ISTANBUL MEDENIYET UNIVERSITY
INSTITUTE OF GRADUATE STUDIES
DEPARTMENT OF MOLECULAR BIOLOGY AND GENETICS

Genetic Manipulation of Shikimate and Ehrlich Pathway Genes Involved in Acute Polystyrene Toxicity in *Saccharomyces cerevisiae*

Master's Thesis

Hande Güzel

June 2025

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Asst. Prof. Dr. Cihan Aydın

June 2025

THESIS JURY APPROVAL

This Master thesis titled "Genetic Manipulation of Shikimate and Ehrlich Pathway Genes Involved in Acute Polystyrene Toxicity in *Saccharomyces cerevisiae*" written by Güzel, Hande at the Department of Molecular Biology and Genetics was accepted by our jury.

JURY MEMBERS **SIGNATURE**

Supervisor:

Asst. Prof. Dr. Cihan Aydın

Istanbul Medeniyet University

Other Jury Members:

Asst. Prof. Dr. Özge Tatlı

Istanbul Medeniyet University

Assoc. Prof. Dr. Beste Turanlı

Marmara University

Date of Defense: 26 / 06 / 2025

STATEMENTS

Style and Reference Manual Statement

Having reviewed this thesis written under my supervision, I confirm that it has been written in accordance with APA Manual of Style and used its [footnote/in text] reference format consistently throughout the entire text.

Signature
Asst. Prof. Dr. Cihan Aydın

Declaration of Originality

I hereby declare that all information in this dissertation has been obtained and presented in accordance with academic rules and ethical conduct. I also declare that, as required by these rules and conducts, I have fully cited and referenced all material and results that are not original to this work.

Signature
Hande Güzel

GENİŞ ÖZET

Saccharomyces cerevisiae'de Akut Polistiren Toksisitesinde Rol Alan Şikimat ve Erlich Yolu Genlerinin Genetik Manipülasyonu

Güzel, Hande

Yüksek Lisans Tezi , Moleküler Biyoloji ve Genetik Departmanı

Danışman: Dr. öğ. üyesi Cihan Aydın

Haziran 2025

Polistiren, yaygın olarak kullanılan petrokimyasal temelli bir plastik çeşididir. Doğada zor bozunabilen yapısı nedeniyle çevrede uzun süre kalıcılığını korur ve canlı organizmalar üzerinde olumsuz biyolojik etkiler yaratabilir. Özellikle nano boyuttaki polistiren parçacıklar, hücresel düzeyde çeşitli toksik etkiler oluşturarak organizmaların metabolik dengesini bozabilir. Bu çalışmada, model organizma olarak kullanılan *Saccharomyces cerevisiae* üzerinde, polistiren nanoparçacıkların potansiyel toksik etkileri değerlendirilmiş ve bu etkilere karşı hücresel stres toleransını etkileyebilecek genetik faktörler araştırılmıştır. Çalışmada, polistirenin neden olduğu toksisiteye karşı maya hücresinin verdiği yanıtları anlamak amacıyla, özellikle amino asit metabolizmasında görevli olan ARO4, ARO9 ve ARO10 genleri hedef alınmıştır. Bu genler, hem hücrenin temel metabolik faaliyetlerinde rol oynayan hem de çevresel stres faktörlerine karşı savunma mekanizmalarının parçası olabileceği düşünülen genlerdir. Genetik müdahaleler kapsamında bu genler için CRISPR-Cas9 sistemiyle gen nakavtı yapılmış, aynı zamanda rekombinant DNA teknolojisiyle aşırı ekspresyon sağlayan suşlar da oluşturulmuştur. Böylece, hem genlerin susturulması hem de yüksek seviyede ifade edilmeleri durumunda hücresel stres yanıtlarındaki değişimler karşılaştırmalı olarak değerlendirilmiştir. Bu kapsamda oluşturulan genetiği değiştirilmiş S. cerevisiae suşları**,** 25 nm boyutunda ve 250 mg/L konsantrasyonunda polistiren nanoparçacıklarla muamele edilmiştir. Deney gruplarında; vahşi tip suş, ARO4, ARO9 ve ARO10 genlerinin nakavt edildiği suşlar ile bu genlerin aşırı ifade edildiği suşlar yer almıştır. Toksisiteye bağlı stres tepkilerinin belirlenebilmesi amacıyla, büyüme eğrileri ve hücre canlılığı analizleri yapılmıştır. Hücre canlılığı değerlendirmesinde metilen mavisi boyama yöntemi kullanılmıştır. Elde edilen verilere göre, polistiren nanoparçacıklara maruz bırakılan genetiği değiştirilmiş suşlarda, büyüme eğrileri açısından vahşi tip suşla karşılaştırıldığında anlamlı bir fark gözlenmemiştir. Bu durum, polistirenin doğrudan büyüme hızına ciddi bir baskı oluşturmadığını göstermektedir. Ancak, metilen mavisi boyası ile yapılan hücre canlılığı ölçümlerinde, genetik olarak manipüle edilen bazı suşlarda hücresel canlılık oranlarında minimal bir artış tespit edilmiştir. Özellikle genlerin aşırı ifade edildiği suşlarda bu artış daha belirgindir. Bu sonuçlar, ARO gen ailesinin üyelerinin polistiren kaynaklı stres koşullarında hücreyi kısmen koruyucu bir rol üstlenebileceğine işaret etmektedir. Buna rağmen, gözlemlenen koruyucu etkinin sınırlı düzeyde kalması, polistirenin oluşturduğu toksisitenin çok daha karmaşık hücresel

mekanizmalarla bağlantılı olduğunu düşündürmektedir. Stres yanıtı sadece belirli genlerin düzenlenmesiyle değil, aynı zamanda hücrenin genel metabolik dengesini, zar geçirgenliğini, redoks homeostazını ve protein katlanma süreçlerini etkileyen çok yönlü bir sistemle kontrol edilmektedir. Dolayısıyla, ARO genlerinin tek başına bu sistemi tamamen düzenlemesi mümkün değildir. Bu çalışma aynı zamanda, çevresel toksinlerin hücreler üzerindeki etkilerini sadece fenotipik düzeyde değil, genetik ve moleküler düzeyde değerlendiren yaklaşımların önemini vurgulamaktadır. Çevresel biyoteknoloji ve toksikoloji alanlarında bu tür sistematik analizler, toksik etkilere karşı dirençli organizmaların tasarlanması açısından büyük önem taşımaktadır. Tez kapsamında kullanılan CRISPR-Cas9 sistemi, *S. cerevisiae*'de genetik manipülasyonların hızlı ve etkili bir şekilde gerçekleştirilmesini sağlamış ve bu teknolojinin toksikoloji araştırmalarında kullanılabilirliğini göstermiştir. Ayrıca, bu çalışmada kullanılan yaklaşım, ileriye dönük olarak farklı çevresel stres faktörlerine karşı hücresel yanıtların araştırılmasına da temel oluşturabilir. Ağır metaller, farklı plastik türleri veya oksidatif ajanlar gibi toksik bileşiklerin etkileri de benzer genetik müdahalelerle analiz edilerek daha kapsamlı bir stres yanıt haritası oluşturulabilir. Bu sayede, hem çevresel kirliliğin organizmalar üzerindeki etkisi daha net anlaşılabilir hem de biyoteknolojik uygulamalarda kullanılabilecek dayanıklı mikroorganizma suşları geliştirilebilir. Sonuç olarak, bu tez çalışması ile *S. cerevisiae*'nin polistiren nanoparçacıklara karşı verdiği yanıtlar genetik düzeyde değerlendirilmiş, stres koşullarına karşı ARO4, ARO9 ve ARO10 genlerinin potansiyel rolleri ortaya konmuştur. Elde edilen bulgular, çevresel kirleticilere karşı hücresel savunma mekanizmalarının anlaşılmasına katkı sunmakta; aynı zamanda bu bilginin çevresel biyoteknoloji, toksikoloji ve sürdürülebilir mikrobiyal üretim alanlarında uygulanabilirliğine dair önemli ipuçları vermektedir.

Anahtar Kelimeler: polistiren, toksisite, CRISPR-Cas9, nakavt, aşırı ifade

ABSTRACT

Genetic Manipulation of Shikimate and Ehrlich Pathway Genes Involved in Acute Polystyrene Toxicity in *Saccharomyces cerevisiae*

Güzel, Hande

Master's Thesis, Molecular Biology and Genetics Department

Supervisor: Asst. Prof. Cihan Aydın

June 2025

Polystyrene is a widely used petrochemical plastic and has a long-term persistence due to its structure that is difficult to decompose in the environment and can cause toxic effects on biological organisms. In this context, this study on polystyrene toxicity on the model organism *Saccharomyces cerevisiae* is based on the evaluation of the toxic effects of polystyrene on this yeast and the determination of potential genetic targets that may affect cellular stress tolerance against toxicity and the manipulations targeting various metabolic pathways at the gene level. In particular, the ARO4, ARO9 and ARO10 genes, which have role in amino acid metabolism in yeasts, were focused on. In these genes, both knockout using the CRISPR and overexpression of the genes using strategies based on recombinant DNA technologies were achieved. Genetically modified strains were treated with 25nm and 250mg/L PS and their possible effects on cell stress tolerance were investigated by growth curves and cell viability analyses. According to the results, while no significant difference was observed in the growth curves for knockout and overexpression strains, when the cell count results with methylene blue were compared with the wild type strain, it could be said that cellular stress tolerance to polystyrene was minimally increased in gene manipulated strains. In this context, this thesis contributes to the literature in terms of understanding the genetic responses of *S.cerevisiae* to environmental toxic agents and sheds light on the potential applications of genetic engineering approaches in areas such as environmental biotechnology, toxicology and sustainable microbial production.

Keywords: Polystyrene, toxicity, CRISPR-Cas9, knockout, overexpression

ACKNOWLEDGEMENTS

First of all, I would like to thank my advisor Asst. Prof. Dr. Cihan Aydın, who guided me throughout my thesis work, helped me overcome the difficulties I encountered with his knowledge and experience, and supported me with his endless understanding and patience during this process.

I would like to thank my professors at Marmara University, Prof. Dr. Bülent Mertoğlu, Assoc. Prof. Dr. Beste Turanlı and Assoc. Prof. Dr. Ceyhun Bereketoğlu, who supported me throughout my master's degree and thesis process.

Finally, I would like to thank my family and my fiancé, who were always with me throughout my education life and especially during my master's degree and were my morale throughout the whole process.

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LIST OF ABBREVIATIONS

DNA	Deoxyribonucleic acid
RNA	Ribonucleic acid
YPD	Yeast Peptone Dextrose Medium
gDNA	Genomic DNA
YE	Yeast Extract
PS	Polysytrene
PS- NPs.	Polysytrene Nanoparticles
PCR	Polymerase Chain Reaction
dNTP	Deoxynucleotide triphosphate
EDTA	Ethylenediamine tetraacetic acid
TAE	Tris- Acetate- EDTA
TE	Tris-EDTA
HR	Homologous Recombination
NHEJ	Non-homologous End Joining
CRISPR/Cas	Clustered regularly interspaced palindromic repeats
TracrRNA	Trans-activating CRISPR RNA
CrRNA	CRISPR RNA
PAMs	Protospacer adjacent motif
FAE	Formamide-EDTA extracts
RT	Room Temperature

LIST OF SYMBOLS

°C: Degree Celsius

µ: Micro

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1. INTRODUCTION

1.1. Saccharomyces Cerevisiae

Saccharomyces cerevisiae functions as the primary yeast species used among eukaryotic model organisms for sugar conversion into ethanol and carbon dioxide. The organism known as "baker's yeast" contains a membrane-enclosed nucleus together with membrane-bound organelles and demonstrates facultative anaerobic properties. *S.* cerevisiae serves conventional food processing functions while researchers use it extensively for important biotechnological research activities. (Karathia et al., 2011) These studies can be listed specifically as metabolism, cell cycle, stress responses, manipulation of gene expression mechanisms, and gerontological studies.(Bilinski et al., 2017) As a eukaryotic organism, it has also been preferred in toxicology and environmental interaction studies with its features similar to human cells. In this context, *S.cerevisiae* offers a biological system suitable for studying environmental pollutants, such as microplastics and nanoplastics, at the cellular level (Nielsen et al., 2019).

1.1.1. *Saccharomyces Cerevisiae* as a Model Organism

Scientists have used *Saccharomyces cerevisiae* as their primary model organism for research during multiple years. The environmental sciences field frequently selects this organism for genetic and molecular biological research studies.Many reasons for its preference can be stated as its eukaryotic cell structure, the possibility of working in the laboratory, and its short reproduction period (Sharma et al., 2020). In addition, one of the most important reasons for its frequent use in biotechnology is the possibility of easy genetic manipulation (Anju et al., 2020). The methods used in genetic engineering such as knock-out, overexpression and site-directed mutagenesis are quite easy to apply in *S.cerevisiae*. In addition, its rapid multiplication in simple media (such as YPD: Yeast Peptone Dextrose) in the laboratory environment and its high transformation efficiency are effective in the field of recombinant DNA technologies, which are frequently used in

recombinant protein production.(Shen et al., 2019) Following these advantages, it is also used in biofuel production.(Agathokleus et al., 2021)

In addition to all these concepts, *S.cerevisiae* is an important organism in understanding human diseases and developing treatments because it is a eukaryotic organism that shares many basic biological processes with human cells. There are considerable similarities between human cells and yeast cells in terms of DNA repair, intracellular mitochondrial functions, protein coding, cell death mechanism (apoptosis) and basic cell cycle control. With the complete sequencing of the genome of *S.cerevisiae* in 1996, its genes have been understood functionally and it has become a suitable organism for the application of genetic manipulations.(Kaluç et al., 2024) In addition, it is quite advantageous in terms of ethics, ethical restrictions such as the use of mammalian model organisms do not apply to this organism In this context, it is a eukaryotic organism that is frequently preferred for use in cancer research, neurodegenerative disease, metabolic diseases and aging studies.

S.cerevisiae is also frequently used as a model organism in environmental sciences. The fact that the responses of this yeast to environmental cell factors have been well characterized is the most important reason for *S.cerevisiae* to be frequently studied in environmental sciences and toxicology. In these studies, *S.cerevisiae* enables the molecular level examination of adaptations or stress responses to pesticides, heavy metals, organic pollutants and micro- and nanoplastics and toxic effects on yeast.(Braconi et al., 2016) As mentioned in the previous paragraphs, *S.cerevisiae* is a valuable microorganism in many scientific studies, including environmental sciences, because it is a member of the eukaryotic group, is open to genetic manipulation, can be safely studied under laboratory conditions and has ethical advantages over mammalian cells.

1.1.2. Nanoplastics and Their Toxicological Impact on Yeast

In recent years, the prevalence of nanoplastics and the negative effects they can have on living organisms have made studies in these areas popular in the scientific world. The review study at 2019 stated that nanoplastics negatively affect the basic processes of living organisms, which are growth, development and reproduction, and cause abnormalities in normal metabolism. In this context, it has been stated that specifically positively charged and small-sized nanoplastics can pass through the cell membrane and form intracellular accumulation. In addition, the possibility of various additives carried

by nanoplastics and the pollutants they adsorb being released into the organism poses a serious threat to the organism. (Shen et al., 2019)

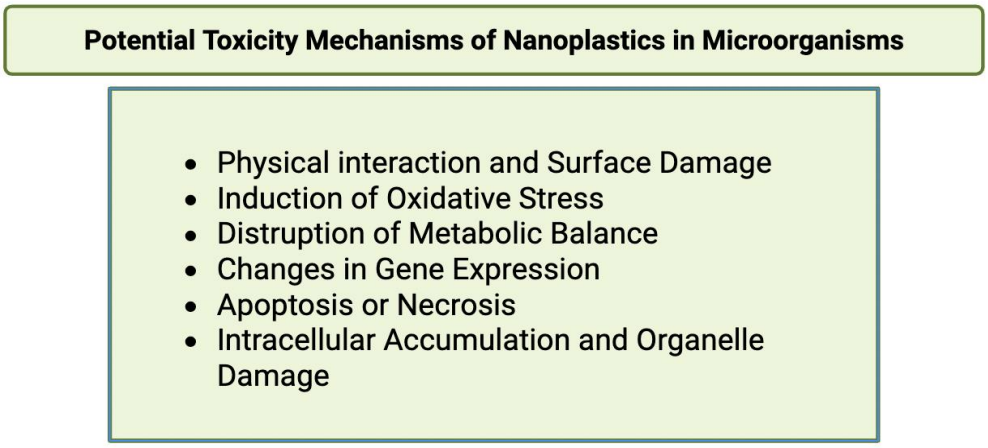


Figure 1. Potential Toxicity Mechanisms of Nanoplastics(NPs) in Microorganisms.

The unique surface characteristics and hydrophobic properties of nanoplastics enable them to bind with environmental pollutants including persistent organic pollutants and heavy metals. The resulting interactions between these substances produce intensified toxic effects. (Ozbek et al., 2021) As listed in Figure 1, nanoplastic toxicity can be observed in eukaryotic cells through various mechanisms. Firstly, physical interaction and surface damage may occur when nanoplastics directly adhere to the cell membrane and negatively affect membrane permeability. In addition, nanoplastics can be taken into the cell by mechanisms such as endocytosis and accumulate in intracellular organelles and cause damage. As a result, cellular functions are negatively affected and imbalances occur in energy metabolism pathways. The disruption of metabolic balance is the main reason for disruptions in the cell's energy production pathway and in addition to amino acid metabolism.(Zahmatkesh et al., 2022)Such stress responses caused by nanoplastics can be observed as changes in gene expression, overexpression of genes or gene suppression. In very high concentrations or long-term exposures, programmed cell death mechanisms may be activated.(De Felice et al., 2022)

1.1.3. Polystyrene structure and Its Use in Investigating Nanoplastic Toxicity in *S.cerevisiae*

Styrene is a valuable molecule that has both industrial and environmental importance and has been frequently used by environmental scientists in recent years. In these days, the effects of styrene on eukaryotic cell metabolism have been a matter of curiosity. Styrene, when it comes to its chemical properties, is one of the simplest alkenylbenzenes and is also known by different names such as phenylethylene or vinylbenzene. The main environmental threat stems from styrene emissions which release into air and water and soil during industrial manufacturing of polystyrene and styrene-butadiene and styrene-based resins (Mooney et al., 2006; Nomura et al., 2013). Environmental scientists now focus on microbial systems to study the toxic effects of styrene because of this emerging issue.

