. They were inoculated both in the YMM and YMM that contained $CoCl_2$ (5mM). After 48 h incubation at 30ºC and 150 rpm, these cultures named as first generation and from the control samples, YMM and YMM with $CoCl_2$ (5mM) were inoculated again. After 48 h, these cultures control that were grown on YMM called as second generations. The same procedures were repeated until 6^{th} generation. OD_{600} of the control and 5mM $CoCl_2$ stress applied samples were measured.

3 RESULTS

3.1. Stress Application and Creation of Generations

In order to obtain generations, $CoCl_2$ stress was applied to *101* in early growth phase ($OD_{600} \approx 0.5$-0.6) for 90 min, the culture was then incubated overnight in YMM. At the end of that incubation, the new generation was obtained. Since the stress was applied for a short time to a culture at a specific growth phase, the type of the stress application was named as "pulse stress". Two different strategies in terms of stress application level were applied in order to obtain generations. Constant stress generations were obtained by applying constantly 0.5mM $CoCl_2$ for each generation (Table 3.1). However, $CoCl_2$ concentrations were increased at each successive generation while obtaining increasing stress generations.

In Table 3.2, the nomenclature for pulse increasing stress generations and the applied cobalt stress level are shown. Between first and 14^{th} increasing stress generations $CoCl_2$ stress levels were increased by 0.5mM for each generation, from 18^{th} to 22^{nd} pulse stress generations, stress levels were increased by 10mM $CoCl_2$ for each generation, and from 14^{th} to 18^{th} and from 22^{nd} to 36^{th} increasing stress generations, $CoCl_2$ stress levels were increased by 5mM (Table 3.2).

Table 3.1 Nomenclature of pulse constant stress populations

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

Table 3.2 Nomenclature for pulse increasing stress populations

Increasing Stress Generation Name	Code	Stress condition (mM $CoCl_2$)
1st increasing stress generation	*1I*	0.5
2nd increasing stress generation	*2I*	1.0
3rd increasing stress generation	*3I*	1.5
4th increasing stress generation	*4I*	2.0
5th increasing stress generation	*5I*	2.0
6th increasing stress generation	*6I*	3.0
7th increasing stress generation	*7I*	3.5
8th increasing stress generation	*8I*	4.0
9th increasing stress generation	*9I*	4.5
10th increasing stress generation	*10I*	5.0
11th increasing stress generation	*11I*	5.5
12th increasing stress generation	*12I*	6.0
13th increasing stress generation	*13I*	6.5
14th increasing stress generation	*14I*	10.0
15th increasing stress generation	*15I*	15.0
16th increasing stress generation	*16I*	20.0
17th increasing stress generation	*17I*	25.0
18th increasing stress generation	*18I*	30.0
19th increasing stress generation	*19I*	40.0
20th increasing stress generation	*20I*	50.0
21st increasing stress generation	*21I*	60.0
22nd increasing stress generation	*22I*	70.0
23rd increasing stress generation	*23I*	75.0
24th increasing stress generation	*24I*	80.0
25th increasing stress generation	*25I*	85.0
26th increasing stress generation	*26I*	90.0
27th increasing stress generation	*27I*	95.0
28th increasing stress generation	*28I*	100.0
29th increasing stress generation	*29I*	105.0
30th increasing stress generation	*30I*	110.0
31st increasing stress generation	*31I*	115.0
32nd increasing stress generation	*32I*	120.0
33rd increasing stress generation	*33I*	125.0
34th increasing stress generation	*34I*	130.0
35th increasing stress generation	*35I*	135.0
36th increasing stress generation	*36I*	140.0

3.2 Selection of Individual Mutants from Final Mutant Populations

The final generation obtained by pulse increasing stress application strategy was the 36th increasing stress generation obtained under 140mM $CoCl_2$ pulse stress conditions. This culture was regrown in liquid YMM, after which it was inoculated to solid YMM, and 12 single colonies were randomly chosen as mutant individuals of increasing stress selection strategy (Table 3.3).

Table 3.3 Nomenclature for mutant individuals selected from increasing stress final population *(36I)*

Individual Mutant of 36th Increasing Stress Generations	Code
1st increasing stress individual mutant	*1I*
2nd increasing stress individual mutant	*2I*
3rd increasing stress individual mutant	*3I*
4th increasing stress individual mutant	*4I*
5th increasing stress individual mutant	*5I*
6th increasing stress individual mutant	*6I*
7th increasing stress individual mutant	*7I*
8th increasing stress individual mutant	*8I*
9th increasing stress individual mutant	*9I*
10th increasing stress individual mutant	*10I*
11th increasing stress individual mutant	*11I*
12th increasing stress individual mutant	*12I*

Thirty six constant stress generations were obtained parallel to the increasing stress generations. The final constant stress generation was the 36th constant stress generation. All these generations were obtained by applying 0.5mM $CoCl_2$ pulse stress to the previous culture. Finally, 36th constant stress generation was inoculated to solid YMM and incubated at 30ºC. Twelve single colonies were picked and they were inoculated to liquid YMM, and incubated at 30ºC and 150 rpm. The nomenclature for the individuals of the 36th constant stress generation is shown in Table 3.4.

Table 3.4 Nomenclature for mutant individuals selected from the constant stress final population *(36C)*

Individual Mutant of 36th Constant Stress Generation	Code
1st constant stress individual mutant	*C1*
2nd constant stress individual mutant	*C2*
3rd constant stress individual mutant	*C3*
4th constant stress individual mutant	*C4*
5th constant stress individual mutant	*C5*
6th constant stress individual mutant	*C6*
7th constant stress individual mutant	*C7*
8th constant stress individual mutant	*C8*
9th constant stress individual mutant	*C9*
10th constant stress individual mutant	*C10*
11th constant stress individual mutant	*C11*
12th constant stress individual mutant	*C12*

There were three other mutant individuals that were selected (Table 3.5). The 33rd increasing stress generation was inoculated to solid YMM containing 20mM $CoCl_2$. These mutants appeared on the Petri plate as single colonies with different colours.

Table 3.5 Nomenclature for mutant individuals selected from 33rd increasing stress generation.

Individual Mutant of 33rd Increasing Stress Generation, Name	Code
Super (white)	*CNVR*
White	*W*
Brown	*B*

3.3 Characterization of Populations and Individual Mutants

3.3.1 Determination of cobalt stress resistance

3.3.1.1 Cobalt stress resistances of constant and increasing stress generations

All generations were obtained in parallel with their control group to which pulse $CoCl_2$ stress was not applied. When the overnight incubation of the cultures was finished, optical densities (OD_{600}) of the generation and its control group were measured. By dividing OD_{600} value of the stress-applied generation to that of its control, the survival ratio of this generation was obtained. The survival ratios of both

constant and increasing stress generations were determined (Figure 3.1 and Figure 3.2)

