ISTANBUL TECHNICAL UNIVERSITY ★ GRADUATE SCHOOL OF SCIENCE ENGINEERING AND TECHNOLOGY

MOLECULAR CHARACTERIZATION of OXIDATIVE STRESS RESISTANT YEAST

M.Sc. THESIS

Nazlı KOCAEFE

Department of Advanced Technologies

Molecular Biology- Genetics and Biotechnology Programme

MAY 2012

ISTANBUL TECHNICAL UNIVERSITY ★ GRADUATE SCHOOL OF SCIENCE ENGINEERING AND TECHNOLOGY

MOLECULAR CHARACTERIZATION of OXIDATIVE STRESS RESISTANT YEAST

M.Sc. THESIS

Nazlı KOCAEFE
(521091097)

Department of Advanced Technologies

Molecular Biology- Genetics and Biotechnology Programme

Thesis Advisor: Prof. Dr. Zeynep Petek Çakar

MAY 2012

İSTANBUL TEKNİK ÜNİVERSİTESİ ★ FEN BİLİMLERİ ENSTİTÜSÜ

OKSİDATİF STRESE DİRENÇLİ MUTANT MAYANIN KARAKTERİZASYONU

YÜKSEK LİSANS TEZİ

Nazlı KOCAEFE
(521091097)

İleri Teknolojiler Ana Bilim Dalı

Moleküler Biyoloji- Genetik ve Biyoteknoloji Programı

Tez Danışmanı: Prof. Dr. Zeynep Petek ÇAKAR

MAYIS 2012

Nazlı Kocaefe, a **M.Sc.** student of ITU **Graduate School of Science Engineering and Technology** student ID 521091097, successfully defended the **thesis/dissertation** entitled "**MOLECULAR CHARACTERIZATION of OXIDATIVE STRESS RESISTANT YEAST**", which she prepared after fulfilling the requirements specified in the associated legislations, before the jury whose signatures are below.

Thesis Advisor :	**Prof. Dr. Zeynep Petek ÇAKAR** (Istanbul Technical University)	.....
Jury Members :	**Assoc. Prof. Dr. Ayten YAZGAN KARATAŞ** (Istanbul Technical University)	.....
	Assist. Prof. Dr. Behice Şebnem SESALAN (Istanbul Technical University)	.....

Date of Submission : 04 May 2012
Date of Defense : 06 June 2012

To my family,

FOREWORD

I would like to thank to my supervisor Prof. Dr. Zeynep Petek Çakar for giving me the chance to study in this thesis project. She gave me valuable advices and support always when I needed. I also thank to her to accept me to Yeast Group.
I want to thank to specially Ceren Alkım of being great help during the development of this thesis. She encouraged me when everything gone badly. She was always with me not only in laboratory but also in all life, weekdays and weekends, morning and night.
I also want to thank to Bahtiyar Yılmaz for his successful experiments. His mutant is the basis of my thesis.
What's more, I thank to members of Yeast Group. Gökhan Küçükgöze, Gülçin Balaban, Hande Tekarslan, Ülkü Yılmaz, Seçil Erbil, Mevlüt Arslan, Burcu Turanlı Yıldız, Özge Özmeral. Especially I owe to thank to Can Holyavkin and Arman Akşit for motivation to experiments and life. I also thank Caner Nazlı for technical assistance in primer design for PCR experiments.
I also thank to Hilal Saraç, Ceren Göral, Murat Üstün and Naz Kanıt for their contributions.
I also want to thank to Levent Üge, for his technical support.
Finally, I would like to thank to my family being with me all the time. Fortunately, I have your supports.

May 2012

Nazlı KOCAEFE
(Molecular Biologist)

TABLE OF CONTENTS

Page

ABBREVIATIONS

CDW	**:** Cell Dry Weight
EMS	**:** Ethyl Methane Sulphonate
YMM	**:** Yeast Minimal Medium
YPD	**:** Yeast Extract Peptone Dextrose
KAc	**:** Potassium acetate
YNB	**:** Yeast Nitrogen Base
HPLC	**:** High Pressure Liquid Chromatography
PCR	**:** Polymerase Chain Reaction
qPCR	**:** Quantitative Polymerase Chain Reaction
DNA	**:** Deoxyribo Nucleic Acid
RNA	**:** Ribo Nucleic Acid
cDNA	**:** Complementary Deoxyribo Nucleic Acid
OD	**:** Optical Density
w/t	**:** Wild Type
PBS	**:** Phosphate Buffer Saline
Glc6P	**:** Glucose-6-phosphate
UTP	**:** Uracil triphosphate
Glc-1P	**:** Glucose-1-phosphate
PPi	**:** Pyrophosphate
UDP-Glc	**:** Uridine diphosphate glucose
Tre-6-P	**:** Trehalose-6-phosphate
Pi	**:** Inorganic phosphate
dNTP	**:** Deoxyribonucleotide triphosphate
ROS	**:** Reactive Oxygen Species

LIST OF TABLES

Page

LIST OF FIGURES

Page

MOLECULAR CHARACTERIZATION of OXIDATIVE STRESS RESISTANT YEAST

SUMMARY

Oxygen is both essential and harmful for aerobic organisms. Oxygen and oxygen derived oxidants cause cellular damages and diseases. As a cellular aspect, cellular components like lipids, proteins and DNA are affected by reactive oxygen species. Antioxidant systems and repair mechanisms protect cells and organisms from their harmful effects.

Reactive oxygen species (ROS) are different states of dioxygen. Superoxide anion, hydrogen peroxide and reactive hydroxyl radical constitute in ROS. These are created during oxidative respiration.

To understand the protection mechanisms of organisms, a model organism is required. The yeast, *Saccharomyces cerevisiae* is a suitable organism for oxidative stress studies, because of its properties like being a eukaryotic and unicellular organism. It provides high amounts of information about eukaryotic organisms. It is also easy to work with *S.cerevisaie*. Furthermore, it has an interesting life cycle. It can be both haploid and diploid. Besides, it undergoes sporulation in starvation conditions. Due to these properties, *S. cerevisiae* is the most used model organism for oxidative stress, cancer and aging research.

In our study, *S. cerevisiae* was used as a model organism. Oxidative stress resistant mutant had been obtained previously by evolutionary engineering method. EMS mutagenesis had been applied to make random mutation on yeast genome. Hydrogen peroxide was then applied continuously and increasingly, and the most resistant mutants were selected. Following cross-resistance experiments, a more resistant individual was selected as an oxidative stress resistant mutant.

To understand resistance mechanism, this resistant mutant was used. Phenotypical experiments were carried out to observe its resistance and sensitivity to different stress factors. For this purpose, spot assay was used with different stress containing solid media. Such as hydrogen peroxide, metal ions or different carbon sources. Furthermore, effects of calcium and glutamic acid on oxidative stress tolerance were investigated for wild type and oxidative-stress resistant mutant.

Glucose, maltose, glycerol, ethanol and acetate amounts were measured by HPLC and, trehalose and glycogen amounts were determined via enzymatic analysis. Physiology of oxidative stress resistant mutant was observed via these studies. Glucose utilization, maltose, glycerol, ethanol and acetate production results were reported. The results gave information about differences in metabolite levels and described genrally the metabolism of H_2O_2 stress resistant mutant.

In addition, deletion of *YAP1* gene, in the mutant gave on the potential role of this gene in H_2O_2 resistance mechanism, of the mutant.

For gene expression analysis, qPCR was carried out. Expression of some genes which have been desired in the literature to be potentionally important in oxidative stress, was investigated. *OCA1*, *MSN2* and *MSN4* expression levels were analyzed by qPCR.

For a detailed and global investigation of gene expression, microarray analysis was carried out. Gene expression levels were determined for both wild type and oxidative-stress resistant mutant, and compared to each other. Up-regulated and down-regulated genes were determined and their pathways were investigated.

OKSİDATİF STRESE DİRENÇLİ MUTANT MAYANIN KARAKTERİZASYONU

ÖZET

Oksijen aerobik solunum yapan bütün canlılar için hem gerekli hem de zaralıdır. Oksijen ve oksijenden türevlenen oksidantlar hücresel zarara yol açtıkları gibi bazı hastalıklara ve kansere de neden olmaktadır. Lipidler, proteinler ve DNA, reaktif oksijen türlerinin (ROS) oluşturduğu oksidatif stresten etilenmektedir. Canlılar; antioksidant sistemler ve enzimatik mekanizmalar ile oksidatif stresin zararlı etkilerinden korunabilmektedirler. Bunun yanı sıra organizmalar, meydana gelen hasarı düzeltmek adına çeşitli tamir mekanizmaları da geliştirmişler.

Reaktif oksijen türleri dioksijenin (O_2) farklı durumlarından meydana gelmektedirler. Süperoksit anyonu, hidrojen peroxit, reaktif hidroksil radikalleri en önemli reaktif oksijen türleridir. Bunlar, oksijenli solunum sırasında üretilirler.

Reaktif oksijen türevlerinin, oksidatif stresin etkilerinden korunmak için canlıların geliştirdiği antioksidant sistemler, enzimatik yollar ve tamir mekanizmaları gibi korunma yollarını anlamak; hastalıkların, yaşlanmanın ve kanserin doğasını anlamak için çok önemlidir. Bu mekanizmaların anlaşılması için bir model organizmaya ihtiyaç duyulur. *S. Cerevisiae* mayası, çeşitli özelliklerinden dolayı oksidatif stress çalışmaları için uygun bir model organizmadır.

S. cerevisiae ökaryotik ve tek hücreli bir canlıdır. Tek hücreli olması genetik manipulasyonu kolay kılarken, ökaryotik olması diğer ökaryotlar hakkında bilgi edinilmesine olanak sağlamaktadır. Bunun yanında, *S. cerevisiae*'nin yaşam döngüsünde araştırmalarda oldukça önemli bir yer kaplar. *S. cerevisiae* yaşam döngüsünde hem haploid hem diploid olan ve doğada poliploidi olarak bulunabilen bir canlıdır. Buna ek olarak diploid formu açlık durumunda mayoz bölünme ile sporulasyona giderek haploid sporları oluşturmaktadır. Bu özelliği araştırmalar için farklı metodlar geliştirilmesine olanak sağlar. Bu özelliklerinden dolayı *S. cerevisiae* oxidatif stres, kanser ve yaşlanma araştırmalarında en fazla tercih edilen organizmalardan biridir.

Bu çalışmada model organizma olarak *S. cerevisiae* kullanılmıştır. Evrimsel mühendislik yöntemiyle daha once elde edilmiş olan oksidatif strese dirençli mutantın fizyolojik ve moleküler karakterizasyonu yapılmıştır. Mutantın elde edilmesi için başlangıç kültürü öncelikle EMS (etil metan sülfonat) ile rastgele mutasyona tabi tutulmuş, daha sonra sürekli ve giderek artan giden bir hidrojen peroksit stresine maruz bırakılmıştır. Bu rastgele mutasyon ve stres koşulundaki seleksiyon sayesinde oluşan bireylerden oksidatif strese ve yapılan çapraz direnç testlerine en fazla dayanan birey deneylerde kullanılacak mutant olarak seçilmiştir. Çapraz direnç testi; bireylerin ağır metaller, yüksek tuz, yüksek etanol gibi farklı stres koşullarına verdiği tepkiler, üreme durumlarını görmeye yarayan testlerdir.

Bu çalışmada *S. cerevisiae*'nin oksidatif strese direnç mekanizmasını araştırmak için, bu mutant kullanılmıştır. Fenotip analizi için katı besi yerinde yapılan çapraz direnç testi ile mutantın dirençli ya da duyarlı olduğu stres koşulları belirlenmiştir. Bunun için farklı konsantrasyondaki hidrojen peroksit içeren katı besiyerleri ve farklı stres koşullarına sahip (metal, iyon ve farklı bileşikler) katı besiyerlerinde mayalar üretilmiş ve sonuçları gözlenmiştir. Ayrıca kalsiyum ve glutamik ait içeren hidrojen peroksitli katı besiyerlerinde mayalar büyütülmüş, bu sayede kalsiyumun ve glutamik asidin, oksidatif stres direnci üzerine etkileri gözlenmiştir.

Ayrıca HPLC (Yüksek Basınçlı Sıvı Kromotografi) ile, üretilen ve tüketilen metabolitler ölçülmüştür. Hem mutant hem yaban tipin farklı üreme koşullarında ve farklı zamanlarda, besi yerindeki glikoz tüketimi, maltoz, etanol, gliserol ve asatet üretimi belirlenmiştir. Hem yaban tip hem de mutant için yapılan bu ölçümler sonucunda, her iki suş için de üretilen metabolit konsantrasyon farklılıkları belirlenmiş, besiyerinde bulunan glikozun bitiş zamanlarındaki fark gözlenmiştir.

Ayrıca, enzimatik yöntemlerle hücrede oluşturulan trehaloz ve glikojen konsantrasyonları ölçülmüştür. Trehaloz ve glikojen *S. cerevisiae*'de stres durumunda üretilen metabolitlerdir. Trehaloz bir disakkarit, glikojen ise polisakkarittir. Hücrede rolü açık olarak bilinmemesine karşın, açlık ve stres durumuna karşı hücrenin depoladığı besin kaynakları olarak bilinir. Bunu yanında protein ve hücre membranı yıkımına karşı saperonlar gibi işlevde gördüğü bilinmektedir. Bu sebeplerden ötürü mutantın ve yaban tipin trehaloz ve glikojen üretim seviyeleri belirlenmiş ve karşılaştırılmıştır. Aynı zamanda HPLC ile trehaloz ve glikojen deneylerinde suşlara hidrojen peroksit uygulanmış ve sonuçta stress faktörünün de bu metabolitlerin üretimi üzerindeki etkisi de gözlenmiş ve karşılaştırılmıştır.

Fizyolojik analizlerin yanında gen delesyonu ile hem yaban bireyde, hem de mutant bireyde *YAP1* geni silinmiş mutant suş elde edilmiş ve bu suş da farklı konsantrasyona sahip katı besiyerinde üretilerek bu genin üreme ve stres üzerindeki etkisi gözlemlenmiştir.

Moleküler analiz kapsamında qPCR (quantitative PCR) ve mikroarray ile yaban tip ve mutanttaki gen ekspresyon düzeyleri belirlenmiştir. Yaban tipin ve mutantın, hem stresli koşulda hem de kontrol koşulunda *OCA1*, *MSN2* ve *MSN4* genlerinin ekspresyon düzeyleri belirlenip karşılaştırılmıştır. *OCA1* geni hücre döngüsünde kontrol noktasında görev aldığı ve oksidatif stres durumunda merkezi bir etkinliğe sahip olduğu bilinmektedir. Daha önce yapılan araştırmalarda *OCA1* hücre döngüsünde anahtar bir rol oynadığı görülmüş. *MSN2* ve *MSN4* ise *Saccharomyces cerevisiae* için en önemli genel stres yanıt genlerindendir. Bu sebeplerden ötürü oksidatif strese dirençli mutant mayada bu genlerin ekspresyon düzeyleri incelenmiştir.

Daha kapsamlı gen ekspresyon analizi olarak mikroarray yapılmıştır. Yaban tip ve mutantın tüm gene ekspresyon düzeyleri karşılaştırılmıştır. Mutantta ekspresyonu artan ve azalan genler belirlenmiş ve bu genlerin hangi yolaklarda işlev gördüğü araştırılmıştır. Bu sayede organizmadaki bütün genlerin transkripsiyon seviyeleri belirlenerek, organizmanın oksidatif stres durumunda hayatta kalmasını sağlayan yolaklar aydınlatılmıştır. Aynı zamanda, bu çalışmanın verileri yapılan fizyolojik ve fenotipik testleri de desteklemektedir. Çıkan sonuçlara gore, karbon metabolizmasında görev yapan genlerin oksidatif strese dirençli mayada öne çıktığı, bu genlerin transkripsiyon düzeylerinin oldukça yüksek olduğu belirlenmiştir.

