ISTANBUL TECHNICAL UNIVERSITY★INSTITUTE OF SCIENCE AND TECHNOLOGY

INVERSE METABOLIC ENGINEERING OF ALUMINIUM-RESISTANT *Saccharomyces cerevisiae*

M.Sc. Thesis by

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Department of Molecular Biology – Genetics and Biotechnology

Molecular Biology – Genetics and Biotechnology Programme

DECEMBER 2015

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Thesis Advisor : Prof.Dr. Zeynep Petek ÇAKAR

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İSTANBUL TEKNİK ÜNİVERSİTESİ ★ FEN BİLİMLERİ ENSTİTÜSÜ

TERSİNE METABOLİK MÜHENDİSLİK YÖNTEMİ İLE ALUMİNYUMA DİRENÇLİ *Saccharomyces cerevisiae* ELDESİ

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To my mom and dad,

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ABBREVIATIONS

EMS : Ethyl Methane Sulfonate

LP : Last Population

µg : Microgram

µL : Microliter

mM : Millimolar

mg : Milligram

mL : Milliliter

MPN : Most Probable Number

WT : Wild Type

YMM : Yeast Minimal Medium

YPD : Yeast Extract Pepton Dextrose

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INVERSE METABOLIC ENGINEERING OF ALUMINIUM-RESISTANT *Saccharomyces cerevisiae*

SUMMARY

Although Al^{3+} is non-essential and toxic to life, it is commonly encountered in water, foods, medicines and beverages. Al^{3+} has been reported to interact with organic molecules *in vitro*, however, molecular mechanisms of aluminium toxicity/tolerance are not well-known. Aluminium is also related with human neurodegenerative diseases such as Alzheimer and Parkinson.

The yeast *Saccharomyces cerevisiae* is a model organism which has been used widely in molecular biology and biotechnology for a long time. In addition to research, it has also been used for many industrial applications. It can make fermentation which is very important in baking and wine industry.

S.cerevisiae can be in haploid and diploid form and its genome has a high degree of homology with those of higher eukaryotes. This makes it important in understanding molecular mechanisms of human-related diseases.

In this study, *S. cerevisiae* was used as a eukaryotic model organism to investigate aluminium tolerance mechanisms. An evolutionary engineering strategy based on batch selection at increasing Al^{3+} concentrations was employed to obtain aluminium-resistant *S. cerevisiae* mutants: initially, *S. cerevisiae* CEN.PK 113-7D wild type strain was chemically mutagenized to increase the genetic diversity of the initial population for selection. 905 (Wild Type) and 906 (EMS mutagenized wild type) were screened under different aluminium levels such as; 0.05, 0.1, 0.4, 0.6, 1, 2, 5, 10, 25, 50 mM to determine the initial aluminium stress level to be used during increasing stress level selections. A batch selection strategy was then applied to obtain aluminium-resistant *S. cerevisiae* mutants by gradually increasing aluminium-stress levels from 0.5 to 21.5 mM $AlCl_3$ through 43 passages.

The 43^{th} last population was spread on plates and 12 individual mutants were randomly chosen. They were tested for their aluminium resistance by using spot assay procedure. According to spot assay results, 4 highly resistant mutants (named as Alu9, Alu10, Alu11, Alu12) were chosen for further analysis.

In order to quantify the aluminium resistances of the mutants in detail Most Probable Number method was applied to those 4 mutant individuals. They were then tested for the genetic stability of the Al-resistance.Results showed that the mutants are genetically stable.

Cross-resistance tests were also applied using a variety of other metal and non-metal stress types to test if the aluminium-resistant mutants developed cross-resistance or sensitivities to other stress types. For this purpose, the following stresses were tested: 8%(v/v) Ethanol, 0.2 mM $NiCl_2$, 0.7 mM H_2O_2 , 0.25 mM $CuSO_4$, 1 ml/L phenyl ethanol, 1.3 M $MgCl_2$, 2.5 mM $CrCl_3$, 10 mM $MnCl_2$, 30 mM $FeSO_4$, 80 mM H_3BO_3, 300 mg/ml Propolis, 0.5 M $CaCl_2$, 2 mM $GaNO_3$, 75 μM $AgNO_3$, 0.20

$NiCl_2$, 10 mM NaCl, 3 mM $ZnCl_2$, 1 mM $CoCl_2$, 2 mM LiCl, pH 4, 25 mM $FeCl_2$. However, no significant cross-resistance or sensitivities to other stress types were detected, except for a slight level of LiCl,$GaNO_3$ and ethanol tolerance, and a slight sensitivity to $AgNO_3$.

According to the experimental results, the best individual mutant (Alu10) was chosen for further detailed analyses at physiological and molecular levels.

TERSİNE METABOLİK MÜHENDİSLİK YÖNTEMİ İLE ALUMİNYUMA DİRENÇLİ *Saccharomyces cerevisiae* ELDESİ

ÖZET

Aluminyum (Al^{+3}) toksik olmasına rağmen suda, ilaçlarda, yiyecek ve içeceklerde aluminyum ile karşılaşılmaktadır. Aluminyumun organizmada organik moleküllerle etkileştiği ortaya konmasına rağmen toksisite veya tolerans mekanizması yeterince bilinmemektedir. Günlük hayatta birçok şekilde maruz kalınan aluminyum, kalp kapakçığında birikerek hastalık nedeni olabilmektedir. Bununla beraber, beyinde plak oluşturarak Parkinson ve Alzheimer gibi nörodejeneratif hastalıklarla da ilişkili olduğu bilinmektedir. Buna karşın, böbrek hastalarına diyaliz ile birlikte tedavi amaçlı verilen ilaçlar gibi aluminyum içeren ilaçlar da mevcuttur. Bu durum birçok tartışmayı beraberinde getirmektedir.

Saccharomyces cerevisiae moleküler biyolojide ve biyoteknolojik uygulamalarda uzun süredir kullanılan bir model organizmadır. Hızlı üremesi ve üreme koşullarının ekonomik olması başlıca tercih sebeplerindendir. Haploid ve diploid olarak bulunabilir ve insan gibi yüksek ökaryotlarla büyük oranda genom homolojisine sahiptir. Bu yönüyle *S.cerevisiae* insanla ilişkilendirilmiş hastalıklar üzerinde yapılan çalışmalarda kullanılmaktadır. Hastalık mekanizmalarının moleküler düzeyde incelenmesine ve metabolik yolakların anlaşılmasına katkıda bulunmaktadır. *S.cerevisiae* bilimsel araştırmaların yanı sıra, endüstride de büyük ölçüde tercih edilmektedir. Fermentasyon özelliği nedeniyle, fırıncılıkta ve alkollü içecek endüstrisinde yaygın olarak kullanılmaktadır.

Bu çalışmada, *S.cerevisiae* mayası aluminyum tolerans mekanizmasının incelenmesine yönelik model organizma olarak kullanılmıştır. Bu amaçla tersine metabolik mühendislik yöntemi kullanılarak, *S.cerevisiae* giderek artan aluminyum stres düzeylerinde, ardarda yapılan kesikli kültürlerle üretilerek, aluminyuma yüksek direnç gösteren mutant maya suşları, seleksiyon yoluyla elde edilmiştir. Çalışmada *S.cerevisiae* CEN.PK 113-7D suşu kullanılmıştır. Bu suş kimyasal mutajen (EMS) kullanılarak rastgele mutasyona uğratılmıştır. Bu işlem sonucunda, yüksek genetik çeşitliliğe sahip bir populasyon elde edilmiştir. Kimyasal mutasyona uğratılmayan yaban tip suş *905* olarak adlandırılırken, mutasyona uğratılmış populasyon *906* olarak adlandırılmıştır.

Çalışmaya başlamadan önce aluminyumun bu suşlar üzerindeki inhibisyon etkisinin belirlenmesi amacı ile aluminyum varlığında tarama testi yapılmıştır. Bunun için *905* ve *906* suşları, 0.05mM, 0.1mM, 0.4mM, 0.6mM, 1mM, 2mM, 5mM, 10mM, 25mM, 50mM $AlCl_3$ varlığında inkübe edilmiştir. Bu deneyin sonuçlarına göre seleksiyon deneyleri için başlangıç konsantrasyonu 0.5 mM, adım büyüklüğü de 0.5 mM olarak belirlenmiştir.

906 populasyonu ilk olarak 0.5 mM $AlCl_3$'e maruz bırakılmıştır. 24 saatlik inkübasyondan sonra hayatta kalan bireyler 1mM $AlCl_3$'e maruz bırakılmıştır. Bu işlem, hayatta kalma oranı kritik seviyeye düşene kadar tekrar edilmiştir. Bu şekilde

aluminyuma dirençli (0.5 mM-21.5 mM) 43 populasyon elde edilmiştir. 43. populasyonun 10^{-6}'ya kadar dilüsyonu yapılmış ve katı YMM besiyerine ekilmiştir. 72 saat sonra oluşan kolonilerden 12 tanesi rastgele seçilmiştir. Seçilen kolonilerin aluminyum direnci açısından kıyaslanması ve daha yüksek direnç gösterenlerin seçilmesi amacı ile spot assay yöntemi uygulanmıştır. Bu yöntemde seçilen 12 birey, farklı konsantrasyonlarda aluminyum içeren katı besiyerine 10^{-8}'e kadar seyreltilerek ekilmiştir. Spot assay sonucuna göre Alu9, Alu10, Alu11 ve Alu12 adlı mutant bireyler diğer 8 mutanta göre daha yüksek seviyede aluminyum direnci göstermiştir. Bu nedenle detaylı analizler için bu dört mutant seçilmiştir.

Mutant bireylerin aluminyum direncinin nicel olarak belirlenmesi için En Muhtemel Sayı (MPN) yöntemi uygulanmıştır. Bu yöntemde seçilen 4 birey, 10 mM ve 15 mM $AlCl_3$ varlığında 96-well plate içerisinde 5 tekrarlı olarak inkübe edilmiş ve koloni oluşumlarına göre direnç düzeyleri kıyaslanmıştır. MPN metodu ile istatistiksel olarak %95 güvenilirlikle direnç düzeyleri nicel olarak belirlenmiştir.

MPN sonuçları göz önüne alınarak, bireylerin aluminyum direnci yaban tip suş olan *905* ile kıyaslanmış ve başarılı bir şekilde direnç kazandırıldığı anlaşılmıştır. Gözlenen direncin kalıcı bir mutasyon mu yoksa bir adaptasyon mu olduğunu belirlemek amacı ile genetik kararlılık testi uygulanmıştır. Bu testte seçilen 4 birey aluminyum içermeyen YMM besiyerinde büyütülmüştür. 24 saat inkübasyondan sonra aluminyum içermeyen taze besiyerine aktarılmış ve bu işlem 7 gün(7 pasaj) boyunca devam ettirilmiştir. Her gün alınan örneklerin aluminyum direnç seviyeleri MPN metodu uygulanarak incelenmiştir. Bireyler aluminyum içermeyen besiyerinde inkübe edildikten sonra aluminyum içeren besiyerine aktarılmış, hayatta kalma oranları belirlenmiş ve zamanla bu oranın düşmediği görülmüştür. Sonuç olarak; seçilen bireylerdeki direncin ortam adaptasyonu değil, genetik düzeyde kalıcı mutasyonlar olduğu anlaşılmıştır.

Aluminyum direnci kazanan bireylerin farklı stres türlerine karşı direnç veya hassasiyet kazanıp kazanmadıklarını araştırmak amacı ile çapraz direnç testi uygulanmıştır. Bu testte Alu 9, Alu10, Alu11, Alu12, 905 (WT) ve 43. Nesil (LP) stres içeren katı besiyerine seyreltilerek ekilmiş ve 72 saat inkübe edilmiştir. Üreme durumlarına göre bireyler kıyaslanmıştır. Bu amaçla; hacmen %8 Etanol, 0.2 mM $NiCl_2$, 0.7 mM H_2O_2, 0.25 mM $CuSO_4$, 1 ml/L feniletanol, 1.3 M $MgCl_2$, 2.5 mM $CrCl_3$, 10 mM $MnCl_2$, 30 mM $FeSO_4$, 80 mM H_3BO_3, 300 mg/ml Propolis, 0.5 M $CaCl_2$, 2 mM $GaNO_3$, 75 µM $AgNO_3$, 0.20 $NiCl_2$, 10 mM NaCl, 3 mM $ZnCl_2$, 1 mM $CoCl_2$, 2 mM LiCl, pH 4, 25 mM $FeCl_2$ denenmiştir. Ancak alüminyum direnci kazanan bireylerin, test edilen farklı streslere karşı önemli ölçüde direnç veya hassasiyet geliştirmedikleri gözlenmiştir. Yalnızca LiCl, $GaNO_3$ ve etanole karşı hafif düzeyde çapraz direnç; $AgNO_3$'e karşı ise hafif düzeyde hassasiyet gözlenmiştir. Spot assay, genetik kararlılık ve çapraz direnç sonuçları göz önüne alınarak, Alu10 mutantı detaylı moleküler ve fizyolojik analizler yapılmak üzere seçilmiştir.