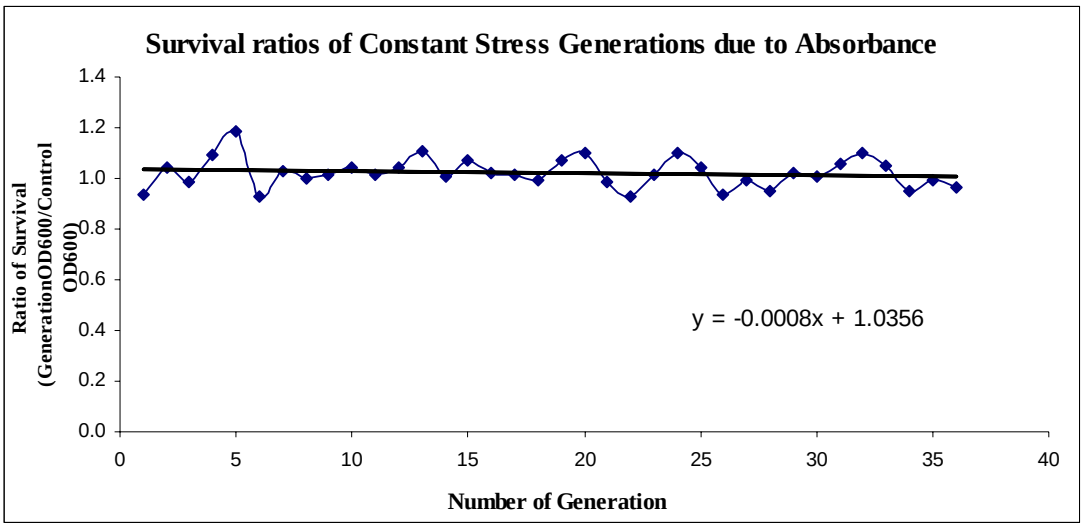


Figure 3.1 Survival analysis of constant stress generations

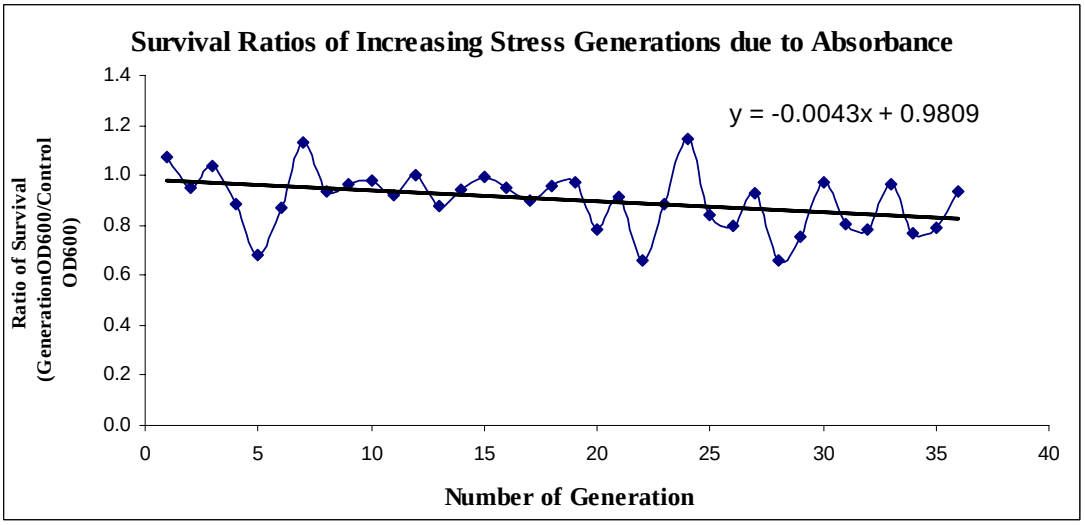


Figure 3.2 Survival analysis of increasing stress generations

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

The 36th constant stress individual was grown on solid YMM and 12 single colonies were selected randomly. Cobalt stress tolerance of constant stress individuals, *100, 101* and 36th constant stress generation were tested with 5 tube MPN methodology. While applying 5 tube MPN method, 0.5 and 5mM $CoCl_2$ stress were applied continuously. At the end of the appropriate incubation at 30ºC, their survival ratios were determined by dividing number of "cells per ml" of the stress applied samples

by number of “cells per ml” of the control group. Table 3.6 shows the per cent survival ratio values of the individuals and populations.

Table 3.6 Per cent survival ratio of constant stress individuals and populations

Population / individual name	% survival at 0.5mM $CoCl_2$	% survival at 5mM $CoCl_2$
100	49.00	0.10
101	667.00	0.23
36C	100.00	0.10
36G	59.00	0.10
1C	23.00	0.02
2C	26.00	0.00
3C	49.00	0.01
4C	486.00	0.26
5C	263.00	1.54
6C	225.00	0.01
7C	22.00	0.02
8C	191.00	0.08

The average of per cent survival ratio of individuals was calculated and named as “C”. 36^{th} constant stress generation, “C” and the individuals per cent survival ratio was divided to per cent survival ratio of *100*. These survival ratios as fold of wild type are demonstrated both in Table 3.7 and Fig. 3.3.

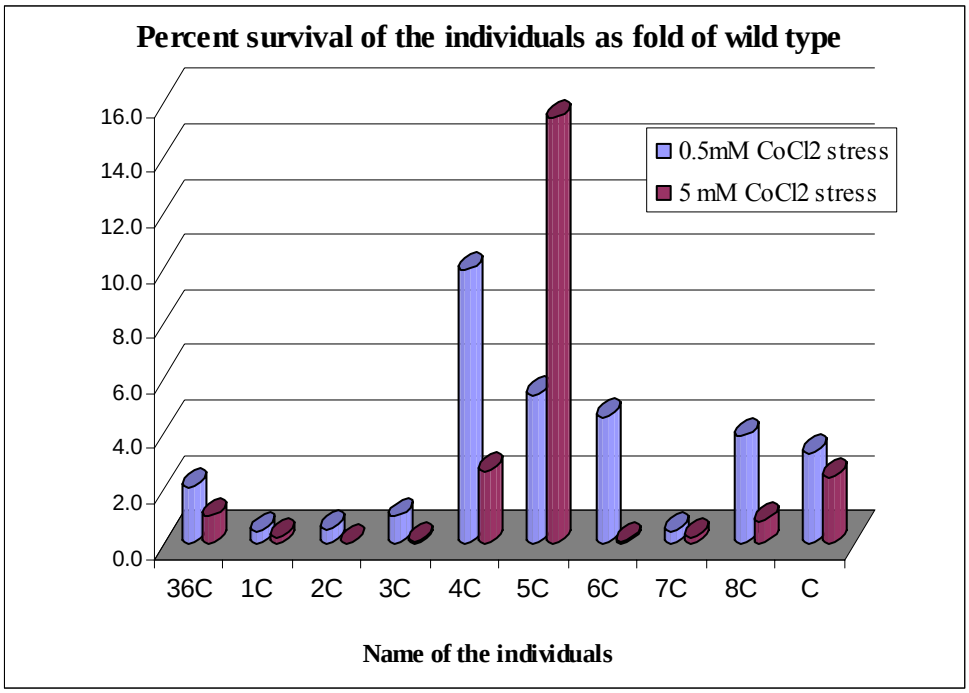


Figure 3.3 36th constant stress generation, "C" and the individuals per cent survival ratio as fold of wild type after 0.5 and 5mM $CoCl_2$ continuous stress

Table 3.7 36th constant stress generation, "C" and the individuals per cent survival ratio as fold of wild type after 0.5 and 5mM $CoCl_2$ continuous stress

Name of the individuals and population	Survival as fold of wild type (after 0.5mM $CoCl_2$)	Survival as fold of wild type (after 5mM $CoCl_2$)
36C	2.04	1.00
1C	0.47	0.23
2C	0.53	0.01
3C	1.00	0.10
4C	9.92	2.63
5C	5.37	15.43
6C	4.59	0.10
7C	0.45	0.22
8C	3.90	0.84
C	3.28	2.44

Under continuous 0.5mM $CoCl_2$ stress, 4C and 5C could survive 9.92 and 5.37 fold of wild type, respectively. Moreover, under continuous 5mM $CoCl_2$ stress, 5C had 15.43-fold of wild type per cent survival ratio.

The individuals of the 36th constant stress generation showed rather heterogeneous cobalt stress resistance (Table 3.7 and Fig. 3.3). Some of the individuals (C4, C5)

could tolerate $CoCl_2$ stress much better than the population; whereas some others could not.

Twelve individuals were obtained from the 36th increasing stress generation. In order to determine their resistances to continuous $CoCl_2$ stress (0.5 and 5mM $CoCl_2$), 100, 36th increasing stress generation and these individuals were tested by using 5 tube MPN method. Their survival ratios were determined after incubation at 30ºC and these values are demonstrated as per cent survival ratio (Table 3.8).