Özellikle, *S. cerevisiae*'de stres savunmasında önemli rol oynayan trehaloz ve glikojenin üretiminde rol oynayan genlerin ekspresyon düzeylerinde oldukça yüksek artış gözlenmiştir.Sonuç olarak da, ortaya çıkan bu transkriptomik veriler ile sonraki yapılacak çalışmalar için vizyon oluşturulmuştur.

1. INTRODUCTION

1.1 *Saccharomyces cerevisiae* as a Model Organism

Eukaryotic cells, from yeast to mammals, respond and adapt to environmental stress by evolutionarily conserved systems (Popa *et al.,* 2010). The choice of the budding yeast as a model organism for studying complex processes is understandable. It has inherent, advantageous attributes that make it very attractive for modern biological studies (Gershon and Gershon, 2000). *Saccharomyces cerevisiae* is a species of yeast. It is used yeast to use both industry and scientific research as a eukaryotic model organism. It is used as a model organism to study aging, regulation of gene expression, signal transduction, cell cycle, metabolism, apoptosis, neurodegenerative disorders and many other biological processes (Karathia *et al.,* 2011).

1.1.1 *Saccharomyces cerevisiae* and its properties

Saccharomyces cerevisiae is a unicellular eukaryote and has subcellular organelles commonly found in eukaryotes. For this reason, *Saccharomyces cerevisiae* is attractive for researchers. In the mid nineties, the whole genome of *S. cerevisiae* was sequenced. It contains 16 chromosomes. The complete genome sequence revealed 5885 protein-coding genes on *S. cerevisiae* genome. *S. cerevisiae* taxonomic hierarchy is given on the Table1.1 (Anderegg *et al.,* 1988; Schaechter 2009).

Table 1.1 : Classification of *Saccharomyces cerevisiae.*

Kingdom	Fungi
Phylum	Ascomycota
Class	Hemiascomycetales
Order	Saccharomycetales
Family	Saccharomycetacae
Genus	Saccharomyces
Species	*Saccharomyces cerevisiae*

In normal culture conditions, *Saccharomyces cerevisiae* is ellipsoidal/ovoid in shape and approximately 5-10µm long by 3-7µm wide. Diploid cells are larger than haploid cells and that can be observed under light microscope. Figure 1.1 shows a scanning

electron micrograph (SEM) of *S. cerevisiae*. Small daughter cells can be seen budding off from the larger cell. Diploid cells are generally about two fold of haploid cells in size. Cell wall (200 nm thick) maintains plasma membrane of *Saccharomyces cerevisiae*. Above all, of the other important functions, the most important role of cell wall is the protection of cell unity. Cell wall of yeast occurs during budding and mating (Michels 2002). The yeast cell wall is composed of glucan, mannan and protein with small amount of chitin. The bud wall is not derived from material of mother cell wall, it is newly synthesized (Hartwell 1974). The plasma membrane surrounds plasma and cytoskeleton of cell containing a nucleus, mitochondrion, endoplasmic reticulum, vacuole, Golgi, secretory and endocytic vesicles. Like other eukaryotic organisms, ER, Golgi, vesicle systems and cell membrane provide cell communication to cell (Michels 2002).

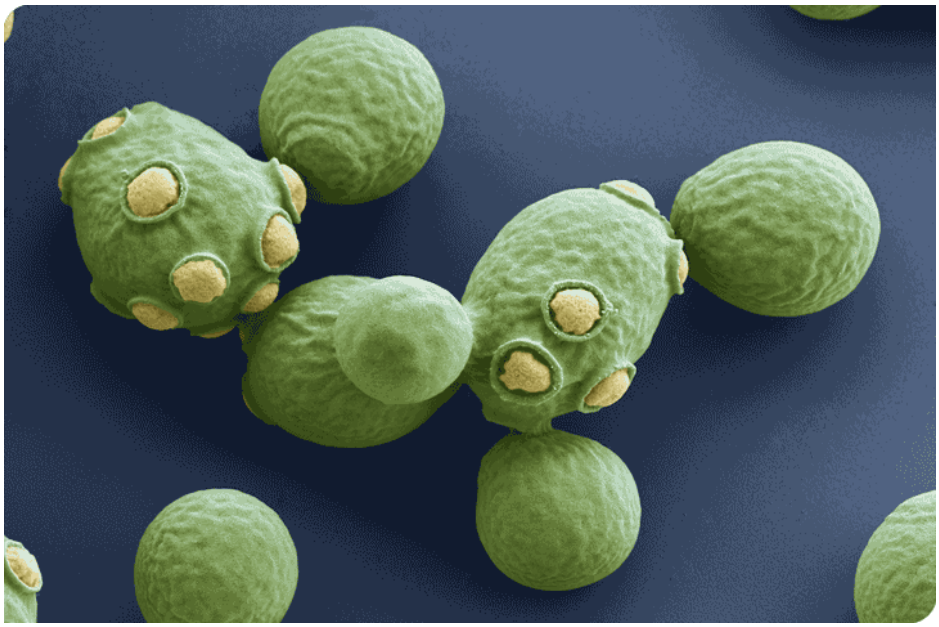

Figure 1.1 : *Saccharomyces cerevisiae* image. Photographed by SEM (Scanning Electron Microscope).

S. cerevisiae cells have two mating types in haploid form. These are described as ‘a’ and ‘α’ mating types. Mating type is determined by *MAT* locus on the right arm of chromosome III (Astell *et al.*, 1981). It is interesting in this system; these mating types can switch in normal condition as reversible. Feature of yeast creating both ‘a’ and ‘α’ generation was explained by homothallic nature of organism. HO gene is responsible for mating switching. HO gene is coded a restriction endonuclease. However, mating switching is not desired for experiments. For scientific studies, to maintain of strains mating type, HO gene is inactivated (Jennings 1993).

In haploid phase, two cell types secrete a hormone (pheromone) that acts specifically on cells of the opposite mating type. α mating type cells secrete α- factor (tridecapeptide), a mating type cells secrete a- factor (it is associated with two modified peptides a2 and a3) (Anderegg *et al.*, 1988).

Vegetative cell division of yeast occurs by budding, in which a daughter is initiated as an outgrowth the mother cell, followed by nuclear division, then cell wall formation and finally cell separation. Haploid cells budding is observed adjacent to the previous one (radial budding), whereas diploid cells budding is seen at opposite pole (axial budding)(Schaechter 2009). Normal laboratory haploid strains have a doubling time of approximately 90 min in rich medium YPD (Yeast Extract-Peptone-D-glucose medium) and approximately 140 min in YMM (Yeast Minimal Medium). These division times observed only in the presence of glucose and at 30°C.

Both haploid form and diploid form exist in life cycle of *S. cerevisiae*. For mating and creating diploid cell, *MATa* and *MATα* type haploid strains are carried out by mixing approximately equal amount on YPD medium and incubated at 30°C for 6 hours. Diploid cells can be observed under light microscope and selected. In starvation conditions diploid cells undergoe sporulation, this condition is created by using potassium acetate medium in laboratory. Meiosis occurs and haploid spores appear. Meiosis provides homologous recombination and changes on the genome. Spores are encapsulated within sac-like structure, this structure is called ascus. Generally ascus can include three or four spore cells. When YPD medium is provided and spores separated with micromanipulator, spores go to normal haploid cells (Roth and Halvorson 1969) (Figure 1.2). *S. cerevisiae* has a typical eukaryotic cell cycle (including G_1, S, G_2 and M).

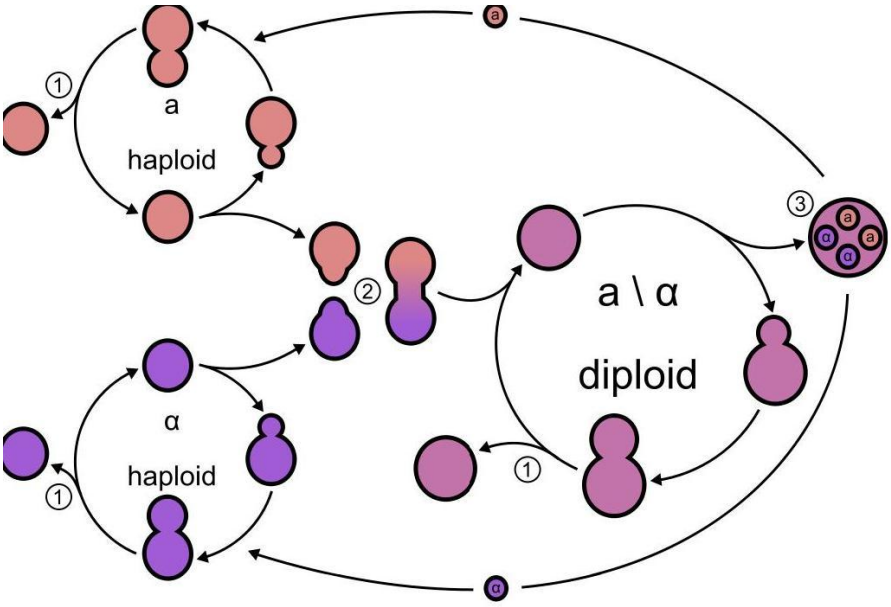

Figure 1.2 : Life cycle of *S. cerevisiae*. 1 haploid cell, 2 diploid cell (mating), 3 spores in ascus (meiosis).

1.1.2 Importance of *Saccharomyces cerevisiae* in experiments

The use of model organisms for research is important. It is not possible to conduct some experiments on humans because of ethical issues. Thus, studying with *S.cerevisiae* provide useful information on more complex eukaryotic organisms, including humans (Karathia, Vilaprinyo et al. 2011).

As a model organism *Saccharomyces cerevisiae* has important properties. Firstly, it is a unicellular eukaryotic organism with simple has uncomplicated structure and short life cycle. Its genome is composed of 6000 genes, which have been sequenced and mapped (Goffeau *et al.*, 1996). Its uncomplicated structure allows easy understanding of major processes in the complex organisms, It provides essential molecular genetics tools to study signal transduction, control cell cycle progression, understand the basis of switch from mitosis to meiosis, genetic recombination, intracellular trafficking of proteins, response to stress (heat shock proteins, ethanol resistance, oxidative stress and heavy metal resistance etc.) and protein degradation (Stahl 1996; Glover and Lindquist 1998; Whitmarsh and Davis 1998; Gershon and Gershon 2000).

Yeast has a lot of ortholog gene in human genome, including some disease-causing genes. Some genes are transducted to yeast to find out effects of these genes

expressions on uncomplicated eukaryote to solve much easier than study on human. Plasmid constructs and mutations help to understand working mechanism of these genes in multi-cellular organisms. These observations make the budding *S. cerevisiae* as an attractive model organism. Working on target organisms like human and other higher eukaryotic organisms is too difficult as they have longer life span, anatomical complexity, longer cell cycle, and expensive maintenance. however, *S. cerevisiae* is a desirable organism due to its ability to grow in a cheap and simple medium. Its phenotype and growth can be observed on agar plate easily (Gershon and Gershon 2000).

The phenotype of any organism is defined by interaction of genome with environment. With few exceptions, the genome is not altered in response to environmental change, but an organism may appear phenotypically distinct in different environmental conditions. As a result, gene expression changes. Thus, studies of gene expression regulation are an important area of genetic research (Jennings, 1993). Stress also affects cell proliferation, sensing and signaling in yeast. For instance, stress treatments cause a transient arrest of the cell cycle, commonly in G1, under osmotic stress and oxidative stress condition. This prevents cells from being damaged during cell cycle phases where they are more vulnerable (S and M phase)(Jennings 1993).

Diploid form of *S. cerevisiae* undergoes meiosis and is separated to haploid cells by micromanipulator. *S. cerevisiae* has an advanced homologous recombination and it contributes studying simple knock-out of individual genes. Additionally cells can be maintained and studied in lightly (Gershon and Gershon 2000). As a haploid for *S. cerevisiae* occurs in a two distinct morphological form. These are ‘a’ and ‘α’ mating types. Experiments on both haploid and diploid form of *S. cerevisiae* give some information about genes like dominancy.

Whole organisms from unicellular to multi-cellular are exposed to stress in environmental conditions. Thus they develop responses which require a complex network of sensing and signal transduction leading to adaptations of cell growth and proliferation via adjusting of gene expression program, metabolic activities, and other properties of cell. These harsh environmental conditions push the limits of survival of a cell, obstruct it from performing optimally. Cells or organisms response

and adapt to harsh conditions with their defense mechanism. These response and adaptation mechanisms are highly complicated. Stress response researches provide cell biology information. How cells responds to stress condition give fundamental information about molecular and cellular biology. For instance, heat shock proteins are part of the cellular response to heat stress. They have homeostatic function and control protein folding as chaperons. In addition, some responses are common to all stress condition, general response genes have a role to protect cell against to environmental stress condition (Hohmann and Mager 2003).

Stress research has a wide impact on medical issues. As an example, stress responses are related to aging, apoptosis, cancer and immunological responses. Additionally, stress has an important role in applied biotechnology. These studies in biotechnology aim at improvement of resistance of organisms. One of the most known studies is development of ethanol-resistnce in microorganisms. Besides, osmotic resistance and protection of food stuff from spoilage are also important, because these applications are industrially beneficial. Thus, there are several good reasons to study yeast responsesagainst a variety of strm conditions. (Hohmann and Mager 2003).

To understand the role of stress response genes in living organisms, particularly *S. cerevisiae* is studied. *S. cerevisiae* has been adapting to new environmental conditions. *S. cerevisiae* cells gain cross resistance against different stress(Mitchel and Morrison 1982). This idea induced the thought of a general mechanism for cellular stem protection response. As mentioned before, heat shock genes are induced not only by high temperatures but also by other stress conditions (Kurtz, Rossi et al. 1986). As a second example, Msn2p and Msn4p are general stress response proteins. It is known that stress induced genes are controlled by common mechanisms. Stress Response Elements (STRE) are common promoters of stress response genes (Schmitt and McEntee 1996). Hypotetically, STRE binding factor is related two zinc-finger transcription factors, Msn2p and Msn4p. Deletion studies showed that in the absence of these genes (*MSN2* and *MSN4*) cells show sensitivity to different environmental conditions. It was showen that Msn2p and Msn4p induce a great number of genes in response to many different stresses. Thus these factors were known as the "general stress" transcription factors that were activated by stress condition (Martinez-Pastor *et al.*, 1996; Hohmann and Mager 2003).