Sonuç olarak; yüksek konsantrasyonda $AlCl_3$'a direnç gösterebilen bir *S.cerevisiae* suşu elde edilmiştir. Bu suşun alüminyum direncinin genetik açıdan kararlı olduğu da görülmüştür. Çapraz direnç testi uygulanarak alüminyum ile birlikte farklı streslere karşı hassasiyet veya direnç geliştirip geliştirmediği analiz edilmiş ve bazı stress türleri için hafif düzeyde direnç/hassasiyet gözlenmiştir. Daha sonra yapılacak çalışmada, elde edilen bu dirençli suşun transkriptomik ve/veya genomik analizi yapılacaktır. Bu sayede alüminyuma direnç sağlayan moleküler mekanizma

anlaşılmaya çalışılacaktır. Alüminyuma dirençli mutantın alüminyum iyonlarını tutma kapasitesi de belirlenerek biyoremediasyon veya diğer endüstriyel uygulamalarda kullanılma potansiyeli de belirlenecektir. Ayrıca elde edilen alüminyuma dirençli *S. cerevisiae,* ökaryotik bir model organizma olarak alüminyum ile ilişkilendirilen nörodejeneratif hastalıkların mekanizmalarının aydınlatılmasına da katkıda bulunabilecektir.

1. INTRODUCTION

1.1. The Yeast '*Saccharomyces cerevisiae*' and its Industrial Importance

Saccharomyces cerevisiae, also known as baker's or brewer's yeast, has been used in many studies. It is easy to grow, and it is a unicellular eukaryote. Yeast can grow sexually or asexually and this process can be controlled biochemically in laboratory. Sexually, two haploid cells mate and produce the diploid form of zygote. This zygote can reproduce mitotically or divide by meiosis and produce haploid cells. Asexually, it reproduces haploid cells by budding (Lewis, Raff, and Roberts 2014).

Taxonomic classification of *S. cerevisiae* is shown in Table 1.1. Yeast can be classified according to the characteristics listed below (Glaser and Nikaido 2007).

- Certain physiological features
- Mode of sexual reproduction
- Microscopic appearance
- Biochemical features

Table 1.1 : Taxonomic classification of *Saccharomyces cerevisiae.*

Kingdom	Phylum	Class	Order	Genus	Species
Fungi	Ascomycata	Saccharomycetes	Saccharomycetales	Saccharomyces	*S.cerevisiae*

S. cerevisiae has also been playing a very important role in several biotechnological applications (Laget and Cantley 2001). It has been used for making bread, wine etc. for a long time. In more recent years, yeast has gained an important role as a eukaryotic model organism in biotechnology, molecular biology and biochemistry. In 1996, the whole genome sequencing of the yeast was completed (Akada 2002). That was the first whole genome sequencing project applied to *S. cerevisiae* (Nevoigt 2008). *S.cerevisiae* is the most common yeast, which has been used as a eukaryotic model organism for the last decades (Laget and Cantley 2001).

S. cerevisiae cell wall is an important source of β-D-glucan which is a glucose homopolymer with many functional, nutritional and human health benefits. Yeast β-D-glucan had good potential for use as a prebiotic ingredient in food, also as a medicinal and pharmaceutical product (Borchani et al. 2016).

The effect of copper stress on the fermentation performance of *Saccharomyces cerevisiae* and its copper adsorption pathway during alcoholic fermentation were investigated. *S. cerevisiae* has a non-biological adsorption of copper, but compared with biological (living yeast) adsorption, the non-biological adsorption was very low (Sun et al. 2016).

Engineering the fatty acid metabolic pathway in *S.cerevisiae* for advanced biofuel production is a recent biotechnology application of *S.cerevisiae* (Figure 1.1). The metabolically engineered yeast strains provide a platform for the production of important fatty acid-derived chemicals and fuels. This application for industrial use has advantages of high productivity, high capacity and tolerance to environmental changes, and strong resistance to inhibitors (Tang, Lee, and Ning Chen 2015).

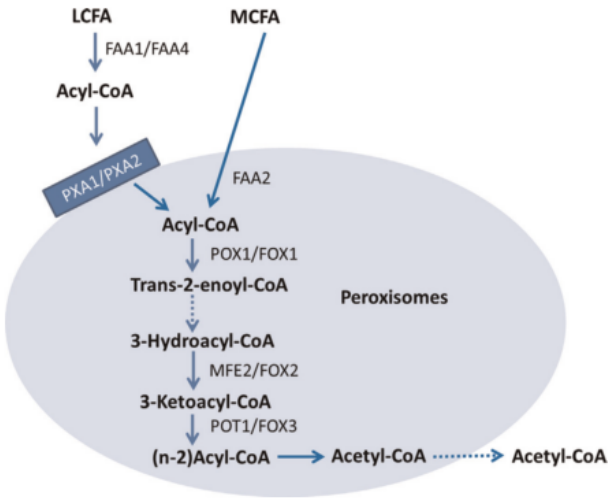

Figure 1.1: Schematic diagram of β-oxidation in yeast peroxisomes. LCFA: long chain fatty acid; MCFA: medium chain fatty acid (Tang et al. 2015).

1.2. Aluminium Characteristics and Industrial Importance

The most commonly found metal in the Earth's crust is Aluminium. This metal is available in the environment naturally and is used in cooking foils, pots, pans, airplanes automobiles and construction materials. It has unstable forms which are

highly reactive, like liquid and gas (J. R. Walton 2011). Al, Ca and Fe can be used as a coagulation factor of *S. cerevisiae* in UASB reactors (Kalyuzhnyi et al. 2004).

Aluminium binds electrostatically and has high affinity for oxygen in ligands of phosphate and carboxylate groups such as deoxyribonucleic acid (DNA), ribonucleic acid (RNA), and proteins. Thus, aluminium has a potential effect of toxicity in environment and human health (J. R. Walton 2011). Aluminium compounds have different solubility properties. The solubility is pH-dependent, which is shown in logarithmic pH scale in Figure 1.2. Thus, one pH unit decrease represents a 10 times increase in acidity. At neutral pH, Al^{3+}, which is the most toxic form of this element, is insoluble (J. R. Walton 2011). In acidic environments, the free Al^{3+} form can be soluble, thus it can be toxic for living organisms; like plants growing in acidic soils.

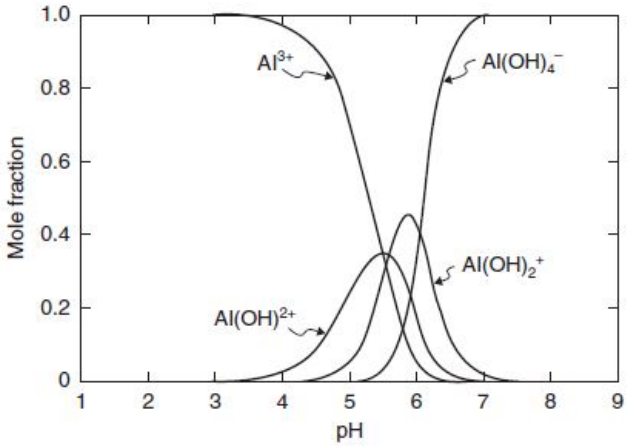

Figure.1.2 : Aluminium compound solubilities at different pH values. $Al(H_2O)_6^{3+}$ is abbreviated as Al^{3+} (J. R. Walton 2011).

Aluminium has no known function in biological processes and it is highly toxic for organisms. The toxic effect mechanism of Al^{+3} is not well understood. The toxicity of Al^{+3} has been suggested to result from binding of highly reactive Al^{+3} to various cellular components or from substituting for some cations $Fe^{2/3+}$, Mg^{2+}, Ca^{2+} in essential cellular reactions. Some plant species, especially those adapted to acidic soils are highly Al^{-}tolerant. Natural tolerance mechanisms depend on chelation or sequestration of Al^{+3}. For example, in tea plants (*Camellia sinensis L.*) Al^{+3} is taken up and stocked in vacuoles with organic acids as complexes. In Al^{-}tolerant kind of wheat, Al^{+3} is chelated at the external part of the cells by releasing malic acid.

Although still not yet documented, there are other suggestions about Al tolerance mechanism which include transmembrane pumps of Al-binding molecules. This molecule has been shown to contribute to tolerance to other toxic metals like Cd^{2+} and Cu^{2+}. Also, it has been documented that Al^{+3} cause extensive components of general stress response in plants, involving phenylalanine ammonia lyase, protease inhibitors and metallothionein. The nature of the stress is still unclear, but there are indications that oxidative stress may have been induced in both animal and plant tissues which are exposed to Al (Schott and Gardner 1997). General industrial applications of Al are shown in Figure 1.3.

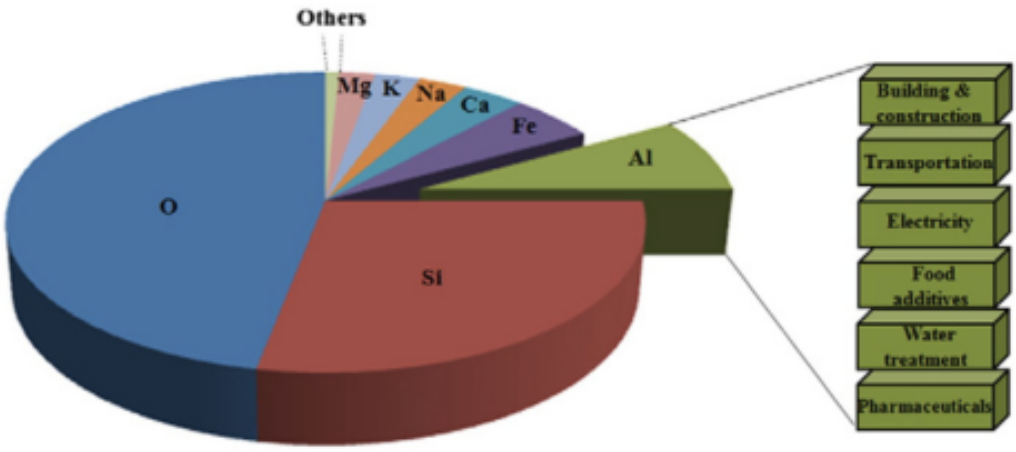

Figure 1.3 : Elemental composition of the earth's crust and the industrial applications of Al (Auger et al. 2013).

1.3. Mechanism of Aluminium Toxicity

In cells, Al binds to ligands as Al^{+3}, not Al^{+4}. Thus, Al^{+3} has significant biological importance. Bioavailable Al^{+3} has two main types of toxic action in living cells. Firstly, Al^{+3} competes with some other cations like, Mg^{+2}, Ca^{+2} and ferric iron Fe^{+3}, disturbing some cellular functions. Secondly, Al^{+3} disrupts iron metabolism, thus free radicals can increase and oxidative damage occurs by this disregulation. Al^{+3} can elevate the level of lipid peroxidation induced by iron or copper. Apart from these, Al^{3+} can cause oxidative damage by itself, involving superoxide anion, stimulating antioxidant activity by superoxide dismutase.

Al^{+3} is smaller than Mg^{+2}, thus it can easily displace Mg^{+2} at catalytic sites in enzymes like kinases, phosphatases and polymerases. ATP-Mg^{+2} complex is used as a substrate in all ATP-requiring reactions. But Al^{+3} binds to phosphate chain on ATP about 10^3 times more strongly than Mg^{+2} because of its high charge ratio. Nanomolar quantity of free Al^{+3} affects all these molecules. Thus, Al^{+3} has a high potential effect for disrupting functions of living organism (J. R. Walton 2011). An overview of the cellular process of Al is shown in Figure1.4.