Polystyrene is a linear, amorphous polymer with an atactic structure, consisting of the monomeric building block styrene. Styrene is formed by treating ethyl benzene with steam at high temperature and pressure or by the reaction of benzene and ethylene with aluminum chloride as a catalyst. The chemical reaction of the polymerized styrene, shown in Figure 2, results in the conversion of styrene to distyrene in an acidic environment and the formation of polystyrene by heating the product.(Arfin et al., 2015)

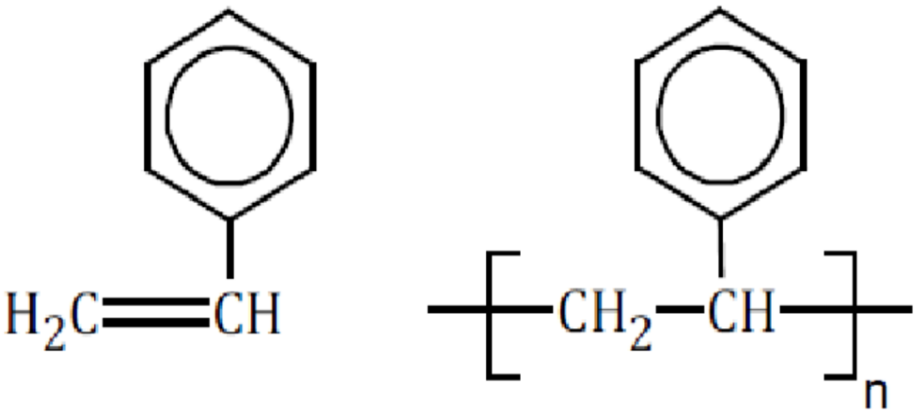


Figure 2. Chemical diagram of styrene polymerization to polystyrene. Polystyrene is a thermoplastic polymer obtained by styrene polymerization. In the polymerization of styrene, an aromatic vinyl monomer, the carbon-carbon π bond in the vinyl group is broken and a new σ bond is created, thus establishing σ bond between the monomers. Each repeating unit of polystyrene, whose chemical formula is (C_8H_8)□, contains a phenyl group. (Arfin et al., 2015)

1.1.4. Metabolic Responses to Polystrene Toxicity in Yeast: Shikimate and Ehrlich Pathway

Polystyrene is a type of plastic frequently used in packaging and electronics industry due to its high chemical stability, low density and long environmental permanence. When polystyrene's long environmental permanence and unique physicochemical properties are evaluated, it can be considered that it will cause toxic effects in microorganisms. Especially when it is decomposed into micro and nano sizes, polystyrene increases the reactive surface area that can affect living microorganisms and thus its effect increases. PS toxicity can cause changes in gene expression levels, affect cellular stress responses and affect metabolic pathways. PS exposure leads to direct or indirect impacts on the shikimate and ehrlich pathways which serve as essential metabolic pathways for aromatic amino acid synthesis..(Kojima et al., 2023)

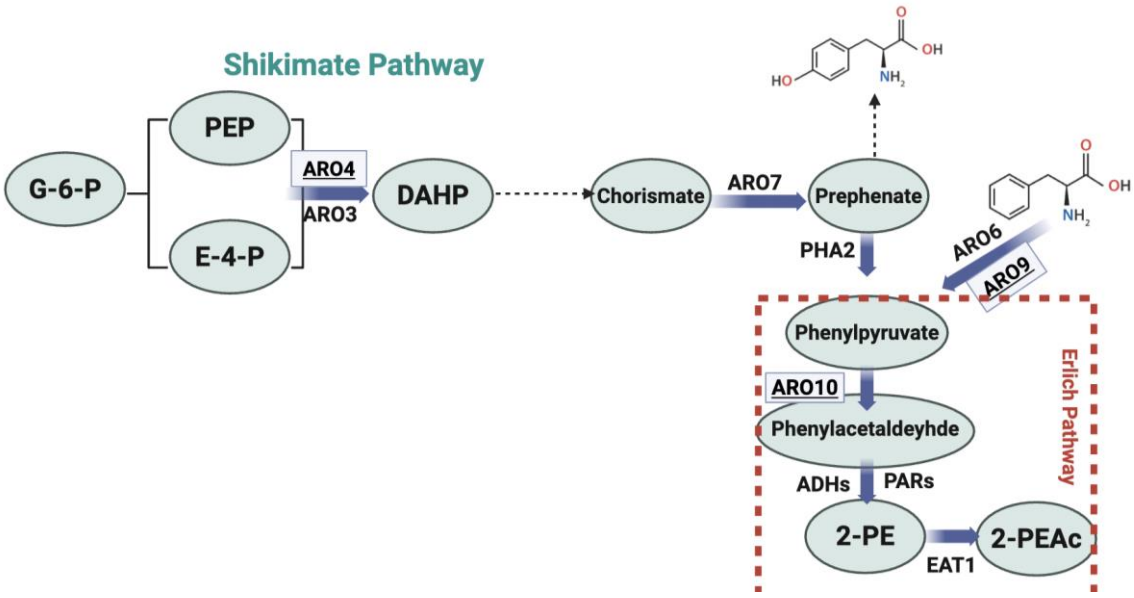


Figure 3. Shikimate and Ehrlich Pathway on Yeast. The shikimate pathway has duty on synthesis of aromatic amino acids like tyrosine, phenylalanine and tryptophan in *S.cerevisiae.* As seen in Figure 3, the beginning of this pathway is precursors derived from the glycolysis pathway such as phosphoenolpyruvate and erythrose-4-phosphate.(Averesch et al., 2018) The first step, catalyzed by DAHP synthase enzymes via ARO3 and ARO4 genes, is subject to feedback inhibition by phenylalanine and tyrosine. Phenylalanine is synthesized via the pathway named shikimate pathway and the next step, the Erlich pathway, begins, which can produce aromatic

alcohols. The first step performed in this pathway, performed by ARO6 and ARO9, is the conversion of phenylalanine to phenylpyruvate. This is followed by the decarboxylation of phenylpyruvate to phenylaceraldehyde by ARO10. The last step of the Erlich pathway is the reduction of phenylacetaldehyde to 2-PE, which is provided by ADH enzymes.(Li et al., 2021)

It is predicted that environmental toxins and stress factors may cause transcriptional changes in *S.cerevisiae*. For example, in a study conducted in 2017 on nonylphenol exposure in *S.cerevisiae*, this was recorded that the expression of ARO9 and ARO10 genes, which are involved in the biosynthesis and catabolism of aromatic amino acids, was significantly suppressed due to nonylphenol exposure. In addition, this study discussed that nonylphenol can cause cell membrane damage and affect the amino acid mechanism, and may also indirectly lead to disruptions in phenylalanine metabolism. (Bereketoglu et al., 2017)

In a diversified study conducted in 2017, it was examined how BPA affected gene expression profiles in yeast cells. When the findings were examined, it was seen that BPA, which functions in the Erlich pathway in yeast cells and is important in the catabolism of aromatic amino acids, and specifically alpha-keto acids such as phenylpyruvate. Downregulation was recorded in the ARO10 gene, which provides decarboxylation. This downregulation may have negative effects on cell metabolism. (Bereketoglu et al., 2017)

Environmental stress factors can cause metabolic changes in *S.cerevisiae* and trigger gene transcriptional responses. Environmental stress factors such as heat shock can affect the transcriptional response in the cell in general, as well as causing changes in the expression of ARO10 gene, which has duty on in amino acid catabolism. In addition, the type or quality of nitrogen sources can also has impact the expression of some genes that play an important role in aromatic amino acid catabolism pathways. For example, it was recorded that the expression of ARO9 and ARO10 genes, which have an important role in the Erlich pathway, increased in poor nitrogen source environments, while it was suppressed in strong nitrogen sources. (Vuralhan et al., 2003) To summarize, chemicals such as noniphenol or bisphenol A, which may cause toxic effects, or environmental stress factors such as heat and nitrogen, have been effective in the expression of ARO genes located in the shikimate and erlich pathways, which involve the biosynthesis and

catabolism of aromatic amino acids in *Saccharomyces cerevisiae*. In this context, the possibility that these metabolic pathways are affected by the toxicity that polystyrene may cause should also be taken into consideration.

1.2. CRISPR-Cas9 Technology: From Discovery to Gene Editing

In 1987, scientists first discovered Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) as repetitive sequences of DNA within the Escherichia coli genome, though their purpose remained a mystery back then (Ishino et al., 1987). It wasn't until the early 2000s that research revealed these sequences takes important place in the adaptive immune system of prokaryotes, acting as a defensive mechanism against invasive genetic material. (Mojica et al., 2005). In a study conducted in 2007, *Streptococcus thermophilus* bacteria were exposed to phage attack, and it was subsequently observed that these bacteria integrated new spacer sequences derived from their phage genomes into their CRISPR regions. The presence of these new spacers showed that the bacteria gained resistance to the relevant phage. In this context, it was experimentally demonstrated that the CRISPR system constitutes a natural defense mechanism against bacteriophages in bacteria and archaea. (Barrangou et al., 2007) Following this, in a study conducted in 2008, it was experimentally shown that the CRISPR-Cas system prevented plasmid-based gene transfer in the Staphylococcus epidermidis model. Accordingly, the idea that the CRISPR system is an immune system that controls the horizontal transfer of genetic elements, not just antiviral defense or only against phages, was demonstrated in this article. (Marraffini et al., 2008) The CRISPR system was defined as a programmable gene editing system that performs target DNA cutting using the Cas9 endonuclease in 2012, with the work of Jennifer Doudna and Emmanuelle Charpentier, which can be considered revolutionary in terms of genetics (Jinek et al., 2012). This invention earned Doudna and Charpentier the Nobel Prize in Chemistry in 2020.

After discussing the discovery of the CRISPR system and its evolution leading to gene editing and its development from a system that provides adaptive immunity in prokaryotes to genetic engineering, it is necessary to discuss the CRISPR mechanism in

detail. The components that make up the CRISPR system are mainly CRISPR loci containing short repeat sequences, spacer DNAs and Cas proteins that enable the cutting of the target DNA. This mechanism has three basic stages; these can be listed as adaptation, expression and interference. As seen in Figure 4, in the first stage, adaptation, a piece of the viral DNA that enters the cell integrates into the CRISPR locus named as a "spacer". In expression, this locus is transcribed and crRNA is formed and crRNAs form a complex with the Cas9 prorein and this complex recognizes the target DNA sequence thanks to the spacer sequence in the crRNA and the Cas9 protein cuts the target DNA double-stranded with its endonuclease activity. With this cut, DNA repair mechanisms in the cell can be stimulated and gene silencing, gene insertion or point mutations can be performed. What makes CRISPR-Cas9 superior in genetic modifications is that the recognition of the target DNA is carried out via guide RNA and the presence of PAM motifs. Accordingly, this system targets the target locus with high specificity and provides a convenient mechanism for genetic modifications. (Doudna et al., 2014)

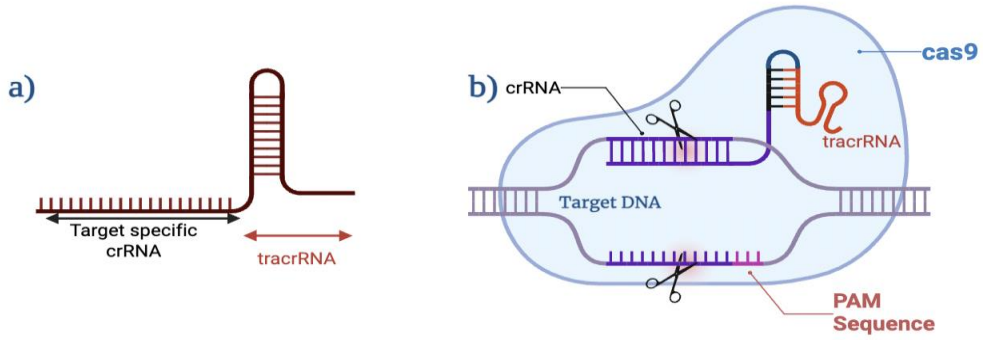


Figure 4. CRISPR-Cas9 Mechanism. This system consists of an RNA molecule (sgRNA) that specifically guides the target DNA sequence and the Cas9 endonuclease. The guide RNA base-pairs with the target DNA, allowing Cas9 to create a double-stranded break at a specific location. This break is processed by cellular DNA repair mechanisms, allowing editing of the target gene. (Doudna et al., 2014)

The CRISPR-Cas9 system is a gene editing tool with high target specificity, based on the recognition and double-stranded cutting of target DNA regions by the cas9 protein via a short gRNA. This system can be frequently used to make edits in living genomes due to its high target specificity and easy designability. This technology can be used in varied

areas like modeling of diseases and the development of potential treatments for various diseases and transcriptional regulation. It has the potential to support successful developments in areas such as rare hereditary diseases, clinical gene therapies, immunotherapies developed for cancer and therapies that can be created for viral infections. In addition to all these, it is a very suitable technology for use in applications such as gene editing to increase product productivity in agricultural biotechnology, development of resistance to stress responses in plants and elimination of unwanted traits in plants through gene editing. (Hsu et al., 2014)

1.2.1. Gene Expression Systems and Gene Manipulation in *Saccharomyces Cerevisiae*

Understanding the gene expression systems of *Saccharomyces Cerevisiae* is very important for understanding the basic biological processes in eukaryotic organisms and for biotechnological developments. *S.cerevisiae* is frequently used as a model organism in scientific researches such as genetic engineering, protein engineering, synthetic biology and molecular biology.

The main elements of the gene expression system in *S.cerevisiae* are promoters, terminators, and selective markers, such as elements contained in a plasmid. Promoters that regulate the expression level and determine the timing of this system can be classified as constitutive promoters and promoters sensitive to environmental factors in this yeast. Promoters that are not sensitive to intracellular signals or environmental factors can be exemplified as TEF1, ADH1, and PGK1 in this yeast, and these promoters provide continuous expression. From another perspective, promoters sensitive to environmental factors, such as GAL1, CUP1, and MET25, are used in inducible systems. For example, the GAL1 promoter is an inducible promoter of the GAL1 gene, which is involved in galactose metabolism. The GAL1 promoter used in controlled gene expression is active in the asset of galactose and inactive in the asset of glucose. (Mumberg et al., 1995) Terminators, another major element of the gene expression system, are responsible for the termination of transcription and also ensure the stability of mRNA transcripts. Terminators are situated in the 3'UTR part of the gene, are responsible for the cutting and polyadenylation of the mRNA, and enable the transcript to enter translation. The most

commonly used terminators in this yeast are ADH1, TEF1, CYC1 and PGK1. (Yamanishi et al., 2013) Other important elements of the gene expression systems of *S.cerevisiae* are selective markers and signal sequences. Selective markers ensure that the cell with gene modification can be correctly selected. These markers can be examined under two main headings as auxotrophic and antibiotic resistance selection markers. Auxotrophic markers such as URA3, LEU2, HIS3, and TRP1 are markers that allow only carrier cells to grow in a nutrient-deficient such as -LEU environment and are selected using drop-out media. In addition, markers such as KanMX, which provide antibiotic resistance, allow genetically modified cells to be selected in environments where the G418 antibiotic is present. (Wach et al., 1994) Another element, signal sequences, allow proteins to be directed out of the cell or to certain organelles. Thanks to these sequences, the target protein can be correctly oriented and folded after translation. For example, a-factor signal peptides are a signal sequence that allows recombinant proteins to be secreted from the yeast cell. (Brake et al., 1984)

Gene manipulation applications in *S.cerevisiae* are quite popular applications for today's molecular biology, synthetic biology and protein engineering. Homologous recombination, which has been used for many years, is used in methods such as gene deletion and gene insertion and is a system that works by using DNA fragments that are specifically integrated into the target gene region. (Baudin et al., 1993) CRISPR-Cas systems are a tool frequently used in gene editing in bacterial systems, and can also be used quite successfully in yeast. Various methods have been developed that increase the usability of this method and facilitate the gene editing system. One of these methods is the Cas9 protein, which is expressed with an expression cassette containing a 2μ replicon and controlled by the TEF1 promoter, while the target-specific guide RNA (gRNA) of approximately 20 bases is encoded with a separate cassette under the SNR52 promoter. The main feature that makes this method easy, fast and efficient is that gRNAs are obtained directly by PCR without requiring cloning. Plasmid containing the Cas9 gene and gRNA primers create the gRNA expression cassette by PCR. Approximately 40-60 bases long oligonucleotide is used to direct the gene to the region where it will be edited. With these ODNs, genes in the target region can be deleted (knock-out) (Generoso et al., 2016)

Gene editing in *S.cerevisiae* is quite valuable in basic genetic studies as well as in medicine, biotechnology, environmental sciences and industry. Specifically, in terms of recombinant protein production, the gene expression system of *S.cerevisiae* can be used for the production of medical products such as insulin, vaccine antigens, growth hormones and therapeutic antibodies. (Porro et al., 2011) In addition, with CRISPR-Cas9 technologies applied in yeast cells, it is possible to work in the field of cancer biology, research on neurodegenerative diseases and drug toxicity studies. (Tenreiro et al., 2013) From different angle, gene manipulation systems on yeast have also been used in industrial biotechnology in the production of biofuels, production of aromatic compounds and synthesis of bioplastics. (Nevoigt, 2008)

2. MATERIALS&METHODS

2.1. MATERIALS

Table 1. List of instruments

Device Type	Model	Brand
-86˚C ULT Freezer	DW-86L486E	Haier
Autoclave	OT 90L	Nüve
Centrifuge (Cold)	-	Beckman Coulter
Centrifuge (Cold)	Centrifuge 5418 R	Eppendorf
Centrifuge	Allegra X-15R Centrifuge	Beckman Coulter
Incubator	M3025P	Electro-mag
Magnetic Stirrer	MI0102003	Four E'S Scientific
Microcentrifuge	WN-CMV6000 microcentrifuge	Weightlab instruments
Micropipette 1 ml	PIPETMAN P1000	GILSON
Micropipette 200 uL	PIPETMAN P200	GILSON
Micropipette 100 uL	PIPETMAN P100	GILSON
Micropipette 20 uL	PIPETMAN P20	GILSON
Micropipette 10 uL	PIPETMAN P10	GILSON
Micropipette 2 uL	PIPETMAN P2	GILSON
pH Meter	XS 201 T pH Electrode	Vio Labs
Shaking incubator	NCI55	Nükleon
Spectrophotometer	DS-11+Spectrophotometer	DeNovix
Ultra-Low Temperature Freezer	Haier DW86L388	Haier Biomedical
Ultra-pure water Purification System	Milli-Q	Merck
Vortex	MI0101002	Four E'S Scientific

Waterbath	VWB 18	Nüve
Electroporator	The GenePulser Xcell 1652666	Bio-Rad
RT-qPCR Machine	Quant-Studio	Thermo Fisher
PCR Machine	Fast Thermal Cycler	A300

Table 2. List of chemicals

Name	**Brand**	**Catalog**
Tryptone	BioShop Canada	-
Agar	BioShop Canada	-
Ammonium Sulfate		-
Yeast Extract	BioShop Canada	-
Yeast Nitrogen Base	BioShop Canada	YNB404
Lithium Acetate(LiAc)	BLDpharm	BD136428
SDS	Multicell	-
Dextrose (Glucose)	Isolab	-
Triton X-100	Sigma Aldrich	-
MgCl2	BioShop Canada	-
Salmon Sperm DNA	BioShop Canada	DNA002.1
PEG3350	ERLAB	-
HCl 37%	TEKKİM	TK.911011
Sorbitol	BioShop Canada	SOR508.1
Dithiothreitol (DTT)	Biomatik	A2452-25G
Calcium Chloride	Isolab	-
Potassium Chloride	Isolab	-
Trizole	Sigma Aldrich	419338
Sodium Chloride (NaCl)	KimyaLab	-
Sodium Hydroxide (NaOH)	Isolab	-
Ethanol/ Teksoll (95%)	Honeywell	-

Isopropanol	TEKKİM	TK.200650
EDTA	BLDPharm	-
Polystrene	Kisker BioTech	-
Formamide	BioShop Canada	
G-418 sulfate	GoldBio	G-418-25
Diethyl pyrocarbonate (DEPC)	Sigma	-
Chloroform	Fluka	-
Ethidium bromide	Sigma	-
Potassium Acetate	Sigma	-
PEG3350	Sigma	-
Methylene Blue	Sigma	-

Table 3. List of Molecular Biology Reagents and Tools

Name	**Brand**	**Catalog**
10X FD Green Buffer	ThermoScientific	-
5X Q5 Reaction Buffer	NewEnglandBiolabs	-
NEB T4 Buffer	NewEnglandBiolabs	-
5X Reaction Buffer	ThermoScientific	-
2X BEAR Mix	CALab	-
dNTP	ThermoScientific	-
RNAase A	ThermoScientific	-
Q5 HF DNA Polymerase	NewEnglandBiolabs	-
NEB T4 Ligase	NewEnglandBiolabs	-
Revert-Aid RT	ThermoScientific	-
Taq	CALab	-
RiboLock RNAase Inhibitor	ThermoScientific	-
Random Primer	ThermoScientific	-

SYBR Green II	Beyotime	-
ROX	Vazyme	-
Orange G	Sigma	-
ApaI	ThermoScientific	-
BamHI	ThermoScientific	-
BcuI	ThermoScientific	-
BsaI-v2-HF	ThermoScientific	-
Eco31I	ThermoScientific	-
Eco32I	ThermoScientific	-
HindIII	ThermoScientific	-
KpnI	ThermoScientific	-
NheI	ThermoScientific	-
SalI	ThermoScientific	-
SmaI	ThermoScientific	-
PdmI	ThermoScientific	-
PstI	ThermoScientific	-

2.2. METHODS

2.2.1. Cultivatition and gDNA Extraction of *S.cerevisiae* By4742

2.2.1.1. Media Preparation and Cultivation of *S.cerevisiae* By4742

Saccharomyces cerevisiae By4742 (his3Δ1; leu2Δ0; lys2Δ0; ura3Δ0) strain was used in all experimental applications for this thesis. YPD (Yeast Extract Peptone Dextrose) medium was prepared for the cultivation of yeast cells. The medium was prepared to contain 10 g/L YE, 20 g/L tryptone/ peptone and 2% glucose.In addition, YPD medium which is solid was prepared comprised 10 g/L YE, 20 g/L tryptone, 20 g/L agar and 2% glucose. After the components except glucose were dissolved in pure distilled water and autoclaved, glucose was prepared separately and added to the media after filter sterilization. All cultures were waited at 30°C, and shaked at 200 rpm for overnight. Main cultures were taken in log phase.