Different environmental conditions and stress factors affect cells (from bacteria to animals) which induce a small group of genes strongly (expression of stress response gene). Heat stress especially induces expression of heat shock proteins. These heat shock proteins (hsp70, hsp83/90, hsp100, the α-crystalline-related, hsp60 and ubiquitin are so common heat shock proteins) help cells to adapt to high temperatures (Morano K.A, 2011). They are also produced at normal temperature. Heat shock transcription factors were identified and characterized. Heat shock protein genes of *S.cerevisiae* were sequenced and their expression regulation was well studied. In addition to this, osmotic stress, heavy metal stress, ethanol and oxidative stress have been studied as stress response resistance studies. High osmotic environment affect stress response genes' expression and ion channels (Nadal E., 2011). Organism tries to exclude ions from cytoplasm and adapt to this environment. Osmotic stress studies on yeast can be applied on agriculture. Understanding osmotic stress responsible genes and mechanism of stress resistance was important for agriculture, when widespread problem of salinization of soil is considered. Metal tolerance is an important issue because the environment contains metals. Furthermore, organisms' structures also include metal, for example, enzymes contain metal ions as co-factor (Festa R.A., 2011). Therefore, investigations of metal stress response genes have importance to understand the role of metals in the metabolism. The other environmental factor is ethanol. Ethanol is produced because of microbial fermentation of carbohydrates. Produced ethanol accumulates in medium and this ethanol becomes a stress factor to yeast cells (You K. M., 2002). *S. cerevisiae* (because it produces more alcohol than other organisms) strains that are resistant to ethanol, are tried to be obtained in order to develop ethanol production yield. They can be used in alcohol industry and biofuel production (Anderegg *et al.*, 1988; Jennings 1993). One of the most important stress factors studied is oxidative stress factor. *S. cerevisiae* is known as a facultative anaerobe. Therefore, whether the environment has oxygen, it reciprocates, as a result it produces reactive oxygen species that look like other eukaryotic aerobic organisms. Furthermore, it is required for synthesis of unsaturated fatty acids and glycerol (Gibson *et al.*, 2008). ROS produced by cellular systems causes damage and disease and is balanced by antioxidants and repair systems.

1.2 Relation of Oxidative Stress with *Saccharomyces cerevisiae*

Oxygen is known as an essential and toxic compound for aerobic organisms. Oxygen and oxygen derived oxidants cause cellular damage and disease. These are balanced with antioxidants and repair systems. Oxidative stress is of crucial importance in biomedicine, because of its relation to a number of neurodegenerative, inflammatory and cardiovascular diseases, cancer and the process of aging (Ames *et al.*, 1993; Hohmann and Mager, 2003).

1.2.1 Oxidative stress elements

S. cerevisiae has become a unique system in which to study oxidative stress and redox reactions in eukaryotes. ROS damage cellular components such as lipids, proteins and DNA. Additionally, ROS toxicity can result in growth inhibition and cell death can be monitored in yeast cells.

In aerobic organisms, production of reactive oxygen species is tried to be balanced with antioxidant defense systems. Having too many reactive oxygen species (ROS) than antioxidants could cause a system failure. This condition causes damage to organism and this situation is called oxidative stress. It can be defined as the biomolecular damage caused by attack of ROS upon the constituents of living organisms (Halliwell and Gutteridge, 2007).

ROS represent different oxidation states of dioxygen (O_2). It includes singlet oxygen, the superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) and highly reactive hydroxyl radical (•OH) (Hohmann and Mager, 2003). Reactive oxygen species is a collective term that includes not only the oxygen radicals but also some non-radical derivatives of O_2 such as hydrogen peroxide and ozone. The reason is that a free radical is any species capable of independent existence that contains one or more unpaired electrons. For the purpose of this definition singlet oxygen and superoxide radicals are oxygen radicals (Halliwell and Gutteridge, 2007).

Superoxide anion (O_2^-) is created by one electron reduction of O_2. Dismutation of two molecules of superoxide anion by two electrons constitute to hydrogen peroxide. Reactive hydroxyl radical (•OH) can be formed by hemolytic fission of water or by the iron-catalyzed Fenton reaction. The latter reaction involves oxidation of Fe^{2+} to Fe^{3+} with reduction of hydrogen peroxide to •OH and OH^-. O_2^- can also participate

to•OH formation by its dismutation to hydrogen peroxide and by reducing Fe^{3+} to Fe^{2+} via Haber-Weiss reaction. These reactions are shown on Table 1.2 (Hohmann and Mager 2003).

Table 1.2 : Redox reactions in *S.cerevisiae*

	Reaction
Haber-Weiss reaction	$Fe^{3+} + O_2^- \rightarrow Fe^{2+} + O_2$
Fenton reaction	$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + \bullet OH$
Dismutation (SOD)	$2O_2^- + 2\ H^+ \rightarrow O_2 + H_2O_2$
Dismutation (Catalase)	$2\ H_2O_2 \rightarrow 2\ H_2O + O_2$

In general, hydrogen peroxide is used as oxidative stress reagent. It belongs to the non-radical group of the ROS and can be converted into other more reactive ROS by various means including enzymes. Furthermore, hydrogen peroxide is relatively stable and less reactive compared to other specific chemical reactions (Bienert *et al.*, 2006). Hydrogen peroxide can easily mix with water and diffuse into cell. Sometimes water can use channels (aquaporin) for passing through the lipid bilayer and hydrogen peroxide can also pass from these channels (Kawada, Khan et al. 2004). Adaptation of yeast to hydrogen peroxide seemed to involve changes in plasma membrane lipid bilayer composition to slow hydrogen peroxide entry (Antunes *et al.*, 2002). Membrane lipid composition regulates hydrogen peroxide transportation (Figure 1.3).

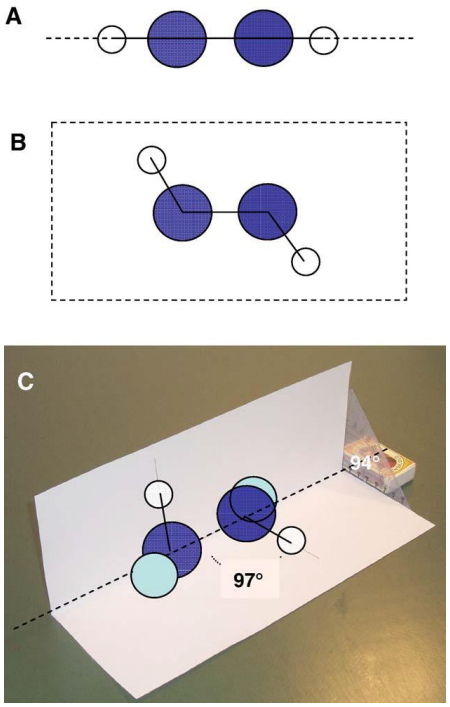

Figure 1.3 : Three possible conformation of hydrogen peroxide. Oxygen atoms are represented with blue and hydrogen atoms are white. (A) Linear conformation, (B) Trans-planar conformation, (C) Skewed cis conformation.

1.2.2 Effects of oxidative stress on *Saccharomyces cerevisiae*

ROS are highly reactive and have ability to damage cellular constituents such as proteins, lipids and DNA. They affect cell proliferation, cell adaptation, damage the cellular component and its high volume causes cell death (Halliwell and Gutteridge 2007).

Stress treatment causes a transient arrest of cell cycle, commonly G_1 or at also G_2-M boundary. Cell cycle arrest may be needed to prevent damage during cell cycle phases. For example, *OCA1* gene expression increases under oxidative stress condition. *OCA1* gene product provides cell arrested in G_1. It also causes peroxidation of lipids and directly affects cell membrane (Alic *et al.*, 2003). Oxidative stress can damage proteins involved in maintenance of essential ion gradients cell and environment. Radical group of enzymes are attacked by ROS.

ROS damage to nucleoproteins produces base and sugar damage, single-strand breaks, abasic sites and DNA-protein cross-links. Superoxide radicals and hydrogen peroxide do not disturb DNA directly. Fenton and Haber-Weiss reactions catalyse

the formation of •OH and induce DNA damage in yeast cells (Frankenberg *et al.*, 1993 and MoradasFerreira *et al.*, 1996). 8-hydroxyguanine is one of the most general base damage, this damage occur in lethal concentrations of hydrogen peroxide, not sublethal concentration. In addition, hydrogen peroxide enhance incidence of intrachromosomal recombination in *S. cerevisiae* via created hydroxyl radicals. Thus, it can be said that oxidative stress plays an important role in DNA recombination in yeast (MoradasFerreira *et al.*, 1996).

The chemistry of oxidativw protein damage is more complex than for DNA. There are 20 or more amino acid rsidues instead of four based and a sugar.Each aminoacids capable of forming several oxidation products.Further more, peptide bonds can also be attacked, for instance •H abstraction by •OH (Halliwell *et al.*, 2007).

High concentration of hydrogen peroxide extend the malondialdehyde compound resulting from lipid peroxidation in yeast. It is immediately increased unsaturated fatty acid on the lipid-bilayer membrane. Furthermore, some products of lipid peroxidation are able to damage DNA and inactivate some proteins (Steels *et al.*, 1994 and MoradasFerreira *et al.*, 1996).

1.2.3 Oxidative stress response of *Sacchoromyces cerevisiae*

Under normal physiological conditions, antioxidant defense mechanisms are certainly adequate to maintain ROS at basal/unharmful levels and to repair cellular damage. Primary defense system is related with ROS neutralization as well as secondary defense system repair or remove the products of oxidation damage to cellular components (MoradasFerreira *et al.*, 1996).

S. cerevisiae has two defense systems upon oxidative stress exposure. Non-enzymatic (antioxidant defense system) and enzymatic ways help to defense against oxidative stress. Antioxidant defense systems and enzymatic defense systems are shown at Table1.3.

Table 1.3 : *Saccharomyces cerevisiae* defense systems against oxidative stress.

Gene (Product)	Function
***SOD1* (cytoplasmic SOD)**	Dismutation of superoxide anion
***SOD2* (mitochondrial SOD)**	
***CTA1* (catalase A, peroxisomal)**	Decomposition of hydrogen peroxide
***CTT1* (catalase T, cytoplasmic)**	
***CCP1* (cytochrome peroxidase)**	Reduction of hydrogen peroxide
***GSH1* (glutathione)**	Reduction of protein disulphidase; scavenging of free radicals
***GLR1* (glutathione reductase)**	Reduction of oxidized glutathione

Glutathione, trehalose, glycogen, ascorbic acid, and antioxidants make up the non-enzymatic defence systems. Cytosolic and mitochondrial catalases, mitochondrial and peroxisomal superoxide dismutases build up enzymatic defense systems of *S. cerevisiae*. *S. cerevisiae* accumulates carbohydrates such as trehalose and glycogen to cope with stress condition. These carbohydrates act as storage factors. Moreover, it is known that trehalose and glycogen have a stabilizer effect on cellular structures under stress condition (Crowe *et al.*, 1984). Trehalose prevents denaturation and aggregation of proteins. These fuctions of trehalose resemble the role of chaperone proteins (Arguelles, 2000). Nonetheless, their roles have not been explained clearly yet. Furthermore, they have a possible role in cell cycle progression. Trehalose and glycogen levels increase during G_1 phase and degrade when cell goes to S phase. This result shows trehalose and glycogen may be required under low sugar to temporarily increase the sugar flux in order to complete a cell division cycle (Sillje *et al.*, 1999)

Trehalose is a disaccharide and has a function as a regulator of glycolysis during maltose utilization. Glycogen is a polysaccharide and has a function as nutrition storage. Trehalose and glycogen pathways are shown in Figure 1.4. Glycogen catabolism pathway is given separately in Figure 1.5.

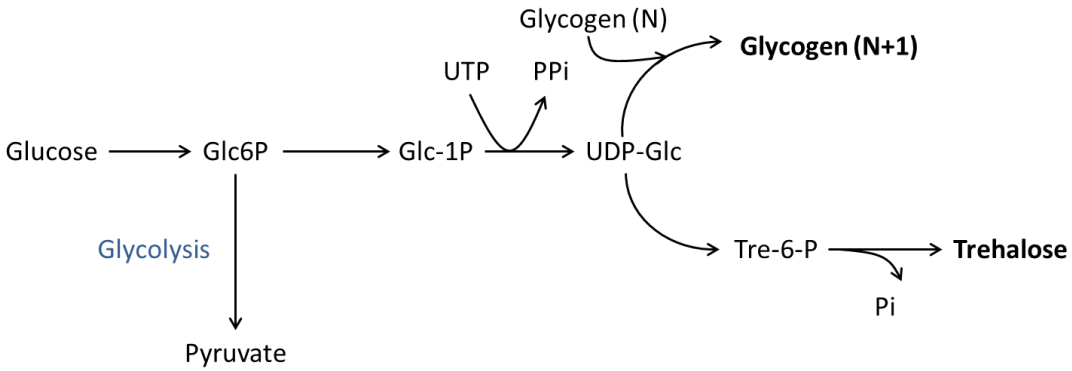

Figure 1.4 : Trehalose and glycogen pathway in *S. cerevisiae.*

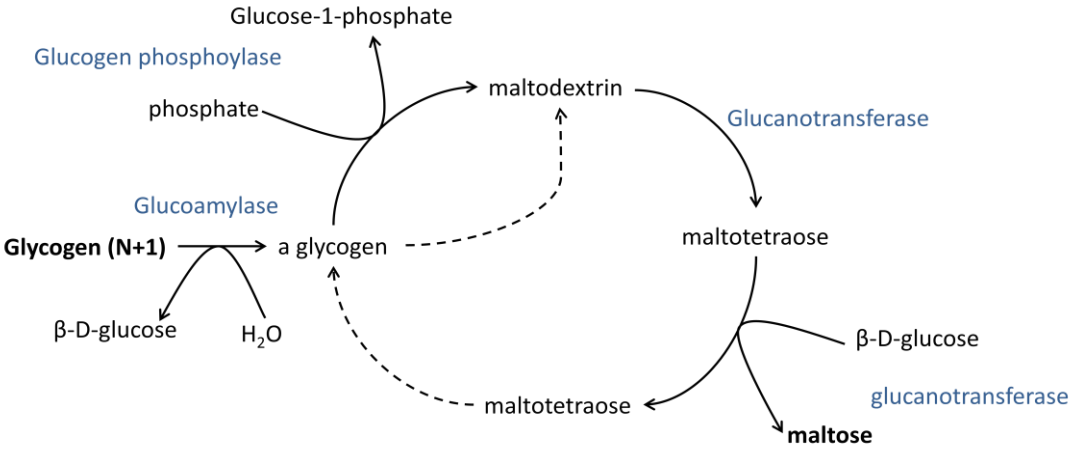

Figure 1.5 : Glycogen glucose-6-phosphate, UTP: uracil triphosphate, PPi: pyrophosphate, UDP-Glc: uridine diphosphate glucose, Tre-6-P: trehalose-6-phosphate, Pi: inorganic phosphate).

As genomic dimension, response of yeast to oxidative stress is regulated by these genes' expression. Transcription factors have a major role. *YAP1* gene encodes an important transcription factor. It regulates enzymatic and non-enzymatic reactions. It controls expression of *GSH1* (related with glutathione synthetase) and thioredoxin metabolism as well as SOD, catalase and cytochrome c peroxidase. In oxidative stress condition, Yap1p is oxidized to a disulphide bridge by peroxides, protein goes to nucleus and increase gene expression (Hon *et al.*, 2003). Deletion studies showed that *YAP1* deleted strains have hypersensitivity to hydrogen peroxide and are not able to induce 32 proteins. In addition, *MSN2* and *MSN4* are general stress response genes. They are functionally related and encode Cys_2His_2 zinc finger proteins. They bind to STRE (stress-response elements) sequence and have a role binding factor of STRE (Hasan *et al.*, 2002).