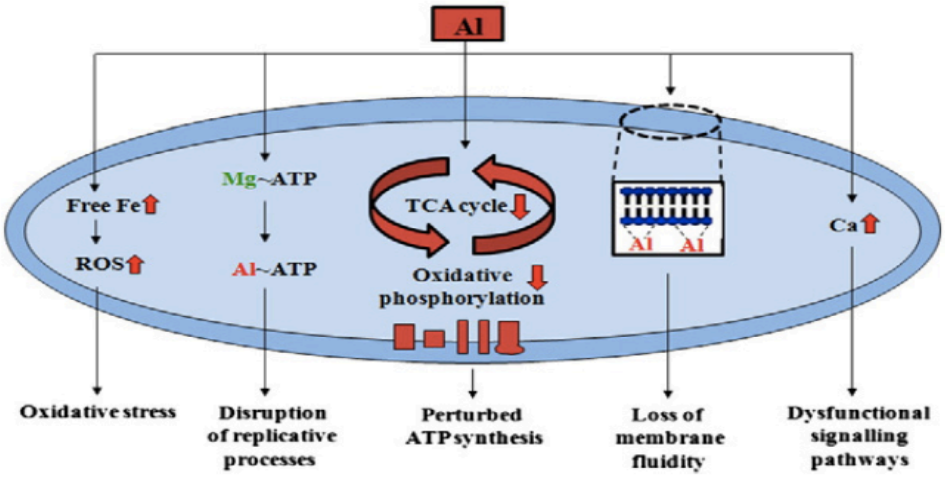

Figure 1.4 : An overview of the cellular processes affected by an Al-rich microenvironment (Auger et al. 2013).

For thermodynamic reasons, Al^{+3} is unlikely to substitute for Ca^{+2} in proteins. But on the contrary, Al^{+3} competes with Ca^{+2} and Mg^{+2} for small ligands like carboxylate and phosphate groups, nucleotides, polynucleotides, and inorganic phosphate to form insoluble complexes. This reaction disturbs the cell metabolism. Al^{+3} binding to inorganic phosphate can cause the latter's depletion (J. R. Walton 2011). Al toxicity induces programmed cell death in yeast, plants and animals (Tani et al. 2008).

Aluminium does not participate in biological processes but on the contrary, aluminium has many positive contributions to health. For example, it increases the efficiency of vaccines, it supports body's immune system against the active agent. Aluminium is a useful component of phosphate binders, buffered aspirins, antimicrobials and antidiarrheals; irrigants for bladder hemorrhage and vaginal douches; topical powders and creams for diaper rash, athlete's foot, and anorectal pruritis; and toothpastes, dental cements, styptic pencils, cosmetics, deodorants and

sunscreens. It is also used in alloy form as some prosthetic hip, shoulder, and knee replacements. Repetitious usage of Al^{+3}-including products has negative side effects like irritation and organ failure (J. Walton 2011).

Al has been associated with some brain diseases like dialysis encephalopathy, amyotrophic lateral sclerosis, Parkinson, Dementia, Alzheimer disease and multiple sclerosis (Crisponi et al. 2012).

In biological systems, Al has been reported to interact with organic molecules, including proteins, polynucleotides, lipids and glycosides *in vitro*, but this literature does not give information about the Al toxicity mechanism. There are also some suggestions that Al interacts with membrane-associated proteins and participates in signal transduction in yeast and animal cells.

In *S.cerevisiae* cells, Colin (1996) hypothesized that Al is responsible for blocking of Mg uptake, because of the following reasons:

1) When Mg is present, Al is less toxic. But Ca, K and PO_4^{-3} do not affect as Mg does. They have less effect on Al toxicity.
2) Uptake of Co is blocked by Al, maybe Mg uptake is also blocked because of the inhibition of the non-specific divalent cation transport system.
3) The *cot2* mutation in yeast causes decrease in the activity of the divalent cation uptake system and increase in sensitivity to Al.
4) Lack of Mg and Al toxicity cause similar morphological changes in growing cells, for both *cot2* mutated strains and the wild-type yeast (MacDiarmid and Gardner 1996).

1.4. Microbial Aluminium Tolerance

Metal pollution concerns all organisms, particularly in an increasingly industrialized environment. Effect of metal toxicity has been well documented. Heavy metals such as mercury, lead and cadmium, as well as toxic metals like Al and chromium, in addition to metalloids like arsenic are known to cause major damage to the ecosystem. Researchs dealing with Al exposure have demonstrated that Al concentrations lower than 3mM inhibit the growth of *Escherichia coli.* Certain microorganisms indicate natural resistance to high levels of Al concentration. Design bioreactors can help improve the efficiency of such processes (Figure 1.5). Metabolic

engineering is also important for biotechnological processes aiming at decontaminating pollutants (Auger et al. 2013).

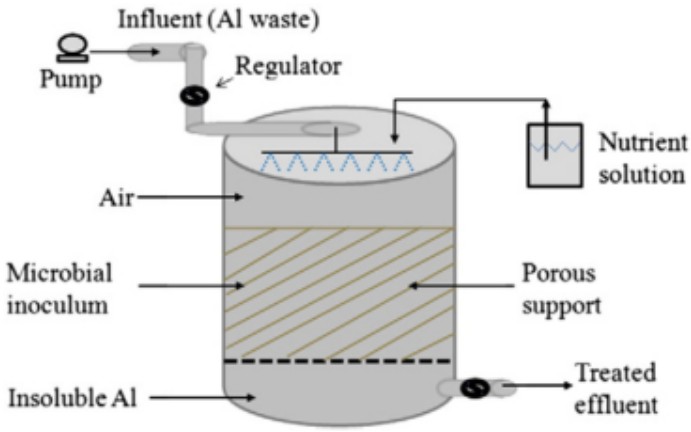

Figure 1.5 : Proposed bioreactor design. A trickling biofilter which feeds in the wastewater and a nutrient source for the microorganism permits the bioprecipitation of Al, which is filtered out of the system (Auger et al. 2013).

Al is directly toxic, but it can also reduce the bioavailability of phosphorus (P) in the ecosystem by formation of stable chemical complexes (Clivot et al. 2014).

Al toxicity affects mitochondrial respiratory functions, alters the redox status, and causes the programmed cell death in tobacco cells. Figure1.6 shows the toxicity related with Reactive Oxygen Species (ROS) production and oxidative stress (Panda et al. 2008).

In *E.coli,* Al is accumulated intracellularly at a single binding site with 0.4 mM K_m, and the surface binding mechanism is not known clearly. Also in *Anabaena cylindrica*, aluminium localizes in polyphosphate granules and in cell walls, but not in the cytoplasm (Guida et al. 1991).

S.cerevisiae deletion mutants were used to investigate the role of genes in aluminium uptake mechanism. Aluminium transport-related genes were identified by screening aluminium tolerant mutant phenotypes.

Cross resistances of those deletion mutants to other metals were also investigated and mutants with significant resistance and tolerance were determined (Tun et al. 2014).

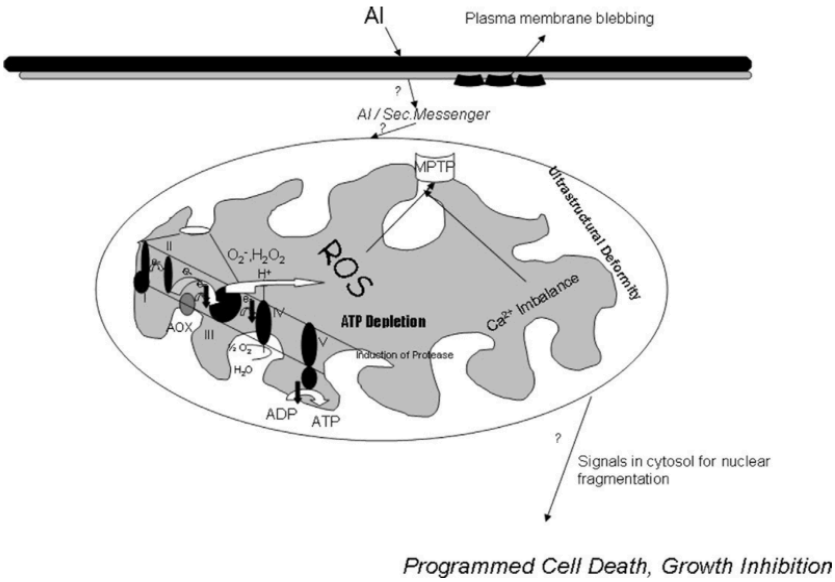

Figure 1.6 : Model of toxicity in tobacco cells for intracellular mechanism. Al is related with iron pump inhibition, membrane rigidification, lipid peroxidation. Al causes ROS production, respiratory dysfunction and loss of inner membrane potential (Panda et al. 2008).

Genome-wide screening of the aluminium tolerance genes in *S.cerevisiae* has determined some of the tolerance mechanisms in yeast. The PKC1-MAPK cascade signalling pathway is an important Al-tolerance factor in the signal transduction pathway (Kakimoto et al. 2005)(Figure 1.7).

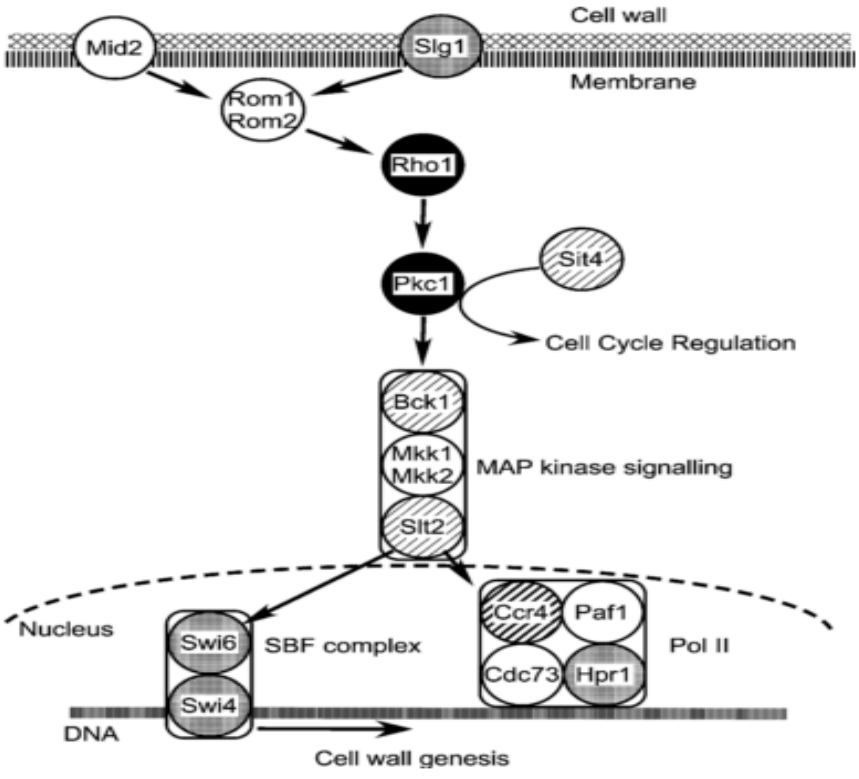

Figure 1.7 : Model for cell integrity signalling pathway (Kakimoto et al. 2005).

1.5. Inverse Metabolic Engineering: A Powerful Approach for Microbial Strain Improvement

The term "metabolic engineering" was defined as "The improvement of cellular activities by manipulation of enzymatic, transport, and regulatory functions of the cell with the use of recombinant DNA technology"(Bailey, 1991). However, limitations in rational metabolic engineering such as the need for extensive information on the organism of interest led to the introduction of a "bottom-up" approach called "inverse metabolic engineering"(Bailey et al. 1996). In Figure 1.8, a schematic diagram of inverse metabolic engineering is summarized.

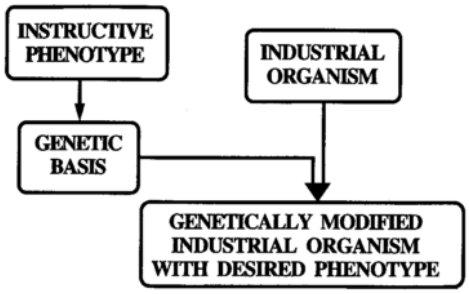

Figure 1.8 : Schematic diagram of inverse metabolic engineering (Bailey et al. 1996).

Evolutionary engineering is an inverse metabolic engineering strategy that is based on random mutation and selection of desired phenotypes under conditions that favor those phenotypes(Alkım et al.,2014). It is a successful method for microbial strain improvement. It is usually difficult to apply rational metabolic engineering to improve genetically complex phenotypes. This difficulty could be overcome by applying random and combinatorial approaches, such as evolutionary engineering (Cakar et al., 2005).