2.2.1.2. gDNA Extraction of By4742 by LiOAc-SDS Method

Genomic DNA extraction was performed using the LiOAc-SDS method for three samples from Saccharomyces cerevisiae By4742 cells. 1.5 mL of culture from cells grown in log phase was moved to centrifuge tubes and precipitated at 11,000 × g for 3 min and the liquid lying above a precipitate was discarded. Cell sediments were suspended with lysis buffer that have components such as 0.2 M LiOAc and 1% SDS. The mixture was homogenized by vortexing and after the mixture was waited at 70 °C for 5 min. After this process of incubate, 300 µL pure ethanol which is 100% was supplemented to the tubes and centrifuged at 15,000 × g for 3 min by vortexing vigorously for phase separation and the liquid part was thrown away. The precipitate was resuspended with 600 µL 70% ethanol for washing and precipitated at 15,000 × g. After separated the liquid part, the precipitate was dissolved in 100 µL of TE (TRIS-EDTA) buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). It was spun at 15,000 x g for 15 seconds, and the liquid part was moved to a different tube and gDNA samples were stored at -20°C. (Looke et al.,2011)

2.2.1.3. gDNA Extraction of By4742 by Bust'n Grab Method

One of the methods used to obtain genomic DNA from Saccharomyces Cerevisiae By4742 yeast cells quickly, easily and with high yield is Bust'n Grab. Three samples of By4742 cells were incubated overnight until expansied to log phase at 30°C and 200 rpm. The one day after, 1.5 mL of sufficiently grown cells were moved to a 2 mL tube and precipitated with centrifuge at 3,600 × g. Then, this precipitate was re-dissolved with 200 µL of lysis buffer containing 10 mM TRIS-HCl pH 8.0, 1 mM EDTA, 100 mM NaCl, 2% Triton X-100 and 1% SDS. Then, it was incubated in ice-isopropanol for 2 min and then immediately at 95°C for 1 minute and this step was performed again. Then it was swirled for 30 sec and 200 µL chloroform/phenol/isoamyl mixture was added to the solution and the mixture was swirled for 2 minutes. After the swirling step, it was settled at 20,000 x g at RT and the liquid lying above was transferred to a different tube containing the same amount of 100% ethanol as the solution and swirled slowly. This mixture was left at room temperature for 5 minutes and subsequently centrifuged at 20,000 x g at RT for 5 minutes. Afterwards, after the liquid part was thrown away, the precipitate was washed with 500 µL of 70% ethanol and the liquid part was thrown away. Then, the cap of the tube that contains the precipitate was opened and left at 60°C for 5 minutes and left to dry in air. Finally, the precipitate was re-dissolved with 30 µL TRIS-EDTA buffer and these gDNA samples were stored at -20°C. (Harju et al., 2004)

2.2.1.4. Direct Boiling Method with TE(Tris-EDTA) Buffer for gDNA Extraction

Genomic extraction of other three samples of By4742 cells carried out with direct boiling method with TE buffer. By4742 cells were expansied overnight until they achieved sufficient enlargement phase at 30°C incubator with 200 rpm shaker. The other day, 1 mL of saturated By4742 cells precipitate with 4,000 x g centrifugation for 5 min. Then, precipitates were resuspended 500 µL of TRIS-EDTA buffer and incubated at 95°C for 10 minutes. Afterwards, the mixture was precipitate by centrifuge at 4,000 x g for 5 minutes and the upper phase of mixture was relocated to the different tube and stored at 20°C. (Shin et al., 2021)

2.2.2. Determination of ARO4, ARO9 and ARO10 genes by PCR and PCR purification

2.2.2.1. Determination of ARO4, ARO9 and ARO10 genes by PCR

For the amplification of ARO4, ARO9 and ARO10 genes, PCR reactions were prepared in 25 µL reaction volume using NEB® Q5 High-Fidelity DNA Polymerase. Specific primer pairs were used for each gene which are gDNAs extracted with LiOAc-SDS method, primer sequences are given in Table 4. This reaction mixture is shown in Table 5.

Table 4. Primer Sequences for Forward and Reverse of ARO4, ARO9 and ARO10.

ARO4_CE_F	GGTCTCTAATGAGTGAATCTCCAATGTTC
ARO4_CE_R	GGTCTCAAAGCCTATTTCTTGTTAACTTCTCTTC
ARO9_CE_F	GGTCTCTAATGACTGCTGGTTCTG
ARO9_CE_R	GGTCTCAAAGCTCAACTTTTATAGTTGTCAAAAAA
ARO10_CE_F	GGTCTCTAATGGCACCTGTTACAATTG
ARO10_CE_R	GGTCTCAAAGCCTATTTTTTATTTCTTTTAAGTGC

Table 5. PCR components

Component	**Volume(µL)**
5X Q5 Reaction Buffer	4 µL
dNTP Mix (10 mM)	0.5 µL
Forward/Reverse Primers (10 µM)	2.5 µL
Q5 High-Fidelity DNA Polymerase	0.5 µL
Template gDNA (~100 ng/µL)	1 µL
DEPC (No nuclease) Water	16.5 µL
Total	**25 µL**

Table 6. Thermal Cycler Program

Step	Temperature (°C)
Initial denaturation	98°C
Denaturation	98°C
Annealing	55–65°C
Extension	72°C
Final extension	72°C

2.2.2.2. Agarose Electrophoresis and PCR Purification

The products of ARO4, ARO9 and ARO10 genes amplified by PCR were electrophoresed in 1% agarose gel in 1X TAE buffer at 100 V for approximately 30–40 minutes. The bands were collected by cutting DNA bands of appropriate sizes with the help of a UV transilluminator. DNA purification from the gel was performed using NTI buffer. Gel pieces were weighed precisely and 300 µL of NTI buffer was used for each 100 mg of gel. Subsequently, the tubes containing the gel fragments and NTI buffer were maintained at a temperature of 50°C for around 15 minutes, with periodic vortexing to ensure complete dissolution of the gel. Once dissolved, the gel mixture was then transferred to spin columns (biocomma® Micro-Scale DNA Purification Columns) and subjected to centrifugation at 11,000 x g for a duration of 1 minute. 700 µL PE buffer was added to the column, centrifuged at 11,000 x g, and this wash step was performed again. Afterwards, the columns were spined down as empty at 11,000 x g for 2 minutes and ethanol residue was aspirated. DNA in the column was eluted with 15 µL Elution Buffer. The columns are incubated at 70°C for 2 minutes.Then, purified DNAs were analyzed with Nanodrop for concentration measurement and stored at -20°C.

2.2.3. Cloning ARO4, ARO9 and ARO10 genes into v2_pJET Vector

2.2.3.1. Cut v2_pJET Vector and Ligation with ARO genes

ARO4, ARO9 and ARO10 genes, which were detected by PCR and gel purified, were cloned into the v2_pJET plasmid to create a suitable expression system. In the cloning process, the vector DNA was cut with Thermo Scientific Eco32I restriction enzyme. The cutting reaction was established as shown in Table 7.

Table 7. Restriction Digestion Reaction of v2_pJET with Eco32I

Component	**Volume (µL)**
v2_pJET plasmid (1 µg)	X µL *(1000ng)*
X µL *(1000ng)*	2 µL
Eco32I enzyme (Thermo)	1 µL
dH_2O	Up to 20 µL
Total	**20 µL**

2.2.3.2. Cloning ARO genes into v2_pJET Vector

The v2_pJET plasmid, which was cut with Eco32I enzyme and purified, and the ARO4, ARO9 and ARO10 genes, which were purified after PCR, were subjected to each ligation reactions using NEB T4 DNA Ligase (New England Biolabs). The amount of insert and vector to be added to the ligation reactions was added to the reaction in accordance with the calculations seen in Table 8, and a total volume of 10 µL was prepared as in Table 9. After the reactions were set up, they were kept overnight at 16°C. Then, they were utilized for transformation into suitable competent cells.

Table 8. Calculation of Ligation Reaction

Ligation Reaction Calculation;
Vector length x 6 = ng of vector add into reaction
Insert lenght x 6 x 3 = ng of insert add into reaction

	Length	Calculation
ARO4	1113 bp	1.11kb x 6 x 3 = 19.98 ng
ARO9	1542 bp	1.54kb x 6 x 3 = 27.72 ng
ARO10	1908 bp	1.9 kb x 6 x 3 = 34.2 ng
v3_pJET	3415 bp	3.4 kb x 6 = 20.4 ng

Table 9. Ligation Reactions

Components	**V2_ARO4**	**V2_ARO9**	**V2_ARO10**	**V2Ø**
NEB T4 Buffer	1µL	1µL	1µL	1µL
NEB T4 Ligase	0.5µL	0.5µL	0.5µL	0.5µL
V2_pJET(vector)	20.4ng	20.4ng	20.4ng	20.4ng
ARO4	19.98ng	-	-	-
ARO9	-	27.72ng	-	-
ARO10	-	-	34.2ng	-
ddH_2O	Up to 10µL	Up to 10µL	Up to 10µL	Up to 10µL

2.2.3.3. Transformation into NEBStable Competent Cell

Ligated v2_pJET_ARO4, v2_pJET_ARO9 and v2_pJET_ARO10 and v2_pJET vector without any insert plasmid structure were transformed into one of the competent E. coli strains by heat-shock method using methods based on bacterial transformation. For each transformation reaction, 50 µL NEBStable competent cells were waited on ice for use. Following this, 5 µL of ligated reactions prepared and kept at 16C overnight were slowly added to 50 µL of NEBStable cells. These samples were waited on ice for 30 minutes. Then, these samples were exposed to heat in a 42C water bath for 30 seconds, thus performing the heat shock application. After 30 seconds, the samples were quickly put back on ice and frozen for 5 minutes. After this step, 450 µL of room temperature liquid LB broth was added for every sample and grown by shaking at 200 rpm for 1 hour at 37°C. Finally, since ampicillin resistance reactions were transformed, 200 µL of

transformed cells were seeded onto LB solid medium containing ampicillin antibiotic and allowed to grow overnight at 37°C. (Chang et al., 2017)

2.2.3.4. Inoculate Colony and Plasmid Isolation (MiniPrep)

The following day, ampicillin was selected by being careful to select only one of the transformant colonies growing on the plate for a culture and these cultures were seeded into 4mL ampicillin-containing liquid media and these cultures were grown overnight at 37C by shaking with 200 rpm. The next day, the cultures that reached a sufficient growth phase were sedimented by centrifuging in 2mL tubes at 11,000 x g for 3 minutes. After the cells were sedimented, the remaining liquid, i.e. the medium, was aspirated and each of the sediments was dissolved with 250 µL of P1 resuspension buffer containing 50 mM Tris-HCl pH 8.0, 10 mM EDTA, 100 µg/mL RNaseA. Then, 250 µL of P2 lysis buffer containing 200 mM NaOH, 1% SDS and thymolfetelain for coloring was added to the resuspended samples taken from ice. After mixing with a flip-flop, it was waited at RT for 5 minutes for the lysis buffer to work. As soon as 5 minutes were completed, 350 µL of 3M Potassium Acetate containing buffer, namely neutralizer (N3), was added to the samples and blended gently with a flip-flop until the color of the samples turned white. Then, these samples were sedimented at 16,000x g for 10 minutes until cell debris was thoroughly separated and the supernatant was transferred to columns designed for plasmid isolation as approximately 800 µL. Then, these columns were carefully placed in the centrifuge and spun at 11,000 x g for 1 minute. For the washing steps, first PB buffer containing guanidine hydrochloride, which is a binder, was added as 500 µL and precipitated again at 11,000xg to ensure that the buffer passed through the column. The second washing step was performed by adding 700 µL of PE buffer, an ethanol-based buffer, to the centrifuged columns. After the buffer residue remaining in the reservoir of the plasmid isolation columns was discarded, they were spun down at 11,000xg for 2 minutes to sure that the silicone-based layer holding the plasmids in the column was freed from ethanol residues. Then, the upper parts of the columns containing the plasmid were placed in a different tube and 60 µL of elution buffer containing TRIS-HCl pH 8.0 was added to the columns freed from ethanol residues and kept at 70C for 2 minutes. Then, it was centrifuged again at 11,000xg for 1 minute to ensure that the plasmids passed into a new tube. The samples with plasmid isolation were stored at -20°C for later experiments.

2.2.3.5. Verification of Ligation Plasmids with Restriction Enzymes

In order to verify the recombinant plasmids obtained after the cloning process, the digestion process was carried out using appropriate restriction enzymes. The v2_ARO4 plasmid was digested with Eco31I and the v2_ARO9 and v2_ARO10 plasmids were digested with HindIII and ApaI enzymes. The components of the reactions are as shown in Table 10. After the reactions were set up, they were incubated at 37C for 1h, and after incubation, they were run on 1% agarose gel and visualized under UV transilluminator.

Table 10. Components of Restriction Digestion Reactions

Component	**V2_ARO4**	**V2_ARO9**	**V2_ARO10**
10x FD Green Buffer	2 µL	2 µL	2 µL
Eco31I (ThermoScientific)	0.5 µL	-	-
ApaI(ThermoScientific)	-	0.5 µL	0.5 µL
HindIII(ThermoScientific)	-	0.5 µL	0.5 µL
Plasmid	1-1.5 µg	1-1.5 µg	1-1.5 µg
ddH_2O	Up to 20µL	Up to 20µL	Up to 20µL

2.3. GENE MANIPULATION OF *Saccharomyces cerevisiae*

2.3.1. PCR-based gRNA Cassette Assembly

The CRISPR-Cas9 strategy was used to knockout of the ARO4, ARO9 and ARO10 genes in the *S. cerevisiae* genome. In the first stage, the pRCC-K plasmid, which contains the Cas9 endonuclease gene and a single guide RNA (sgRNA) cassette and was obtained from Addgene, was used. Plasmid number 81191 which shown in Figure 5 was linearized by cutting with BamHI and NotI (ThermoScientific) restriction enzymes with the reaction shown in the Table 11 and purified by following the PCR product purification protocol. The PCR reaction was set up as shown in the Table with the sgRNA oligos specifically designed for ARO4, ARO9 and ARO10 (Table 12) and the pRCC-K plasmid carrying the Cas9 gene, cut with BamHI and NotI and purified. In this way, DNA products containing

specific cas9-sgRNA cassettes for the ARO4, ARO9 and ARO10 genes were obtained. (Genereso et al., 2016) These were labeled as ARO4_g1, ARO4_g2, ARO9_g1, ARO9_g2, ARO10_g1 and ARO10_g2.

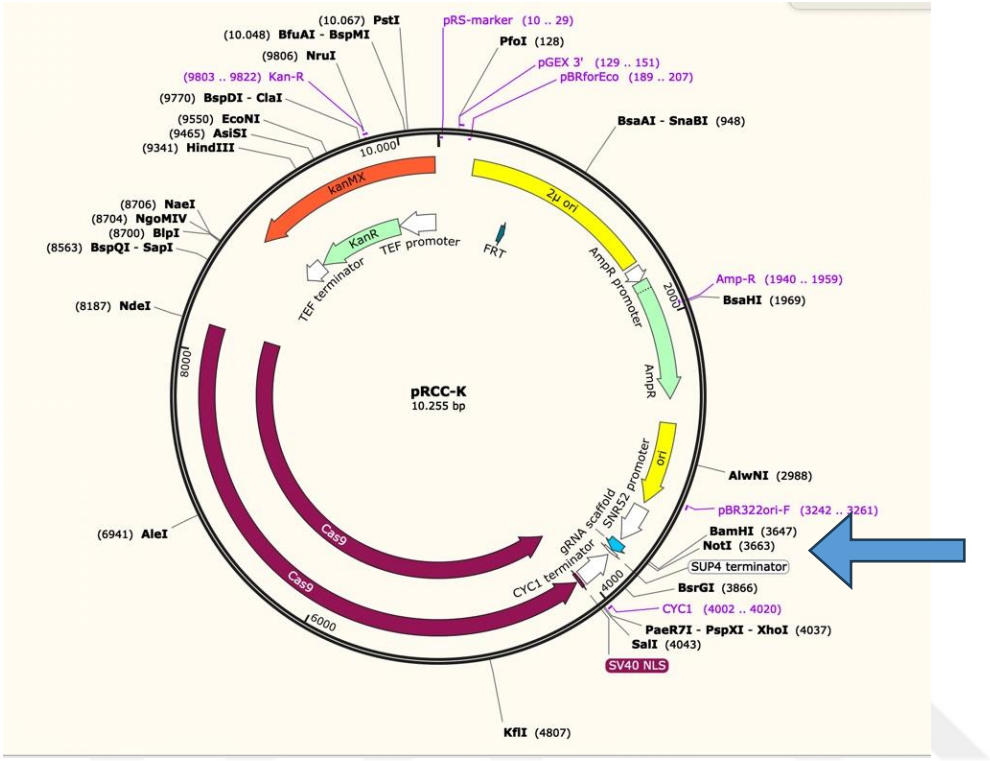


Figure 5. pRCC-K plasmid and Restriction Digestion with BamHI and NotI.

Table 11. Restriction Digestion of pRCC-K plasmid

Component	**Volume**
10X FD Green Buffer(Thermo)	2 µL
BamHI	0.5 µL
NotI	0.5 µL
pRCC-K(81191) plasmid	1 µg
ddH_2O	Up to 20 µL
Total	20 µL

Table 12. sgRNA oligos of ARO4, ARO9 and ARO10

ARO4_g1_F	gatc**GAAATGGTGAGAATGAGCAG**gttttagagctagaaatagcaag
ARO4_g1_R	**CTGCTCATTCTCACCATTTC**gatcatttatctttcactgcg
ARO4_g2_F	gatc**GAGAAGCCAAGAACAACCGT**gttttagagctagaaatagcaag
ARO4_g2_R	**ACGGTTGTTCTTGGCTTCTC**gatcatttatctttcactgcg
ARO9_g1_F	gatc**ACCTTCGACAGAGAGTCCCA**gttttagagctagaaatagcaag
ARO9_g1_R	**TGGGACTCTCTGTCGAAGGT**gatcatttatctttcactgcg
ARO9_g2_F	gatc**CTAGCGACCTGACTACTGAA**gttttagagctagaaatagcaag
ARO9_g2_R	**TTCAGTAGTCAGGTCGCTAG**gatcatttatctttcactgcg
ARO10_g1_F	gatc**TTGCTCGGTAGCCTACTTGG**gttttagagctagaaatagcaag
ARO10_g1_R	**CCAAGTAGGCTACCGAGCAA**gatcatttatctttcactgcg
ARO10_g2_F	gatc**CACAAACGACAACATCACCA**gttttagagctagaaatagcaag
ARO10_g2_R	**TGGTGATGTTGTCGTTTGTG**gatcatttatctttcactgcg

Table 13. PCR Reaction of Cas9-sgRNA Casette Assembly

Component	**Volume**
5X Q5 Reaction Buffer (NEB)	5 μL
Q5 Polymerase Enzyme	0.5 μL
sgRNA oligo F/R mix (for each its own)	2.5 μL
p-RCC-K (81191) plasmid (BamHI/NotI Digested)	≅ 25 ng
dNTP (10mM)	0.5 μL
ddH_2O	Up to 25 μL
Total	25 μL

2.3.2. Yeast Transformation and Verification

2.3.2.1. By4742 Competent Cell Preparation and Transformation with LiAc/ss Carrier DNA/PEG Method

Saccharomyces cerevisiae By4742 strain was incubated in 5 mL of YPD medium at 30°C overnight at 200 rpm. Afterwards, 2.5 mL of the overnight culture was transferred to 50 mL of 2X YPD medium and grown for 5 hours at 30°C at 200 rpm to reach log phase. After the cells reached log phase, they were precipitated with centrifuge at 3,000 x g for 5 minutes. At the same time, ss Carrier DNA was waited at 95°C for 5 minutes and fastly accommodated on ice to ensure denaturation. Precipitated cells were cleaned down two times with sterile ice-cold water and after precipitation at 3,000 x g at 4°C, the liquid part was thrown away and the solution was resuspended with 1 mL sterile water. Then, 1 mL of cells resuspended with sterile DEPC water were relocated to 1.5mL tubes and centrifuged at max g for 30 seconds at 4°C. After discarding the liquid, the sediment was resuspended with 500 µL amount of sterile DEPC water and distributed into 1.5mL tubes as 50 µL aliquots. Each 50 µL of cells was spun at 10,000 x g for 30 seconds at 4°C. TRAFO mix prepared in the composition seen in the Table 14 was added to each tube as 360 µL. ARO4_g1, ARO4_g2, ARO9_g1, ARO9_g2, ARO10_g1, ARO10_g2 plasmids at a concentration of 1 µg and appropriate ODN oligos (Table 15) at a concentration of 2 µg were supplemented to this mixture at the end. Then, these tubes were waited in a 42°C water bath for 1h. After the incubation of mixture, the tubes were spined down at 10,000 x g and the liquid part was removed. Transformant precipitates were dissolved with YPD and recovered by incubating at 30°C for 2h and spread as 100 µL on YPD agar plates containing G418 antibiotic with concentration of 200mg/mL and waited at 30°C for 3 days. (Gietz&Schiestl 2007; Gietz, 2014)

Table 14. TRAFO Mixture

Component	Volume
PEG 3350 (50% [w/v])	240 µL
LiAc 1.0 M	36 µL
SS Carrier DNA (2.0 mg/mL)	50 µL
Plasmid DNA plus d/d sterile water	34 µL
Total volume	360 µL

Table 15. Oligodeoxynucleotide Sequences

ARO4_ODN	TTTAACCGCTAAATTTAGTAAACAAAAGAATCTATCAGAAATGTTTTTTTAATGATATATGTAACGTACATTCTTTCCTC
ARO9_ODN	AAATACACACATACCACAATTACACTCTCTCATCGACTCAACAGTGAAGATTAAACCTATGTGTTATTTGCATATCATAT
ARO10_ODN	TAAAGTTTATTTACAAGATAACAAAGAAACTCCCTTAAGCAAACTTGTGGGCGCAATTATAAAACACTGCTACCAATTGT

2.3.2.2. Verification of Knockout Plasmids

In order to verify the knock-out processes, colonies that acquired G418 resistance after transformation were selected and analyzed. First, the selected colonies were grown in YPD broth containing 200 mg/mL G418 by shaking at 200 rpm at 30°C overnight. The another day, cells were precipitated and genomic DNA (gDNA) was extracted by the LiAc-SDS method. The obtained gDNAs were used as DNA template in the PCR for knock-out verification. The F/R primers used in the verification PCR were designed to distinguish the regions around the targeted gene from the relevant knock-out plasmids, as

seen in the Table 16. The PCR reaction mixture was prepared in 25 ul, containing 2X BEAR Mix (The mixture contains 100mM TRIS-HCl pH 8.5, 150mM KCl, 20% Trehalose, 6mM $MgCl_2$, 20mM DTT, 0.2mM EDTA, 1.6mM) (Graham et al., 2021) and Taq DNA polymerase enzyme, as seen in the Table 17. The amplified PCR products were run by electrophoresis in TAE buffer at 100 V for 40 minutes on a 1% agarose gel. DNA bands were visualized under UV transilluminator.