1.3 Metabolic Engineering

Metabolic engineering was defined as the improvement of cellular activities by manipulation of enzymatic, transport, and regulatory functions of the cell with the use of recombinant DNA technology (Bailey 1991 and Cakar *et al.*, 2012). Genetic manipulations are required for desired phenotype with knowledge about metabolic pathways and chemical networks. However, this aspect had limitations to study. As mentioned previously, requirement of a lot of information about metabolic pathways of interest and/or regulatory systems could be impeding for the study. Moreover, complexity of cellular physiological response may make it difficult to reach desirable results. For instance, to activate a pathway a high number of gene are stimulated. Besides, it is difficult to genetically manipulate industrial strains (Bailey *et al.*, 1996 and Cakar *et al.*, 2012). To bypass these limitations, a "bottom-up" approach named inverse metabolic engineering was suggested (Bailey et al,1996).

1.3.1 Inverse metabolic engineering

The aim of this approach is obtaining a mutant organism with a desired phenotype first, and then characteize this strain by using high throughput technologies in genomics, as well as proteomics. The most important advantage of this approach is that it is not necessary to have knowledge about all and/or whole metabolic mechanism. Any identified phenotypic property could be transferred to a target organism.

1.3.2 Evolutionary engineering

Evolutionary engineering can be used to obtain desired phenotypes by continuous environmental treatment and selection. A classical method for strain development is physical or chemical mutagenesis followed by cultivation on cultured on solid/liquid media containing selective condition such as high salt, oxidative agent, high metal concentrations and high/low temperatures. Repeated cycles of this treatment select for highly resistant mutants.

1.4 Aim of the Study

The aim of this study was molecular and physiological characterization of an oxidative stress resistant *Saccharomyces cerevisiae* mutant, that was previously obtained by evolutionary engineering methods. Random mutation had been carried out by EMS and selection procedure was performed with continuous and increasing H_2O_2 application. One of the most resistant individual mutant had been selected with respect to oxidative stress resistance and cross-resistance to other stress types (Yilmaz 2009). Regarding cross resistance and previous studies on gene expression level determination and catalase enzymatic activity data (Yilmaz 2009), experiments were designed. Diploid cell and knock-out strains were generated. Phenotypical analyses of wild type, mutant, *yap1* and diploid cell of mutant were performed under different levels of hydrogen peroxide exposure. Furthermore, physiological analysis of the mutant was carried out by HPLC analyses, trehalose and glycogen determination including cell dry weight measurements. Glucose consumption and metabolite production rates were measured. Considering previous expression level determination studies on this mutant, expression levels of some genes thought to be important, were determined by q-PCR. In these studies, by applying hydrogen peroxide, effects of oxidative stress were observed. Besides, microarray analysis was performed to investigate whole genome expression levels of oxidative stress resistant mutant to study the all mechanisms of oxidative stress resistance.

2. MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 Strains

S. cerevisiae CEN.PK 113-7D (*Mata*, *MAL2*-g^c, *SUC2*) and *S. cerevisiae* CEN.PK 113-1A (*Matα*) were used as wild type strains which obtained kindly provided by L.Benbadis from INSA-Toulouse. Hydrogen peroxide-resistant strain 'H7' which was obtained via evolutionary engineering was also used from *S. cerevisiae* CEN.PK 113-7D (*Mata*, *MAL2*-g^c, *SUC2*) background (Yilmaz, 2009). Diploid cells (2n) were obtained by mating H7 and opposite mating type wild type strain.

2.1.2 Culture media and preservation conditions of strains

Media were prepared as described in Sections 2.1.2.1-2.1.2.4. They were then autoclaved at 121°C and 1.2 atmospheric pressure for sterilization.

Yeast strains were stored for a short period of time at 4°C on YPD medium an Petri plates. Strains could be kept alive nearly for one month. Additionally, strains were stored in microcentrifuge tubes in 30% (v/v) at -80°C. For this storage method, strains were grown on solid YPD medium, cultured in liquid YPD medium in test tubes for overnight at 30°C. Culture was centrifuged, supernatant was thrown away, and pellet was resuspended in 30% (v/v) glycerol.

2.1.2.1 YPD (Yeast Extract Peptone Dextrose) Medium

Yeast Extract Peptone Dextrose Medium, also abbreviated as YPD, is a complete medium for yeast growth. It contains 2% D-glucose, 1% yeast extract, 1% peptone and 2% agar (if it is used as solid media). As a selective medium (for gene knock out determination), Geneticin (G418) was added to YPD solid medium. Geneticin concentration was adjusted 100µg/ml. YPD contents are shown in Table 2.1.

Table 2.1 : Contents of YPD Medium. *Agar is used for solid media.

Content "w/v"	Amount
2% D-Glucose	20g
1% Peptone	10g
1% Yeast Extract	10g
2% Agar*	20g
dH_2O is added up to final volume 1000ml.	

2.1.2.2 YMM (Yeast Minimal Medium)

Yeast Minimal Medium was used for regular growth and stress analyses. It is known also as SD (Synthetic-Defined) medium. It includes 2% D-glucose, 1% Yeast Nitrogen Base without amino acids and agar (if solid media is desired).

Table 2.2 : YMM contents. *Agar is used for solid media.

Content "w/v"	Amount
2% D-Glucose	20g
0,67% Yeast Nitrogen Base without aminoacids	6.7g
2% Agar*	20g
dH_2O is added up to final volume 1000ml.	

2.1.2.3 KAc (Potassium Acetate) Medium

Sporulation medium (KAC) consists 1% 'w/v'anhydrous potassium acetate and 2% 'w/v' agar. It serves starvation condition for cell and makes them to undergo sporulation. Strains undergo several divisions on sporulations medium and then sporulate after 3 to 5 days incubation (Roth and Halvorson 1969).

2.1.2.4 Non-fermentable carbon source supplemented medium

Yeast can use some carbon sources. A different non-fermentable carbon source was used to observe growth of *S. cerevisiae* wild type and mutant, 'H7'. Different non-fermentable carbon supplements (ethanol, acetate and glycerol) were added to YNB (Yeast Nitrogen Base without amino acid medium). They contained 0.67% Yeast Nitrogen Base without aminoacid and 2% agar for 1litre medium (Table 2.3).

Table 2.3 : Medium contents for different carbon sources. *Agar is used for solid media.

Content "w/v"	Amount
0.67% Yeast Nitrogen Base without amino acid	6.7g
3% Glycerol	30ml
1% Ethanol	10ml
%2 Maltose	20g
2% Agar	20g
dH_2O is added up to final volume 1000ml.	

2.1.2.5 Plates supplemented with H_2O_2 for stress treatment

YMM which contained different concentrations of H_2O_2 was prepared by using 100 mM hydrogen peroxide main stock. Desired amount of hydrogen peroxide was added to 15000µl of YMM for each plate. These plates were used for spot test to understand effects of different hydrogen peroxide concentration on strains' growth. Besides, these plates supplemented calcium were used to show effect of calcium on growth on medium with hydrogen peroxide.

Table 2.4 : Hydrogen peroxide dilution rates and amounts for treatment.

Final concentrations of H_2O_2	H_2O_2 amounts (100mM)	Media amount for each plate
0mM - control	---	15000µl
0.5mM	75µl	14925µl
0.75mM	112.5 µl	14887.5µl
1mM	150 µl	14850µl
1,5mM	225 µl	14775µl
2mM	300 µl	14700µl

2.1.2.6 HPLC stock solutions and HPLC standards

HPLC was used for quantitative determination of glucose, maltose, ethanol, glycerol and acetate. Standard solutions were prepared only for these metabolites. Stock solutions, for standards, included glucose and metabolites. Glucose and each metabolite are listed in Table 2.5. Besides, 5mM sulphuric acid (H_2SO_4) was used as an "eluent" and the mobile phase. Firstly, Solution A was prepared with glucose. Solution B was thenprepared including ethanol, glycerol and acetate. Solution A and solution B were mixed at different rates. The last solution'C', contained only maltose. Contents of stock solutions and standard solutions are shown on Table 2.5 and table 2.6.

Table 2.5 : Stock solutions of HPLC standards.

Solution A	
Glucose	120g
Add to dH_2O up to Final volume 1000ml	
Solution B	
Ethanol	30g
Glycerol	2g
Acetate	4g
Add to dH_2O up to Final volume 1000ml	
Solution C	
Maltose	20g
Add to dH_2O up to Final volume 1000ml	

Table 2.6 : Standard solutions of HPLC standards (Std: Standard solution).

Standard Solutions	**Mixing Volumes**	**Eluent Volume**	**Final Volume**
Standard1	1ml Solution A 3ml Solution B	2ml Eluent	6ml
Standard2	0.75ml Std1	0.25ml Eluent	1ml
Standard3	0.50ml Std1	0.50ml Eluent	1ml
Standard4	0.25ml Std1	0.75ml Eluent	1ml
Standard5	0.125ml Std1	0.875ml Eluent	1ml
Standard6	0.063ml Std1	0.937ml Eluent	1ml

Table 2.7 : Standard solutions of HPLC standards.

Standard Solutions	**Mixing Volumes**	**Eluent Volume**	**Final Volume**
Standard1	1ml Solution C	-	1ml
Standard2	0.75ml Std1	0.25ml Eluent	1ml
Standard3	0.50ml Std1	0.50ml Eluent	1ml
Standard4	0.25ml Std1	0.75ml Eluent	1ml
Standard5	0.125ml Std1	0.875ml Eluent	1ml
Standard6	0.063ml Std1	0.937ml Eluent	1ml

On Table 2.8; glucose, ethanol, glycerol and acetate amounts (g/L), and retention time (min) are indicated.

Table 2.8 : Concentrations and retention time of metabolites in HPLC standards. Retention times were given in ± 0.2 min range.

Metabolite(g/L)	**Glucose**	**Ethanol**	**Glycerol**	**Acetate**	**Maltose**
Standard 1	20	15	1	2	20
Standard 2	15	11.25	0.75	1.5	15
Standard 3	10	7.5	0.5	1	10
Standard 4	5	3.75	0.25	0.5	5
Standard 5	2.5	1.875	0.125	0.25	2.5
Standard 6	1.125	0.9375	0.0625	0.125	1.25
Retention Time (min)	8.43	19.95	12.5	13.81	6.95

2.1.3 Laboratory equipment, chemicals, kits/enzymes, solutions/buffers, programs/ databases, primers for PCR

Materials that were used for the experiments are listed according to their trademarks, and country of origin. Laboratory equipment, chemicals, solutions, kits, buffers, enzymes, computer programs and databases are indicated Tables 2.9-2.13 respectively.

Table 2.9 : Equipment used in the experiments.

UV-Visible Spectrophotometer	NanoDrop2000, Thermo Scientific (USA)
	Shimadzu UV-1700 (Japan)
	Bio-Rad Benchmark Plus™Microplate Reader Spectrometer (USA)
HPLC System	Shimadzu (Japan)
HPLC Controller	Shimadzu SCL 10A (Japan)
HPLC Liquid Chromatography	Shimadzu LC- 10AD (Japan)
HPLC Degasser	Shimadzu DGU-14A (Japan)
HPLC Column	Bio-rad HPX-87H Aminex ion-exclusion column, 300x7.8mm (USA)
HPLC Refractive Index Detector	Shimadzu RID-10A (Japan)
HPLC Auto Injector	Shimadzu SIL-10AD (Japan)
HPLC Column Oven	Shimadzu CTO-10AC (Japan)
Laminar Flow Cabinet	Faster BH-EN 2003 (Italy)
Rotary Shaker	Techne- Roller Blot Hybridizer HB-3D (UK)
Centrifuge	EppendorfMicrocentrifuge 5424 (Germany)
	Beckman Coulter Allegra™ 25R Centrifuge (USA)
Orbital Shaker	Sartorius-cermotat SII (Germany)
Thermomixer	EppendorfThermomixer Compact
Real-Time PCR	Roche Light-Cycler 480 (Switzerland)
Pipette	Eppendorf (Germany)
Water Bath	Julabo SW22 (Germany)
Thermal Cycler	BIO-RAD T100 Thermal Cycler (USA)
	TC-512 / TC-412/ TC-312 Techne (UK)
Bioanalyzer	Agilent 2100Bioanalyzer (USA)
Rotary Shaker	Techne- Roller Blot Hybridiser HB-3D, (UK)
Transilluminator	VilberLourmat
pH Meter	Mettler Toledo NP220 (Switzerland)
Autoclave	TOMY SX700E (Japan)
-80°C Refrigerator	Sanyo Ultra Low (Japan)
-20°C Refrigerator	Arçelik 2031D (Turkey)
Bio Analyzer 2100	Agilent Technologies, UK
+4°C Refrigerator	Arçelik 3061 Plus (Turkey)
Magnetic Stirrer	Magnetic StrirrerLabworld-Online (German)
Incubator (+80°C)	Nüve EN400 Incubator
Microscope	Olympus CH30 (USA)
Ultrasonicator	Transsonic TP690
Vortex mixer	Heidolph (Germany)
Gel electrophoresis	BioRad (UK)

Table 2.10 : Chemicals.

Peptone	MERCEK (Germany)
Yeast Extract	MERCEK (Germany)
D-Glucose	VWR BDH PROLABO (UK)
Glycerol	DuchefaBiochemie (Holland)
Sulphuric acid	Riedel-de Haen (Germany)
Yeast Nitrogen Base without Aminoacid	BD Difco™ (USA)
NaCl	Carlo Erba (Italy)
H_2O_2	MERCEK (Germany)
$CoCl_2$	Fluka (USA)
$NiCl_2.6H_2O$	MERCEK (Germany)
$CySO_4.5H_2O$	Fluka (USA)
$ZnCL_2.6H_2O$	Sigma ALDRICH (USA)
Lithium Acetate (LiAc)	Sigma ALDRICH (USA)
$MnSO_4H_2O$	Sigma ALDRICH (USA)
Ethanol	J. T. Baker (the Netherland)
$(NH_4)_2Fe(SO4)_2.6H_2O$	MERCEK (Germany)
$MnCl_2.4H_2O$	MERCEK (Germany)
$CaCl_2.2H_2O$	Carlo Erba (Italy)
Acetic acid	Riedel-de Haen (Germany)
Ammonium Acetate	Carlo Erba (Italy)

Table 2.11 : Kits and enzymes used in studies.

DNA Isolation Kit	Roche High Pure PCR Template Preparation Kit (Switzerland)
RNA Isolation Kit	Roche High Pure RNA Isolation Kit (Switzerland)
	Qiagen RNA Purification Kit
cDNA Synthesis Kit	Roche Transcriptor First Strand cDNA Synthesis Kit (Switzerland)
SYBR Green	Roche Light Cycler 480 SYBR Green I Master Mix (Switzerland)
SYBR Gold	Roche SYBR Gold Nucleic Acid Gel Stain (Switzerland)
Trehalase (from porcine kidney)	Sigma T8778-5UN (Germany)
A-Amyloglucosidase	Roche 3500U (Switzerland)
Glucose Oxidase/ peroxidase	Sigma (Germany)
o-Dianisidinedihydorochloride	Sigma (Germany)
Gluose Standard Solution	Sigma (Germany)
Amyloglycosidase (3500U)	Roche (Switzerland)
Master mix	GML Master Mix Kit

Table 2.12 : Solutions and buffers.

Solutions/Buffers	**Contents/Amounts**
TBE (electrophoresis buffer)	Tris (Hydroxymethyl) amino methane, boric acid, EDTA
PBS (Phosphate Buffer Saline)	pH 7.4 50mM
Agarose gel (%1.5)	1,5% agar in TBE
Glycerol (-80°CStrain Stock)	30%
Ethanol (Sterilization)	70%
HPLC Eluent (Mobile Phase)	0.5mM Hydrogen Sulphur
H_2O_2 Stock Solution	100mM stock solution
SYBR Gold Solution	2µl SYBR Gold in 50ml TBE

Table 2.13 : Programs/ databases and their functions.