Table 3.8 Per cent survival ratio of increasing stress individuals and populations

Population / individual name	% survival at 0.5mM $CoCl_2$	% survival at 5mM $CoCl_2$
100	38	2
101	34	2
36G	30	3
I1	261	26
I2	30	2
I3	486	154
I6	38	2
I8	150	22
I9	66	10
I10	69	10
I11	31	2

The average per cent survival ratio of the increasing stress individuals was calculated and named as "I". The 36th increasing stress generation, individuals and "I" per cent survival ratios were determined as fold of wild type and are shown on Table 3.9 and Fig. 3.4.

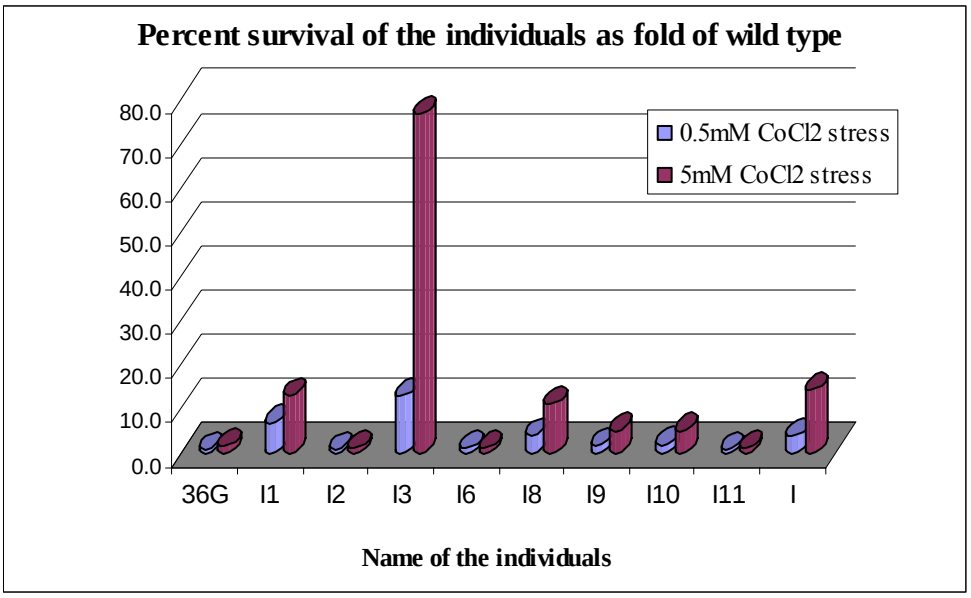


Figure 3.4 36th increasing stress generation, "I" and the individuals per cent survival ratio as fold of wild type after 0.5 and 5mM $CoCl_2$ continuous stress

Table 3.9 36th increasing stress generation, "I" and the individuals per cent survival ratio as fold of wild type after 0.5 and 5mM $CoCl_2$ continuous stress

Name of the individuals and population	Survival as fold of wild type (after 0.5mM $CoCl_2$)	Survival as fold of wild type (after 5mM $CoCl_2$)
36G	0.79	1.50
I1	6.87	13.00
I2	0.79	1.00
I3	12.79	77.00
I6	1.00	1.00
I8	3.95	11.00
I9	1.74	5.00
I10	1.82	5.00
I11	0.82	1.00
I	3.72	14.25

Upon continuous stress of 0.5 and 5mM $CoCl_2$, the individual I3 showed 12.79 and 77 fold survival of wild type, respectively.

The individuals that were selected from 20mM $CoCl_2$ containing solid YMM, and *100* were tested in 5 tube MPN method against continuous 5mM $CoCl_2$ stress. These cultures per cent survival ratios and survival ratio as fold of wild type are shown on Table 3.10 and 3.11 and Fig. 3.5.

Table 3.10 Per cent survival ratios of *100, Cnvr, W, and B* for 5mM $CoCl_2$ continuous stress application

Name of the individuals and population	5mM $CoCl_2$ stress applied samples per cent survival
100	0.00
Cnvr	4.44
W	218.75
B	0.22

These individuals of 33rd increasing stress generation showed highes cobalt stress resistance.

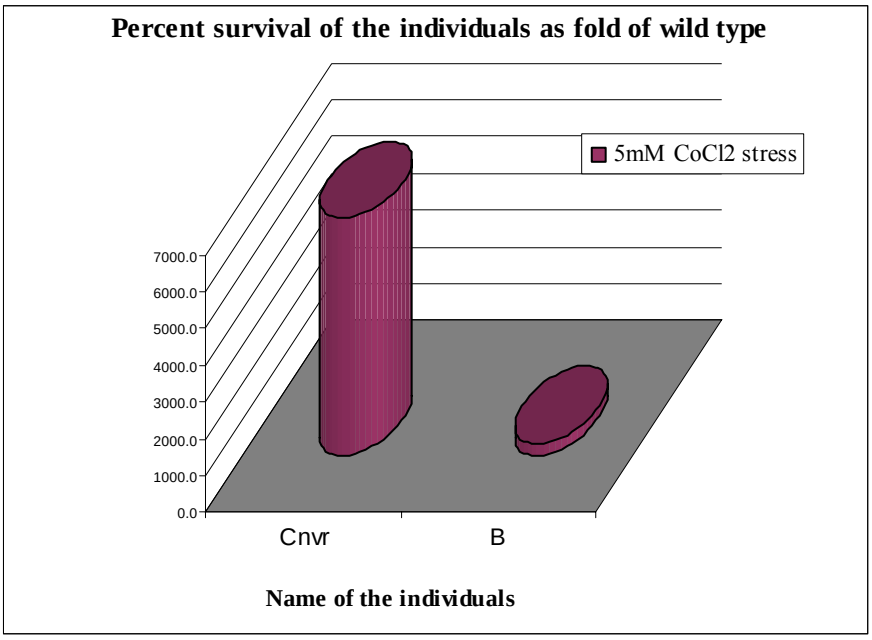


Figure 3.5 *Cnvr*,and *B* per cent survival ratio as fold of wild type after continuous 5mM $CoCl_2$ stress

Table 3.11 *Cnvr*,and *B* per cent survival ratio as fold of wild type after continuous 5mM $CoCl_2$ stres

Name of the individuals and population	Survival as fold of wild type (after 5mM $CoCl_2$)
Cnvr	6441
B	316

Cnvr and B had 6441 and 316 fold of wild type survival. Neither constant stress individuals nor increasing stress individuals could reach these higher fold survival values.

3.4. Determination of various other stress resistances

3.4.1 Copper stress

These precultures of the selected individuals and final population were tested in order to see their copper stress resistances. Five tube MPN method was used in order to determine their continuous $CuCl_2$ stress resistance. As stress levels 0.5 and 1mM $CuCl_2$ were selected. The cultures were incubated at 30ºC, for 96h, upon which per cent survival ratios of the cultures were calculated (Table 3.12).

Table 3.12 Per cent survival ratios of individuals and populations for copper stress after 96 h incubation at 30ºC

Population / individual name	% survival at 0.5mM $CuCl_2$	% survival at 1mM $CuCl_2$
100	0.00	0.00
36C	0.01	0.00
36G	0.03	0.00
5C	1.74	0.00
6C	0.04	0.00
I1	0.00	0.00
I3	0.59	0.00
I8	0.44	0.00
I10	0.07	0.00
Cnvr	44.44	0.01
W	0.02	0.15
B	0.00	0.01

The per cent survival ratios demonstrated that *5C, I3, I8* and *Cnvr* were also resistant to copper stress (Table 3.12). Not only these individuals but also the others had higher per cent survival ratio when compared with wild type that could not survive in the presence of $CuCl_2$ at all.

3.4.2 Chromium stress

By applying 5-tube MPN method, continuous $CrCl_3$ stress resistance of *100,* final population and the individuals were investigated. Two different stress levels (2.5 and 5mM $CrCl_3$) were applied to these cultures and they were incubated at 30ºC for 96 h. Their per cent survival ratios are shown in the Table 3.13.