Table 16. Primers of Verification of gRNA

ARO4_ Ver_F	ACTGCGAAATGACTCAACGATG
ARO4_ Ver_R	TCCCAAAGCTACGATATGACTC
ARO9_ Ver_F	AAAAGAGAGAGCCGTACCGC
ARO9_ Ver_R	GCGAGTACGGTGCTAAATGC
ARO10_ Ver_F	GGGCTAGTTTGCATCGTCAC
ARO10_ Ver_R	TCAAGTGGTGAGCCGAAAGC

Table 17. PCR Reaction for Each Gene of Knock-out System Verification

Component	**Volume**
2X BEAR Mix	12.5 µL
Taq Polymerase	1 µL
gDNA Template	1 µL
Verification Primers F/R	2.5 µL
ddH_2O	8 µL
Total	25 µL

2.3.3. Overexpression Vector Design and Golden Gate Assembly

2.3.3.1. Design of Auxotrophic Selectable (-LEU) Yeast Expression Vector

For the expression of ARO4, ARO9 and ARO10 genes, the pOpen_Yeast (pYeast) vector, which is a galactose-inducible expression vector and provides leucine protrophy, was used in this method. Golden Gate Assembly was established with the pYeast vector with ARO4, ARO9 and ARO10 genes (inserts) and the reaction components in the Table 21. This mixture, which contained BsaI and T4 ligase enzymes at the same time, was kept waiting at 37°C for 4 hours. Afterwards, the pYeast_ARO plasmids transformed into bacterial competent cells were cultured on LB media that contains Kanamycin antibiotic and grown overnight at 37°C. After the day, the colonies that grown on the plate were grown in LB liquid culture containing Kanamycin at 37°C at 200rpm in incubator with shaker. After one day, plasmid isolation was performed and verification was performed by cutting with appropriate restriction enzymes. (Tables 22.1, 22.2, 22.3) After plasmid verifications were done, synthetic defined media without leucine was prepared for selection after yeast transformation, containing the components shown in the Table 19, and the amino acid mixture was prepared as in the Table 18.

Table 18. Yeast AA Mixture without LEU

Aminoacid ve Nucleic Acids	**Volume**
Adenine	400mg
Arginine	400mg
Histidine	400mg
Lysine	600mg
Methionine	400mg
Phenylalanine	1000mg
Threonine	4000mg
Tryptophan	400mg
Tyrosine	600mg
Uracil	400mg

Table 19. Synthetic Defined Media/Drop-out Media Components

Component	Amount
Yeast Nitrogen Base	1.7 g per L
Ammonium Sulfate	5 g per L
Dextrose	%2
Agar	20 g per L
Yeast AA mix	430 mg per L

2.3.3.2. Design of KanMX Selective Yeast Overexpression Vector

Overexpression plasmids containing ARO4, ARO9 and ARO10 genes were constructed using the Golden Gate assembly method. Each plasmid was designed to contain the components in Table 20 using modular parts obtained from the Open Yeast Collection (Freegenes) library. A Golden Gate Assembly reaction containing the components in Table 21 was set up and incubated at 37°C for 4 hours. Bacterial competent cells (NEBStable) were transformed by heat shock method and grown overnight at 37°C in LB media containing Chloramphenicol. The following day, colonies were selected and grown in liquid LB media containing Chloramphenicol at 37°C at 200 rpm with shaking incubator. Next day, plasmid isolation was performed with miniprep buffers and verification reactions were set up with appropriate restriction enzymes. (Tables 23.1, 23.2, 23.3)

Table 20. Parts of Overexpression Vector

Assembly Plan	Parts (Open Yeast Collection:Freegenes)
Yeast Origin	Sc2micron
Yeast Selection	ScKanR-marker
Left Connector	AConL-start/ AConL1/ AConL2
Promoter	Sc-pTDH3
Yeast Coding Seq	ARO4/ ARO9/ARO10
Yeast Terminator	Sc-tTDH1
Right Connector	AConR1/ AConR2/ AConR-end
Bridge	OYC-bridge-AGAC-GCAA

Table 21. Golden Gate Assembly Reaction

Component	Volume
10X T4 Buffer (NEB)	1 µL
T4 Ligase	0.2 µL
BsaI-v2-HF	0.6 µL
Vector	30 ng
Insert	30 ng
ddH_2O	Up to10 µL
Total	10 µL

2.3.4. Restriction Verification of Expression Vectors

2.3.4.1. Restriction Verification of pYeast_ARO Vectors

After bacterial transformation, colony selection and plasmid isolation, the reactions shown in the Table 22.1 for pY_ARO4, Table 22.2 for pY_ARO9 and Table 22.3 for pY_ARO10 were set up with appropriate restriction enzymes selected. Then, these reactions were waited at 37°C for 1 hour and run at 100V for 30 minutes on a 1% agarose gel.

Table 22.1 Reaction of Restriction Verification of pYeast_ARO4

Component	Volume
10X FD Green Buffer	2 µL
KpnI	0.2 µL
Eco32I	0.2µL
SmaI	0.2 µL
PstI	0.2 µL
pY_ARO4 plasmid	1-1.5 µg
ddH_2O	Up to 20 µL
Total	20 µL

Table 22.2 Reaction of Restriction Verification of pYeast_ARO9

Component	Volume
10X FD Green Buffer	2 µL
Eco32I	0.2 µL
KpnI	0.2 µL
SmaI	0.2 µL
PstI	0.2 µL
pY_ARO9 plasmid	1-1.5 µg
ddH_2O	Up to 20 µL
Total	20 µL

Table 22.3 Reaction of Restriction Verification of pYeast_ARO10

Component	Volume
10X FD Green Buffer	2 µL
Eco32I	0.2 µL
KpnI	0.2 µL
SmaI	0.2 µL
PstI	0.2 µL
SalI	0.2 µL
pY_ARO10 plasmid	1-1.5 µg
ddH_2O	Up to 20 µL
Total	20 µL

2.3.4.2. Restriction Verification of Antibiotic Selective Overexpression Vectors

Overexpression plasmids prepared by combining Open Yeast Collection (Freegenes) fragments with Golden Gate Assembly were cut and verified with appropriate restriction enzymes after bacterial transformation, colony selection and miniprep. The reactions shown in the Table 23.1, 23.2, 23.3 were set up and incubated at 37°C for 1 hour. These reactions were carried out on 1% agarose gel with 100V 40 minutes electrophoresis.

Table 23.1 Reaction of Restriction Verification of Overexpression ARO4 Plasmid Samples

Component	Volume
10X FD Green Buffer	2 µL
PdmI	0.2 µL
NheI	0.2 µL
OE_ARO4 plasmid	1-1.5 µg
ddH_2O	Up to 20 µL
Total	20 µL

Table 23.2 Reaction of Restriction Verification of Overexpression ARO9 Plasmid Samples

Component	Volume
10X FD Green Buffer	2 µL
PdmI	0.2 µL
BcuI	0.2 µL
OE_ARO9 plasmid	1-1.5 µg
ddH_2O	Up to 20 µL
Total	20 µL

Table 23.3 Reaction of Restriction Verification of Overexpression ARO4 Plasmid Samples

Component	Volume
10X FD Green Buffer	2 µL
PdmI	0.2 µL
BcuI	0.2 µL
OE_ARO10 plasmid	1-1.5 µg
ddH_2O	Up to 20 µL
Total	20 µL

2.3.5. Electrocompetan Cell preparation and Transformation

High-efficiency transformation of *Saccharomyces cerevisiae* strains was carried out based on the electroporation-based protocol for (Lewis et al., 2023). Within the scope of this method, yeast cells to be used in transformation were first incubated individually in 5 ml volume of YPD liquid medium at 30 °C overnight to obtain a saturated culture. Cell density was determined according to the OD_{600} nm value and diluted into 100 ml of 2X YPD medium to become 2 OD_{600} units and incubated for 4 hours at 220 rpm at 30 °C. Necessary buffers for the transformation process were prepared during the incubation period. Electroporation buffer was prepared from 2 M sorbitol, 100 mM $CaCl_2$ and sterile distilled water; The conditioning buffer was prepared using 2 M LiOAc, 1 M DTT and ddH_2O and the components were added sequentially, first ddH_2O, then LiOAc and lastly DTT, to prevent precipitation.

After incubation, the cells were precipitate by centrifuge at 1,500 x g for 3 minutes and the liquid part was thrown away. The cells were cleaned up twice with cold sterile water, then with electroporation buffer. Afterwards, each cell pellet was suspended with conditioning buffer and waited at 30°C at 220 rpm for 30 min. Following this process, the cells were spinned again and washed with ice-cold electroporation buffer. Finally, the cell pellet was suspended with electroporation buffer to a total volume of 1 ml and made ready for electroporation.

For each transformation reaction, 1 µg plasmid DNA and 2 µg ODN oligos each were mixed up with 400 µl of concentrated cells and moved to pre-cooled electroporation cuvettes. Electroporation procedure was performed at voltage of 2.5 kV, capacitance of 25 µF and resistance of 200 Ω parameters, and after the procedure, cells were transferred to 8 ml of outgrowth (1:1 Sorbitol, 2X YPD) medium. In order for the cells to recover,

they were waited at 30°C for 1 hour without shaking and the tubes were gently inverted and mixed every 15 minutes. At the end of the keep waiting, cells were precipitated by centrifugation and resuspended with appropriate selection medium. They were cultured in YPD medium that contains G418 antibiotics and kept waiting at 30°C for 2 or 3 days. (Hood& Lewis, 2023)

2.3.6. RNA Extraction with FAE and RNA Purification

Total RNA extraction from *Saccharomyces cerevisiae* colonies selected by antibiotic selection was performed using a formamide-based one-step method. The OD_{600} value was checked for each colony grown under appropriate conditions, and 40 µL of formamide solution (98% Formamide, 10mM EDTA) was added for every 500ul OD_{600} =2.0 value. Following the OD_{600} value calculations, each colony was suspended in 160 µl of a formamide-containing solution within a 1.5 ml tube. The samples were then kept waiting at 70°C for 10 minutes and subsequently vortexed. This step ensured the swift lysis of cell walls and the release of nucleic acids. Afterwards, the tubes were placed on ice to cool and spun down at 14,000 x g for 2 minutes, causing the cellular debris to precipitate. The liquid part, that contains the RNA extract, was then carefully moved into a separate tube.(Shedlovskiy et al., 2017) The liquid part is resulting was mixed with 560 µL TRIzol reagent, 280 µL of ddH_2O and 140 µL of supernatant and after homogenization, an equal volume (980 µL) of chloroform was supplemented. The mixture was shaken vigorously and kept at RT for 5 minutes to ensure separation of phase. The samples were spun down with centrifuge at 12,000 x g for 10 minutes at 4°C and the upper aqueous phase was relocated to a different tube. In order to precipitate the RNA, 1.1 times 100% isopropanol was added, kept at 4°C for 60 minutes and then spun down again at 12,000 x g at 4°C and the liquid part was thrown away. The resulting RNA precipitate was cleaned with 70% ethanol and spun again at 12,000 x g, and the precipitate was air-dried after removing the remaining ethanol. Finally, the RNAs were dissolved with nuclease-free (DEPC) water and some of them were used for cDNA synthesis, while the remaining part was stored at -80°C. (Chomczynski et al., 2006)

2.3.7. cDNA Conversion and qPCR

Following the isolation of RNAs via the one-step formamide method and subsequent purification using Trizol-chloroform extraction, cDNA synthesis was perfomed by utilizing the RevertAid First Strand cDNA Synthesis Kit from Thermo Scientific, adhering strictly to the manufacturer's guidelines. As detailed in Table 24, each reaction incorporated 1 µg of RNA sample. The pre-denaturation step involved a mix of random hexamer primers and dNTPs, alongside the addition of RiboLock RNAase inhibitor. Subsequently, reverse transcriptase enzyme was introduced, and the mix of reaction was kept waiting at 25°C for 10 minutes followed by 42°C for one hour. The enzyme was then deactivated at 70°C for 10 minutes.

Table 24. cDNA Synthesis with Revert-aid First Strand cDNA Synthesis Kit

Content	**Volume**
5X Reaction Buffer	4 µL
Template RNA(1000ng)	1 µL
Random Primer (100 µM)	2 µL
RiboLock RNAase Inhibitor (20 U/µL)	1 µL
10mM dNTP	2 µL
Revert-Aid RT (200 U/µL)	1 µL
DEPC ddH_2O	9 µL
Total	**20 µL**

The products obtained from cDNA synthesis were used in qPCR analysis to analyze the expression levels of target genes. qPCR reactions were prepared in a total reaction volume of 20 µL, as shown in the Table 26, containing 2× BEAR Mix, Taq DNA polymerase, SYBR Green (100x) and ROX reference dye (50x). Forward (F) and reverse (R) primer pairs specifically designed for each gene were used. ACT1 gene was used as an internal reference to normalize expression data. Reactions were run in duplicate, and the results were analyzed using the comparative ΔΔCt method according to Ct values.

Table 25. Forward and Reverse Primers of qPCR

ARO4_qPCR_F	aaccaccgaatctcaactgc
ARO4_qPCR_R	tgacaagcatccacagcaac
ARO9_qPCR_F	ttaatcgcccagccttttcc
ARO9_qPCR_R	ggtcgattcgtcgcaaattg
ARO10_qPCR_F	tgaactgaacgccgcttatg
ARO10_qPCR_R	aggcgcttaattcaccaacg

Table 26. qPCR Reaction Components

Content	Volume
2X BEAR mix	10 µL
Forward/Reverse Primers (10 µM)	4 µL
Template (cDNA)	1 µL
Taq Polymerase	1 µL
100x SYBR	0.1 µL
50x ROX	0.2 µL
dNTP (10mM)	2 µL
DEPC ddH_2O	2 µL
Total	**20 µL**

Table 27. qPCR Machine Protocol

Temperature	Time	Cycle
50°C	**5 min**	**1**
95°C	**2 min**	**1**
95°C	**15 min**	**50**
60°C	**30 sec**	
95°C	**15 sec**	**1**
60°C	**15 sec**	**1**
95°C	**15 sec**	**1**

2.3.8. Cultivation and 250mg/ml Polystrene Exposure

Saccharomyces Cerevisiae By4742 cells were cultivated at a speed of 200 rpm and a temperature of 30°C in a shaking incubator. Initially, a single-cell colony from a YPD plate was transferred into 25 ml of YPD liquid medium to establish pre-cultures, which were then incubated overnight. Subsequently, 1% of the pre-culture by volume was used to inoculate 100 ml of YPD contained within 500 ml flasks. The cultures were allowed to grow until they reached mid-logarithmic phase, as measured by an OD600. At this point, polystyrene was introduced at a concentration of 250 mg/L. In parallel, a culture without polystyrene served as the control.

2.3.9. Methylene blue staining and cell counting

Following exposure to polystyrene, the viability of cells was assessed by using a methylene blue solution (0.01% methylene blue, 2% sodium citrate) to evaluate inhibition rates. Yeast culture samples exposed to polystyrene for 3 hours were appropriately diluted and combined with an equal volume of methylene blue solution. The count of dead (blue stained) and living (unstained) cells was performed microscopically within 10 minutes of the mixture.

3. RESULTS

Saccharomyces Cerevisiae is an organism that is highly sensitive to environmental stress factors or various chemicals that may cause toxic effects. Thanks to its adaptation potential, this yeast can develop various defense methods against environmental stress factors. Nanoparticles that are hydrophobic and difficult to biodegrade, such as polystyrene, can cause various changes in metabolic pathways in the yeast cell and significant changes in gene expression profiles. (Sendra et al., 2021) Under the influence of various environmental stress factors, it can cause many cellular changes such as oxidative stress in yeast, distruption of metal-ion homeostasis, changes in energy metabolism, changes in the cell wall and protein misfolding.