Fast PCR	**Primer Design**
Microsoft Office Programs	Data and result order
Fast PCR	Primer design
2100 Expert	Program of Bioanalyser for RIN measurement
www.yeastgenome.org	Genome and gene data base of *S. cerevisiae*
Gene Spring	Microarray data analysis

Table 2.14 : Primers for q-PCR.

Primer name	**5' ➔ 3'**
***ACT1* forward**	CTTTCAACGTTCCAGCCTTC
***ACT1* reverse**	TCACCGGAATCCAAAACAAT
***OCA1* forward**	GAGGACGTTCCAGAGGATGA
***OCA1* Reverse**	TTCTTGTGGGGCATGTGATA
***MSN2* forward**	GAACGCAAATGCAGACTCAA
***MSN2* reverse**	AAAGAACTTGGGCAAGCAGA
***MSN4* forward**	GGTGATGGCAAATCCTCTGT
***MSN4* reverse**	AGCCGGAGATGACTTGAGAA

Table 2.15 : Primer sequences for PCR-based gene deletion. KANmx cassette sites are shown in bold, underlined and homologous recombination region in italics.

Primer name	**5' ➔ 3'**
***YAP1* forward**	*GTTTTTTGCCACCCAAAACGTTTAAAGAAGGAAAAGTT GTTTCTTAAACC*GGGTTAATTAAGGCGCGCCA
***YAP1* reverse**	*ATAGAAAAAGTTCTTTCGGTTACCCAGTTTTCCATAAAG TTCCCGCT*ATTATCATCGATGAATTCGAGCT
***YAP1* for verification**	TGTACCATTGAGACGAAGTG
***KanMX* forward for verification**	TAATGGCTGGCCTGTTGAAC
***KanMX* Reverse for verification**	GAGGCATAAATTCCGTCAGC

2.2 METHODS

2.2.1 Cultivation of cells

Cultures were incubated at 30°C and 150 rpm for all experiment in this study unless otherwise stated.

2.2.2 Mating of yeast

S. cerevisiae CEN.PK 113-1A (*Matα*,) was used as a wild type with opposite mating type (named as 934 throughout in this study). 934 and H7 strains were grown on

Yeast Extract Peptone Dextrose (YPD; yeast extract 1%, glucose 2%, peptone 1%) agar plate at 30°C overnight. These cultures were mated on YPD agar plate the next day. For this process, strains of equal volume were mixed on YPD solid medium. Diploid cells (zygotes) were monitored by the end of 4-6 h of incubation at 30°C under the light microscope. Fresh YPD solid medium was streaked with these diploid cells to select single colonies. For sporulation the pre-grown single colonies were inoculated on potassium acetate agar plate (anhydrous potassium acetate, 1% 'w/v') and grown for 24-48 h at 30°C. The cells were named as diploid by observing the presence of a tetrad under light microscope.

2.2.3 Deletion of *YAP1* on wild type and H7 strains

Deletion was carried out as described by Gietz and Woods (2002). First of all, deletion cassette for *yap1*::KAN_{MX} was constructed by PCR. Genomic DNA In a first set of experiment, *S.cerevisiae* CEN.PK *Mat a* and oxidative stress resistant mutant of this wild type strain was used as a recipient strain. *KanMX* marker was used as a cassette source. Special primers were designed nearly ~70bp long. Each oligonucleotide used contains two distinct regions, one (50bp part) which permits homologous recombination at the target locus. It is named the deleting sequence. The second part (20bp) allows the PCR amplification of selectable marker (Baudin A. 1993). The sequence of the primers were shown on the Table 2.15.

Using these targeting primers, antibiotic resistant cassette is amplified and resulting PCR product contains this *KanMX* sequence and 50bp of genomic DNA sequence to the left and right of the gene deletion of interest. This deletion cassette was purified with electrophoresis on agarose gel. Gel was stained with SYBR Gold and imaged under UV light. *yap1*::*KanMX* deletion cassette were used for transformation with Li-Ac method (Gietz and Woods 2002, Brachmann CB 1997).

This product is transformed into yeast. Firstly, strain was grown in 10 mL YPD in 50 ml test tube as a pre-culture at 150 rpm shaking speed and 30°C. Next day, cultures were inoculated with an initial OD_{600} of 0.3 to fresh YPD. When culture reached 1.6 optical densities at 600 nm, one ml of culture was transferred to 1.5 ml microfuge tube, centrifuged at 10'000 *g* for 2 min and supernatant was removed. Pellets were incubated with 300 µl (100 mM) lithium acetate buffer for 30 min at room

temperature. Cells were centrifuged at 10'000 *g* for 2 min and transformation mix was added to pellets. Transformation mix components were given on table 2.16. Cells were incubated at 30°C for 60 minutes. To open pores of cell wall (Gietz and Woods 2002), 42°C heat shock was applied for 20 min in water bath. Cells were then spread to Geniticine (G418) selective medium plate and incubated at 30°C for 2 days.

Table 2.16 : Li-Ac. transformation mixture.

Content	Concentration	Amount
Polyethylene glycol	50 % 'v/v'	90 µl
Lithium acetate	1 M	15 µl
ss carrier DNA	2 mg/ml	4 µl
Deletion cassette		5 µl
H_2O	To 130 µl	

Transformants include those cells in which two crossover events occurred, one between each end of the 50bp target sequence of PCR product and the corresponding genomic DNA sequence, thereby replacing the gene of interest with the cassette (Brachmann CB 1997). Transformant colonies for wild type and 2 transformant colonies for mutant was observed on solid YPD medium all transformant for wild type and 15 transformant was scanned and their DNAs were isolated using High Pure PCR Template Preparation Kit (Roche) and analyzed by PCR. Verification PCR was carried out to prove deletion presence. For verification, three primers were used. One of them binds to upstress of deleted site. Others complement inside *KanMX* maker sequence.

Deletion cassette construction, transformation, homologous recombination and verification steps are shown in Figure 2.1.

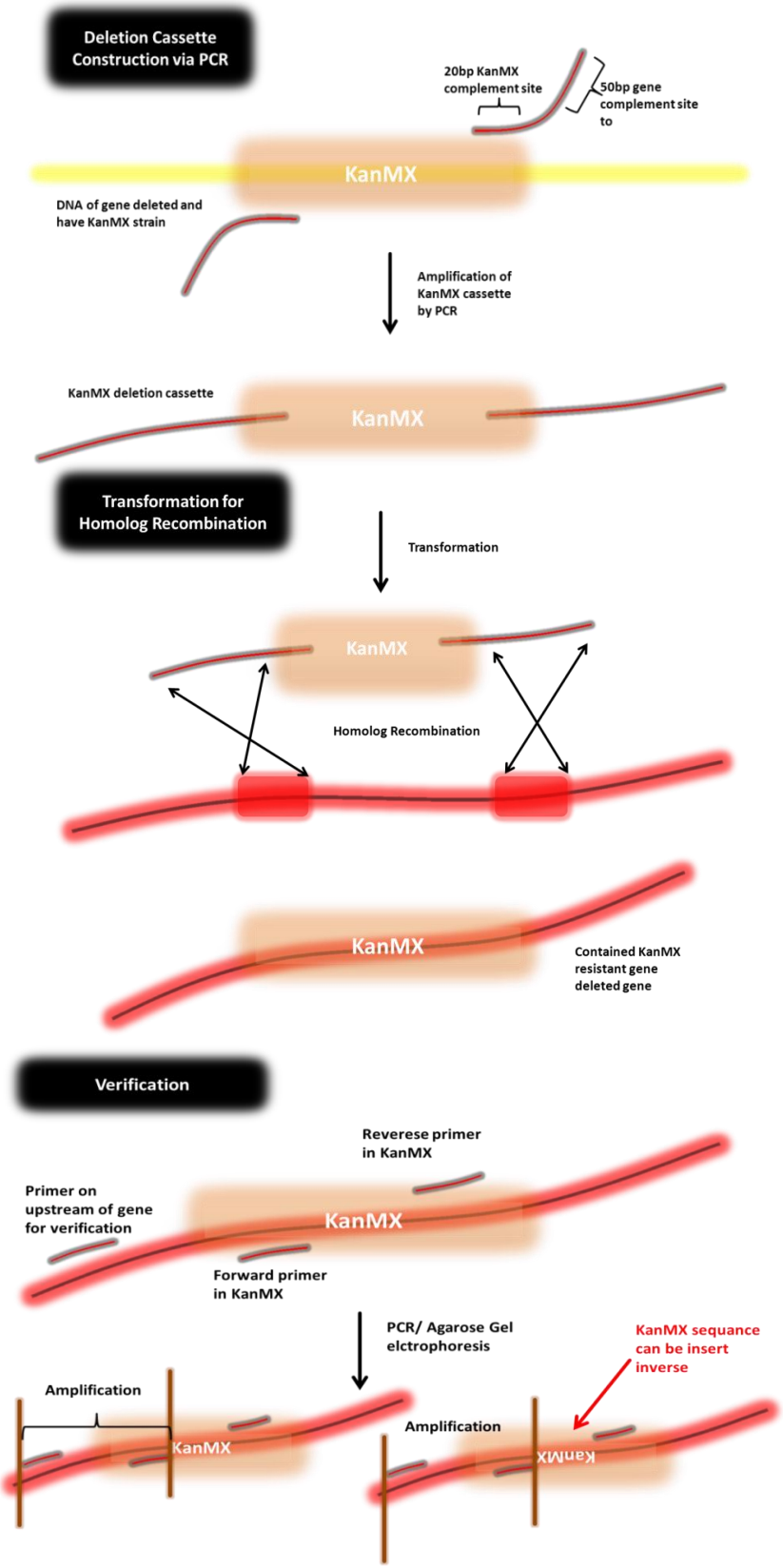

Figure 2.1 : Deletion cassette construction, insertion of this cassette into the organism of interest selection of correct transformants.

Deletion verification PCR contents and program are shown on Tables 2.17 and 2.18, respectively.

Table 2.17 : Verification PCR contents. Three primers were used. Two of them bind into *KanMX* site, one of them bind back of deleted region.

Content	Amount
i-taq	0.5 µl
Buffer (with $MgCl_2$)	2 µl
Template	2 µl
$MgCl_2$	1 µl
Glycerol (30%)	5 µl
Primer	1 µl + 1 µl +1 µl
dNTP	2 µl
Water is added up to final volume 20µl	

Table 2.18 : PCR program for deletion verification.

Initial denaturation	94°C 2min	1 cycle
Denaturation	94°C 20 sec	35 cycle
Annealing	50°C 20sec	
Extension	72°C 1.5 min	
Final extension	72°C 5 min	1 cycle

Finally, wild type *yap1::KanMX* and H7 *yap1::KanMX* strains were spotted to YMM medium containing different concentrations of hydrogen peroxide to observe effect of *YAP1* deletion on the strains.

2.2.4 Phenotype test for *S. cerevisiae* w/t strain, oxidative stress-resistant mutant 'H7' and diploid cells

To observe growth of wild type, H7 and diploid of oxidative stress mutant and wild type were spotted to YMM supplemented with different chemical as spot assay.

2.2.4.1 Determination of effect of calcium on growth of w/t and H7 by spot assay

Pre-cultures of w/t and H7 were incubated overnight at 150 rpm and 30°C. Next day, cultures were inoculated at an initial OD_{600} of 0.3 to 10 ml medium in 50 ml test tube. When cultures reached OD_{600} of 3, 4 OD_{600} unities of cells were harvested with centrifuge (at 5'000 *g* and 3min) and suspended in 50 µl water. Cultures were serially diluted (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}) and each dilution was spotted on YMM. Each plate was supplemented with calcium, hydrogen peroxide or both calcium and hydrogen peroxide with different concentrations of them (100mM and 500mM

$CaCl_2$, and 0.5, 0.75, 1 mM H_2O_2). Plates were incubated at 30°C for 24 h and visualized.

2.2.4.2 Spot assay of diploid cell (2n, 934 x H7)

Firstly, w/t *Matα* (934), H7 and 2n (934xH7) cells were incubated in 10 ml YPD overnight as pre-culture. Pre-cultures were inoculated with an initial OD_{600} of 0.5 and when they reached 2.5 OD_{600}, they were harvested with micro centrifuge at 10'000*g* and 5 min. Cells were concentrated to 4 OD unities of cells and 50 µl water was added to the pellets. Cultures were serially diluted (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}) and each dilution was spotted on YMM agar plate (% 0.67 'w/v' yeast nitrogen base without amino acids, agar %2 'w/v' and D-glucose %2 'w/v'). Besides, hydrogen peroxide (0.5, 0.75, 1, 1.5 mM H_2O_2), sodium chloride (0.5, 0.75, 1M) and 1mM glutamic acid were added to plates to observe growth of diploid cells and their corresponding parental strains. Plates were incubated at 30°C for 48 h and photographed.

2.2.4.3 Spot assay of wild type, wild type *yap1::KanMX*, H7, H7 *yap1::KanMX*

To observe growth of deletion strains, they were spotted on YMM plate. Pre-culture and spot test were carried out as explained in Section 1.2.4.2.

2.2.5 Physiological analysis for wild type and oxidative stress resistant mutant

Pre-culture of wild type and H7 were inoculated in 50 ml YMM at 30ºC and 200 rpm stirring speed overnight. Cultures were started at 0.3 OD_{600} with 400 ml culture volume in YMM and YMM containing 0.5 mM hydrogen peroxide (H_2O_2) in 2L flask. Cultures were incubated at 30ºC and 150 rpm shaking speed during 30 h. Samples were harvested at every 1.5 h for OD_{600} measurements, cell dry weight determination and metabolite analysis (glucose, ethanol, glycerol, acetate, and trehalose/glycogen content) by HPLC and enzymatic methods, respectively.

2.2.5.1 Monitoring OD_{600} for growth curve

While obtaining the growth curve of wild type and mutant, samples were taken to measure OD_{600} every 1.5 h of batch cultivation.

2.2.5.2 Metabolite analysis by HPLC

One ml sample was collected by centrifugation. Supernatants were filtered through a 0.2 µm pore-size membranes. Ethanol, acetate, glycerol, maltose and residual glucose were determined by HPLC using an HPX-87H Aminex ion-exclusion column (300 x 7.8 mm; Bio-rad, USA) at 60°C. The column was eluted with 5mM sulphuric acid at a flow rate of 0.6 ml/min, using a Shimadzu RID-10A refractive-index detector. Three samples were harvested for each measurement to verify the accuracy of this experiment.

2.2.5.3 Trehalose and glycogen content determination by enzymatic reaction

Trehalose and glycogen content were determined according to the Parrou and Francois method (1997). 25 optical density unities of cells were collected and centrifuged at 10'000 *g* speed for 5 min (Eppendorf centrifuge, Germany). Supernatant was thrown away and pellet was stored at -20ºC. 250µl 0.25 M sodium carbonate was added to the pellets and cells were placed to 95°C for 4 h. Cell suspensions were then adjusted to pH 5.2 with 1 M 150µl acetic acid and 0.2 M 600 µl sodium acetate buffer (pH 5.2). Subsequently, 500µl of the samples were transferred to new tubes for trehalose determination and the other 500µl was used for glycogen analysis.

For trehalose determination, 10µl trehalase (Sigma, T8778-5UN, and Germany) was pipetted into 500 µl cell suspensions and the tubes were placed to 37°C for overnight incubation without shaking.