Evolutionary engineering depends on three main steps (Petri and Schmidt-Dannert 2004):

- Improved strain synthesis,
- Analysis of the strains' productivity under desired conditions,
- Designing of the next target for further optimization

Evolutionary engineering starts with random mutagenesis to obtain an initial population with increased genetic diversity.

UV light and chemicals such as ethyl methane sulfonate can be used for this random mutagenesis step. Selection of the desired phenotype is then applied under cultivation conditions which favor that desired phenotype (Otero, Panagiotou, and Olsson 2007).

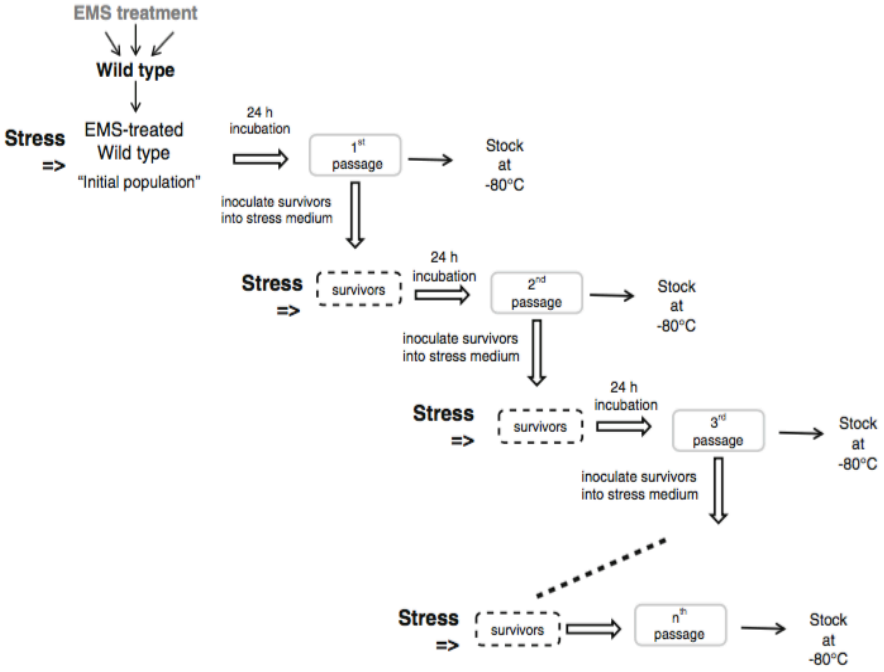

Figure 1.9 : Experimental protocol for selection under continuously applied condition (Alkım et al. 2014).

A variety of selection strategies can be applied in evolutionary engineering: based on how the stress factor is applied, one may choose continuously applied stress conditions or pulse stress conditions as two different selection strategies. In Figure 1.9, an overview of selection under continuously applied stress conditions is given as an evolutionary engineering strategy. Here, the stress conditions are present continuously, e.g. throughout the whole cultivation.

In pulse selection strategy, stress conditions are applied only for a limited period of time during the cultivation. Another way of varying selection strategies is based on selection at mild, constant stress conditions versus selection at gradually increasing stress levels throughout the selection.

1.6. Aim of the Study

The aim of the present study was to obtain highly aluminium resistant and genetically stable *S. cerevisiae* using an inverse metabolic engineering strategy, evolutionary engineering, and to determine the aluminium stress resistance levels and

potential cross-resistances of the aluminium-resistant mutants against other metal and non-metal stress types. The cross-resistance results could provide insight into the common resistance mechanisms between aluminium stress and other stress types. The ultimate aim is to analyze the aluminium hyper –resistant yeast mutants obtained in this study at physiological, genomic, transcriptomic and/or proteomic levels, to gain insight into the molecular mechanisms of aluminium resistance in yeast as a eukaryotic model organism and to exploit this information for applications such as bioremediation and/or aluminium-related human neurodegenerative diseases.

2. MATERIALS AND METHODS

2.1. Materials

2.1.1. Strain

Saccharomyces cerevisiae CEN.PK113-7D (*MATa*) strain was provided by Dr. Laurent Benbadis (INSA-Toulouse, France) and named as *905*. *906* was obtained by treatment of *905* with a chemical mutagen ethyl methane sulfonate.

2.1.2. Growth media

2.1.2.1. Yeast complex medium(YPD)

Yeast peptone dextrose medium was used for regular growth as a complex medium. Chemicals are shown in Table 2.1.

Table 2.1 : Contents of yeast extract peptone dextrose medium (YPD).

Chemicals	Amount
Yeast nitrogen base without aminoacids	10 g
Dextrose	20 g
Peptone	10 g
Water	1 L

2.1.2.2. Yeast minimal medium(YMM)

Yeast minimal medium was used before stress applications and during selection experiments. Its ingredients are shown in Table 2.2.

Table 2.2 : Contents of yeast minimal medium (YMM).

Chemicals	Amount
Yeast nitrogen base without aminoacids	6.7 g
Dextrose	20 g
Water	1 L

2.1.2.3. Yeast minimal agar medium(YMM-agar)

Ingredients of YMM-agar medium used in this study is given in Table 2.3.

Table 2.3 : Contents of solid yeast minimal medium (YMM-agar).

Chemicals	Amount
Yeast nitrogen base without aminoacids	6.7 g
Dextrose	20 g
Agar (for solid media)	20 g
Water	1 L

2.1.3. Buffers and solutions

List of buffers and solutions are shown in Table 2.4.

Table 2.4 : The buffers and solutions used in this study.

Buffers and solutions	Concentrations
$CoCl_2$ solution	1 M
$CrCl_3$ solution	1 M
$CuCl_2$ solution	1 M
$FeCl_2$ solution	500 mM
$AlCl_3$ solution	500 mM
Glycerol	60%(v/v)
H_2O_2 solution	5 M
$MnCl_2$ solution	1 M

2.1.4. Chemicals

All chemicals which were used in this study are listed in Table 2.5.

Table 2.5 : The chemicals used in this study.

Chemicals	Supplier
Nickel chloride hexahydrate ($NiCl_2.6H_2O$)	MERCK (Germany)
Cobalt chloride hexahydrate ($CoCl_2.6H_2O$)	Fluka (USA)
Copper (II) sulphate pentahydrate ($CuSO_4.5H_2O$)	Sigma ALDRICH (USA)
Hydrogen peroxide (H_2O_2)	MERCK
Chrome chloride ($CrCl_3$)	Acros Organics (USA)
Zinc chloride ($ZnCl_2$)	Carlo Erba (Italy)
Magnessium chloride hexahydrate $MgCl_2.6H_2O$	MERCK (Germany)
Ammonium iron (II) sulphate hexahydrate $(NH_4)_2Fe(SO_4)_2.6H_2O$	MERCK (Germany)
Manganese (II) chloride tetrahydrate $MnCl_2.6H_2O$	MERCK (Germany)
Ethanol (C_2H_6O)	J.T Baker (The Netherlands)
Aluminium chloride hexahydrate ($AlCl_3 .6H_2O$)	MERCK (Germany)
Sodium chloride (NaCl)	MERCK (Germany)
Acetic acid	MERCK (Germany)
Acetone (C_3H_6O)	MERCK (Germany)
Agar	BDDifco™ (USA)
Glycerol ($C_3H_8O_3$)	Duchefa Biochemie (The Netherlands)
Yeast Extract	MERCK (Germany)
EMS	Alpha-Aeasar (Germany)
Peptone	Riedel-de Haen
Dextrose	Riedel-de Haen

2.1.5. Laboratory equipments

All equipments which were used in this study are listed in Table 2.6.

Table 2.6 : Laboratory instruments that were used in this study.

Equipment	Supplier
UV Visible Spectrophotometer	Shimadzu UV-1700 (Japan)
Vortex Mixer	Nüve NM 100 (Turkey)
Autoclaves	Zealway Gr110df (China)
Light Microscope	Olympus CH30 (Japan)
Microfuge	Eppendorf Microcentrifuge-5424 (Germany)
Micropipettes	Eppendorf (Germany)
Balance	Precisa BJ 610 C (Switzerland)
Microbalance	Precisa 620C SCS
Laminar Flow Hood	Biolab Faster BH-EU 2003
Magnetic Stirrer	Labworld (Germany)
Benchtop Centrifuge	Eppendorf 5424 (Germany)
pH Meter	Mettler Toledo MP220 (Switzerland)
Deep-freezer	-80 ^{o}C Sanyo Ultra Low MDT-U40865 (Japan)
Refrigerators and Deep-freezers	-20 ^{o}C Arçelik 3011 NY (Turkey)
Shaker	Thermo Scientific Orbital Shaker (USA)
Orbital Shaker Incubators	Certomat S-2 Sartorius (Germany)
Ultrapure Water System	TKA(Germany)

2.2. Methods

2.2.1. Incubation procedure

Strains were grown in 50 mL culture tubes with 10 mL YMM and YPD medium at 30^0C , and 150 rpm. 15 mL YMM-agar medium was used for inoculation in plates.

2.2.2. Selection strategy

Evolutionary engineering was used as a selection strategy to obtain aluminium-resistant mutants from 906 (EMS-mutagenized strain).

Prior to selection experiments, 905 (WT) and 906 (EMS mutagenized strain) were grown at different aluminium stress levels, such as; 0.05, 0.1, 0.4, 0.6, 1, 2, 5, 10, 25, 50 mM to determine the initial aluminium stress level for selection experiments. Cultures were incubated in 50 mL culture tubes with 10 mL YMM at 30^0C and 150 rpm, both in the presence and absence of aluminium stress. At 24^{th} and 48^{th} hour of incubation, OD_{600} values were determined. Survival rates were calculated by dividing the OD_{600} value of the stress-exposed culture to that of the non-stress culture.

A batch selection strategy was then applied to obtain aluminium-resistant *S. cerevisiae* mutants by gradually increasing aluminium-stress level from 0.5 to 21.5 mM $AlCl_3$ through 43 passages. For each passage, stock cultures were prepared.

2.2.3. Stock culture preparation

For each passage, a frozen stock culture was prepared and stored at -80^0C. One ml culture was centrifuged at 14000 rpm for 3 min. The supernatant was removed and 1 mL 30% (v/v) glycerol was added onto the cell pellet. The pellet was then mixed with glycerol by vortexing.

2.2.4. The optical density measurements and determination of the survival rate

A spectrophotometer was used for Optical Density (OD) measurements. The spectrophotometer was adjusted to 600 nm and samples were measured. Survival rates were calculated by dividing the OD_{600} value of the stressed culture to the OD_{600} value of non-stress culture.

2.2.5. Selection of mutant individuals from the final population

In order to select mutant individuals, the final population of selection was diluted until 10^{-6} spread on solid YMM media, and incubated at 30^0C for 48 hours.

The colonies were then randomly picked and transfered into 10 mL YMM, using an inoculation loop. After 24 h of incubation at 30^0C and 150 rpm, stock cultures were prepared for the 12 randomly picked individuals, and stored at -80^0C.

2.2.6. Determination of the mutant individuals with the highest aluminium resistance

In order to determine the mutant individuals with the highest aluminium resistance, two methods were used: spot assay and MPN method. The first one is more qualitative, whereas the last one (MPN Method) quantitatively estimates aluminium stress levels.

2.2.6.1. Qualitative estimation of aluminium resistance of mutant individuals using spot assay

Randomly selected 12 individual mutants, the last population LP (43^{th} passage) and the wild type (905) strains were inoculated onto YMM-agar medium containing 1 mM, 2 mM, 5 mM, 10 mM and 21 mM $AlCl_3$ medium, and on control YMM-agar plates. The plates were incubated at 30^0C and observed at 24^{th}, 48^{th} and 72^{nd} hours of incubation.

2.2.6.2. Quantitative determination of aluminium resistance of mutant individuals using MPN method

According to spot assay results, four highly aluminium-resistant mutant individuals were chosen for quantitative resistance estimation. For quantitatively determining the aluminium resistance of the mutants, MPN method was applied. 96-well plates were used for applying 5-tube MPN methodology.