Table 3.13 Per cent survival ratios of individuals and populations for chromium stress after 96 h incubation at 30ºC

Population / individual name	% survival at 2.5mM $CrCl_3$	% survival at 5mM $CrCl_3$
100	0.26	0.00
36C	0.44	0.00
36G	0.34	0.03
5C	383.33	225.00
6C	1.00	0.01
I1	0.46	0.01
I3	1.00	0.01
I8	0.44	0.00
I10	0.65	0.00
Cnvr	51.85	4.44
W	31.48	0.00
B	0.46	0.01

A cross-tolerance to chromium was observed in *5C, Cnvr,* and *W* with high per cent survival ratios (Table 3.13).

3.4.3 Heat stress

Pulse heat stress was applied at two different levels (52ºC and 80ºC) for 60 min. Survival of the cultures was determined by OD_{600} measurements of the samples which were exposed to pulse heat stress and their corresponding control groups. Table 3.14 shows the per cent survival ratios of the samples after 72 h of incubation at 30ºC and 150 rpm.

None of the mutants have significantly higher tolerance to heat stress than the wild type.

Table 3.14 Per cent survival ratios of population and the individuals for pulse heat stress application

Population / individual name	% survival at 52ºC pulse stress	% survival at 80ºC pulse stress
100	96	4
36C	77	3
36G	89	1
5C	66	0
6C	60	1
I1	98	1
I3	69	1
I8	97	1
I10	89	0
Cnvr	95	1

3.4.4 Ethanol stress

Pulse ethanol stress was applied to the populations and individuals. Cultures were tested according to their 20 % (v/v) ethanol pulse stress resistances.

Continuous ethanol stress was applied in 50 ml centrifuge tubes with 10 ml total liquid volume. Fresh cultures were inoculated to 5 and 10 % (v/v) ethanol containing YMM. After appropriate incubations, OD_{600} measurements were taken.

Table 3.15 Population and individuals per cent survival results upon pulse 20 % (v/v) ethanol stress application followed by incubation at 30ºC, 150 rpm, for 24, 48, and 72 h.

Population / individual name	% survival at 20 % (v/v) ethanol pulse stress after 24 h	% survival at 20 % (v/v) ethanol pulse stress after 48 h	% survival at 20 % (v/v) ethanol pulse stress after 72 h
100	100	92	89
36C	63	50	63
36G	1	56	49
5C	0	38	88
6C	1	77	78
I1	119	79	73
I3	1	32	78
I8	1	18	70
I10	1	35	68
Cnvr	1	64	69

At the end of 24 of incubation, 36th increasing stress generation and the individuals except *I1* could not tolerate pulse ethanol stress.

YMM containing 5 and 10 % (v/v) ethanol was used for continuous ethanol stress application, but the cultures could not tolerate 10 % (v/v) ethanol stress and neither of them could grow at this stress level (data not shown). When the per cent survival ratios were considered upon continuous 5 % (v/v) ethanol stress for 24 h incubation, it was observed that only wild type and 36th constant stress generation could tolerate this stress level.

Table 3.16 Population and individuals per cent survival results upon continuous 5 % (v/v) ethanol stress application after 24, 48 and 72 h of incubation at 30ºC

Population / individual name	% survival at 5% (v/v) ethanol continuous stress after 24 h	% survival at 5% (v/v) ethanol continuous stress after 48 h	% survival at 5% (v/v) ethanol continuous stress after 72 h
100	68	68	80
36C	87	86	63
36G	12	17	18
6C	8	12	16
I1	12	17	23
I3	8	11	11
I8	7	10	12
I10	5	9	9

3.4.5 Oxidative stress

Pulse and continuous oxidative stresses were applied to test for oxidative stress tolerance. When cultures were grown to their late exponential phase (OD_{600} 1.4-1.6), 1M H_2O_2 pulse oxidative stress was applied for 1 h at 30ºC and 150 rpm. This experiment was repeated twice and the average of per cent survival results of cultures from these experiments are shown on Table 3.17.

Table 3.17 Population and individuals per cent survival results after 1M H_2O_2 pulse stress application

Population / individual name	% survival 1M H_2O_2 pulse stress after 24 h
100	61.5
36C	1.5
36G	0.00
5C	0.50
6C	0.00
I1	0.00
I3	0.00
I8	0.50
I10	0.00
Cnvr	1.00

Only, the wild type (*100*) could tolerate 1M H_2O_2 pulse stress application.

Oxidative stress was applied continuously to cultures that were grown overnight. These cultures were inoculated into YMM containing 10mM and 20mM H_2O_2. After 48 and 72 h of incubation OD_{600} measurements were taken. Samples to which 20mM H_2O_2 was applied, could not grow at 24 h of incubation. These experiments were repeated three times.

Table 3.18 Population and individuals per cent survival results for 10 and 20mM H_2O_2 continuous stress application after 48 h

Population / individual name	% survival at 10mM continuous stress after 48 h	% survival at 20mM continuous stress after 48 h
100	8	29
36C	23	18
36G	20	17
5C	82	52
6C	42	37
I1	39	8
I3	23	4
I8	26	7
I10	20	1
Cnvr	29	1

After 48 h of incubation at 30ºC and 150 rpm, the individual *5C* had the highest per cent survival ratio upon continuous 10 and 20mM H_2O_2 stress application.

Table 3.19 Population and individuals per cent survival results after 10 and 20mM H_2O_2 continuous stress application after 72 h

Population / individual name	% survival at 10mM H_2O_2 continuous stress after 72 h	% survival at 20mM H_2O_2 continuous stress after 72 h
100	55	1
36C	48	9
36G	57	31
5C	87	73
6C	56	31
I1	80	19
I3	53	18
I8	37	14
I10	53	20
Cnvr	43	0

Additionally, at the end of 72 h of incubation, *5C* was the most resistant culture that could tolerate either 10mM or 20mM H_2O_2.

3.4.6 Osmotic stress

Osmotic stress was applied by both using pulse and continuous stress strategy. Two stress condition (15 and 30 % (w/v) NaCl) were selected for pulse stress application. Continuous osmotic stress was applied by using 5-tube MPN method, and 5 and 10 % (w/v) NaCl stresses were applied continuously in 96 well plates.

OD_{600} values of pulse stress applied samples were determined after 24 h of incubation and per cent survival values are shown on Table 3.20.

Table 3.20 Population and individuals per cent survival results after 15 and 30 % (w/v) NaCl pulse stress application after 24 h

Population / individual name	% survival at % 15 NaCl pulse stress after 24 h	% survival at % 30 NaCl pulse stress after 24 h
100	104	109
36C	83	108
36G	90	121
5C	121	76
6C	91	82
I1	141	132
I3	106	142
I8	100	150
I10	93	97
Cnvr	61	95

The results showed that individuals and populations could tolerate pulse NaCl stress application. However, under 15 % and 30 % NaCl pulse stress application, *Cnvr, 5C*, and *6C* showed lower per cent survival ratios compared to others. Continuous NaCl stress was applied by using 5-tube MPN method with 5 and 10 % (w/v) NaCl stress levels.

Table 3.21 Population and individuals per cent survival results after 5 and 10 % (w/v) NaCl continuous stress application after 72 h

Population / individual name	% survival at % 5 NaCl continuous stress after 72 h	% survival at % 10 NaCl continuous stress after 72h
100	0	0
36C	0	19
36G	46	46
5C	100	58
6C	174	38
I1	170	170
I3	170	170
I8	58	22
I10	100	170
Cnvr	100	22

The results showed that final population of increasing stress and individuals gained a cross tolerance to continuous NaCl stress as well. Wild type could not survive under these NaCl stress conditions. However, the mutants survival ratios were as high as 100 per cent.

3.5 Determination of Metal Content Associated with Cells Using Flame Atomic Absorption Spectrometer (FAAS)

Five mM $CoCl_2$ stress was applied continuously to final populations and individuals on the 50 ml centrifuge tubes. These cultures were incubated at 30ºC and 150 rpm for 48 h. At the end of incubation, their cell dry weights (CDWs) were determined and the cell pellets were hydrolyzed by using 6M HNO_3. After making 1:2 dilution of the cultures, their cobalt contents were measured by using flame atomic absorption spectrometer (FAAS) with 242.5 nm wavelength and 0.2 nm slit with. Initial cobalt content in the medium that contained 5mM $CoCl_2$ corresponded to 290 ppm.