For glycogen determination 20µl amylo-glycosidase (solubilized in 0.2M sodium acetate pH5.2) (Roche, 3500U, Switzerland) was added to the other 500 µl of cell suspension. The tubes were incubated at 57°C in a rotary shaker (Techne- Roller Blot Hybridiser HB-3D, UK).

For both trehalose and glycogen determinations, glucose standards were prepared. 20 µl of standards and samples were pipetted to different wells of 96-well plate. Then 200 µl of glucose oxidase/peroxidase reagent was added onto samples in wells. The glucose released was determined using the glucose oxidase/peroxidase method

(Cramp 1967). Following sample incubation for 30 min and 37°C, absorbance of the samples was measured at 490 nm by microplate reader.

2.2.5.4 Cell dry weight (CDW) measurement

Firstly, microfuge tubes were weighed for 2 days of incubation at 80ºC. During batch growth of the cultures, 2 ml sample was taken and centrifuged at 14'000 *g* for 5 min. Supernatant was thrown away. Subsequently microfuge tubes containing the pellets were dried at 80°C for 48 h and placed in a desiccator for 30 min. Finally tubes were weighted again and compared with first weight and the cell dry weight was calculated as mg per ml of cell culture. Experiment was carried out triplicate for each measurement.

2.2.6 Expression level determination of *OCA1*, *MSN2* and *MSN4* genes for wild type and oxidative stress-resistant mutant 'H7' by quantitative PCR

Stock cultures from plates were inoculated into 10 ml of YMM and incubated at 30°C on an orbital shaker (Sartorius- cermotat SII, Germany) with 150 rpm agitation. After overnight growth, their OD_{600} values were measured and they were inoculated into 500 ml flasks in 100 ml culture volume by adjusting their OD_{600} to 0.2 (2-4 x 10^6 cells per ml). After incubation, wild-type and mutant cells growing exponentially in YMM were collected at their OD_{600} of 1.0 (1.4 x 10^7 cells per ml), and total RNA was extracted by using the High Pure RNA Isolation Kit (Roche, Switzerland). Then, 0.5mM H_2O_2 stress was added to the rest of the culture and the flask was placed again to the incubator for 90 min at 150 rpm and 30°C. At the end of this incubation, cultures were harvested by setting cell amount as before. To measure total RNA amount in samples Nano Drop 2000 (Thermo Scientific, USA) instrument was used. cDNA was synthesized from wild-type and H7 samples (1 µg of total RNA) by using the Transcriptor First Strand cDNA Synthesis Kit (Roche, Switzerland) with Anchored-oligo $(dT)_{18}$ Primer. cDNA amounts were measured again with Nano Drop 2000. They were used for further analysis by real-time PCR assays. The quantitative-PCR (qPCR) run was performed on a Light Cycler 480 Equipment (Roche, Switzerland) in a 96-well format with a reaction volume of 20 µl with Light Cycler 480 SYBR Green I Master Kit (Roche, Switzerland). Real-time PCR data was presented as individual data points via $2^{-\Delta\Delta CT}$ calculation which is

referred to as delta CT method. ΔCT values were calculated by subtracting the internal control CT value from the gene of interest CT value (Schmittgen and Livak 2008)). First of all, threshold cycle (CT) values were determined for both stress treated and untreated samples, for each gene including the reference gene. Subsequently, ΔCT values were calculated and by assuming the efficiency was 2 for all PCR reactions, $2^{-\Delta\Delta CT}$ values were determined. Throughout these studies, *ACT1* was used as the internal control (hausekeeping) gene.

2.2.6.1 Design of primers

Primers were designed using Fast-PCR program. 171bp, 162, and 160bp were chosen for amplification of *OCA1, MSN2 and MSN4* genes, respectively.

2.2.6.2 RNA extraction of wild type and H7

mRNA extraction was carried out with High Pure RNA Isolation Kit (Roche).

5 OD_{600} unit cells were used for mRNA extraction and harvested at 2000 xg for 5 min in a table top centrifuge. Pellet was suspended in 200 µl PBS (phosphate saline buffer). 10 µl 0,5 mg/ml lyticase (Sigma) was added and incubated for 15 min at 30°C. 400 µl Lysis/ Binding Buffer was added and vortexed for 15 s. High Filter Tube was inserted in one Collection Tube and the liquid sample into the upper buffer reservoir of the Filter Tube. Tube was centrifuged for 15 s at 8'000 *g*. 90 µl DNase Incubation Buffer and 10 µl DNase l (per sample) were mixed in one RNase free micro centrifuge tube. 100 µl mixtures were then added to samples and incubated for 15 min at room temperature. 500 µl Wash Buffer I was added to upper reservoir of the filter tube and then tube was centrifuged for 15s at 8'000 *g*. 500 µl Wash Buffer II was added to upper reservoir of the filter tube and then tube was centrifuged for 15s at 8'000 *g*. 200 µl Wash Buffer I was added to upper reservoir of the filter tube and then tube was centrifuged for 2 minutes at maximum speed to remove residual Wash Buffer. Filter tube was placed in a 1.5 µl micro centrifuge tube. 100 µl Elution Buffer was added to upper reservoir of the filter tube and then tube was centrifuged for 15 s at 8'000 *g*.

Eluted RNA in microcentrifuge tube was measured with nanodrop spectrophotometer and was then stored at -80°C.

2.2.6.3 cDNA synthesis of total RNA extracted from wild type and H7

cDNA synthesis was carried out with Roche High Fidelity cDNA extraction kit (Cat No: 04 707 516 001). The protocol is given below:

RNA amounts were measured with Nano Drop 2000 (Thermo SCIENTIFIC, USA) instrument. All of the initial RNA amount was adjusted according to the sample with the least RNA content. Experiment was carried on ice and on nuclease free environment. 1 µl (2.5 µM) Anchored-oligo $(dT)_{18}$ Primer and injection water (water amount is calculated to final amount of 11.4µl) were added to total RNA. This mixture was incubated for 10 min at 65^{o}C (in order to denature secondary structure of RNA), after which the tubes were immediately cooled cool on ice. Master mixture was prepared with this order (amount were given per test tube); 4µl Transcriptor High Fidelity Reverse Transcriptase Reaction Buffer 5X concentration (final concentration 1X), 0.5 µl Protector RNase Inhibitor 40 U/µl (final concentration 20U), 2µl Deoxynucleotide Mix (final concentration 1mM), 1µl DTT (final concentration 5mM), 1.1µl Transcriptor High Fidelity Reverse Transcriptase (10 U/µl) were added to this tube. The mixture was incubated in thermal cycler TC 412 (Techne, USA) firstly for 30 min at 50^{o}C and then for 5 min at 85^{o}C. The reaction was then stopped on ice. Finally cDNA amounts were measured using Nano Drop 2000 and these tubes were stored at -20^{o}C.

2.2.6.4 Real time PCR

We used SYBR green kit (Cat No: 05 091 284 001) was used for quantitative PCR. The protocol is given below:

For standard curve, wild type (without stress), its dilutions (1:10, 1:100, 1:1000) and the other samples' dilutions (1:10) were prepared. Real Time Mixtures were prepared for all three dilutions (Table 2.19).

Table 2.19 : Component of Real Time PCR mixture.

Component	Amount	Concentration
Primer (Forward)	1µl	10 µM
Primer (Reverse)	1µl	10 µM
cDNA	8µl	
SYBR Green	10µl	

12 µl master mixtures (Primer Forward+ Primer Reverse+ SYBR Green) per test sample were added to Real Time PCR plate wells and 8 µl cDNA was added to PCR plate in dark condition. Experiment was performed three times.

Roche Light Cycler 480 was used as thermal cycler (Roche, Switzerland). PCR program is shown in the Table 1.20.

Table 2.20 : Light Cycler program.

Denaturation	95^0C 10 sec	1 cycle
Amplification	95^0C 10 sec	45 cycle
	55^0C 18 sec	
	72^0C 20 sec	
Melting	95^0C 5 sec	1 cycle
	65^0C 1 min	
	97^0C continuous	
Denaturation	40^0C 1 min	1 cycle

2.2.7 Microarray analysis of wild type and oxidative stress resistant mutant

2.2.7.1 RNA extraction of wild type and mutant

Total RNA of wild type and mutant were extracted with RNeasy Mini kit (QIAGEN). The protocol was carried out according to the suppliers' instructions.

First of all, wild type and mutant (H7) were incubated in 20 ml YMM in 100 ml flask at 30^0C 150 rpm overnight as preculture. Next morning, strains were inoculated to 100 ml YMM in 500 ml flask as nearly 0.1 initial OD_{600}. Culture was grown at 30^0C 150 rpm until reach it to 1 OD_{600} ($5x10^7$ cells/ml). Extraction was then carried out by RNeasy Mini kit (QIAGEN). To disrupt cell wall of yeast, enzymatic lysis method was used with lyticase enzyme (Sigma). One ml cell whose OD_{600} was ~1, were harvested with centrifugation at 1'000 *g* for 5 min at room temperature. Supernatant was removed and pellet was resuspened in 1.5 ml freshly prepared Y1 (contents of Y1 given on Table 2.21) containing lyticase. Cells were incubated for 30 min at 30°C with shaking to disrupt cell wall. Spheroplast was centrifuged for 5 min at 300 *g* and the supernatant was removed. 350 µl Buffer RLT was added and vortexed to lyse spheroplast. 350µl 70% ethanol were added to homogenized lysate and mixed by pipetting.

Lysate was transferred to an RNeasy spin column in 2 ml collection tube and centrifuged for 15 seconds at 8'000 *g*. 700 µl Buffer RW1 added to spin column and centrifuged for 15 seconds at 8'000 *g* to wash the spin column membrane. 500 µl Buffer RPE was added to spin column and centrifuged for 15 seconds at 8'000 *g* to re-wash the column membrane. Last stage was repeated once more but centrifugation was applied for 2 min. Spin column was placed to a new collector tube and centrifuged at full speed (15'000 *g*) for 1 min. Finally, spin column was placed to a new nuclease-free 1.5 ml microfuge tube, 40µl RNase free water was added onto it and centrifuged for 1 min at 8'000 g.

Table 2.21 : Qiagen RNA Extraction Kit buffer preparation contents.

Buffer Name	Content (amount)
RLT Buffer	10 µl β-mercaptoethanol per 1 ml RLT buffer was added
Y1 Buffer	1M sorbitol and 0.1M EDTA is prepared and adjusted pH7.4, just before use 0.1% β-mercaptoethanol and lyticase were added.
RPE Buffer	4 volumes of ethanol was added to RPE buffer

2.2.7.2 RNA quality and quantity measurement by BioAnalyzer and NanoDrop

Purified RNA was measured by Nanodrop 2000 (ThermoScientific) and analyzed by BioAnalyzer 2100 (Agilent technologies). Nanodrop measurements gave concentrations of RNA samples in terms of µg/µl. Moreover 260/280 and 230/280 absorbance ratios were determined by Nanodrop. BioAnalyzer 2100 results gave information about RNA quality.

2.2.7.3 Microarray Experiment

The general procedure for microarray experiments is shown schematically in Figure 2.2. The whole experiment could be divided to 3 steps. cRNA construction and amplification steps were constituted the first step of the experiment. Following the first step, hybridization of the samples to Microarrays was carried out (Agilent yeast microarrays). Slides were washed to prepare them for the final step. At the final step, the microarrays were scanned and signals was detected by the Scanner (Agilent technologies). Finally, data were evaluated by Genespring program.

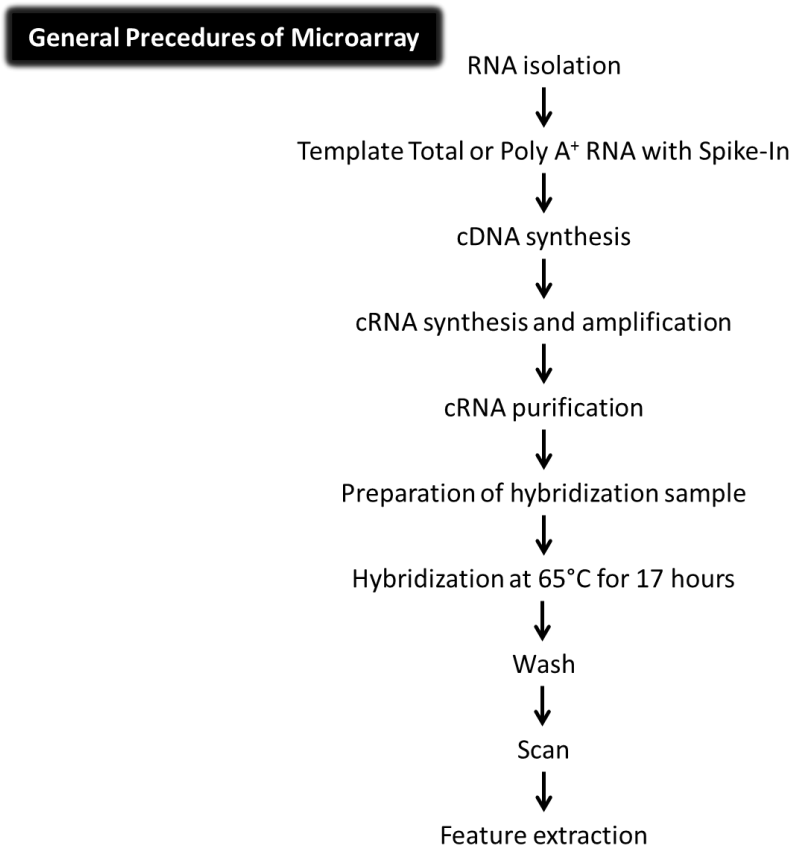

Figure 2.2 : Flowchart of microarray processing.

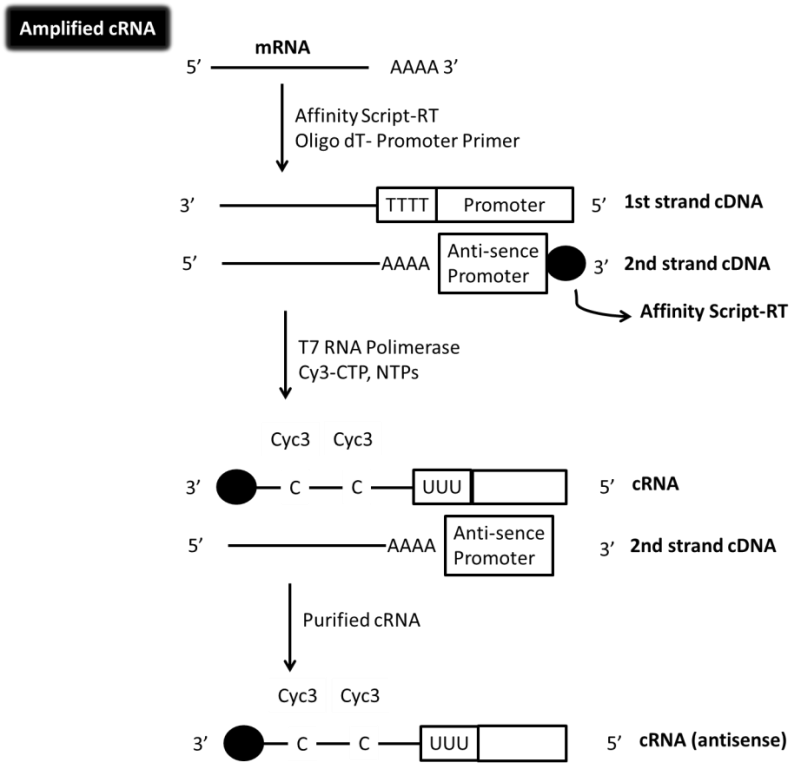

Figure 2.3 : Amplified cRNA and labeling.