YMM (180 µl) was filled into the wells. For control plate, only YMM was filled into the wells, but the other (test) plates filled with 10 mM and 15 mM $AlCl_3$-containing YMM. Culture (20 µl) was added into the first row and mixed. 20 µl culture was transfered from the first row to the second and this was repeated until the 8^{th} row (10^{-8} dilution). Strains were incubated at 30^0C for 72 hours. The number of surviving cells at different aluminium concentrations was estimated by using the MPN table, based on the Poisson regression (Rowe et al., 1977).

2.2.7. Determination of genetic stability

Genetic stability test was applied to the highly aluminium-resistant mutant individuals in order to verify if the acquired aluminium tolerance is resulting from permanent genetic mutations or not (e.g. an adaptation).

Precultures were prepared from each individual's -80^{0}C stock culture and incubated overnight at 30^{0}C and 150 rpm. For each mutant, OD_{600} was then adjusted to 0.25 and the culture was incubated in fresh 10 ml YMM medium. After 24 h of incubation, stock cultures were prepared from the first day sample. Inoculation to fresh media was repeated for 7 passages (days) and from each passage of each mutant, stock cultures were prepared and kept at -80^{0}C.

MPN method was applied to determine the aluminium resistance of individuals during each of the 7 passages, in the presence and absence of 15mM $AlCl_3$ stress.

2.2.8. Determination of cross resistances to other stress types

Cross resistance tests were applied in order to determine whether the mutants have resistance to other metal or non-metal stress types or not.

YMM-agar (15 ml) plates containing different stress factors were prepared. Precultures of each mutant were diluted serially and inoculated onto stress-containing YMM plates . Plates were incubated at 30^{0}C for 72 h and images were taken. Several concentrations were tried for each stress type and the best plate images were taken. The different stress types and their applied concentrations are as follows: 8%(v/v) ethanol, acid stress (pH 4), 0.7 mM H_2O_2, 0.25 mM $CuSO_4$, 1 ml/L phenylethanol, 1.3 mM $MgCl_2$, 2.5 mM $CrCl_3$, 10 mM $MnCl_2$, 30 mM $FeSO_4$, 80 mM H_3BO_3, 300 mg/ml propolis, 0.5 M $CaCl_2$, 2 mM $GaNO_3$, 75 μM $AgNO_3$, 0.2 mM $NiCl_2$, 10 mM NaCl, 3 mM $ZnCl_2$, 1 mM $CoCl_2$, 25 mM $FeCl_2$, 2 mM LiCl.

3. RESULTS

3.1. Determination of the Initial Aluminium Stress Level and Stress Step Size for Selection Experiments

To determine the initial aluminium stress level of selection, both 906 and 905 were incubated under 0 mM (control), 0.05 mM, 0.1 mM, 0.4 mM, 0.6 mM, 1 mM, 2 mM, 5 mM, 10 mM, 25 mM, 50 mM $AlCl_3$ in 10 mL YMM.

Incubation was performed at 30°C and 150 rpm with an initial OD_{600} of 0.25 for 48 h. OD_{600} values of cultures grown at different aluminium stress levels at the 24^{th} hour of incubation are given in Table 3.1. Survival results are shown in Table 3.2.

Table 3.1 : OD_{600} results of cultures grown at different aluminium stress levels at the 24^{th} hour of incubation.

Stress level ($AlCl_3$ concentrations) (mM)	OD_{600} values of *905* culture	OD_{600} values of *906* culture
Control	5.38	5.93
0.05	4.63	6.00
0.10	4.50	5.31
0.40	4.60	4.33
0.60	4.21	3.82
1	4.31	3.13
2	3.20	3.38
5	2.50	2.30
10	1.23	1.26
25	0.48	0.53
50	0.32	0.29

Table 3.2 : Survival rates of cultures grown at different aluminium stress levels at the 24th hour of incubation.

Stress level ($AlCl_3$ concentrations) (mM)	Survival rate of *905* culture (wild type)	Survival rate of *906* culture	Survival rate of *906* culture as fold of wild type (*905*)
Control	0.86	1.01	1.18
0.05	0.84	0.90	1.07
0.1	0.86	0.73	0.85
0.4	0.78	0.64	0.82
0.6	0.80	0.53	0.66
1	0.59	0.57	0.96
2	0.46	0.39	0.83
5	0.23	0.21	0.93
10	0.09	0.09	1.00
25	0.06	0.05	0.82
50	0.86	1.01	1.18

The survival rates of *905* and *906* under increasing aluminium stress levels after 24th h of incubation are shown in Figure 3.1.

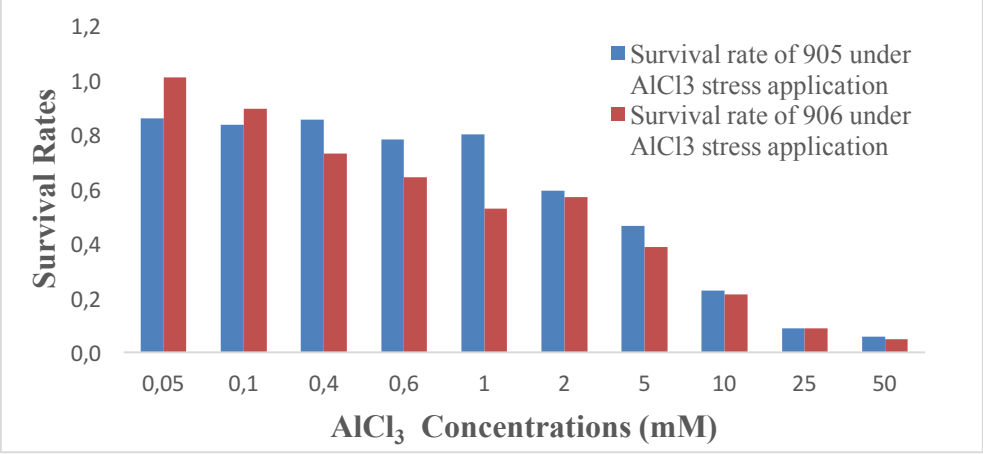

Figure 3.1 : Survival rates of 905 and 906 under $AlCl_3$ stress application at 24th h of incubation.

OD_{600} results at the 48th hour of incubation are shown in Table 3.3 and the corresponding survival rates are given in Table 3.4.

Table 3.3 : OD_{600} results of cultures grown at different aluminium stress levels at the 48^{th} h of incubation.

Stress level ($AlCl_3$ concentrations) (mM)	OD_{600} values of *905* culture (wild type)	OD_{600} values of *906* culture
Control	7.39	7.22
0.05	7.35	6.95
0.1	7.07	6.80
0.4	6.80	6.79
0.6	6.46	6.48
1	6.27	5.58
2	5.47	5.23
5	3.80	3.77
10	1.53	1.53
25	0.57	0.62
50	0.32	0.30

Table 3.4 : Survival rates of cultures grown at different aluminium stress levels at the 48^{th} hour of incubation.

Stress level ($AlCl_3$ concentrations) (mM)	Survival rate of *905* culture (wild type)	Survival rate of *906* culture	Survival rates of *906* as fold of wild type (*905*)
Control	0.99	0.96	0.97
0.05	0.96	0.94	0.98
0.1	0.92	0.94	1.02
0.4	0.87	0.90	1.03
0.6	0.85	0.77	0.91
1	0.74	0.72	0.98
2	0.51	0.52	1.02
5	0.21	0.21	1.02
10	0.08	0.09	1.11
25	0.04	0.04	0.96
50	0.99	0.96	0.97

The survival rates of *905* and *906* under increasing aluminium stress levels after 48th h of incubation are shown in Figure 3.2.

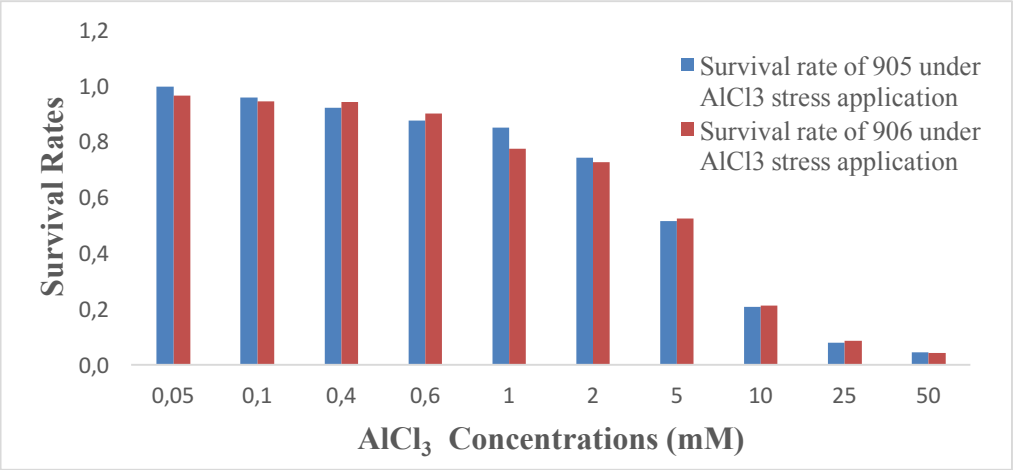

Figure 3.2 : Survival rates of 905 and 906 under $AlCl_3$ stress application at 48th h of incubation.

3.2. Obtaining Continuous Stress Selection Populations

The stock culture of EMS-mutagenized yeast (906) was incubated overnight. The preculture was then transfered to fresh YMM medium and its OD_{600} value was adjusted to 0.25. According to screening results, initial stress level was determined as 0.5 mM $AlCl_3$ and each stress step size was set as 0.5 mM $AlCl_3$. The first population was incubated in 10 mL YMM containing 0.5 mM $AlCl_3$ for 24 h. The same culture was also incubated without $AlCl_3$, as the control culture. Each population was obtained upon incubation under increasing $AlCl_3$ concentrations by 0.5 mM at each step.

3.3. Selection of Individual Mutants From the Final Mutant Population

The last population (43th) population was diluted by 10^{-6} and spread on YMM agar plates to isolate individual mutants. The plates were incubated at 30^{0}C for 72 h. Randomly 12 individual colonies were picked and inoculated into fresh YMM medium. Stock cultures were then prepared for each of the 12 individuals for further analysis.

Table 3.5 : Aluminium concentration of each passage, OD_{600} results of control and populations, survival rates and incubation times of the batch selection experiment at increasing $AlCl_3$ stress levels.

Increasing stress level population numbers	Aluminium concentration applied (mM)	OD_{600} control	OD_{600} population	Survival rates	Incubation time (h)
1	0.5	4.95	4.65	0.94	24
2	1.0	6.19	3.18	0.51	24
3	1.5	4.69	4.73	1.01	24
4	2.0	5.15	3.21	0.62	24
5	2.5	5.63	3.24	0.58	24
6	3.0	4.83	2.03	0.42	24
7	3.5	4.16	1.91	0.46	24
8	4.0	4.32	2.16	0.50	24
9	4.5	4.20	1.57	0.37	24
10	5.0	4.54	3.29	0.72	24
11	5.5	4.43	2.02	0.46	24
12	6.0	4.69	2.28	0.49	24
13	6.5	4.34	2.38	0.55	24
14	7.0	4.91	2.67	0.54	24
15	7.5	4.1	1.92	0.47	24
16	8.0	4.18	1.4	0.33	24
17	8.5	4.40	2.2	0.50	24
18	9.0	4.3	2.45	0.57	24
19	9.5	4.55	3.22	0.71	24
20	10.0	5.13	1.71	0.33	24
21	10.5	5.28	1.58	0.30	24
22	11.0	4.00	1.32	0.33	24
23	11.5	4.13	1.31	0.32	24
24	12.0	4.56	1.53	0.34	24
25	12.5	3.48	1.25	0.36	24
26	13.0	3.85	2.00	0.52	24
27	13.5	4.02	3.54	0.88	24
28	14.0	4.83	2.42	0.50	24

Table 3.5 : Aluminium concentration of each passage, OD_{600} results of control and populations, survival rates and incubation times of the batch selection experiment at increasing $AlCl_3$ stress levels(continued).

Increasing stress level population numbers	Aluminium concentration applied (mM)	OD_{600} control	OD_{600} population	Survival rates	Incubation time (h)
29	14.5	4.54	1.25	0.28	24
30	15.0	4.17	1.12	0.27	24
31	15.5	4.70	0.75	0.16	24
32	16.0	4.10	0.99	0.24	24
33	16.5	4.70	1.90	0.40	24
34	17.0	3.76	1.32	0.35	24
35	17.5	3.62	1.36	0.38	24
36	18.0	4.53	1.08	0.24	24
37	18.5	3.46	1.20	0.35	24
38	19.0	4.10	1.48	0.36	24
39	19.5	4.82	1.19	0.25	24
40	20.0	4.20	0.85	0.20	24
41	20.5	4.30	0.89	0.21	24
42	21.0	3.28	0.59	0.18	24
43	21.5	4.40	0.87	0.20	48

The survival rates of increasing stress selection populations significantly decreased, as the $AlCl_3$ stress levels increased (Table 3.5 and Figure 3.3).