The transcriptome result obtained in the study conducted at Marmara University within the scope of TUBITAK to investigate the toxic effects of polystyrene on *S.cerevisiae* is given in Figure 6. According to transcriptome results, there is a volcano graph (Fig.6a), and heat-map prepared according to z-scores.(Fig.6b) According to these results, when the genes with changing gene expression were examined, a total of 895 of genes with changing expression were detected, 410 of which were up-regulated and 485 of which were down-refulated. The heat-map shown in Figure 6b shows the expression changes of the first 50 genes that showed significant expression level changes. Genes with significant changes in expression levels were classified according to the metabolic pathways in which they function.While genes such as OTO1, DPC7, MPH3 play a role in ribosome biogenesis and energy metabolism (Xia et al., 2006), FIT3 and SIT1 are genes responsible for the uptake of iron ions into the cell. (Lesuisse et al.,2001) URA2 has significant role in pyrimidine biosynthesis that it is generally activated when the need for the cell division and DNA repair increases. The increase in heat shock proteins such as HSP78 and SIT1 shows that stress factors that can create toxic effects such as polystyrene cause distruption in protein folding and mitochondrial stress. (Lesuisse et al.,2001) Besides, the expression change in the ARO10 gene was also recorded in the results in Figure 6b. In this context, other genes involved in aromatic amino acid metabolism may also be affected by polystyrene toxicity. ARO9 and ARO10 genes are activated/ up-regulated in nitrogen-limited environments and provide catabolism of amino acids via Erlich Pathway. (Lee et al., 2013)

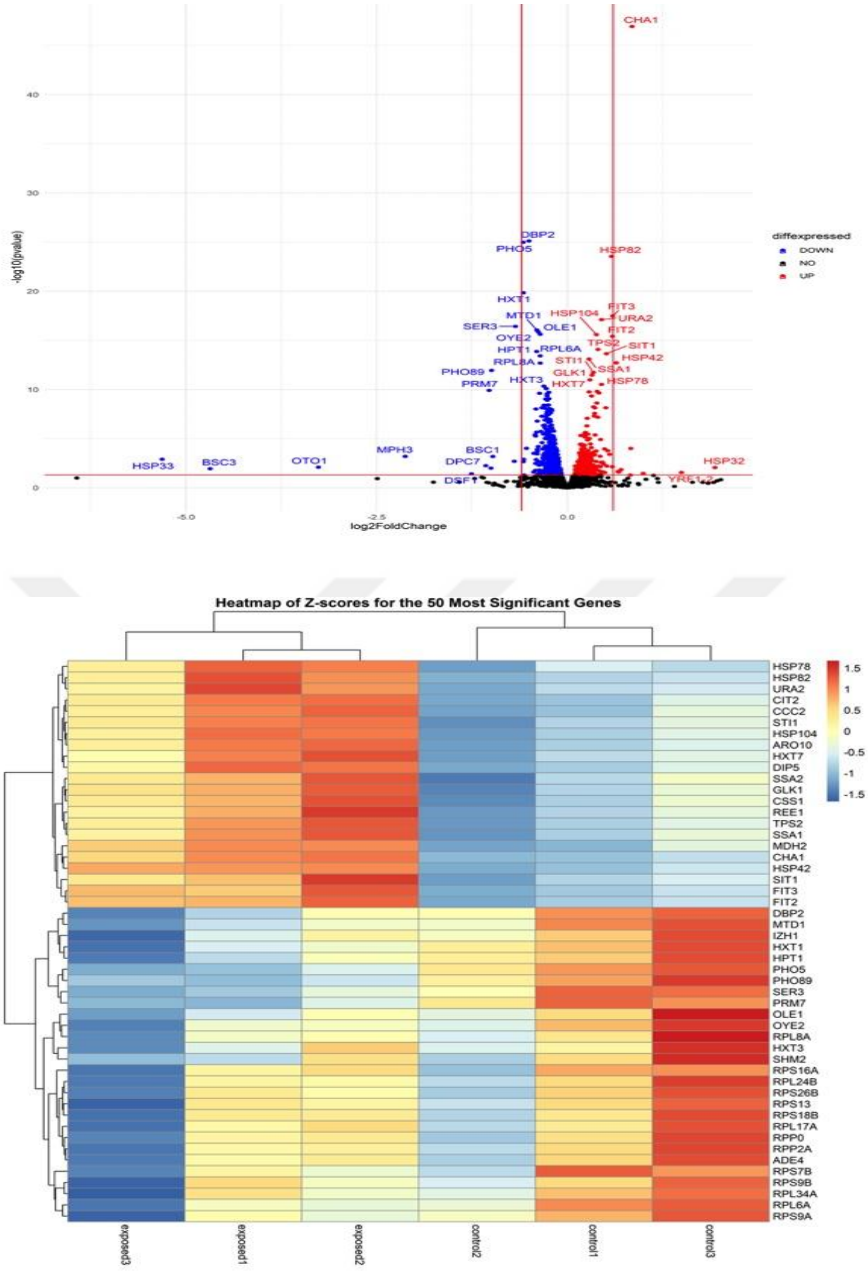


Figure 6. Volcano Graph and Heatmap of Change of Gene Expression Levels .a) The transcriptome analysis data was examined to reveal the log2 fold changes associated with a specific variable, compared to the average of normalized counts across all samples. Genes were considered statistically significant if they fell below a p-value threshold of 0.05. Genes with positive log2 fold changes were marked as upregulated, while those with negative changes were labeled as downregulated. **b)**Additionally, a heat map was generated using z-scores, which involved clustering the top 50 genes with the smallest p-values based on Euclidean distances, with rows scaled accordingly. (Marmara University, TUBITAK Project No: 121Y163)

When the transcriptome results of the polystyrene toxicity experiment were compared with the toxicity-based studies on *S.cerevisiae* carried out by the same project team, it was observed that many of the genes with changed gene expression levels were common. Genes whose gene expressions changed as a result of exposure to different concentrations of bisphenol in *S.cerevisiae* were recorded as ARO10, AIM17, AYR1, CCC2, CIT1, CTR1, CYC7, ENB1, FAA1, FET3, FRE1 ,FRE7, GAD1, GLK1, GPH1 ,GSY2, HXT7, KGD1, KGD2, MDH2, NDI1, PNC1, PRX1, PSK1, SAG1, SDH2, SIT1, SNQ2, TGL5, TIS11, TSL1, URA8, XKS1 (Bereketoglu et al., 2017), and significant expression changes were recorded in these genes as a result of polystyrene toxicity. In addition, in a different toxicity-based study conducted by the same project team, different concentrations of noniphenol exposure were examined on *S.cerevisiae* and changes in the expression of ABF1, ARO10, ARO9, CCC2, CRH1, CTR1, DIP5, ENB1, FAA1, FET3, FRE1, FRE7, GAL11, GUT2, HSP104, HXT7, MDH2, NCE103, PST1, SNQ2, TPO4, URA1, XBP1, YGP1 genes were observed (Bereketoglu et al., 2021).

Polystyrene is a plastic frequently used in packaging materials and disposable products in daily life, but it harms the environment due to its structure that is difficult to decompose in nature and its permanence in nature. Being fragmented into micro and nano-sized and present in the environment causes microorganisms to have direct or indirect toxic effects. Effects such as cell membrane damage, oxidative stress, degradation in metabolic pathways and gene expression changes can be observed. Toxicity-based studies, especially on model microorganisms such as yeast, enable understanding of the effects of polystyrene on biological systems and environmental risk assessments. In this context, toxicity-based studies on *S.cerevisiae* have become quite popular in environmental sciences. When the results of the polystyrene NPs exposure section of the 121Y163 project carried out within the scope of TUBITAK were evaluated and the previous studies of the same project team with materials that could create different toxic effects were evaluated, it was decided to profit by CRISPR-Cas9 system for performing the manipulation of metabolic pathways. Accordingly, the main purpose of this thesis is to evaluate the results obtained from the project number 121Y163 carried out at Marmara University and to prevent the toxicity of polystyrene on *S.cerevisiae* by using gene manipulation techniques with appropriate gene targets.

3.1 *S.cerevisiae* gDNA Extraction and Determination of ARO Genes

3.1.1. gDNA Extraction of *Saccharomyces Cerevisiae* By4742

Three different extraction methods were used to obtain genomic DNA from *S.cerevisiae* cells. These are LiAc-SDS method, Bust'n Grab method and Direct Boiling with TE method. The obtained gDNA samples were stained with Orange G dye and run on 1% agarose gel that contains 0.01% EtBr to appraise the quality, purity and yield of the DNA obtained. The first two samples in Figure 7 were extracted using the LiAc-SDS method, while the 3rd and 4th samples were prepared using the Bust'n Grab method and the last two samples were prepared using the direct boiling method**.** The genomic DNA prepared with the LiAc-SDS method in the second sample was used as a template for the determination of ARO genes. As shown in Figure 7, the sample with the clearest band and no smear is the second sample. High purity and low fragmentation DNAs, which are most suitable for use in PCR reactions, increase the efficiency of DNA polymerase enzymes and reduce the formation of non-specific products.

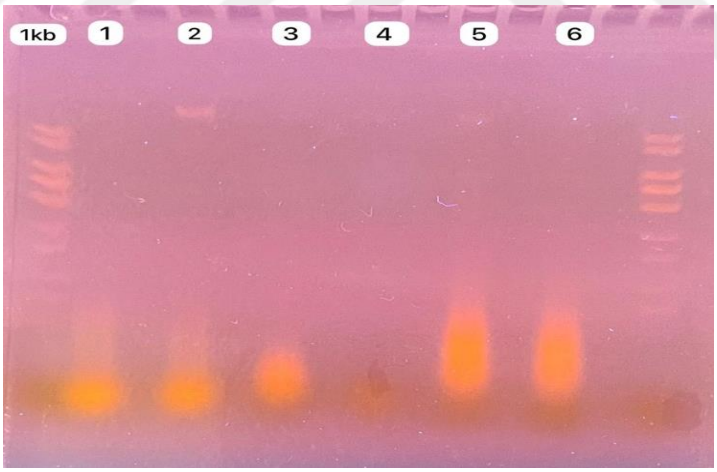


Figure 7. gDNA Extracts run on 1% agarose gel. 1,2. gDNA extracts which were extracted with LiAc-SDS method. **3,4.** gDNA extracts which were extracted with Bust'n Grab method. **5,6.** gDNA extracts which were extracted with direct boiling with TE method.

3.1.2. Amplification of ARO4, ARO9 and ARO10

Among the genomic DNA samples obtained by LiAc-SDS extraction method, the second sample with the highest integrity and purity was used as PCR template. Duplicate reactions established with Q5 high-fidelity DNA polymerase and primer pairs specific to

ARO4, ARO9 and ARO10 genes were evaluated by agarose gel electrophoresis after thermal cycling. In the obtained results, amplicons corresponding to each ARO gene were observed as clear and sharp bands with expected sizes. This shows that the selected DNA sample was of sufficient quality for PCR and the relevant gene regions were successfully amplified. In addition, no non-specific products were observed thanks to the high-fidelity Q5 enzyme used; this supports the suitability of the reaction conditions and primer design.

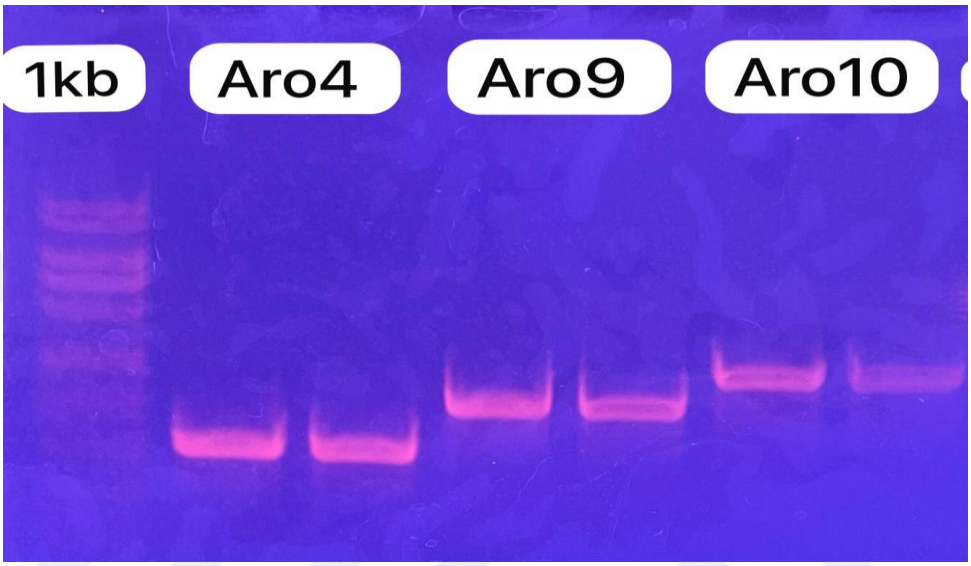


Figure 8. Amplification of ARO4, ARO9 and ARO10 genes with PCR. The length of the ARO4 gene is 1137bp, ARO9 is 1566bp and ARO10 is 1932bp. Two reactions were set up for every ARO gene and run on a 1% agarose gel with TAE buffer. The bands observed in the gel overlap with the lengths of the genes themselves, so the PCR reaction worked.

3.2. Cloning of ARO genes into Amp-resistant Carrier Vector (v2_pJET)

The ARO4, ARO9 and ARO10 genes amplified by PCR were cloned into the v2_pJET vector by using T4 DNA ligase, respectively. The ligation reaction was completed by overnight incubation at 16°C and the resulting recombinant plasmids were transformed into NEBStable chemically prepared competent cells by heat shock the next day. The following day, a single colony selected from antibiotic selection plates was inoculated in LB liquid media containing ampicillin and cultured overnight. Plasmid isolation was performed from the obtained cells. For ligation verification, digestion was performed with appropriate restriction enzymes (ARO4 was cut with Eco31I, ARO9 and ARO10 with HindIII and ApaI) and the digestion products were perused by agarose gel

electrophoresis. In the obtained gel image, bands coincident to both the vector and the fragments of the ARO genes of expected size were clearly observed. These results confirm that the ARO genes were successfully cloned into the v2_pJET vector.

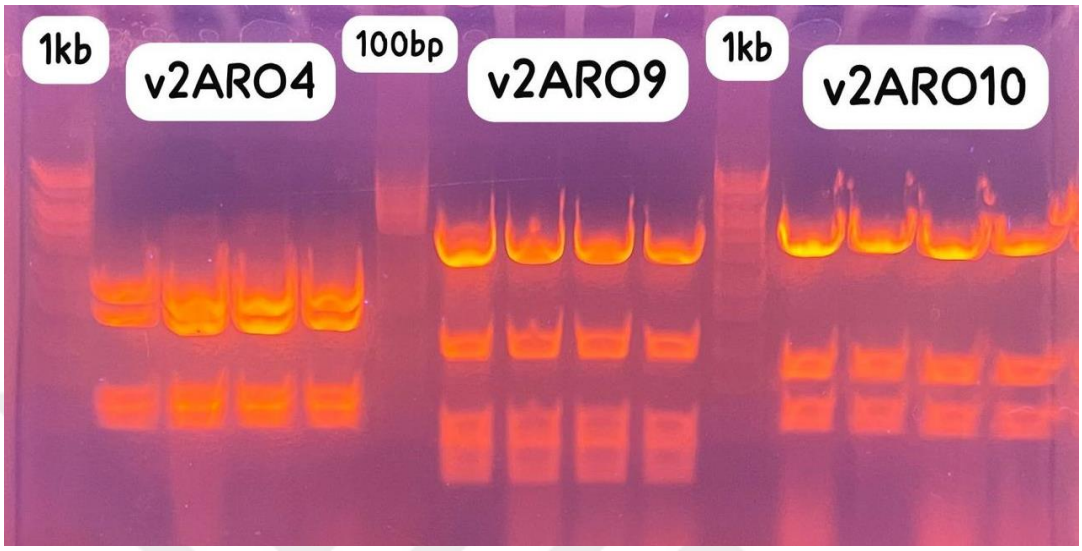


Figure 9. Restriction Verification of Recombinant v2_ARO Vectors. Recombinant vectors formed by ARO4, ARO9 and ARO10 genes cloned into the v2_pJET vector are shown. The v2_ARO4 plasmid was cut with Eco31I restriction enzyme, while the v2_ARO9 and v2_ARO10 plasmids were cut with ApaI and HindIII enzymes. The bands that should be seen after cutting v2_ARO4 with Eco31I are 2282/1815/524, and the bands that should be seen after cutting v2_ARO9 with ApaI and HindIII are 3240/948/440/322. Finally, the bands that should be seen after cutting v2_ARO10 plasmid with ApaI and HindIII are 3962/825/510. Since the lengths of the bands seen in the gel and the required bands are the same, it can be said that the ligation was successful.

3.3. PCR-based gRNA Casette Assembly Verification

Targeted genetic deletion was performed with the homology-based CRISPR/Cas9 system to delete ARO4, ARO9 and ARO10 genes from the genome (Genereso et al., 2016). In this process, PCR products obtained by means of primers specifically designed for the targeted ARO gene regions, together with the pRCC-K vector cut with BamHI and NotI restriction enzymes, were added to the TRAFO mixture containing 2 µg ODN (oligodeoxynucleotide) and 1 µg plasmid and transformed into By4742 cells using the LiAc-PEG-based method. (Gietz et al., 2007) The colonies obtained after transformation were grown in selective YPD medium that contains G418 antibiotic and resistant colonies were selected. gDNA was extracted from these colonies using the LiAc-SDS method. For

knock-out verification, PCR amplification was performed with gene-specific verification primers and the products were analyzed by agarose gel electrophoresis. Band lengths indicating deletion of target genes were clearly observed on the gel. These results indicate that ARO4, ARO9 and ARO10 genes were successfully deleted and removed from the genome.

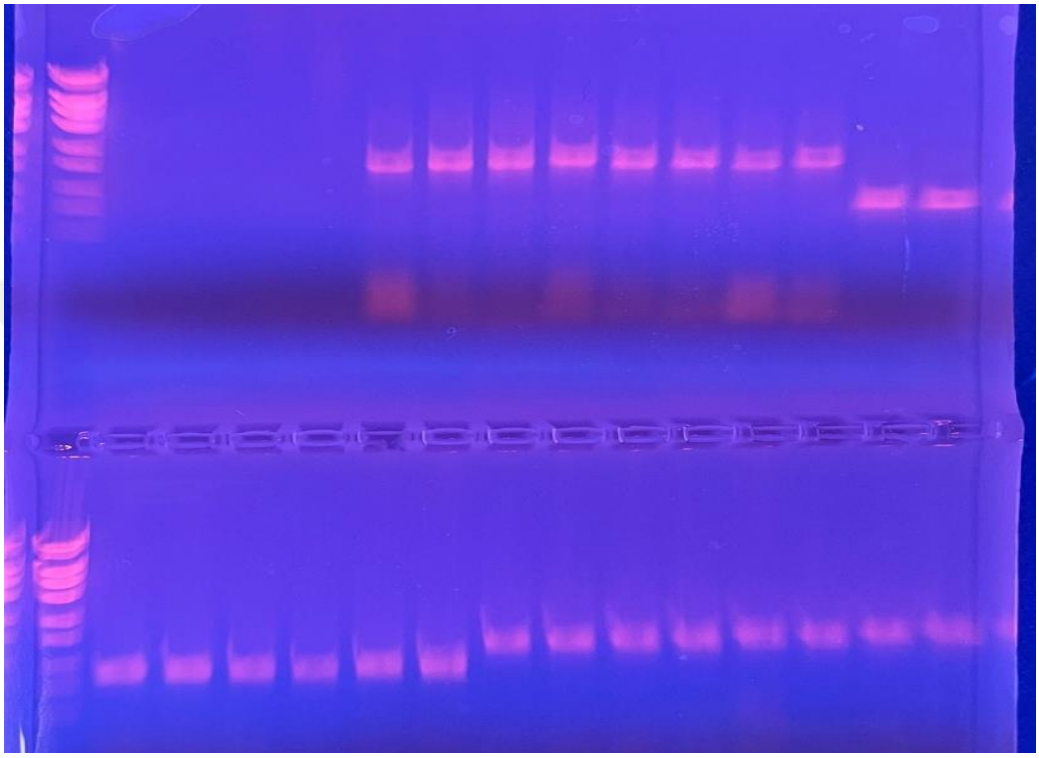


Figure 10. Verification of knockout of ARO4,ARO9 and ARO10 by gene-specific verification primers. Wild-type *S.cerevisiae* By4742 was amplified with verification primers, but no band was seen. (Elongation time may not have been sufficient for amplification) On the other hand, a 1621bp long band was seen as a result of amplification with verification plasmids of ARO4 knock-out plasmids. If the knock-out had not worked, the band length that would have been seen would have been 2758bp, which is the sum of 1621bp and the length of the ARO4 gene, which is 1137bp. For ARO9, the band seen is 874bp, if the knock-out had not worked, it would have been seen as 2440bp. ARO10 is seen as 1273bp, if it had not worked, it would have been seen as 3205bp. In this context, it can be said that the knock-out plasmids worked and the ARO4, ARO9 and ARO10 genes were deleted.

3.4. Verification of p_Yeast Expression Vectors

ARO4, ARO9 and ARO10 genes were successfully inserted into the pOpen_Yeast (pY) vector by using the Golden Gate Assembly method. After the ARO4, ARO9 and ARO10 gene fragments were assembled into the pYeast vector, the recombinant plasmids were transformed into bacterial cells (NEBStable) by following the heat shock strategy. A single colony was inoculated from the colonies obtained in selective medium containing kanamycin to establish a culture and miniprep was applied for plasmid isolation. Digestion analyses were performed with various restriction enzymes to confirm the isolated plasmids. pY_ARO4 plasmid was digested separately with KpnI, Eco32I, SmaI and PstI; pY_ARO9 was digested separately with Eco32I, KpnI, SmaI and PstI; and pY_ARO10 was digested separately with Eco32I, KpnI, SmaI, PstI and SalI enzymes. As seen in Figure 11, the earned products were analyzed by agarose gel electrophoresis. Observation of band patterns corresponding to the predicted cut sites of each enzyme in the gel images revealed that ARO4, ARO9 and ARO10 genes were successfully inserted into the pYeast vector and showed correct localization. The bands were of expected sizes, confirming that both assembly and transformation processes were successful.