3. RESULTS and DISCUSSIONS

3.1 Phenotype test for *S.cerevisiae* wild type, oxidative stress resistant mutant and diploid cells

In this study, growth of wild type (abbreviated as 905), oxidative stress resistant mutant 'H7' and diploid cells was investigated on YNB supplemented with some chemicals and different carbon sources.

3.1.1 Determination of growth of wild type and oxidative stress resistant mutant

Growth of wild type and oxidative stress resistant mutant, 'H7' was investigated in the presence of hydrogen peroxide by treating different concentration of hydrogen peroxide to yeast minimal medium. One and 2 mM hydrogen peroxide concentrations were applied to strains. Control medium, which did not contain hydrogen peroxide, did not affect of growth of both wild type and mutant. Superiority of growth of H7 against wild type appeared in H_2O_2 containing media (Figure 3.1).

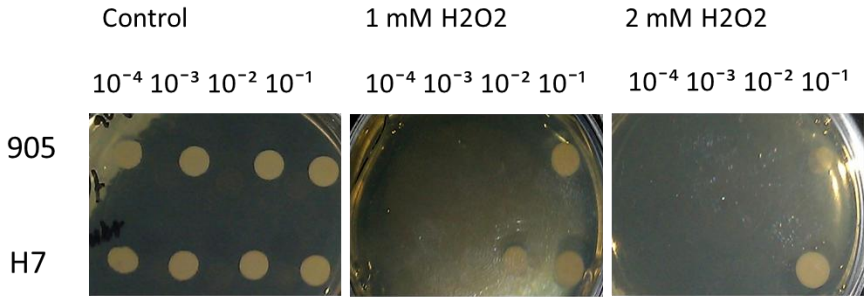

Figure 3.1 : Serial dilutions of wild type and H7 were spotted to minimal medium supplemented with hydrogen peroxide.

3.1.2 Spot assay to observe growth of w/t and H7 in different carbon source

Fermentable (glucose) and non-fermentable carbon sources (ethanol, acetate and glycerol) were supplemented to YNB as solid medium. Besides, the experiments were carried out both in the presence and absence of stress conditions to observe the effects of hydrogen peroxide on growth. 1mM H_2O_2 was applied as stress agent (Figure 3.2). When ethanol was used in medium as the carbon source instead of

glucose, growth of 905 and H7 were less than the growth in medium supplemented with glucose. Besides, hydrogen peroxide prevented the growth of the both strains. However, 905 and H7 growth did not show significant differences (b). Since glycerol was used as carbon source, results were observed like glucose medium assay. H7 was observed to grow more than the wild type in medium with 1mM hydrogen peroxide. (c). When used as a carbon source, acetate limited the growth of strains. When H_2O_2 was added to the medium, strains did not grow (d).

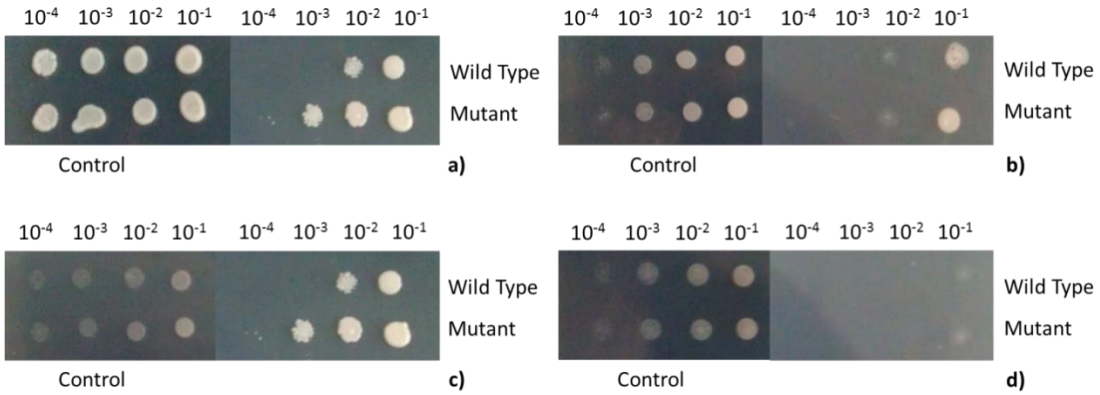

Figure 3.2 : Serial dilutions of wild type and H7 were spotted to glucose (a), ethanol (b), glycerol (c), acetate (d) supplemented YNB solid medium. Photographs of the plates after incubation for 48h at 30°C were shown.

3.1.3 Determination of calcium effect on H7 and w/t growth by spot assay

Budding yeast (*Saccharomyces cerevisiae*) cells use Ca^{2+} as a second messenger when they are exposed to various environmental stress conditions, such as hypotonic and cold stress, hyperosmotic and salt stress, β-phenylethylamine-induced intracellular H_2O_2 generation, or high pH (Popa C.V., 2010). Thus growth physiology of 905 and H7 on calcium supplemented minimal medium was investigated. 10 mM, 100 mM, 500 mM and 1M $CaCl_2$ were used as calcium supplementation and 1mM hydrogen peroxide was used as stress factor. 10 mM and 500 mM calcium addition did not inhibit the growth of the wild type and H7 (Figure 3.3). Besides, these concentrations of calcium help w/t and H7 to survive in 1mM hydrogen peroxide.

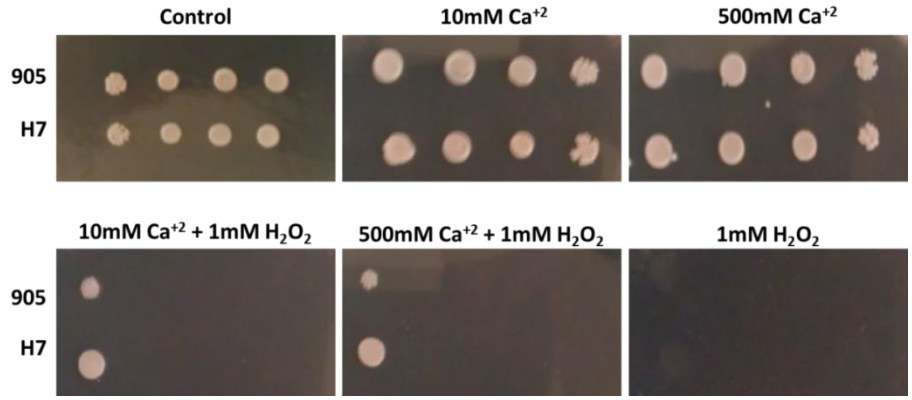

Figure 3.3 : Images of the plates after incubation at 30°C for 60 h in spot assay.

3.1.4 Phenotype analysis of diploid cell (2n, 934xH7)

Diploid cells were formed by mating 934 and H7. Light microscopic images of diploid cell are shown in Figure 2.4. It was observed that H7 mutation/mutations have semi-dominance to their corresponding gene in wild type. Furthermore, H7 showed cross-tolerance to NaCl stress. Additionally, glutamic acid supplementation did not compensate for NaCl stress effect, neither for wild type nor H7 (Figure 3.5)

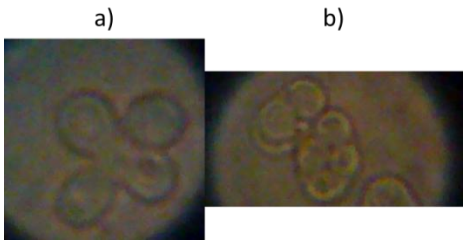

Figure 3.4 : Microscopic image of diploid cell formed via mating H7 and 934. **a)** Diploid form **b)** Tetrad form created with potassium acetate supplemented poor medium.

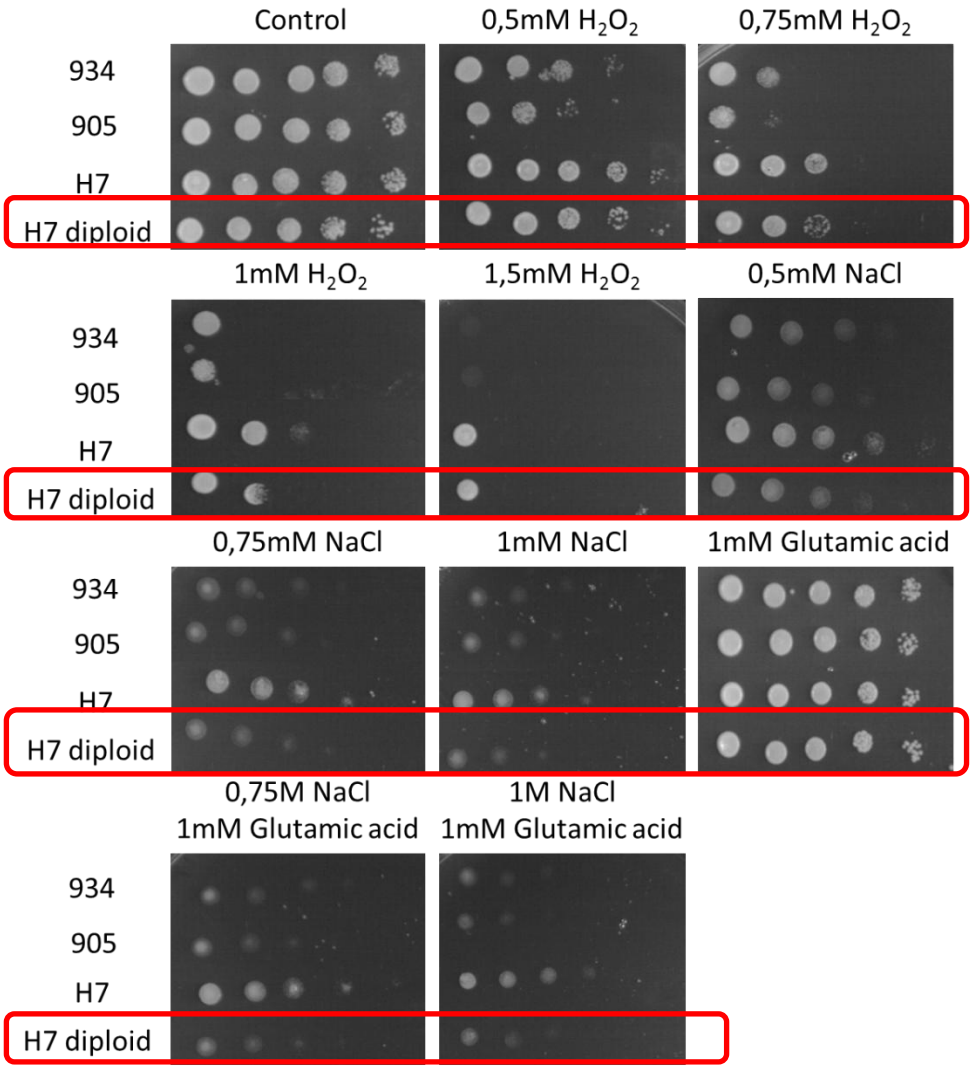

Figure 3.5 : 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4},10^{-5} diluted and spotted cells on YMM supplemented with hydrogen peroxide, sodium chloride and glutamic acid.

3.1.5 *YAP1* deletion in wild type and H7 strains

3.1.5.1 Amplification of *yap1*deletion cassette and transformation

PCR-based gene deletion method was used to obtain *yap1* strains. First of all, *yap1*::*KanMX* cassette was amplified from w/t *zrc1*::*KanMX* genomic DNA. This deletion cassette was transformed to both w/t and H7 as described in Section 2.2.3. *yap1*::*KanMX* cassette is shown in Figure 3.6.

2150bp of *YAP1* sequence was aimed to delete. KanMX has 1483bp to replace to *YAP1* ORF.

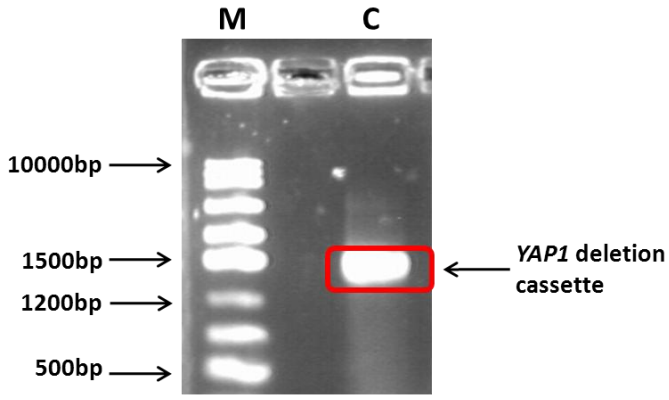

Figure 3.6 : *YAP1* deletion cassette.

3.1.5.2 Verification of *YAP1* deletion

The transformants which appeared on geniticine-containing YPD medium were transferred to another fresh medium and incubated at 30°C. DNA isolation was performed from them and a verification PCR was carried out. The true transformants with *YAP1* deletion gave a ~1200 bp PCR product (Figure 3.7and figure 3.8).

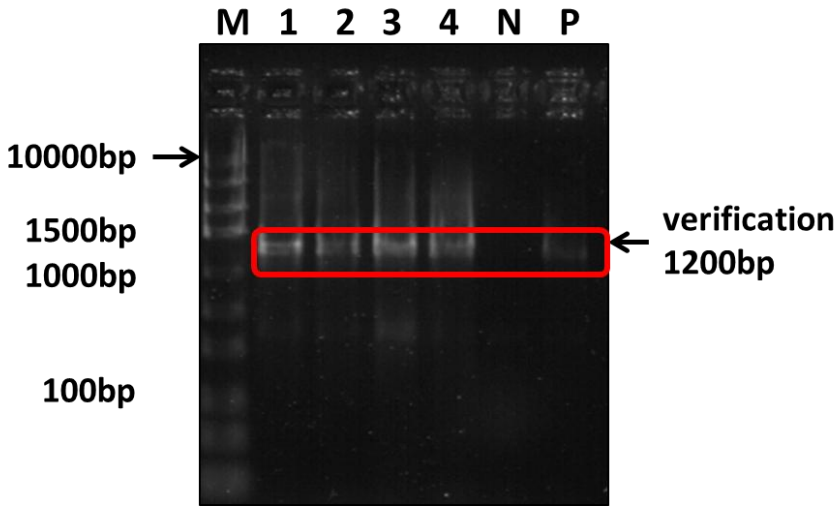

Figure 3.7 : PCR product (enclosed with a rectangle) correct size for the true *YAP1* deleted wild type. M: marker, 1,2,3,4: Verified *YAP1* deleted wild type strains, N: negative control, P: positive control.

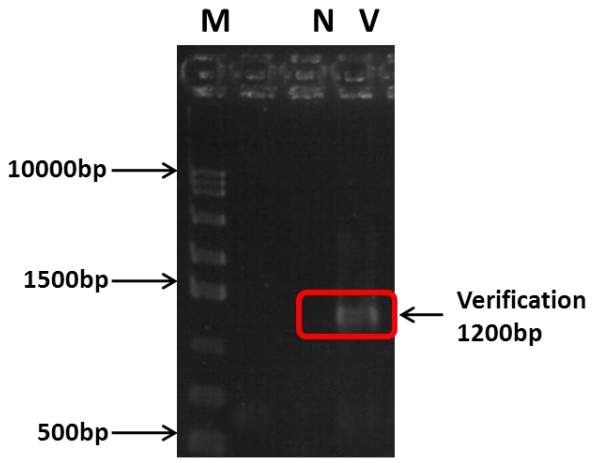

Figure 3.8 : PCR product (enclosed with a rectangle) correct size for the true *YAP1* deleted mutant. M: marker, N: negative control, V: verified Yap1 deleted mutant.