3.5.1 Cobalt content associated with cells

In order to determine cobalt content associated with populations and individuals that were grown both in the YMM and 5mM $CoCl_2$ containing YMM, culture pellets were used. The results that were read on the FAAS were multiplied with the dilution factors and cobalt amount (mg/l) was calculated for the cultures. These values were divided to initial cobalt content in the medium (0 mg/l for YMM and 290 mg/l for 5mM $CoCl_2$ containing medium) in order to calculate per cent cobalt content associated with cells (Table 3.22).

Table 3.22 Per cent cobalt associated with cells that were grown in YMM and in YMM containing 5mM $CoCl_2$ as determined by atomic adsorption spectrometry

Population / individual name	% cobalt hold by the cell on the YMM	% cobalt hold by the cell on the 5mM $CoCl_2$ containing YMM
100	0	0.66
36C	0	0.39
36G	0	0.38
5C	0	0.38
6C	0	0.39
I1	0	0.39
I3	0	0.55
I8	0	0.55
I10	0	0.36
Cnvr	0	0.36

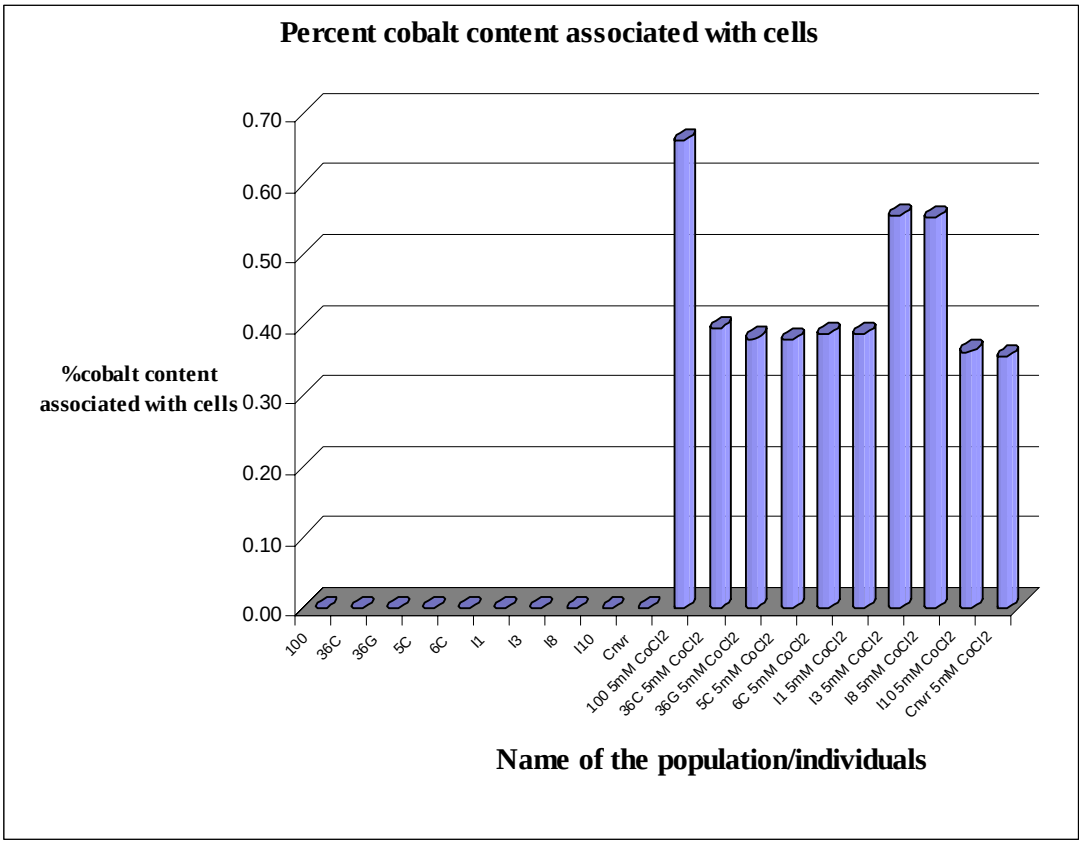


Figure 3.6 Populations and individuals per cent cobalt associated with cells

According to these results, *100, I3* and I8 hold the most cobalt in their pellets. Cobalt content according to culture volume (mg/ml) was divided to cell dry weight per ml (mg/ml) and by this way "cobalt weight" / "cell dry weight" values for each culture were obtained. Populations and individuals per cent cobalt weight / cell dry weight are shown in Figure 3.7.

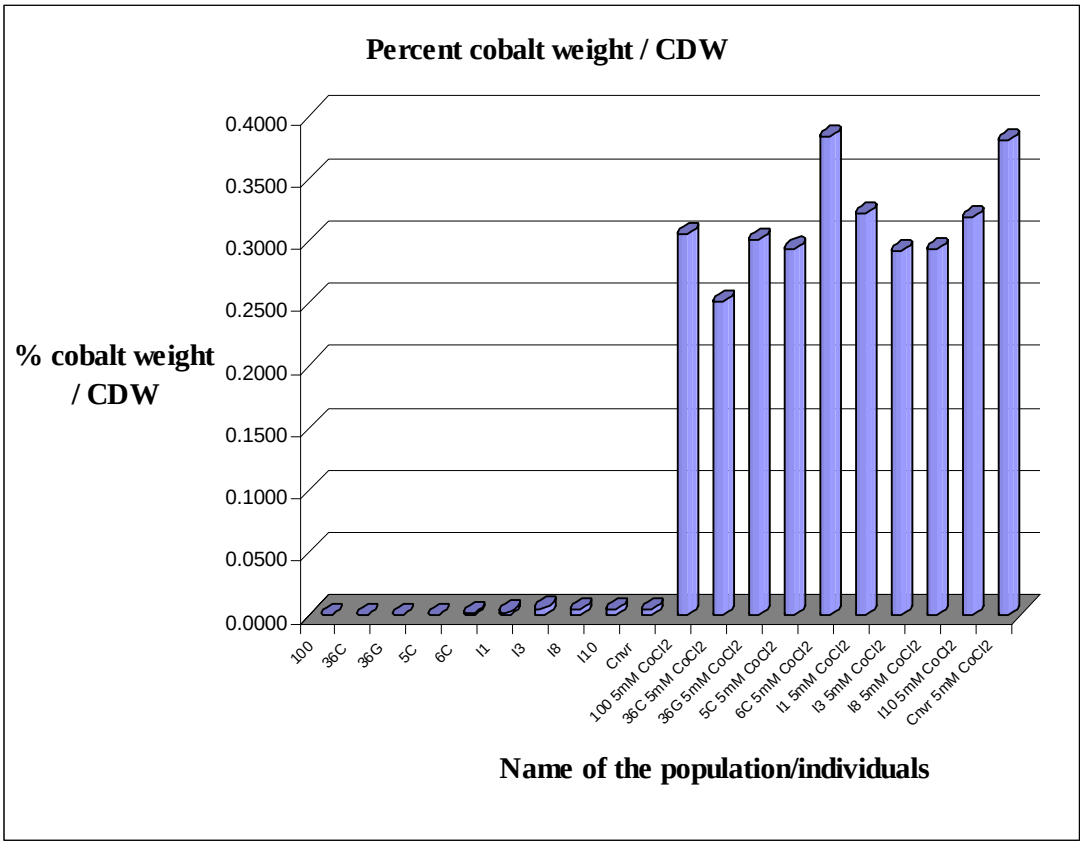


Figure 3.7 Populations and individuals per cent cobalt weight / cell dry weight values

3.6 Colour Changes of Individuals

33rd increasing stress generation was inoculated into solid YMM containing 20mM $CoCl_2$ and incubated at 30ºC. On this plate some colonies with different colours (white and brown) appeared. Two of the white and one of the brown colonies were selected and named "*Cnvr*, *W*, and *B*" as shown on the Table 3.10.