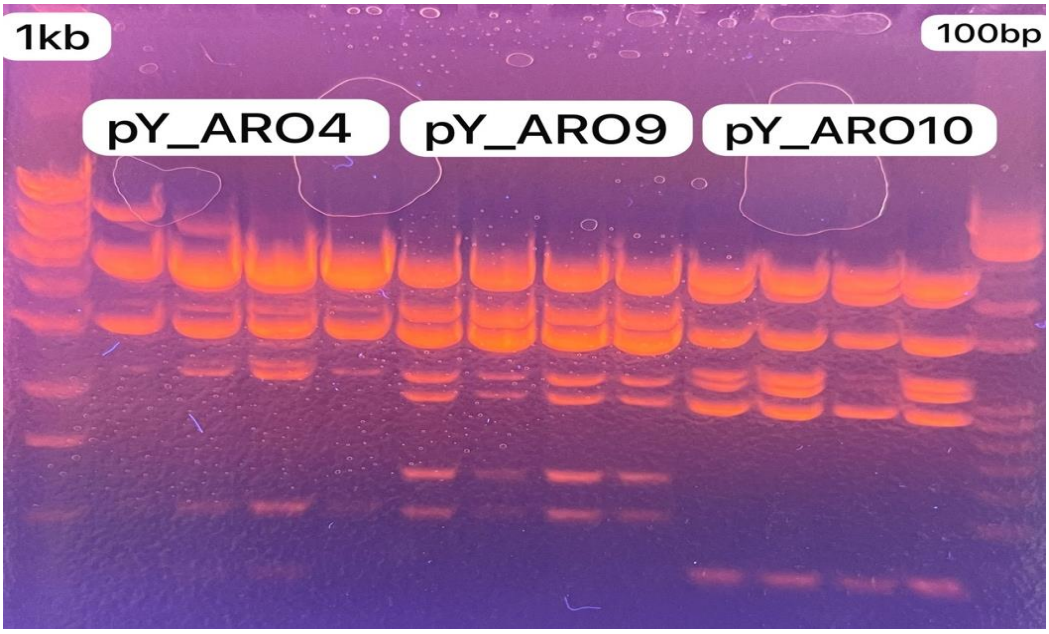


Figure 11. Verification of p_Yeast_ARO recombinant plasmid. pY_ARO4 plasmid was cut with Eco32I, KpnI, SmaI, PstI restriction enzymes. Only the 3rd sample of pY_ARO4 plasmid worked, the bands that should be seen are 2475/1453/1101/1045/358/144

bp. pY_ARO9 samples were cut with Eco32I, KpnI, SmaI, PstI enzymes and all samples worked, the bands that should be seen are 2475/1453/1101/1045/607/496/338 bp. For pY_ARO10, Eco32I, KpnI, SmaI, PstI and SalI enzymes were used for restriction confirmation. The bands that should be seen are 2475/1453/1114/1045/938/364 bp. Therefore, for pY_ARO10, the 1st, 2nd and 4th samples worked.

After their verification, the pY_ARO4, pY_ARO9 and pY_ARO10 recombinant plasmids, which were determined to be working, were successfully transferred to S. cerevisiae BY4742 cells using the high-efficiency yeast transformation protocol.(Gietz et al., 2014) After the transformation of recombinant plasmids, the cells were plated on drop-out selective agar medium (SD–Leu) that did not contain leucine only. Since the pYeast vector used contains the LEU2 selection marker that provides leucine prototrophy, only the cells that successfully received the vector were expected to grow in this medium. As seen in Figure 12 petri dish images, while no colony formation was observed in the blank reactions used as negative control, it was perceived that the cells transformed with the recombinant vectors carrying the ARO4, ARO9 and ARO10 genes grew in the selective medium. This shows that the plasmids carrying all three genes were successfully transferred to yeast cells and that the relevant selection gene (LEU2) was functional. The number and density of colonies after transformation support that the transformation efficiency was high.

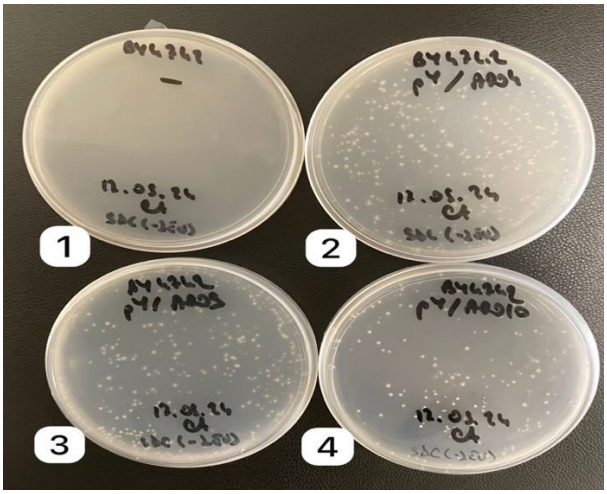
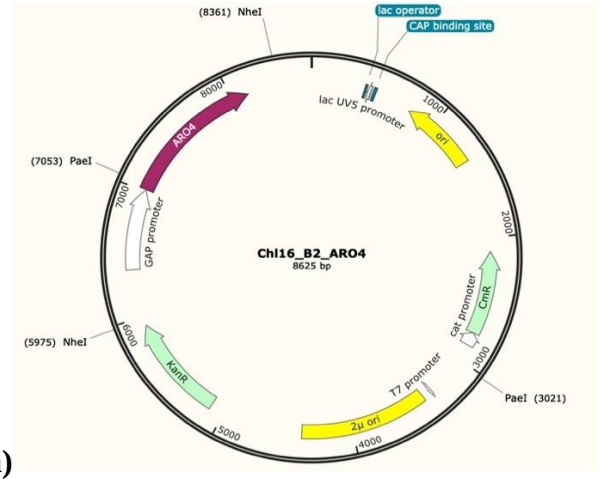


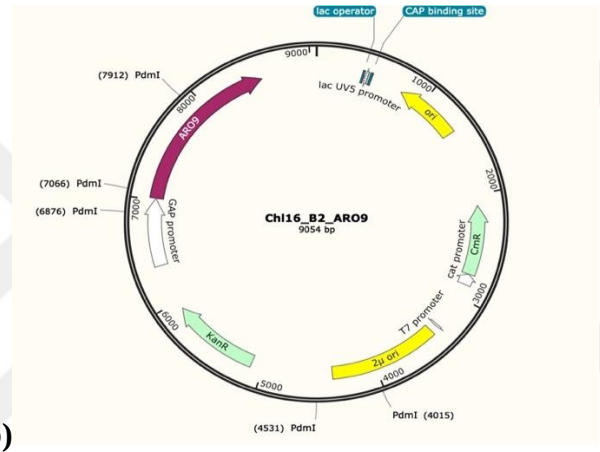
Figure 12. High-efficiency Yeast Transformation of pY_ARO Reactions. **1)**Blank Reaction for Negative Control. **2)** pY_ARO4. **3)** pY_ARO9. **4)** pY_ARO10.

3.5. Verification of CHL-Resistance Overexpression Vectors

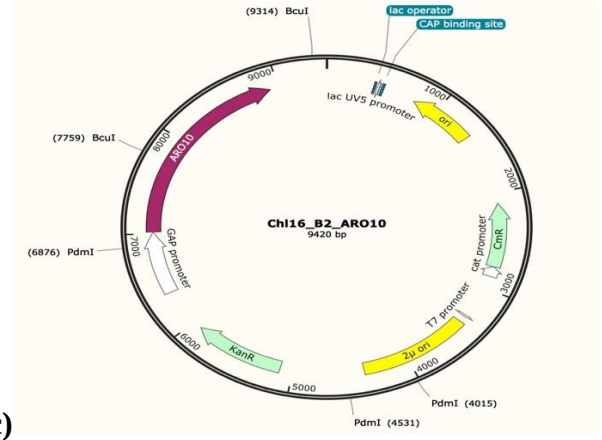
Chloramphenicol resistant overexpression vectors carrying ARO4, ARO9 and ARO10 genes were prepared by Golden Gate assembly. (Table 21) The obtained recombinant plasmids were transferred to competent E. coli cells by using the method based on heat shock, then incubated overnight in LB liquid media containing chloramphenicol and grown overnight, and a single colony was inoculated the following day. The cultures were precipitated the next day and plasmid extraction was performed. In order to confirm the overexpression vectors, ARO4 carrying overexpression vectors were digested with PaeI and NheI, ARO9 carrying vectors with PdmI, and ARO10 carrying vectors with PdmI and ApaI enzymes. The obtained digestion products were persued by agarose gel electrophoresis. Figure 13 indicates the overexpression vectors and the restriction enzymes and cutting sites. When the gel images in Figure 14 are examined, the bands that should be seen when the ARO4_OE vector is cut with PaeI and NheI are 3285/2954/1308/1078 bp. In this context, the working colonies for these overexpression vectors are samples 1, 3, 4, 5, and 6. When the ARO9_OE vector is cut with PdmI, the bands that should be seen are 4121/2345/1036/846/516/190 bp. The working colonies for the ARO9_OE vector are samples 2, 3, 5, and 6. In addition, when the ARO10_OE vector is cut with PdmI and BcuI restriction enzymes, the bands that should be seen are 4121/2345/1555/883/516 bp. Accordingly, only the 6th sample of the ARO10_OE colonies worked. These results indicate that colonies were confirmed in overexpression vectors and can be used for yeast transformation.



a)



b)



c)

Figure 13. Overexpression Casettes. All assembly plans contain few parts such as promoter TDH3, terminator TDH1, selective marker KanR and *S.cerevisiae* origin 2µ as common. **a)**ARO4_OE plasmid digested with NheI/PaeI, the length of bands are 3285/2954/1308/1078 bp. **b)** ARO9_OE plasmid digested with PdmI, the length of bands are 4121/2345/1036/846/516/190 bp. **c)** ARO4_OE plasmid digested with PdmI/BcuI, the length of bands are 4121/2345/1555/883/516 bp.

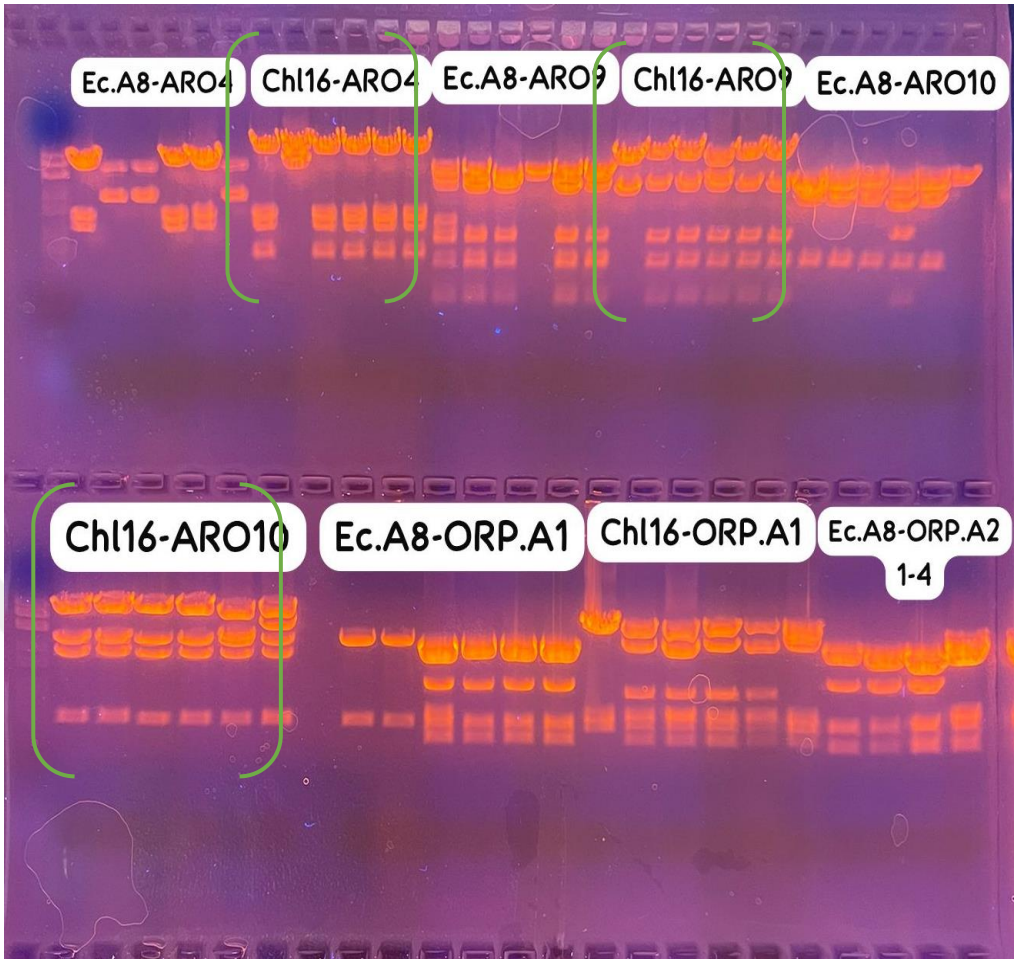


Figure 14. Restriction Verification of Overexpression Vectors. When the ARO4_OE vector is cut with PaeI and NheI, the band lengths should be 3285/2954/1308/1078 bp and the verified ones are samples 1,3,4,5,6. When the ARO9_OE vector is cut with PdmI, the band lengths should be 4121/2345/1036/846/516/190 bp and the verified ones are samples 2,3,5 and 6. When the ARO10_OE vector is cut with PdmI and BcuI restriction enzymes, the band lengths should be 4121/2345/1555/883/516 bp and verified one is only the 6th colony.

3.6. q-PCR Analysis of Overexpression Plasmids Designed with Yeast Plasmid Toolkit

After verification of overexpression vectors, RNA was isolated by formamide extraction method and purified with Trizol-chloroform method and then cDNA synthesis was exerted to analyze mRNA expression levels of ARO4, ARO9 and ARO10 genes transferred to *S. cerevisiae* By4742 cells. qPCR experiments were conducted with appropriate gene-specific primers using SYBR Green dye. ROX passive reference dye was used in the reactions. In this study, quantitative real-time PCR (qPCR) analysis was applied to confirm the overexpression of ARO4, ARO9 and ARO10 genes in

Saccharomyces cerevisiae. The ACT1 gene, known to have constant cellular basal expression, was used as a reference gene in the analyses. As a control group, qPCR was performed with specific primers for ARO4, ARO9 and ARO10 genes using WT genomic DNA; and in the samples representing the overexpression group, overexpression plasmids for each gene were used as templates. In this way, it was aimed to determine the changes in the expression levels of the relevant genes.

As a result of the evaluation of the obtained qPCR data, a smooth exponential increase in the logarithmic phase was observed in the amplification plot graphic (Figure 15). This finding shows that the amplification process was carried out with high efficiency and the obtained Ct values were reliable. In addition, a single and distinct melting peak was detected for each sample in the melting curve analyses; This showed that the amplification represented a specific target sequence and that no primer dimer or non-specific product formation occurred. All these data indicate that the targeted ARO genes were successfully overexpressed by the respective vectors and the validity of the qPCR results was experimentally confirmed.

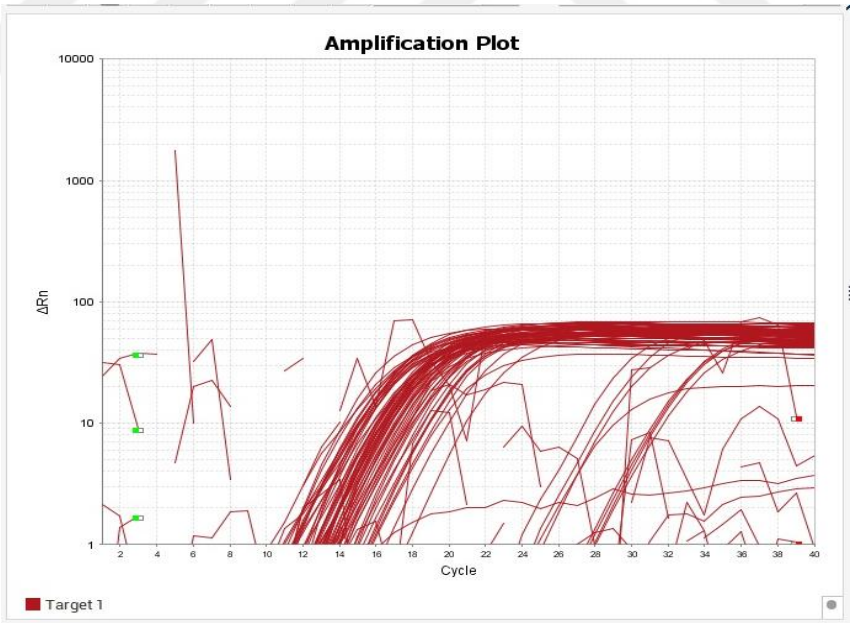


Figure 15. Amplification Plot. Each reaction demonstrates specific and efficient amplification with exponential increase observed in the logarithmic phase.

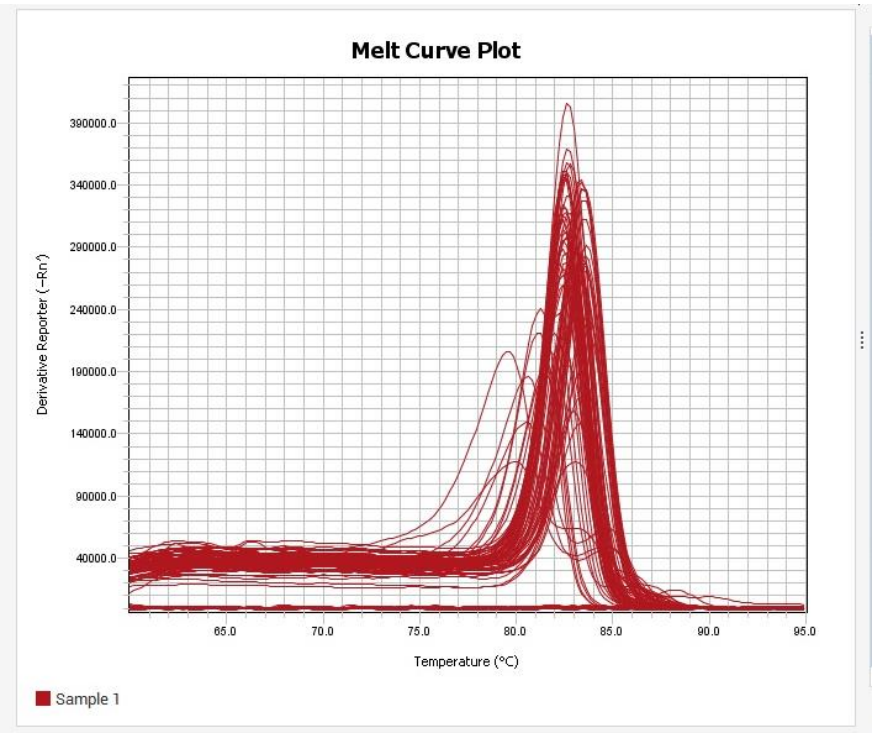


Figure 16. Melting Curve Plot.

3.7. Analyze of Polystrene Exposure on By4742

The results of the step of the investigation of the toxicity of polystyrene on *S.cerevisiae* By4742 (WT) strain, which was carried out in the project number 121Y163, which is ongoing at Marmara University within the scope of TUBITAK, were used for comparison with the polystyrene toxicity experiments in cells with genome editing performed for this thesis. The graph seen in Figure 17 includes the progression of OD600 values of the control group (no polystyrene added) with time when By4742 cell was acutely exposed to polystyrene (25nm) at a concentration of 250 mg/L. Due to various negativities, OD600 measurements could only be taken for 8 hours. In addition, the graph shown in Figure 18 includes the progression of OD600 values of the samples acutely exposed to 200 mg/L polystyrene (395nm) and the control group over time for 24 hours. When the graph of the progression of OD600 measurements over time was examined, OD600 values in WT By4742 cells exposed to 200 mg/L 395 nm polystyrene presented a eloquent increase compared to the control group. In addition, when the cell count was performed, as seen in Table 28, while the total number of cells counted in the control group was 243,

the number of cells in the samples exposed to polystyrene was counted as 196 cells with 5 dead cells and 191 live cells, and accordingly the inhibition rate was calculated as 19.37%.

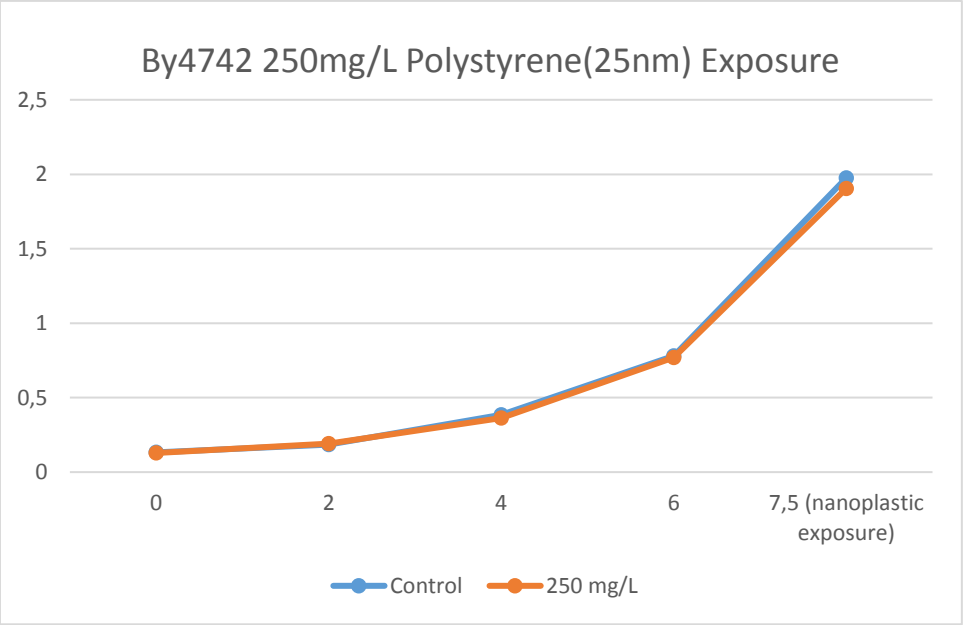


Figure 17. Polystyrene (25nm, 250mg/L) Exposure on *S.cerevisiae* By4742.