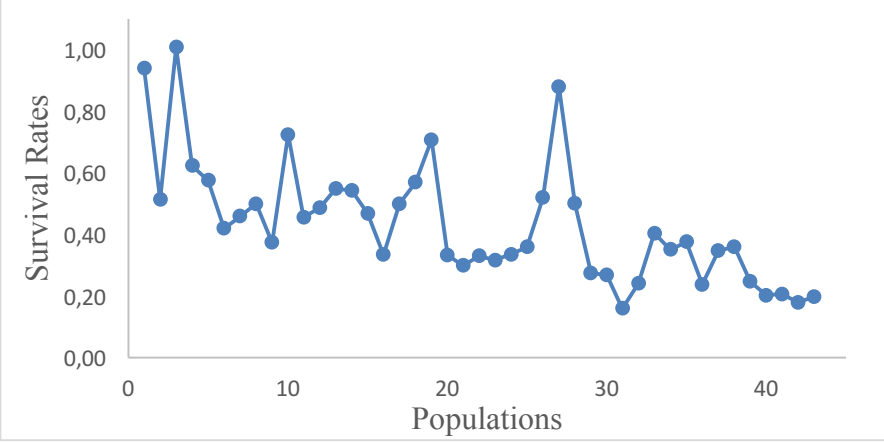

Figure 3.3 : Survival rates of continuously increasing stress level populations.

3.4. Determination of Aluminium Stress Resistances of Mutant Individuals Comparative to Wild Type and The Final Population

To determine the mutant with the highest aluminium resistance 12 individuals, the last population and *905* were tested together. For this purpose, spot assay and MPN method were applied.

3.4.1. Determination of aluminium stress resistances by spot assay

Serial dilutions of mutant individuals (Alu1-Alu12), LP (Last population 43th) and WT (*905*) were inoculated onto YMM agar plates at different aluminium concentrations. The plates were incubated at 30 ^{0}C for 72 h. The results are shown in Figure 3.4. It was observed that, at a very high $AlCl_3$ concentration (21 mM), the wild type could not survive. However, mutant individuals Alu9-Alu12 could grow at that $AlCl_3$ concentration. They seemed to have higher aluminium resistance, compared to the other individuals Alu1-Alu8.

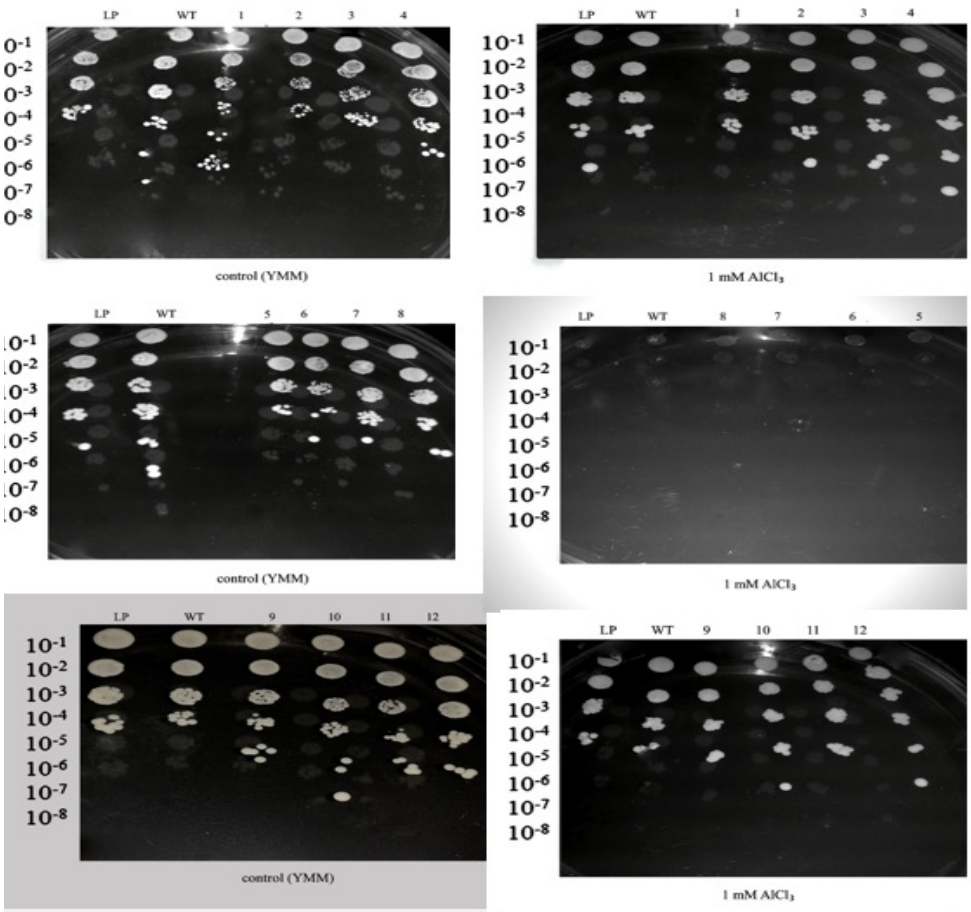

Figure 3.4 : Spot assay results of individuals, last population and wild type at 0 mM and 1mM $AlCl_3$ stress levels.

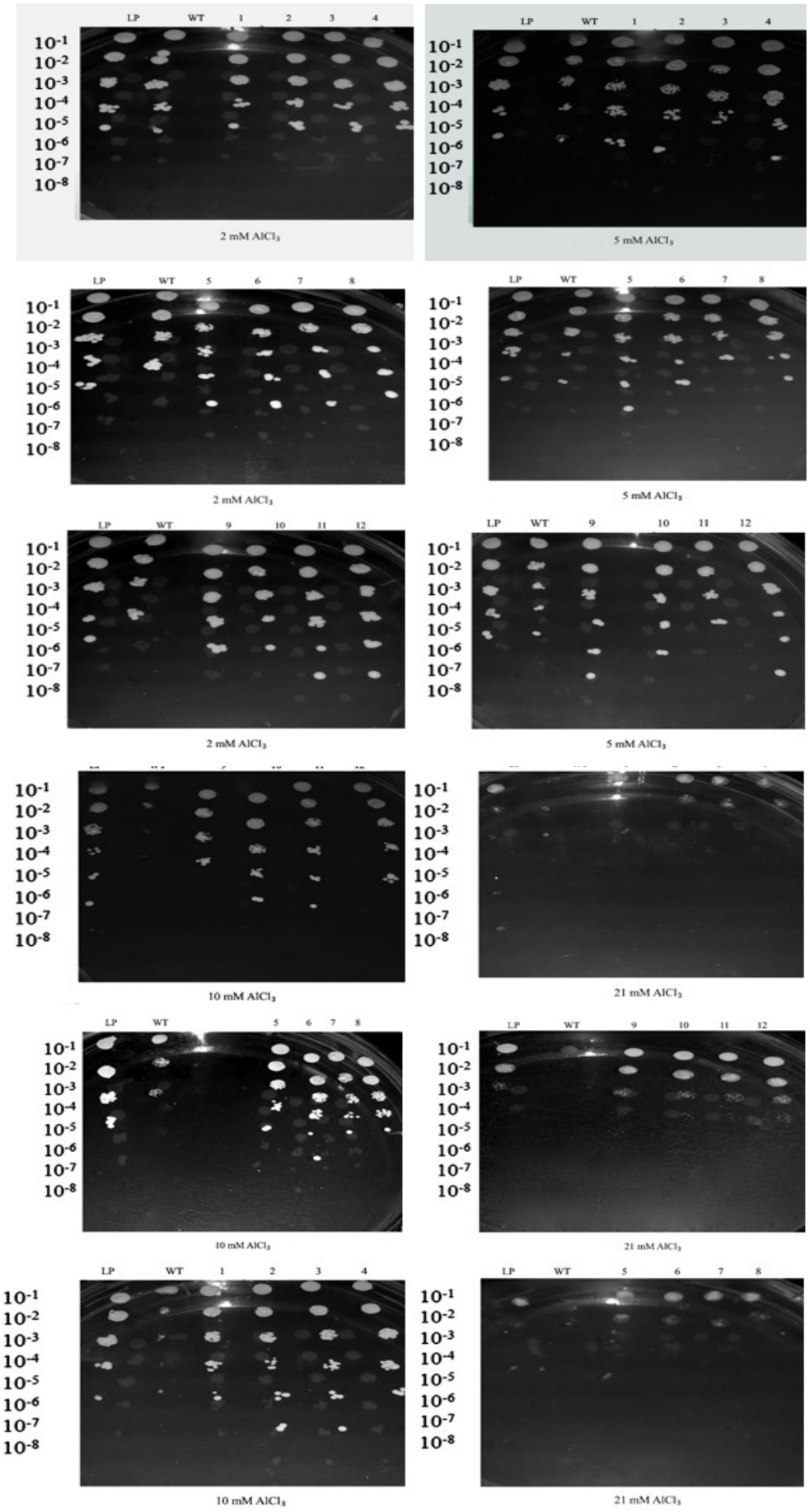

Figure 3.5 : Spot assay results of individuals, last population and wild type at 2 mM, 5 mM 10 mM and 21mM $AlCl_3$ stress levels.

3.4.2. Quantitative determination of aluminium stress resistance levels by MPN method

According to spot assay results, the highest aluminium-resistant individuals Alu9, Alu10, Alu11 and Alu12 were chosen for quantification of their aluminium-resistance levels by MPN method. LP and WT were also analysed for comparison. The cultures were incubated in 96-well plates in the presence and absence of 15 mM and 10 mM $AlCl_3$. At 48^{th} and 72^{nd} h of incubation , survival rates were determined, and the results are shown in Tables 3.6 and 3.7, as well as Figures 3.5 and 3.6, respectively. The results showed that the aluminium stress resistance levels of the four tested individuals were significantly higher than that of the wild type, but they varied significantly.

The mutant individual Alu10 had the highest survival rate at 10 mM $AlCl_3$ upon 48 h of incubation, and it is among the mutants with the highest survival rate at 15 mM $AlCl_3$ upon 72 h of incubation.

Table 3.6 : Percent survival rates and number of viable cells under 10 mM and 15 mM $AlCl_3$ stress at 48^{th} h of incubation.

Culture name	Percent Survival Rates			Number of viable cells		
	at 0mM $AlCl_3$	at 10 mM $AlCl_3$	at 15mM $AlCl_3$	at 0mM $AlCl_3$	at 10 mM $AlCl_3$	at 15 mM $AlCl_3$
WT	-	3%	1%	5400000	140000	35000
LP	-	82%	54%	1700000	1400000	920000
Alu9	-	120%	59%	920000	1100000	540000
Alu10	-	146%	15%	2400000	3500000	350000
Alu11	-	100%	10%	3500000	3500000	350000
Alu12	-	100%	32%	1700000	1700000	540000

According to MPN method results, survival rates were calculated and they are shown in Figure 3.5.

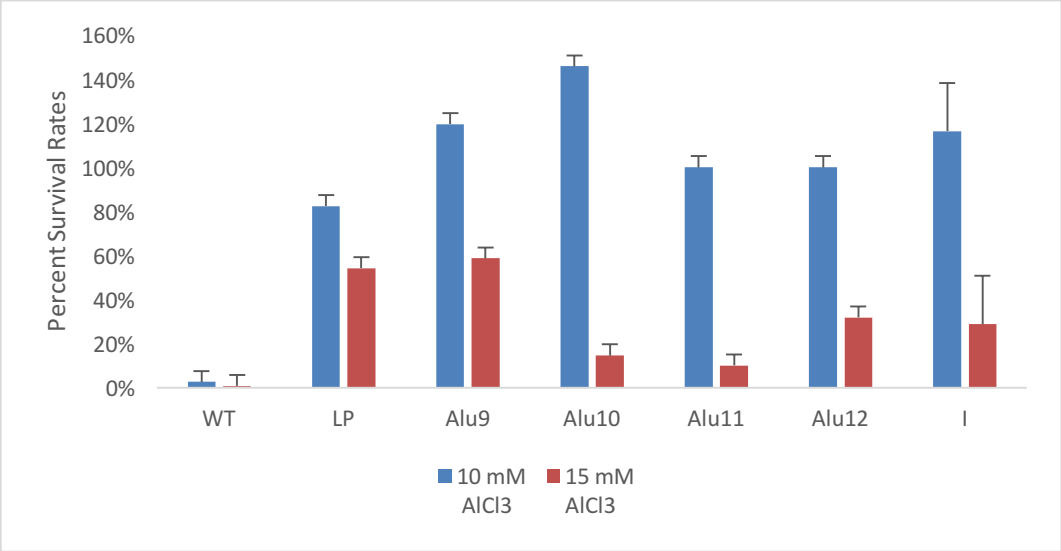

Figure 3.6 : Percent survival rates of individuals, last population and wild type at 48th h of incubation. "I" represents arithmetic average of percent survival rates of four individuals (Alu9-Alu12) tested.