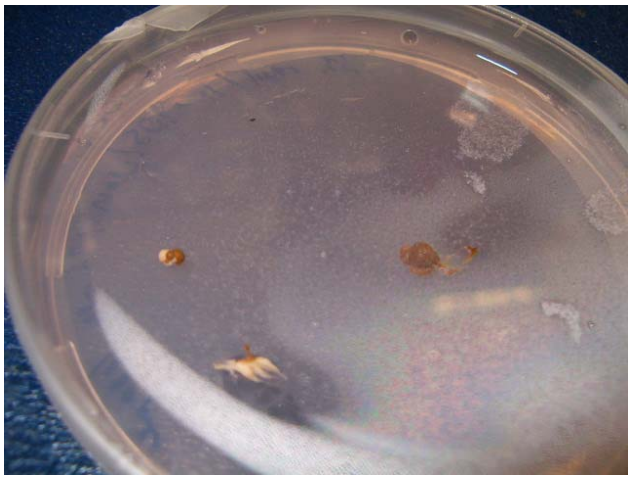


Figure 3.8 YMM containing 20mM $CoCl_2$ where *Cnvr, W*, and *B* were obtained and the other different coloured individuals

These individuals and *100* were inoculated into YMM containing 5mM $CoCl_2$ and 1mM $CuCl_2$. The colour of 5mM $CoCl_2$ and 1mM $CuCl_2$ containing YMM that were inoculated with *B* were changed to brown; whereas, control YMM colour was white (Fig. 3.9). *W* and *100* in YMM containing 5mM $CoCl_2$ were white-pink. YMM containing 1mM $CuCl_2$ was yellow for *W* and white for *100*. Moreover, *100* and *W* in YMM were both white (Fig 3.10 and 3.11).

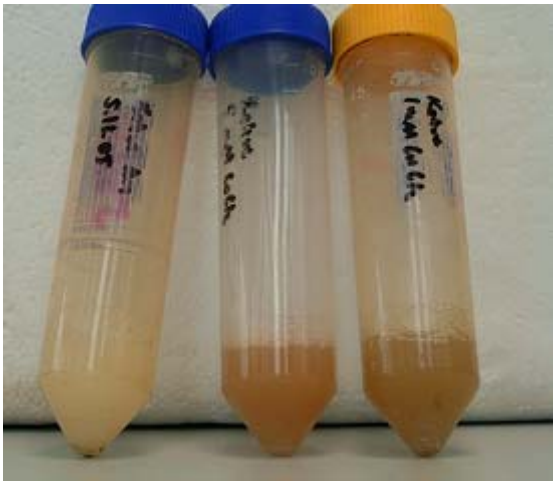


Figure 3.9 *B* grown into YMM, YMM containing 5mM $CoCl_2$ and 1mM $CuCl_2$ (from left to right)

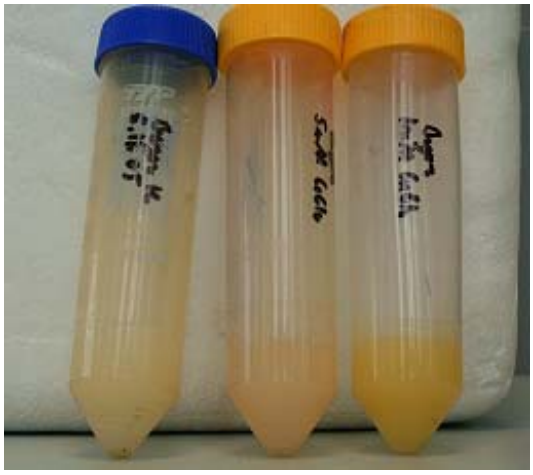


Figure 3.10 *W* grown into YMM, YMM containing 5mM $CoCl_2$ and 1mM $CuCl_2$ (from left to right)

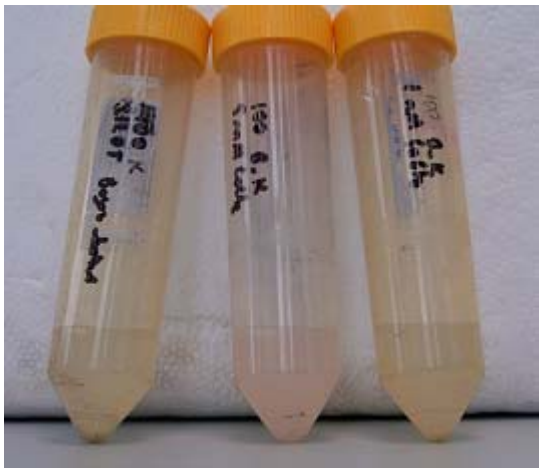


Figure 3.11 *100* grown into YMM, YMM containing 5mM $CoCl_2$ and 1mM $CuCl_2$ (from left to right)

100, 5C, Cnvr, W and *B* were inoculated into solid YMM containing 3mM $CuCl_2$ and 5mM $CoCl_2$ and solid YMM as control. In solid YMM, there were only white colonies for all these cultures. *5C* colonies with brown and white colours were observed on solid YMM containing 3mM $CuCl_2$ and 5mM $CoCl_2$. *Cnvr* brown and white colonies were observed on solid YMM containing 5mM $CoCl_2$ and only white colonies were observed on the solid YMM containing 3mM $CuCl_2$. *W* brown and grey colonies were observed on solid YMM containing 3mM $CuCl_2$. On solid YMM containing 5mM $CoCl_2$, only white colonies of *W* appeared. *B* colonies with brown and white colours were observed on solid YMM containing 5mM $CoCl_2$. Wild type

(*100*) colonies were always white in all metal-containing YMM. On solid YMM containing 5mM $CoCl_2$, the medium colour of *5C* and *Cnvr* turned to bright yellow.

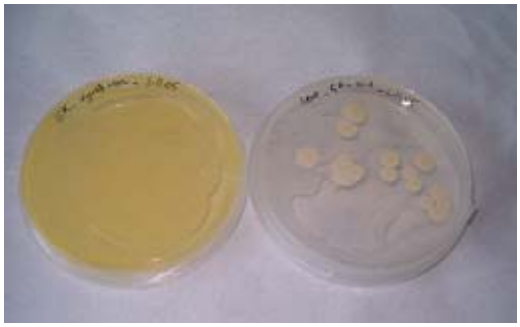


Figure 3.12: *5C* on YMM containing 5 mM $CoCl_2$ and YMM from left to right



Figure 3.13: *Cnvr* YMM containing 5 mM $CoCl_2$ and YMM from left to right

3.7 Genetic stability test

Genetic stability test was applied to final generations and some of the individuals. These cultures were inoculated to YMM and YMM containing 5mM $CoCl_2$. After 48h incubation at 30ºC and 150rpm, OD_{600} measurements were taken in order to determine survival ratios. These cultures that were grown in YMM were named as the 1^{st} generation. Another inoculation was done from this first generation to nonselective medium (YMM without $CoCl_2$), to both YMM and YMM containing 5mM $CoCl_2$ in order to obtain 2^{nd} generation after 48 h incubation. The 3^{rd}, 4^{th}, and 5^{th} generations were obtained by repeating the same procedures. The final generations and individuals OD_{600} values in YMM, YMM containing 5mM $CoCl_2$ and survival ratios after 48 h incubation at 30ºC and 150 rpm are shown in Table 3.23 and Table 3.24, respectively.

After 5 successive cultivations in non-selective YMM (i.e. without $CoCl_2$), no decrease in the survival ratios of the final generations was observed.

The survival ratios of mutants also did not decrease after the same procedure was applied. As a result, by using evolutionary engineering methodology, cobalt stress resistant mutants with genetic stability were obtained.