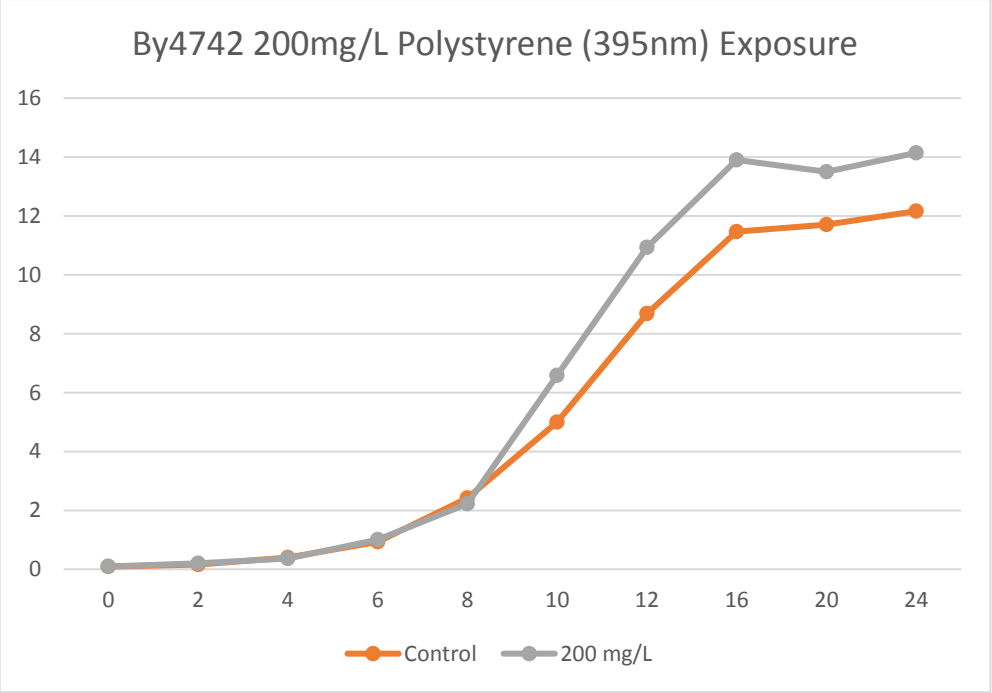


Figure 18. Polystyrene (395nm, 200mg/L) Exposure on *S.cerevisiae* By4742

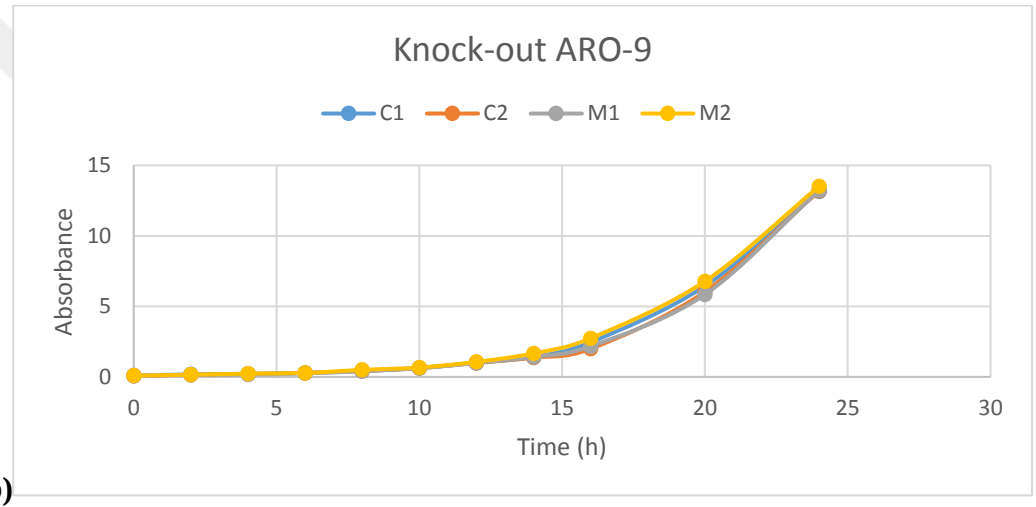
Table 28. Numbers of Live and Dead Cells Determined by Methylene Blue Staining after Exposure to 200mg/L 395nm Polystyrene in Wild-type By4742 Cells

	Control	200 mg/L
Living Cell Number	243	191
Dead Cell Number	0	5
Total	243	196
Inhibition Rate (%)		%19,34

3.7.1. Effect of Polystrene Exposure to Knock-out ARO Genes on By4742

To evaluate the effects of polystyrene exposure on *S.cerevisiae* cells, ARO4, ARO9 and ARO10 knockout genes were treated separately with 395 nm polystyrene at a concentration of 200 mg/L and 25 nm polystyrene with concentration of 250 mg/L and the cultures were monitored by taking OD_{600} measurements at 2-hour intervals for 24 hours. No polystyrene application was made to the control group. Polystyrene treatment was performed after reaching the mid-log phase ($OD_{600} \approx 1.5–1.8$). While ARO4_KO samples reached the mid-log phase at approximately 16 hours, ARO9 and ARO10 reached it at approximately 14 hours. In WT By4742 strains in Figures 17 and 18, it took 7 to 8 hours to reach the mid-log phase. In knockout samples, it was observed that the mid-log phase was reached approximately 2 times slower. When the OD_{600} values recorded for 24 hours were compared between the control group and those treated with 25nm polystyrene at a concentration of 250mg/L, no significant difference was observed.

a)



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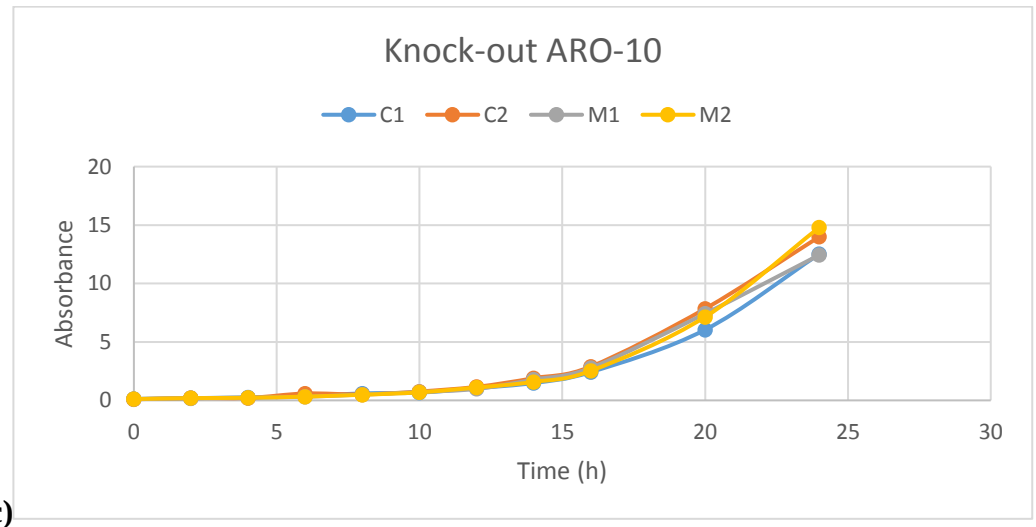


c)

Figure 19. Growth curves (OD600) of Knockout Strains.

After growing the By4742 cells with deleted ARO4, ARO9 and ARO10 genes to mid-log phase, one group of samples were exposed to PS at 200 mg/L concentration of 395 nm and the other group was exposed to PS at 250 mg/L concentration of 25 nm for all gene knockouts. Another group was knockout strains that were not exposed to polystyrene. In the groups with polystyrene added, after the cells achieved mid-log phase, a certain amount of yeast culture and methylene blue solution were mixed in a 1:1 ratio and observed under a microscope 3 hours after the addition of polystyrene at the determined concentration and the number of live cells was recorded. Table 29 shows the cell numbers recorded in the control and polystyrene exposure samples for each gene knockout strain. When the control group and polystyrene exposure samples for the ARO4_KO strain were compared, it was seen that there were more cells in the polystyrene-treated samples. On the other hand, for ARO9 and ARO10 knockout strains, tas a result of exposure to polystyrene, the cell count was recorded to be lower than in the control group.

Table 29. Numbers of Living Cells Determined by Methylene Blue Staining After Exposure to 200 mg/L 395nm and 250mg/L 25nm Polystyrene and Control Group (No PS Exposure) in Knockout By4742 Cells

By4742_KO	Control	200mg/L, 395nm PS	250mg/L,25nm PS
ARO4_KO	172	197	196
ARO9_KO	296	303	249
ARO10_KO	193	162	145

The results of cell counting with methylene blue staining method were recorded to reveal the distributions of the effects of polystyrene nanoparticles (250 mg/L, 25 nm) on cell viability in Saccharomyces cerevisiae wild-type (WT) and ARO4, ARO9, ARO10 gene knockout (KO) strains. While 19.34% inhibition was observed based on cell count between polystyrene application and control group in WT strains, different degrees of damage responses were created in sale gene deletion processes in ARO genes. When Table 30 is examined, determination of the negative aspects of ARO4 knock-out inhibition (-14.24%), i.e. comparison of control group with polystyrene applied sample

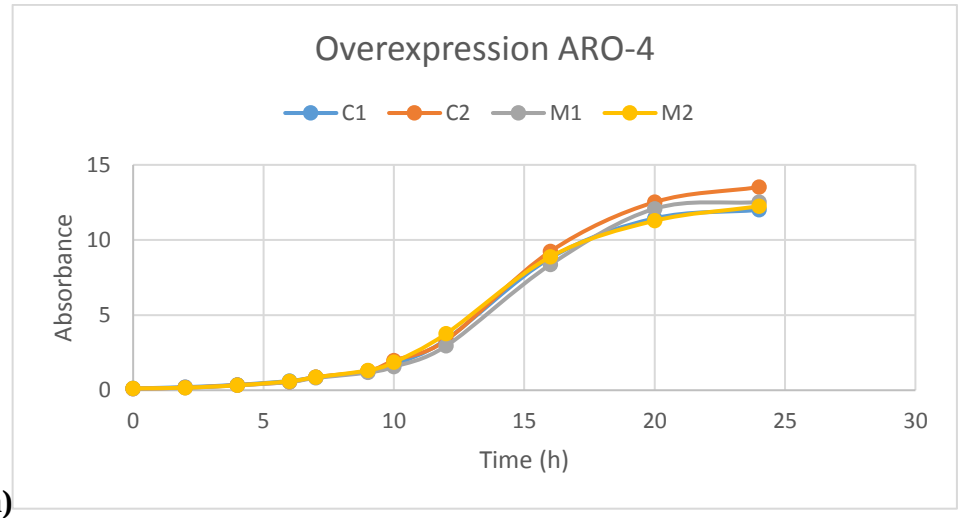
shows that there are more live cells. On the other hand, 6.75% inhibition was observed in ARO9_KO strain and 20.46% in ARO10_KO strain.

Table 30. Inhibition Rate Calculation of Living cell Number of Knock-out Strains which are Control and Polystrene Exposed Group

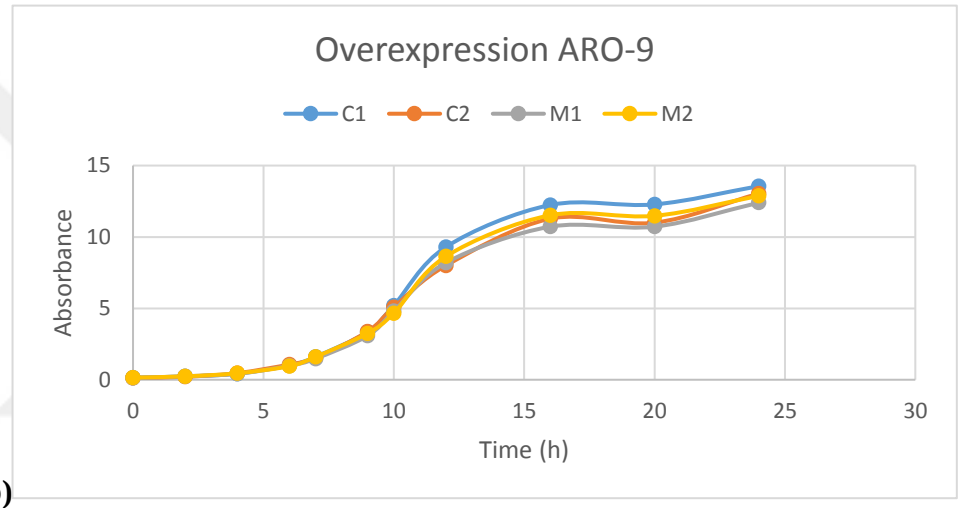
By4742_KO	Avarege Cell Number for Control	Avarege Cell Number of Polystrene Exposed	Inhibition rate (%)
ARO4_KO	172	196.5	-14.24%
ARO9_KO	296	276	6.75%
ARO10_KO	193	153.5	20.46%

3.7.2. Effect of Polystrene Exposure to Overexpressed ARO Genes on By4742

In order to evaluate the effects of polystyrene, the action of overexpression of ARO4, ARO9 and ARO10 genes on cell growth was investigated. Each overexpression strain and vector control group were grown to mid-log phase ($OD_{600} \approx 1.5–1.8$) and then exposed to polystyrene nanoparticles with a concentration of 250 mg/L and a size of 25 nm. Polystyrene exposure experiments using 395nm polystyrene (used as 200mg/L) could not be performed on ARO4, ARO9 and ARO10 overexpression strains because 395nm polystyrene (used as 200mg/L) in laboratory stocks was very low. Experiments were performed using only 25nm polystyrene at a concentration of 250mg/L. OD_{600} values of the non-polystyrene (control) and polystyrene-treated groups were measured every 2 h for 24 h and growth curves were generated. It took approximately 10 h for ARO4_OE strain to reach mid-log phase, while it took around 8 to 9 h for ARO9_OE and ARO10_OE strains. When the growth curve of WT strain in Figures 18 and 19 is examined, it is seen that it takes about 7 hours for the cells to reach the mid-log phase. Overexpressed strains are seen to grow slower compared to the wild-type.



a)



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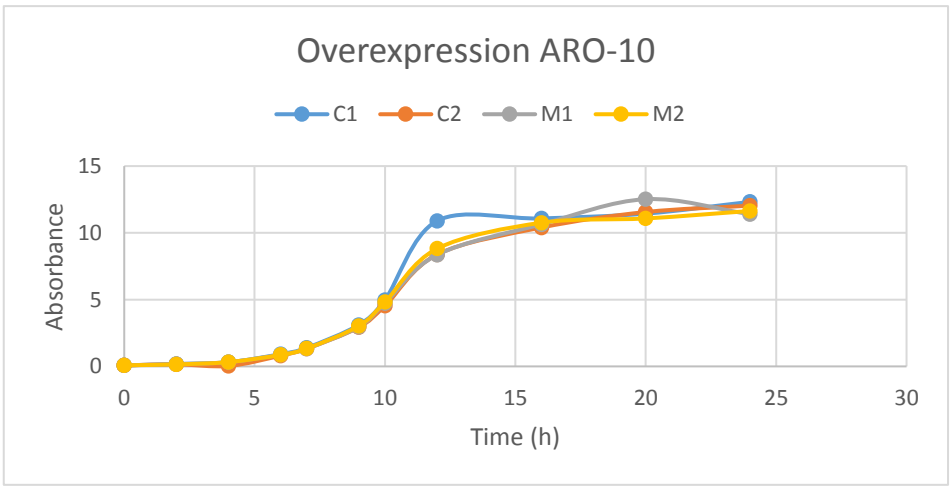

c)

Figure 20. Growth curves (OD600) of Overexpression Strains.

After the By4742 cells with overexpression in ARO4; ARO9 and ARO10 genes were grown to mid-log phase, 250 mg/L polystyrene was added and after growing for 3 hours, a certain amount of yeast culture was mixed with methylene blue solution at a ratio of 1:1 and placed in a hemosymeter and counted under a microscope. This method is dependent on the ability of living cells to reduce methylene blue. While living cells are colorless or have a pale blue color, a clear and dark blue color is observed in dead cells. (Felice et al., 2009) Cells with overexpression that were not exposed to polystyrene (control) and those exposed to 250 mg/L polystyrene were subjected to cell counting separately using the methylene blue solution method. The control group and those exposed to polystyrene contain duplicate samples. When the counting results in Table 31 are investigated, it shows that the number of living cells in the two samples in the control group for ARO4 overexpression cells is 110 and 100, respectively. However, in two samples exposed to polystyrene, the number of live cells decreased to 80 and 94, respectively. In addition, in the comparison of live cells between the control group and cells exposed to polystyrene in cells overexpressed in the ARO9 and ARO10 genes, a diminish in the number of the cells was recorded in the samples exposed to polystyrene.

Table 31. Numbers of Live and Dead Cells Determined by Methylene Blue Staining After Exposure to 250mg/L 25nm Polystyrene and Control Group in Overexpressed By4742 Cells

By4742_OE	**Control 1**	**Control 2**	**PS Exposured 1**	**PS Exposured 2**
ARO4_OE	110	100	80	94
ARO9_OE	327	368	328	269
ARO10_OE	312	325	262	270

In order to evaluate the toxic effects of polystyrene nanoparticles (25 nm, 250 mg/L) on Saccharomyces cerevisiae, cell counts performed by methylene blue staining were analyzed in overexpressed (OE) ARO4, ARO9 and ARO10 strains by comparing them with groups that were not treated with polystyrene. In this analysis, inhibition rates were calculated based on cell number losses resulting from polystyrene treatment and the

obtained rates were compared with the values observed in wild-type (WT) strains. When Table 32 was examined, the inhibition rate was determined as 19.34% in WT strains, while 17.14% in ARO4 OE strains, 14.10% in ARO9 OE strains and 16.48% in ARO10 OE strains.

Table 32. Inhibition Rate Calculation of Living cell Number of Overexpressed Strains which are Control and Polystrene Exposed Group

By4742_OE	**Avarege Cell Number for Control(For 2 samples, C1 and C2)**	**Avarege Cell Number of Polystrene Exposed**	**Inhibition rate (%)**
ARO4_OE	105	87	17.14%
ARO9_OE	347.5	298.5	14.10%
ARO10_OE	318.5	266	16.48%

4. DISCUSSION

Polystyrene is an aromatic vinyl polymer obtained by polymerization of styrene monomers and contains repeating phenyl rings in its chemical structure. In this respect, polystyrene is quite stable and hydrophobic and therefore persistent in environmental conditions and hardly biodegrades. Polystyrene, a frequently used plastic, can be used in disposable packaging materials, laboratory materials and isolation products. (Andrady, 2011) When polystyrene is examined structurally, its hydrophobic structure and high molecular weight limit its direct interaction with microorganisms, but it can reach micro- and nano-sized through environmental effects, UV light or physical abrasion, and this can be a factor for increasing its interaction with microorganisms. (Gewert et al., 2015). In addition, the effects that micro- and nano-sized polystyrene particles will create on microorganisms chronically by maintaining their persistence in the environment are a subject worth investigating. Because, it has been recorded that micro- and nano-sized polystyrene can cause cell toxic effects through cell membrane connectivity and endocytosis. (Chae & An, 2017). Studies in yeasts such as Saccharomyces Cerevisiae show that polystyrene nanoparticles can cause oxidative stress, disrupt metabolic balance, and affect cell wall stress responses. Considering the biochemical structure of polystyrene, one of the metabolic pathways that can be affected by the toxicity that polystyrene will create due to its aromatic structure may be amino acid metabolism. In another study, the effects of PS microplastics on the metabolic pathways of *Pseudomonas aeruginosa* were investigated. This study showed that polystyrene microplastics can affect various metabolic pathways, including metabolism of amino acids. (Tao et al., 2024) These findings bring forward that the aromatic structure of polystyrene in *S.cerevisiae* may also interact with aromatic amino acid metabolism, causing alterations in gene expression levels at the cellular level and leading to biochemical changes.