Table 3.7 : Percent survival rates and number of viable cells under 10mM and 15 mM $AlCl_3$ stress at 72nd h of incubation.

Culture name	Percent Survival Rates			Number of viable cells		
	at 0mM $AlCl_3$	at 10 mM $AlCl_3$	at 15mM $AlCl_3$	at 0mM $AlCl_3$	at 10 mM $AlCl_3$	at 15 mM $AlCl_3$
WT	-	3%	6%	5400000	140000	350000
LP	-	100%	206%	1700000	1700000	3500000
Alu9	-	100%	155%	1100000	1100000	1700000
Alu10	-	146%	138%	2400000	3500000	3300000
Alu11	-	100%	63%	3500000	3500000	2200000
Alu12	-	206%	94%	1700000	3500000	1600000

Percent survival rates at 72nd h of incubation are also shown in Figure 3.6.

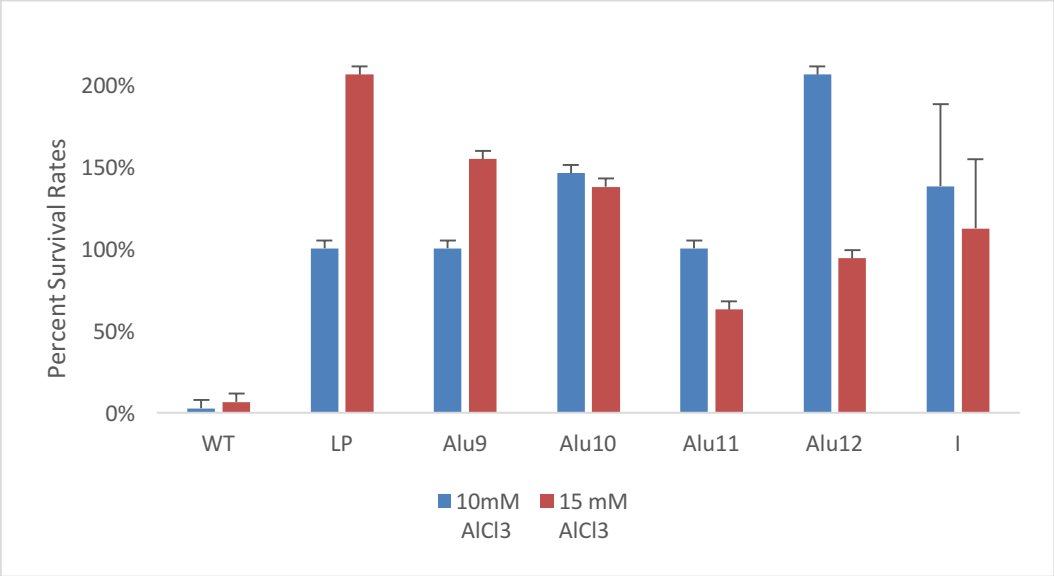

Figure 3.7 : Percent survival rates of individuals, last population and wild type at 72nd h of incubation. "I" represents arithmetic average of percent survival rates of four individuals (Alu9-Alu12) tested.

3.5. Genetic Stability Analysis

Genetic stability test was performed for the four aluminium hyper-resistant mutants by seven successive batch cultivations in the absence of $AlCl_3$ stress and determination of the $AlCl_3$ stress resistance after each of the seven passages by MPN method. The viable cell counts determined by MPN method in the absence and presence of 15 mM $AlCl_3$ stress are given in Tables 3.8 and 3.9

Table 3.8 : Viable cell numbers (per mL) of 7 passages of individual mutants under no aluminium stress.

	Number of cells (cell/mL) under no $AlCl_3$ stress			
Passages	Alu9	Alu10	Alu11	Alu12
1.cultivation	2400000	920000	1700000	700000
2.cultivation	2400000	11000000	2400000	2800000
3.cultivation	1100000	3500000	1700000	1100000
4.cultivation	16000000	1100000	9200000	1100000
5.cultivation	920000	540000	1100000	1700000
6.cultivation	5400000	2400000	540000	1100000
7.cultivation	1600000	3500000	5400000	9200000

Table 3.9 : Viable cell numbers (per mL) of 7 passages of individual mutants upon 15 mM $AlCl_3$ stress.

	Number of cells (cell/mL) upon 15 mM $AlCl_3$ stress			
Passages	Alu9	Alu10	Alu11	Alu12
1.cultivation	2400000	920000	1700000	240000
2.cultivation	1600000	1600000	3500000	3500000
3.cultivation	1700000	2200000	1400000	1600000
4.cultivation	3500000	2400000	3500000	5400000
5.cultivation	2200000	2400000	5400000	3500000
6.cultivation	5400000	3500000	1600000	920000
7.cultivation	2800000	1600000	3500000	4600000

Using the viable cell count data survival rates were calculated for each of the seven passages of the four aluminium hyper-resistant mutants. The results are shown in Table 3.10. The generally high survival rate values imply that the four mutant individuals tested are genetically stable with respect to their aluminium stress resistance characteristics.

Table 3.10 : Survival rates of seven passages of the four mutant individuals upon 15 mM $AlCl_3$ stress.

	Survival rates of mutants upon 15 mM $AlCl_3$ stress			
Passages	Alu9	Alu10	Alu11	Alu12
1.cultivation	1.00	1.00	1.00	0.34
2.cultivation	0.67	0.15	1.46	1.25
3.cultivation	1.55	0.63	0.82	1.45
4.cultivation	0.22	2.18	0.38	4.91
5.cultivation	2.39	4.44	4.91	2.06
6.cultivation	1.00	1.46	2.96	0.84
7.cultivation	1.75	0.46	0.65	0.50

3.6. Cross Resistance to Other Stress Types

The four aluminium hyper-resistant individuals (Alu9, Alu10, Alu 11, Alu12), LP and WT were tested for their potential cross resistance against other stress types, using spot assay method. Upon 72 hours of incubation, images of the control and stress plates were taken (Figures 3.7-3.8-3.9). The results showed that the aluminium hyper-resistant individuals did not generally have a high cross-resistance to any of the stress types tested. However, low levels of cross-resistance against LiCl, $GaNO_3$, and ethanol were observed. Additionally, $AgNO_3$ sensitivity was observed.

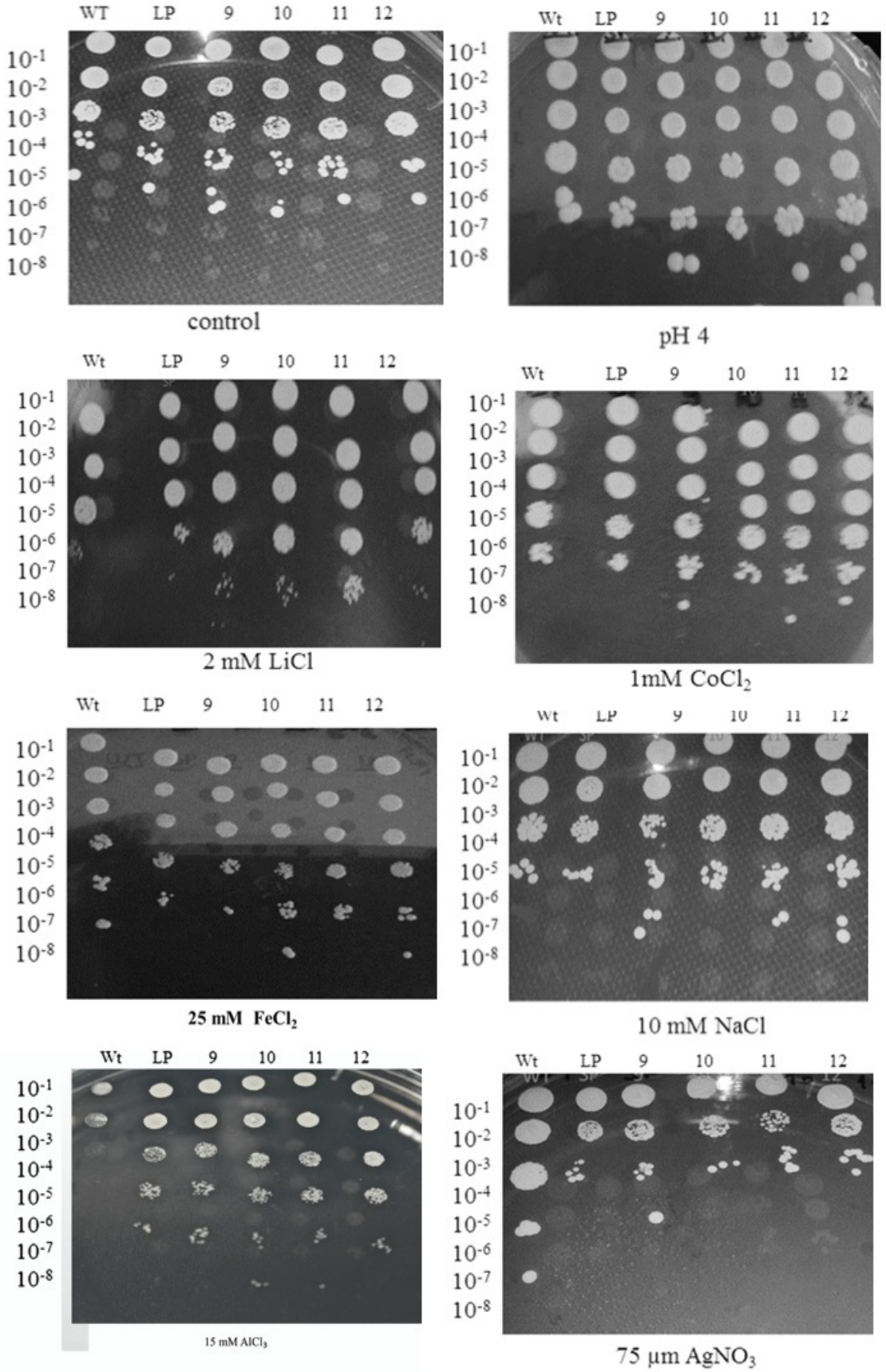

Figure 3.8 : Cross resistance test results (72 hour) of aluminium resistant mutants, LP and WT for pH 4, LiCl, $CoCl_2$, $FeCl_2$, NaCl, $AlCl_3$ and $AgNO_3$.