Table 3.23 OD_{600} values in YMM and YMM containing 5mM $CoCl_2$ and survival ratios of final generations

Culture name	Non-selective cultivation number	OD_{600} (48h) in 5mM $CoCl_2$-containing YMM	OD_{600} (48h) in YMM	Survival Ratio
36th constant stres generation	after 1st non-selective cultivation	9.60	5.85	0.61
	after 2nd non-selective cultivation	10.80	2.80	0.26
	after 3rd non-selective cultivation	11.60	4.00	0.34
	after 4th non-selective cultivation	15.20	6.80	0.45
	after 5th non-selective cultivation	9.20	7.00	0.76
36th increasing stres generation	after 1st non-selective cultivation	14.40	6.78	0.47
	after 2nd non-selective cultivation	13.20	7.60	0.58
	after 3rd non-selective cultivation	9.60	3.80	0.40
	after 4th non-selective cultivation	13.20	6.20	0.47
	after 5th non-selective cultivation	10.40	5.00	0.48

Table 3.24 OD_{600} values in YMM and YMM containing 5mM $CoCl_2$ and survival ratios of individuals

Culture name	Non-selective cultivation number	OD_{600} (48h) in 5mM $CoCl_2$-containing YMM	OD_{600} (48h) in YMM	Survival Ratio
C5	after 1st non-selective cultivation	14.00	7.71	0.55
	after 2nd non-selective cultivation	15.20	6.40	0.42
	after 3rd non-selective cultivation	13.60	5.20	0.38
	after 4th non-selective cultivation	11.60	5.80	0.50
	after 5th non-selective cultivation	10.40	7.75	0.75
C6	after 1st non-selective cultivation	16.40	6.12	0.37
	after 2nd non-selective cultivation	12.80	5.40	0.42
	after 3rd non-selective cultivation	10.00	4.60	0.46
	after 4th non-selective cultivation	12.40	5.00	0.40
	after 5th non-selective cultivation	12.00	7.00	0.58
I1	after 1st non-selective cultivation	12.40	5.99	0.48
	after 2nd non-selective cultivation	13.60	4.80	0.35
	after 3rd non-selective cultivation	10.00	4.00	0.40
	after 4th non-selective cultivation	15.60	5.60	0.36
	after 5th non-selective cultivation	12.00	6.75	0.56
I3	after 1st non-selective cultivation	18.80	4.79	0.25
	after 2nd non-selective cultivation	17.20	5.60	0.33
	after 3rd non-selective cultivation	8.40	4.80	0.57
	after 4th non-selective cultivation	12.80	6.80	0.53
	after 5th non-selective cultivation	11.20	5.25	0.47
I8	after 1st non-selective cultivation	17.60	7.05	0.40
	after 2nd non-selective cultivation	16.00	7.20	0.45
	after 3rd non-selective cultivation	11.20	5.60	0.50
	after 4th non-selective cultivation	14.80	5.40	0.36
	after 5th non-selective cultivation	10.80	6.25	0.58
I10	after 1st non-selective cultivation	16.80	5.59	0.33
	after 2nd non-selective cultivation	12.80	7.40	0.58
	after 3rd non-selective cultivation	14.00	4.60	0.33
	after 4th non-selective cultivation	16.40	6.60	0.40
	after 5th non-selective cultivation	10.40	5.00	0.48
Cnvr	after 1st non-selective cultivation	13.60	6.38	0.47
	after 2nd non-selective cultivation	14.00	6.40	0.46
	after 3rd non-selective cultivation	11.60	6.60	0.57
	after 4th non-selective cultivation	14.00	5.80	0.41
	after 5th non-selective cultivation	16.00	5.50	0.34

4. DISCUSSION AND CONCLUSIONS

The aim of this study was to obtain cobalt-resistant *Saccharomyces cerevisiae* cells by using evolutionary engineering strategies. For this purpose, a genetically diverse initial population of *S. cerevisiae* was obtained by EMS mutagenesis. The resistant generations were selected in the presence of pulse cobalt stresses. For this purpose, two different pulse cobalt stress levels were applied: “constant” and “increasing” stress levels. Mild cobalt stress (0.5 mM $CoCl_2$) was applied in order to obtain constant stress generations and cobalt stress was increased gradually between 0.5 mM, 5 mM and 10 mM to obtain increasing cobalt stress generations. After obtaining final constant and increasing stress generations, individuals were selected randomly from these final generations, and the ones with the highest cobalt resistance were determined and against other stress for the presence of any potential cross-tolerance.

Thirty six constant stress generations were obtained by applying pulse 0.5 mM $CoCl_2$ stress to each generations. The final generation and individuals were tested for their continuous cobalt stress resistances by using 5-tube MPN method. The per cent survival ratios of these cultures were determined as fold of wild type values. The 36^{th} constant stress generation and some of the individuals (for 0.5 mM $CoCl_2$ stress; *4C, 5C, 6C, 8C* and for 5 mM $CoCl_2$ stress; *4C* and *5C*) had higher per cent survivals than the wild type (*100*) for continuous 0.5 and 5 mM $CoCl_2$ stresses; whereas some individuals had lower per cent survivals than the wild type for the same stress conditions (Table 3.3.1.2.1). The most cobalt-resistant individual, for continuous 0.5 $CoCl_2$ stresses, were *4C* and *5C* having 9.92 and 5.37 fold of wild type survival ratio respectively. Additionally, cobalt resistance of *5C* upon 5 mM $CoCl_2$ was 15.43 fold of that of the wild type (*100*). The least cobalt-resistant individual, *7C*, had 0.45 fold of wild type survival ratio, which means that it is less resistant to cobalt than the wild type (Table 3.3.1.2.2). Four of the eight individuals had higher per cent survival than the wild type (*100*) for continuous mild (0.5mM) cobalt stress application. However, this ratio is 2:8 for continuous 5 mM $CoCl_2$ stress application (Table 3.3.1.2.2).

Constant stress generations were obtained by applying pulse 0.5 mM $CoCl_2$ stress and the individuals were selected from the final constant stress generation that had been selected for this stress level for 36 times. Thus, these individuals probably gained resistance to 0.5 mM $CoCl_2$ stress. This could explain the fact that more constant stress individuals had higher per cent survivals for continuous 0.5 mM $CoCl_2$ stress than for 5 mM $CoCl_2$ stress level. Individuals with varying per cent survival significantly different from that of the 36^{th} constant stress population demonstrated that the final population was highly heterogeneous.

Increasing stress generations were obtained by applying pulse $CoCl_2$ at gradually increasing concentrations up to 140 mM. A total of 36 increasing stress generations were obtained. By employing 5-tube MPN method, *100* and mutant individuals obtained from 36^{th} increasing stress generation were tested for their continuous cobalt stress resistance. *I3* had 12.79 and 77.0 fold of wild type survival ratio for continuous 0.5 and 5mM $CoCl_2$, respectively (Table 3.9). *I3* had much higher per cent survival ratio than 36^{th} increasing stress population for both of the stress levels applied. However, some individuals' (*I2, I6* and *I11*) per cent survival ratios were the same as that of the wild type (*100*) and lower than that of the 36^{th} increasing stress generation for continuous 5mM $CoCl_2$ stress application (Table 3.8). Heterogeneity is also valid for the 36^{th} increasing stress population.

The mutant individuals, *Cnvr, W* and *B* had been obtained from the 33^{rd} increasing stress generation that was inoculated on solid YMM containing 20mM $CoCl_2$. According to the results obtained from the continuous 5 mM $CoCl_2$ stress application by 5-tube MPN method, these individuals showed the highest survival ratio as fold of wild type, which were 6441 and 316 for *Cnvr* and *B*, respectively (Table 3.11).

Cobalt-resistant *S. cerevisiae* mutants were obtained by using evolutionary engineering methodology under constant or increasing pulse $CoCl_2$ stress selection conditions. It was observed that the individuals with the highest cobalt resistance were obtained from the increasing stress population, especially when the individuals were selected from a medium containing high cobalt concentrations, as in the case of *Cnvr* and *B*.