It is known that polystyrene can cause changing in gene expression levels, especially in micro and nano-sized microorganisms and therefore on *Saccharomyces Cerevisiae*. According to studies conducted on different microorganisms, polystyrene can affect metabolic pathways related to aromatic amino acid biosynthesis due to its aromatic structure. In Saccharomyces cerevisiae, the production of aromatic aa takes place within shikimate pathway, while their breakdown occurs through the Erlich pathway. Within this

framework, one might anticipate alterations in the activity of genes associated with these pathways. Investigations focusing on toxicity effects in S. cerevisiae reveal shifts in the expression levels of genes participating in both the shikimate and Erlich pathways, particularly under the influence of toxins like bisphenol A and nonylphenol. (especially ARO9 and ARO10) (Bereketoglu et al., 2017) Besides, when the transcriptome analyses of the ongoing study investigating the toxic effects of polystyrene on *S.cerevisiae* at Marmara University were examined (Figure 6), some expression changes were recorded in genes comprised in aromatic amino acid metabolism. In a different research, it was demonstrated that there was an increment in the expression levels of ARO1, ARO2, ARO3 and ARO8 genes in the engineering application for styrene production, and in this context, the production of the targeted metabolite could be optimized by increasing phenylalanine biosynthesis. (McKenna et al., 2014) Considering this study, it can be said that the expression changes in genes comprised in aromatic amino acid pathways show the cell's response to aromatic compounds, while also affecting the cell's tolerance and metabolic response. Accordingly, it can be said that the cell's tolerance to plastics containing aromatic compounds such as polystyrene can also change through varies in the expression levels of ARO genes.

As a result of the transcriptome analysis examining the changes in gene expression levels caused by the toxicity of polystyrene on *S.cerevisiae*, expression changes in ARO4, ARO9 and ARO10 genes, which have duty on aromatic amino acid synthesis and catabolism, were prominent. While the ARO4 gene was up-regulated, ARO9 and ARO10 genes were down-regulated. (TUBITAK Project No: 121Y163) Using these results, this thesis aims to reduce the toxic effect of polystyrene on *S.cerevisiae* by gene manipulations targeting these genes. In order to see how the changes in expression levels will be effective, firstly ARO4, ARO9 and ARO10 genes were knocked out from the genome via CRISPR-Cas9 technology, and then these genes were overexpressed in the *S.cerevisiae* genome.

First of all, knockout application was performed using CRISPR-Cas9 technology for ARO4, ARO9 and ARO10 genes. The main reason for using this strategy is to provide an understanding of the aromatic amino acid metabolism of the cell and the cellular responses associated with it. The method used to knockout ARO4, ARO9 and ARO10 genes is the homologous recombination-based gene deletion method. As an easy, fast and

economical technique, this system can be explained by the system of inserting the pRCC-K plasmid containing the Cas9 gene into PCR with gene-specific designed primers and transforming it together with the ODN oligo to increase transformation into the yeast cell. It is frequently preferred because it is a fast, economical and low off-target system. (Genereso et al., 2016)

The effects of the ARO4, ARO9 and ARO10 genes that were successfully deleted from the *S.cerevisiae* genome on cell metabolism and the cellular response to stress factors are a topic worth discussing. The ARO4 gene is one of the DAHP synthase isoenzymes on the shikimate pathway and takes a role in phenylalanine, tyrosine and tryptophan biosynthesis. Removing the ARO4 gene from the genome significantly disrupts the production of certain amino acids, potentially necessitating external supplementation for cell survival in these conditions (Braus et al., 1991). Conversely, the ARO9 gene acts as an aromatic aminotransferase, playing a role in breaking down aromatic amino acids like phe and tyr. In addition to that, it contributes to the production of volatile aromatic alcohols, such as 2-phenylethanol, through the Erlich pathway.In this context, deletion of ARO9 may reduce the cell's capacity to metabolize toxic aromatic compounds. Some studies have shown that while the production of ARO9 is suppressed in the presence of compounds such as noniphenol, an increase in cellular stress has been observed. (Bereketoglu, 2021) The ARO10 gene functions as a phenylpyruvate decarboxylase and is an important gene in phenylalanine degradation. (Vuralhan et al., 2003) Two different studies have also shown that the expression of the ARO10 gene is suppressed in the presence of bisphenol A exposure, and can be considered as a gene that can be affected by environmental toxins. (Bereketoglu et al., 2017)

When knockout ARO cell samples exposed to 250 mg/L polystyrene were examined, a serious slowdown in cell growth was observed for ARO4, ARO9 and ARO10 deleted from the entire genome. While it took approximately 7 hours to reach the mid-log phase in wild-type By4742, it took approximately 15 hours in By4742 cells with deleted ARO4, ARO9 and ARO10 genes under the same conditions. The significant slowdown in growth observed when the ARO4 gene is removed from the By4742 genome is primarily because ARO4 has significant duty on the initial step of creating phenylalanine, tyrosine, and tryptophan. When this gene is deleted, the shikimate pathway and feedback mechanisms are seriously disrupted and if the cell is not fed with these amino acids from the external

environment, a significant slowdown in growth can be observed. (Luttik et al., 2008) In addition, when the samples in which the ARO4, ARO9 and ARO10 genes were deleted but polystyrene was not added (control) were compared with the samples in which all these genes were knocked out and 250 mg/L polystyrene was added at the mid-log stage, no meaningful difference was observed. Since the deletion of the ARO4, ARO9 and ARO10 genes from the By4742 genome disrupted aromatic amino acid biosynthesis and catabolism, it may have caused intracellular stress and slowed down cell growth without the need for polystyrene addition. On the other hand, the growth rate in samples with added polystyrene was the same as in samples without added polystyrene. In this context, when the results of acute polystyrene exposure were evaluated for By4742 cells with deleted ARO4, ARO9 and ARO10 genes, it was observed that the growth rate slowed down approximately equally in those with and without polystyrene compared to wild-type By4742 cells.

Cell counts of control and polystyrene-treated knockout strains were performed using the methylene blue staining method. The data obtained were recorded for ARO4, ARO9 and ARO10 knockout strains separately for 395nm 200mg/L PS exposure and 25nm 250mg/L PS exposure and for the control group without polystyrene. Upon reviewing the findings, it became clear that ARO4_KO strains exposed to polystyrene (PS) had a greater cell count confronted to the control group. This remarks that removing the ARO4 gene might offer some protection against polystyrene. The ARO4 gene encodes a kinase essential for the initial stages of aromatic aa biosynthesis in the shikimate pathway. (Braus, 1991) A deficiency in ARO4 gene could lead to disruptions in this pathway, potentially lowering the cell's metabolic burden and allowing it to conserve resources during stressful conditions, thereby enhancing survival. In a similar respect, some studies have reported that slowing down growth can also increase cell tolerance to stress. (Zhou et al., 2020) When cell count results were examined in ARO9 and ARO10 knockout strains, those treated with polystyrene showed lower cell numbers than the control group. Considering the duty of these genes in aromatic amino acid metabolism, ARO9 and ARO10 genes encode aminotransferase and decarboxylase enzymes involved in the Erlich pathway. (Hazelwood et al., 2008) Knocking out of ARO9 and ARO10 genes from By4742 cells can cause imbalances in the nitrogen metabolism of the cell and prevent the adaptive response to toxic effects, lthat leads to a decrease in cell proliferation. Besides, some

intermediates in the Erlich pathway maintain intracellular redox balance. Accordingly, the absence of two important genes involved in this pathway may have increased cellular redox stress under stress. (Placzek et al., 2020)

The inhibition rate for knockout strains is determined by the percentage decrease in cell viability when colleting the group treated with polystyrene to the untreated control group. This rate indicates how cell proliferation is hindered by toxicity. The data reveal that wild-type (WT) strains experienced 19.34% inhibition, whereas ARO4 knockout (KO) strains showed a negative inhibition rate of -14.24%. This implies that the absence of the ARO4 gene might enhance resistance to stress caused by polystyrene exposure. The ARO4 gene is crucial in the initial stages of the shikimate pathway, which is essential for the synthesis of aromatic amino acids (Braus, 1991). This metabolic pathway demands a significant amount of energy, so removing the gene could potentially redirect cellular resources toward more critical stress response systems. Likewise, earlier research has demonstrated that altering cellular metabolism can enhance resilience to environmental stresses (Kitano, 2004; Gasch et al., 2000). Conversely, the ARO9 and ARO10 knockout strains exhibited inhibition rates of 6.75% and 20.46%, respectively. Given that ARO10 is involved in nitrogen metabolism and responds to different stress conditions, it is hypothesized that removing this gene could lead to decreased cell growth when stress factors are present (Vuralhan et al., 2003; Boer et al., 2003). The ARO9 gene is crucial for nitrogen source utilization due to its role in aminotransferase activity. Consequently, the deletion of this gene may result in either a growth advantage or disadvantage. Research has demonstrated that modifications in amino acid metabolism can influence how cells respond to environmental stressors across various model systems (Castrillo et al., 2007). These findings underscore the significant impact genetic modifications can have on both specific metabolic pathways and the broader mechanisms of cellular resistance to environmental stress. Comparing the inhibition rates of knock-out strains with wild-type strains is a valuable method for functional analysis of genetic targets in studies of toxicity.

In addition to knockout, ARO4, ARO9 and ARO10 genes, in which changes were desired in gene expression, were overexpressed in By4742 cells. In this study, modular Golden Gate assembly-based plasmid systems were used for overexpression of targeted genes in *S.cerevisiae* cells. The system called "Yeast Toolkit" is a technique that brings together

promoters, selection markers, gene cassettes and terminators. (Lee et al., 2015) Thanks to this system, Golden Gate is used to provide assembly of genetic parts with low error rate and high efficiency. In this study, TDH3 promoter, TDH1 terminator, KanR selective marker, 2-micron yeast origin were mainly used to provide overexpression of ARO4, ARO9 and ARO10 genes. The promoter pTDH3 used to provide overexpression of genes belongs to the glyceraldehyde-3phosphate dehydrogenase gene and can provide high expression both in the asset of glucose and quantitatively. (Mumberg et al., 1995) The tTDH1 terminator used with this promoter meets the desired properties of a terminator with its features that increase the correct termination of transcription and mRNA stability. (de Oliveira Vargas et al., 2013) In addition to these, the 2-micron origin is a high copy number replication origin used specifically for *S.cerevisiae* and is frequently used in yeast to increase target gene expression (Karim et al., 2013). Furthermore, selection markers as example ScKanR provide selection with the G418 antibiotic in *Saccharomyces cerevisiae* and provide in the discrimination of transformants. (Güldener et al., 1996)

Overexpression vectors were tested with qPCR analysis and an S-shape was observed in the amplification plot. In the multicomponent plot, it was observed that SYBR fluorescence was clearly visible in reactions containing SYBR and ROX and a clear peak was observed in the melting point graph. From here, it can be understood that genes were overexpressed by focusing on a single gene.

By4742 cells containing ARO4, ARO9 and ARO10, which were successfully overexpressed, were exposed to 250 mg/L polystyrene. When the results were examined, cells with overexpressed ARO genes reached the mid-log phase faster than the knockout cells and grew faster, but it can be said that their growth slowed down when compared to wild-type By4742 cells. There may be several reasons for this, while it took approximately 7 hours to reach the mid-log phase in cells without genetic editing, it took approximately 10 hours in cells overexpressed in ARO4, ARO9 and ARO10 genes. Proteins produced as a result of overexpression can sometimes fold incorrectly, which disrupts intracellular homeostasis, creating a stress response and slowing down growth. (Hou et al., 2012) Since each of the overexpression cassettes we created contains a 2-micron high-copy plasmid of yeast origin, the cell focuses on replicating the plasmid during division, and the energy and nucleotide consumption required by this process can result in a slowdown in the cell cycle and a decrease in growth rate. (Karim&Jewett,

2016) When the 24-hour growth monitoring (OD600) in the control groups (overexpression strains not treated with polystyrene) and the overexpressed strains treated with 25nm 250mg/L polystyrene was examined, no significant difference was observed between the control group and the polystyrene-treated groups. The study results showed no notable differences in growth between groups exposed to polystyrene and those that were not, indicating that the overexpression of these genes did not provide a distinct advantage or disadvantage in growth when subjected to nanoplastic stress. This suggests that polystyrene may not consistently have a direct inhibitory impact on cell proliferation. Instead, its toxic effects may be more evident in intracellular processes such as oxidative stress, membrane integrity, and protein folding, rather than in overall growth metrics (Bhattacharya et al., 2010; Li et al., 2020; Fadare & Okoffo, 2020). Furthermore, it has been proposed that the overexpression of specific genes, particularly those related to metabolic adaptation, might mitigate cellular responses, leading to stabilized growth patterns under environmental stress conditions (Zhou et al., 2018). Furthermore, it's crucial to understand that growth measurements alone might not capture the complete biological effects of toxic substances. To delve deeper into the cellular stress responses, employing molecular tools like transcriptomic profiling, measuring reactive oxygen species (ROS), or conducting membrane permeability assays is often required (Sendra et al., 2021; Wang et al., 2021). Hence, although the overexpression strains did not exhibit any apparent growth issues, additional molecular examinations are necessary to determine if there are any nuanced toxicological impacts.

Afterwards, the results of cell counting with methylene blue at 3 hours after application of polystyrene nanoparticles (250 mg/L, 25 nm) showed that cell density was lower in polystyrene-applied samples than in the control group. This suggests that polystyrene suppresses cell proliferation or increases cell death rate. Cytotoxic effects of nanoplastics are associated with mechanisms such as disruption of membrane integrity, increase in reactive oxygen species (ROS), uptake into the cell by endocytosis, and metabolic stress (Ramsperger et al., 2020; González-Pleiter et al., 2019).

Upon analyzing the inhibition rate outcomes for overexpressed strains, it was revealed that the inhibition rate was 19.34% in WT strains. In contrast, the ARO4_OE strains showed a rate of 17.14%, ARO9_OE strains exhibited 14.10%, and ARO10_OE strains demonstrated 16.48%. These findings indicate that the three OE strains showed higher

resilience against polystyrene stress compared to the WT strains. Notably, the relatively low inhibition rate in ARO9_OE strains (14.10%) implies that overexpressing this gene might enhance the cells' stress tolerance. Members of the ARO gene family are crucial in aromatic amino acid metabolism and have been linked to various cellular stress responses, including maintaining cell redox balance and nitrogen metabolism (Vuralhan et al., 2003). Earlier studies have highlighted that overexpression can enhance a cell's metabolic adaptability, thereby bolstering its resistance to harmful substances (Feng et al., 2025). By ramping up the activity within the aromatic amino acid biosynthesis pathway, cells can improve their internal metabolic buffering capacity. This allows for a more effective utilization of precursors like phenylalanine and tyrosine, which are crucial for defending against oxidative stress (Lin et al., 2021). When considering this, the reduced inhibition rates seen in OE strains indicate that genetic manipulation could be a promising approach for increasing cellular resilience against environmental toxins.

The molecular responses of *Saccharomyces cerevisiae* to the toxicity caused by polystyrene nanoparticles are thoroughly examined in this thesis, along with the genetic modification of important genes to evaluate their function in stress tolerance. The study offers comparative analyses that advance knowledge of metabolic adaption processes under nanoplastic stress by knocking out and overexpressing the ARO4, ARO9, and ARO10 genes. Several experimental techniques, such as growth kinetics (OD600), methylene blue staining for viability tests, qPCR for gene expression profiling, and inhibition rate computations, provide a multifaceted view of how polystyrene toxicity can be controlled at the genetic level. In particular, it has been demonstrated that the control of genes related to nitrogen metabolism and the manufacture of aromatic amino acids affects the cellular resistance to environmental stress, which has consequences for industrial microbiology and environmental biotechnology.
This strategy can be extended in subsequent research to include the production of toxicant detection biosensor systems, platform cell design for bioremediation, and pollutant-resistant yeast strains. Additionally, this study's demonstration of the effectiveness of CRISPR-based genome editing offers a useful model for accurate and focused gene regulation in yeast systems. In this way, the thesis makes a substantial contribution to the basic science subject of molecular toxicology while also presenting a fresh strategy that could aid in the creation of long-term biotechnological solutions.

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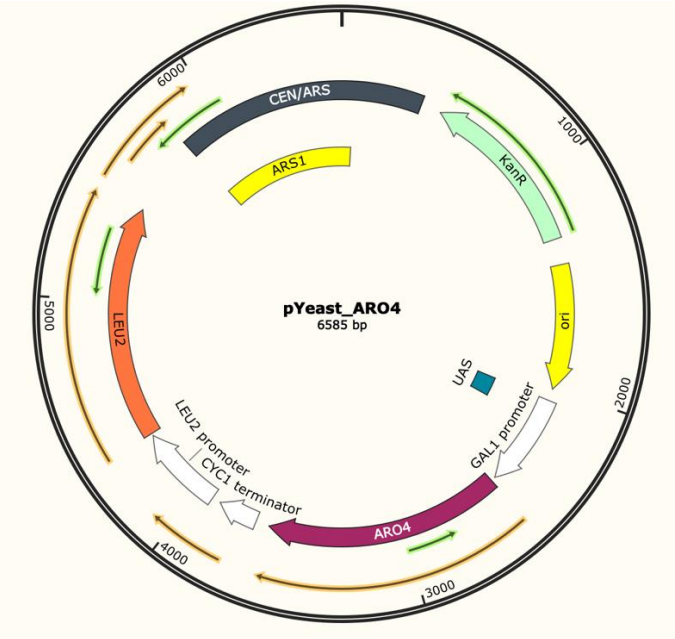
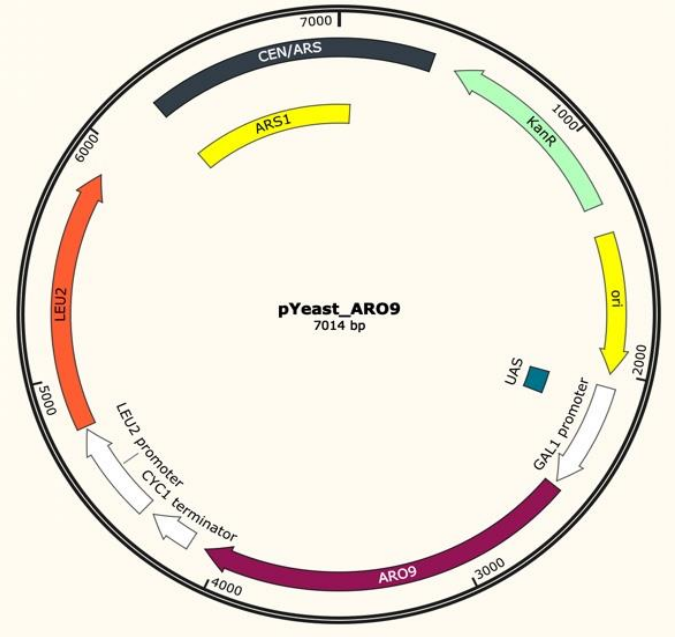
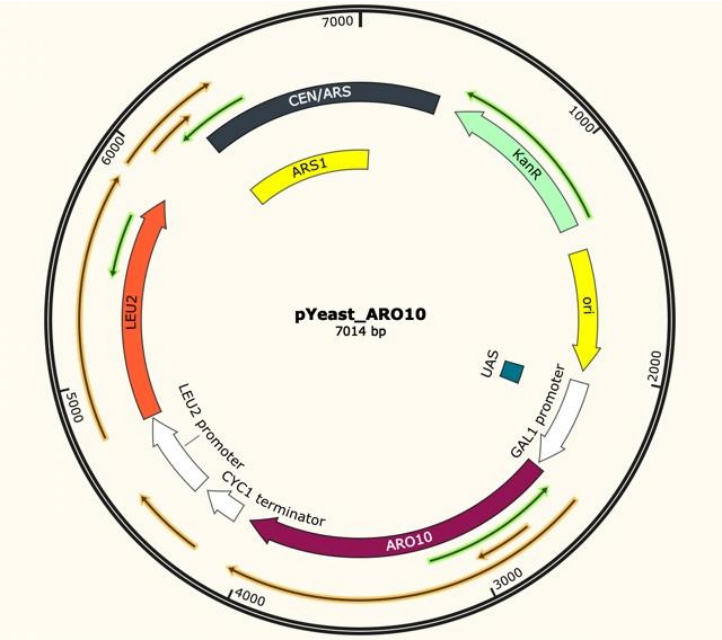
6. SUPPLEMENTARY DATA



Supp. Fig 1. pYeast_ARO4 expression cassette



Supp. Fig 2. pYeast_ARO9 expression cassette



Supp. Fig 3. pYeast_ARO10 expression cassette