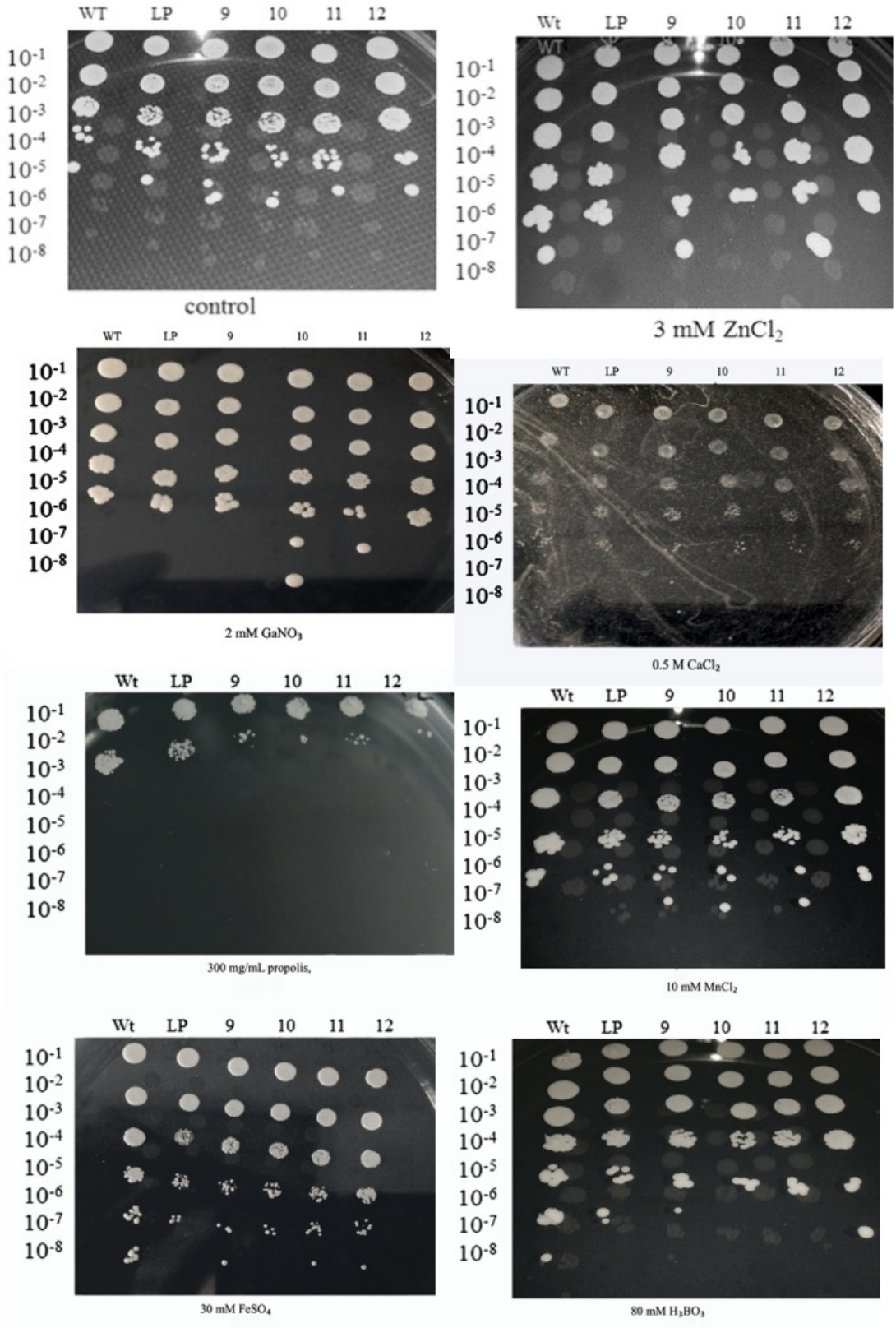

Figure 3.9 : Cross resistance test results (72 hour) of aluminium resistant mutants, LP and WT for $ZnCl_2$, $GaNO_3$, $CaCl_2$, propolis, $MnCl_2$, $FeSO_4$ and H_3BO_3.

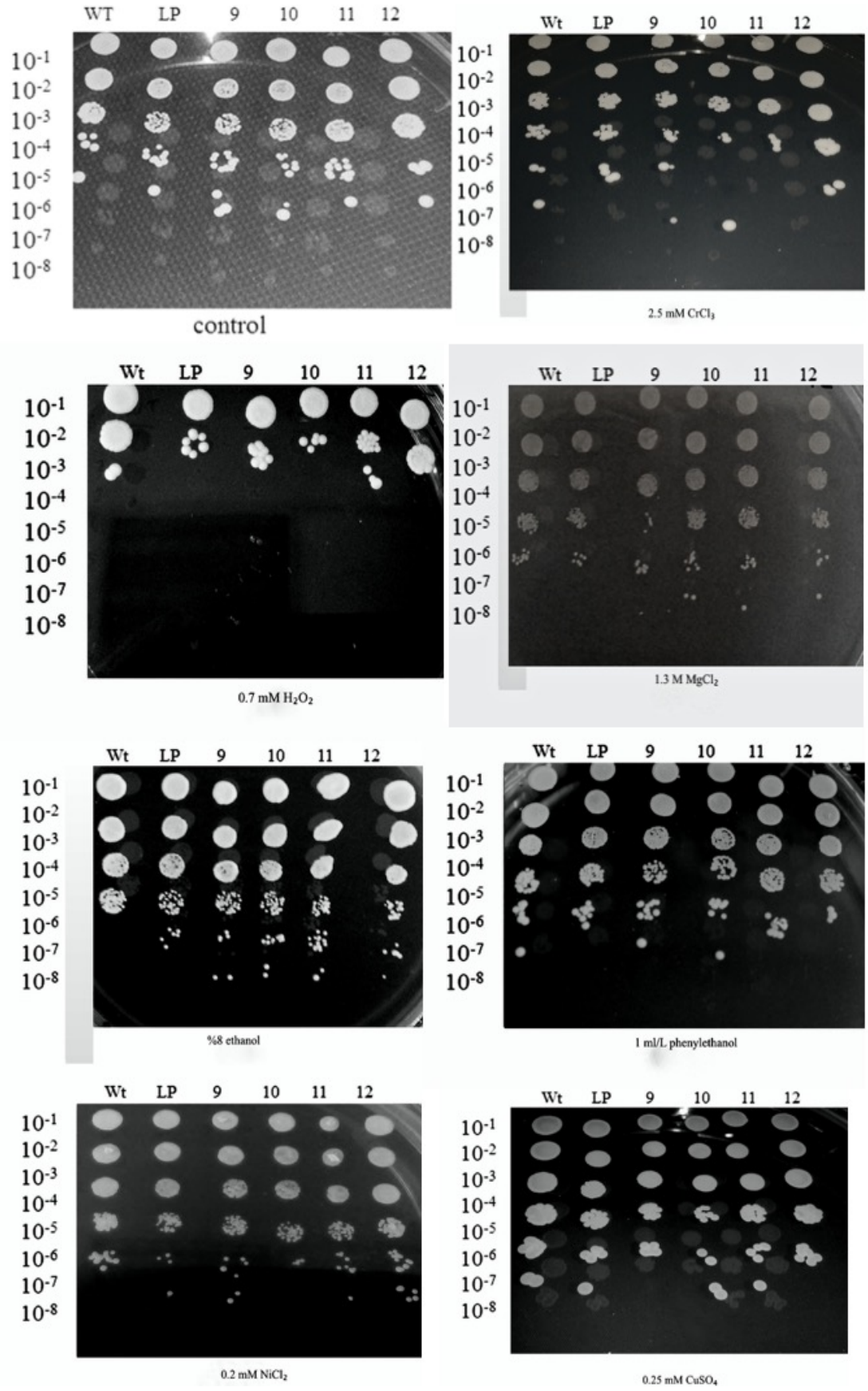

Figure 3.10 : Cross resistance test results (72 hour) of aluminium resistant mutants, LP and WT for$CrCl_3$, H_2O_2, $MgCl_2$, ethanol, phenylethanol, $NiCl_2$ and $CuSO_4$.

4. DISCUSSION AND CONCLUSIONS

In this study, aluminium resistant *Saccharomyces cerevisiae* was obtained by using an inverse metabolic engineering approach, evolutionary engineering. For this purpose, initially, screening was applied to 905 and 906 strains at 11 different $AlCl_3$ concentrations, in order to determine the initial $AlCl_3$ stress level of selection. OD_{600} values were obtained at the 24^{th} and 48^{th} h of incubation and survival rates were calculated for each $AlCl_3$ concentration.

Increasing aluminium concentrations led to dramatically decreasing survival rates (Tables 3.2-3.4). It was observed that between 10 mM and 25 mM $AlCl_3$ 905 cells could barely survive (Figures 3.1-3.2). Thus 15 mM $AlCl_3$ was chosen as the aluminium stress level to be used in resistance determination of the mutants in MPN method, in comparision to the wild type (905) cells.

According to $AlCl_3$ stress screening results, 0.5 mM was chosen as the initial $AlCl_3$ stress level of the selection and the stress level was increased by 0.5 mM at each successive passage.

The EMS-mutagenized, initial population of selection (906) was incubated with 0.5 mM $AlCl_3$ for 24 h. Also a control culture was incubated without aluminium to determine the survival rate. For the second population of selection, 1mM $AlCl_3$ was applied to the culture and $AlCl_3$ levels were increased throughout the 43 populations. At 21.5 mM aluminium concentration, the survival rate dramatically decreased (Table 3.5). Thus, the selection was stopped.

In order to select individual mutants from the final mutant population (43^{th} population), it was spread on solid YMM plate by 10^{-6} dilution. After 72 h of incubation, 12 individual colonies were picked randomly and transferred to liquid YMM for preculture and stock culture preparation. For aluminium stress resistance determination of these 12 individual mutants (Alu1-Alu12), spot assay and MPN method were applied.

In spot assay, 12 individual mutants, WT and LP were diluted from 10^{-1} to 10^{-8} and incubated on aluminium containing solid YMM plates for 72 h until colonies were

observed clearly. Five different $AlCl_3$ (1mM, 2mM, 5mM, 10mM and 21mM) concentrations were applied to all individuals, WT and LP.

At 21 mM $AlCl_3$ stress level which was highly inhibitory to the WT, individual mutants could survive (Figure 3.4). Individual mutants were then compared among each other, based on their aluminium resistance levels and Alu9, Alu10, Alu11 and Alu12 were found to be more resistant than the other mutants at high aluminium concentrations.

Thus, according to spot assay results; Alu9, Alu10, Alu11 and Alu12 were chosen for quantification of their aluminium resistance levels by MPN method. 10 mM and 15 mM were chosen as two aluminium stress levels for this method, as these $AlCl_3$ concentrations caused significant differences in survival between individual mutants and the WT, according to previous experimental all results. Percent survival rates of the individuals and the WT were determined at 48^{th} and 72^{nd} h of cultivation (Tables 3.6-3.7), when the colonies could easily be observed. WT could barely survive, but the survival rates of the individual mutants were significantly higher than that of the WT (Figures 3.5-3.6).

After determination of aluminium resistance levels, cross-resistance tests were performed by applying spot assay to determine any potential cross-resistance to different metal or non-metal stress types. However, no significant cross resistance was generally observed, except for a slight level of LiCl tolerance in all four mutants and $GaNO_3$ tolerance in mutant Alu10, and a slight tolerance to ethanol in all mutants. Additionally, slight sensitivity to $AgNO_3$ was also observed in all mutants tested.

A recent study showed that cross resistance to Fe^{+2} and H_2O_2 were observed in *S.cerevisiae* deletion mutants that were screened for aluminium tolerance (Tun et al. 2014). However, one should note that evolutionary engineering is a different approach than testing deletion libraries, and may involve more genetic diversity. Additionally, the WT strains of the two studies are also different.

Genetic stability test was also performed with Alu9, Alu10, Alu11 and Alu12 in order to determine whether the gained aluminium resistance was stable or not, e.g. an adaptation. Individuals were incubated in YMM without aluminium for 7 successive batch cultivations. MPN assay was applied to each batch culture in order to calculate viable cell numbers and survival rates in the presence of aluminium stress applied during the MPN assay (Tables 3.9-3.10). Survival rates did not decrease significantly

during 7 nonselective cultivations. It was concluded that the tested mutant individuals are genetically stable. Based on all experimental results, Alu10 was chosen as a genetically stable, aluminium hyper-resistant mutant and it will be used in further genetic and transcriptomic analysis.

To conclude, we have succesfully obtained highly aluminium-resistant *S. cerevisiae* mutants by using evolutionary engineering, an inverse metabolic engineering strategy. Successive batch selection strategy at gradually increasing $AlCl_3$ stress levels between 0.5-21.5 mM $AlCl_3$ yielded 43 populations.

Mutant individuals randomly chosen from the last population are highly aluminium-resistant and genetically stable. Detailed comparative physiological, genomic and transcriptomic analyses of the selected highly aluminium-resistant mutant Alu10 and the WT are planned as future studies to gain insight into the molecular mechanisms of aluminium resistance in *S. cerevisiae.*

Additionally, cellular aluminium contents of the Alu10 mutant and the WT will be determined by using atomic absorption spectrometry. Determination of aluminium uptake capacity of the resistant mutant can potentially be useful in bioremediation and industrial applications. The obtained aluminium-resistant *S.cerevisiae* mutant could be used as a eukaryotic model organism to study the molecular mechanisms of human neurodegenerative diseases that have been associated with aluminium.

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