Similar methodologies had been applied in order to obtain organisms with desired phenotypes. Ferea *et al.* (1999) suggested that "Culturing a population of *S. cerevisiae* for many generations under conditions to which it is not optimally adapted selects for fitter genetic variants." and added that "This simple experimental design provides a tractable model of adaptive evolution under natural selection." Moreover, they had continuously grown *S. cerevisiae* cultures aerobically in glucose-limited chemostats and 250 or more generations were obtained. As a result of this process, the final cultures gained the ability to use glucose more efficiently than the beginning cultures.

Cobalt contents associated with wild type (*100*), and the mutants obtained from the final generations (*C5, C6, I1, I3, I8, I10*, and *Cnvr*) were measured by using FAAS. According to these measurements, it was found that the wild type (*100*) had the highest metal content associated with its cells compared to final generations and individuals. The wild type (*100*) had 1.7 and 1.9 times more cobalt in its cells than *C5* and *Cnvr,* respectively, which were the most cobalt-resistant individuals. However, the cobalt content associated with *100* was 1.2 times higher than the most cobalt stress resistant increasing stress individuals: *I3* and *I8* (Table 3.22 and Figure 3.6). According to these results, it can be suggested that those individuals most resistant to cobalt stress, seem to be excluding the metal outside the cells. Gadd *et al*. (1986) trained 3 different strains of *S. cerevisiae* with increasing cobalt concentration in solid medium through successive generations and they called the final cultures as trained cells. Then, through 5 generations (at least), these trained cells were incubated on solid media without metal and named as "detrained". When the control, trained and detrained strains were measured for their cobalt uptake rate in a cobalt-containing medium it was observed that the cobalt uptake rate of the trained cells were much lower than that of the control strain. The same results were also obtained for detrained strains with one exception where the cobalt uptake rate was close to that of the control strain. These results are consistent with our results of FAAS since the wild type holds cobalt much more than the mutant individuals, especially the one with the highest cobalt resistance (*Cnvr*). In another study, Joho *et al.* (1991) used mutant *S. cerevisiae* resistant to Co^{+2} and Ni^{+2} and they showed that this resistant strain had reduced uptake of cobalt compared to wild type. In another study, it was shown that vanadate $(VO_4)^{-3}$ resistant *Neurospora,* $(PO_4)^{-2}$ transport system did not

work and because of this defect $(VO_4)^{-3}$ could not enter the cells, thus the resistance is suggested to be originated from holding metals outside of the organisms (Bowman *et. al*, 1983).

Venkateswerlu *et al*. (1973) obtained a cobalt resistant *Neurospora crassa* strain by subculturing spores on the medium that contained cobalt and they increased cobalt concentration throughout each generation. In this way they obtained a population that could grow in cobalt-containing medium where the wild type could not. They have obtained individuals and determined that one of these individuals had ten-fold cobalt resistance than the wild type. They have determined the cobalt content of the wild type and the cobalt-resistant individual in cobalt containing medium. Cobalt-resistant individual associated 5.77-fold of the cobalt content of the wild type strain. Although the methods to obtain metal-resistant individuals in this study is very similar to our study, the difference in the metal holding pattern between our generations and individuals may result from the fact that *N. crassa* metal tolerance mechanisms may be different than *S. cerevisiae*. Bruins *et al*. (2000) stated that "Active transport of efflux systems represent the largest category of metal resistance systems." They also stated that in order to transport metals from their cytoplasm, active transport systems are used by microorganisms. When this metal resistance mechanism is considered, it can be suggested that the cobalt resistant individuals can be more efficient in transporting toxic metals out of the cells.

Either in the nature or in industrial applications, there are various stresses that *S. cerevisiae* cells are exposed to. The most cobalt resistant individuals (*C5, C6, I1, I3, I8, I10*, *Cnvr*, *W* and *B)*, wild type and the final populations were screened for some of these stress resistance capacities.

Other continuous metal stress resistances of these cultures were also investigated. By using 5-tube MPN method, continuous 0.5 and 1mM $CuCl_2$ stress were applied to these cultures. At the end of the 96 h incubation at 30ºC, *Cnvr* showed 44.4 % survival for continuous 0.5 mM $CuCl_2$ stress application (Table 3.12). The wild type (*100*) could not grow at this continuous $CuCl_2$ stress level and its per cent survival was only 0.00069. For this reason, *Cnvr* had 64412 fold of the wild type survival ratio. *5C* had 1.74 per cent survival which could be considered as a very low per cent

survival, but *5C* had 2520 fold of wild type per cent value which demonstrates that it could tolerate continuous $CuCl_2$ stress much better than the wild type (Table 3.12). Moreover, it can be suggested for these individuals that a cross-resistance for copper exists. Gadd *et al*. (1986) also observed co-tolerance for copper in their cobalt-resistant strains.

Continuous 2.5 and 5 mM $CrCl_3$ stress were applied by using 5-tube MPN method. At the end of 96 h of incubation at 30ºC, *Cnvr* and *W* showed 51.85 and 31.48 per cent survival; whereas the wild type (*100*) had only 0.26 per cent survival for the same stress level (Table 3.13). These values were 199.42 and 121.07 fold of the wild type value, respectively. According to these results, a cross tolerance for chromium can also be pronounced. Yi Hu *et al*. (2003), made *S. cerevisiae* proteome analysis upon 15 different metal stresses by using two-dimensional differential gel electrophoresis. It was observed that different metals can change protein expression similarly and concluded that these proteins were expressed in order to provide an overall cell survival mechanism. However, there were some proteins which were expressed differently only in the presence of one type of metal. They measured 15 different metal effects on protein up/down regulation in yeast proteome quantitatively. According to Yi Hu *et al.* (2003), in the presence of toxic metals like Cu, Cd, Co, Cr, Mn, Ag, Li, and Zn, more upregulation of the analyzed proteins was observed than of downregulation. However, in the case of other (less toxic) metals, the upregulation was similar or lower compared to downregulation for the same proteins. Based on these results, Co^{+2}, Cu^{+2}, and Cr^{+3} could be classified together for their effects on upregulation of some proteins, and the cross resistance for Cu^{+2} and Cr^{+3} can be explained.

A significant cross-tolerance was observed to continuous NaCl stress for all tested individuals and final generations. By employing 5-tube MPN method, NaCl continuous stress was performed. Although wild type could not survive (0.0 per cent survival) after continuous 5 % (w/v) NaCl stress application, *6C* showed 174, *I1* and *I3* showed 170, *5C*, *I10* and *Cnvr* showed 100 per cent survival (Table 3.21).

Pulse and continuous oxidative stresses were also applied to the mutant individuals, final populations and wild type (*100*). Neither individuals nor final populations could

resist pulse 1M H_2O_2 stress; whereas the wild type had 61.5 per cent survival (Table 3.17). Interesting results were obtained when the type of the stress application changed from pulse to continuous. Under continuous 10 mM H_2O_2 stress application, individuals and final populations had higher survival ratios than the wild type, although they could not survive 1M H_2O_2 pulse stress application (Tables 3.18 and 3.19). For all H_2O_2 concentrations tested, *5C* was the most resistant individual to continuous H_2O_2 stress.

The cultures were also tested for a possible cross-resistance to pulse heat stress. However, no cross-resistance to pulse heat stress was observed among the mutant individual (Table 3.14).

Ethanol cross-resistance test were also performed. The results indicated that the mutants did not gain any cross-resistance to pulse and continuous ethanol stress (Tables 3.15 and 3.16).

Genetic stability tests were also performed to the final generations and most cobalt resistant individuals. After five selective cultivation in non-selective medium (YMM without $CoCl_2$), there was no decrease in the cobalt resistances of the mutant individuals. Thus, it can be concluded that the mutants are genetically stable (Table 3.23 and Table 3.24).

To conclude, evolutionary engineering was successfully applied in this system to obtain cobalt-resistant *S. cerevisiae* mutants under pulse cobalt stress as selection conditions. Detailed transcriptomic and proteomic analysis of the mutants should be performed next in order to reveal the genetic basis of this phenotype. These mutants and the information to be obtained from them could potentially be used for practical applications in the fields of bioremediation and/or biomimetics.

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RESUME

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