3.1.5.3 Phenotype of *YAP1* deleted strains upon H_2O_2 treatment

W/t, w/t Δ*yap1*, H7, H7Δ*yap1* and diploid cells were spotted onto YMM plate at different hydrogen peroxide concentrations (Figure 3.9). The cultures were also tested in maltose and H_2O_2 containing solid media as well(Figure 3.10).

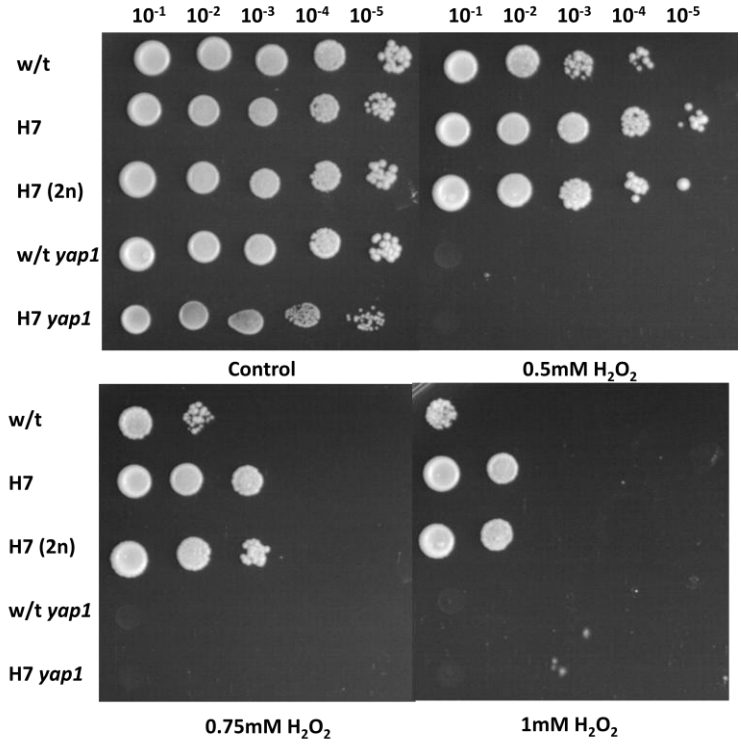

Figure 3.9 : w/t, w/t *yap1*, *H7*, H7 *yap1* and diploid in the presence and absence of H_2O_2.

Deletion of *YAP1* generesulted in complete loss of H_2O_2 resistance in both wild type and H7. It can be concluded that H7 H_2O_2 resistance phenotype depended on *YAP1* gene either directly or indirectly as a regulator gene.

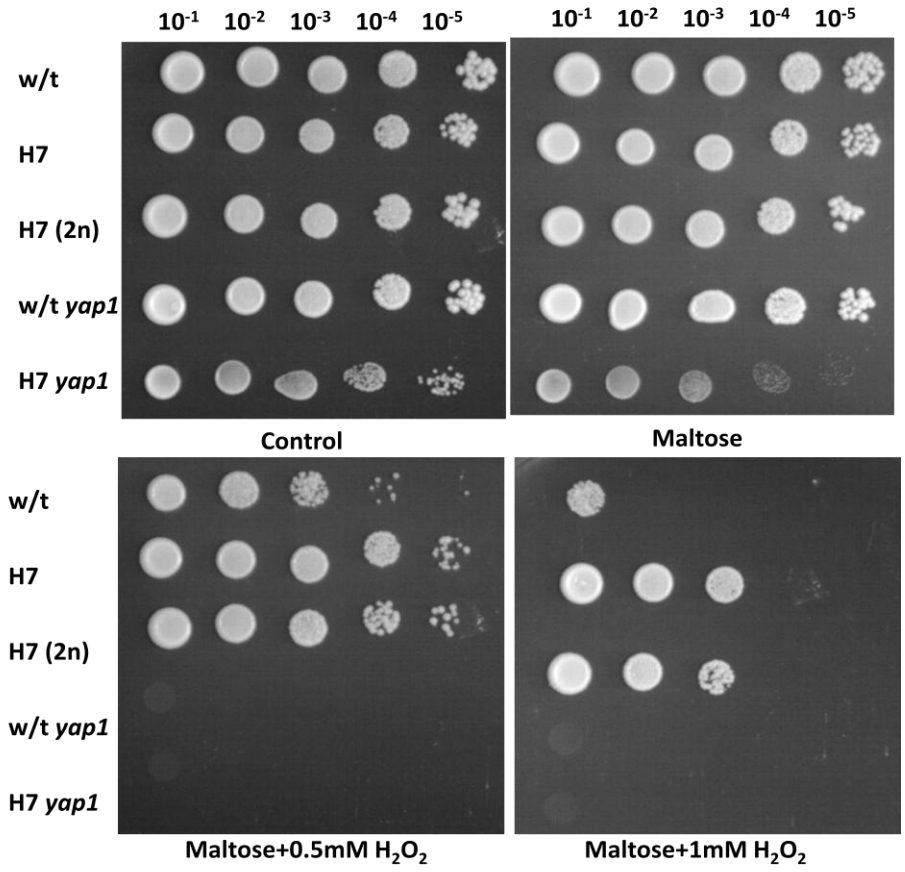

Figure 3.10 : Cultures incubated in minimal medium supplemented with glucose/maltose and H_2O_2.

It was shown that *YAP1* deletion impeded the growth of wild type and H7 in synthetic medium supplemented with maltose and H_2O_2 (Figıre 3.9).

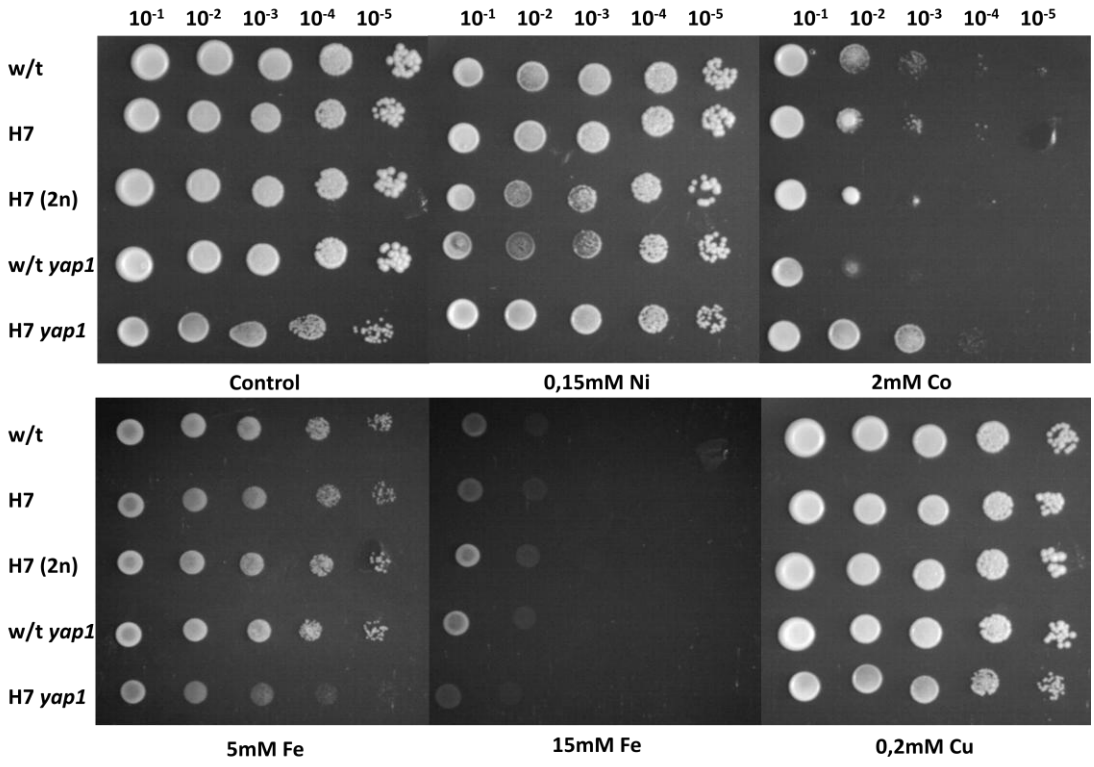

Figure 3.11 : w/t, w/t *yap1*, *H7*, H7 *yap1* and diploid in the presence of different metal stress.

Upon incubation of w/t, w/t *yap1*, *H7*, H7 *yap1* and diploid cells in medium supplemented with cobalt, it was demonstrated that *YAP1* deletion confers cobalt tolerance in H7 cells. However, *YAP1* deletion caused sensitivity of iron in H7 cells (Figure 3.11).

3.2 Physiological analysis for wild type and oxidative stress resistant mutant

As physiological analysis, HPLC and trehalose and glycogen determination experiments were performed. Besides, for each experiment, growth curve was created and cell dry weights were plotted. However, before this experiment, to determine the stress level concentration at which wild type and H7 could survive, growth curve experiment was performed for both wild type and mutant at different H_2O_2 concentrations.

3.2.1 Monitoring OD_{600} of different concentration H_2O_2 treated w/t and mutant

Growth of wild type and mutant were monitored by measuring optical density to determine appropriate concentration of H_2O_2 application for HPLC and trehalose-

glycogen experiments. 0.25 mM, 0.5 mM, 1 mM, 2 mM, 4 mM H_2O_2 were applied to cultures. Cultures were started with nearly 0.3 OD_{600} in 100ml flasks containing 20ml YMM and growth was followed by optical density measurements during 11 h. Both strains survived at 0.5 mM hydrogen peroxide, and could tolerate this concentration (Figure 3.12 – 3.13). Growth rates in 1mM, 2mM and 4mM H_2O_2-containing YMM at shown Table3.1. It can be understand that at 1mM hydrogen peroxide wild type could grow slowly. However, 2 mM and 4 mM hydrogen peroxide prevented growth of both strains. Oxidative stress resistant mutant could survive these concentrations of hydrogen peroxide. Subsequently, 0.5mM and 1mM hydrogen peroxide treatment was chosen for the further experiments.

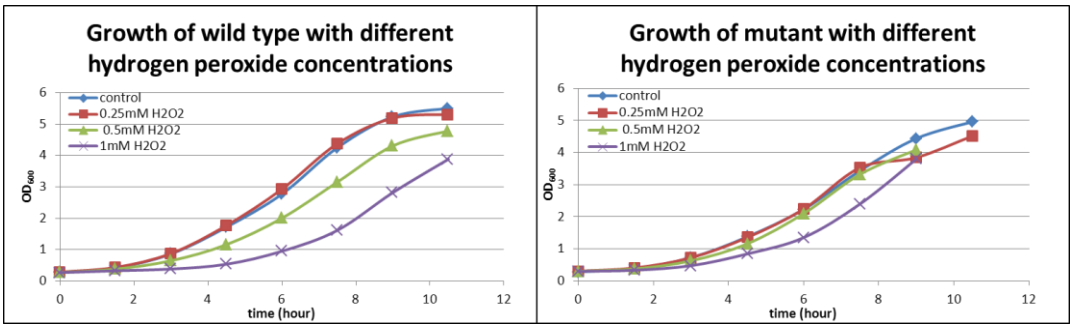

Figure 3.12 : OD_{600} measurements for wild type and mutant. YMM with 0.25mM, 0.5mM and 1mM H_2O_2 were used as different stress conditions.

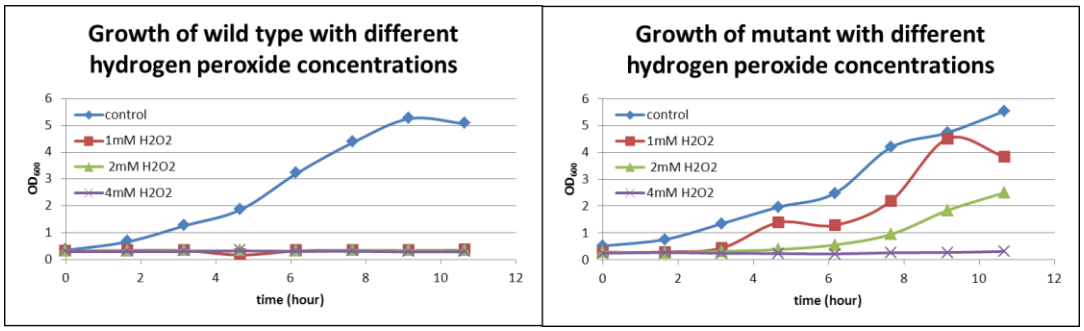

Figure 3.13 : OD_{600} measurements for wild type and mutant. YMM with 1mM, 2mM and 4mM H_2O_2 were used as different stress conditions.

Table 3.1 : Specific growth rate of wild type and H7in the absence and presence of hydrogen peroxide.

Strain (H_2O_2 concentration)	**μ stress/μ control**
w/t (1mM H_2O_2)	0.38
w/t (2mM H_2O_2)	0.04
w/t (4mM H_2O_2)	0.05
mutant (1mM H_2O_2)	1.18
mutant (2mM H_2O_2)	0.83
mutant (4mM H_2O_2)	0.24

3.2.2 Metabolite analysis by HPLC

Growth of w/t and mutant was observed, samples for HPLC and CDW were collected from cultures incubated in the presence and absence of hydrogen peroxide (0.5mM and 1mM). H7 grew more than the wild-type in both cases at the end of 30 h of incubation at 30ºC and 150 rpm shaking speed. Optical density measurements were carried out in triplicate. Average and standard deviation data ware shown Tables 3.2, 3.3 and 3.4. When hydrogen peroxide was present in the medium (0.5mM and 1mM final concentration), it was observed that wild-type grew a little less than the control condition (Figure 3.14 and 3.15). It is interesting that at the end of the 48h, for each strain 1 mM hydrogen peroxide provided much more growth, their OD_{600} values were higher than other cultivation conditions. ln values of OD_{600} were calculated (Table 3.3 and 3.4). Which provided growth information when cells were in log phase (Table 3.5). According to Table 3.5, growth rates of both w/t and H7 were nearly the same in YMM supplemented with 0.5mM H_2O_2. All values were very close because of low hydrogen peroxide concentration (Figure 3.16 and 3.17).

Table 3.2 : Average and standard deviation calculations of OD_{600} values of wild type in the absence and presence of 0.5mM and 1mM hydrogen peroxide.

	Control		**0.5mM H_2O_2**		**1mM H_2O_2**	
Time (h)	**AVG**	**STD**	**AVG**	**STD**	**AVG**	**STD**
0	0.31	0.00	0.30	0.01	0.31	0.00
1.5	0.44	0.03	0.31	0.00	0.30	0.01
3	0.87	0.00	0.36	0.01	0.33	0.01
4.5	1.59	0.04	0.39	0.01	0.35	0.02
6	2.35	0.06	0.43	0.01	0.36	0.02
8	4.32	0.17	0.54	0.02	0.39	0.02
9.5	5.70	0.04	0.80	0.02	0.36	0.01
11	5.93	0.12	1.14	0.02	0.36	0.01
12.5	5.95	0.09	1.77	0.05	0.35	0.02
24	6.37	0.28	5.58	0.25	3.81	0.08
27.5	7.13	0.31	5.87	0.06	6.41	0.28
30	6.81	0.21	5.84	0.35	6.36	0.03
48	7.35	0.26	6.24	0.13	7.91	0.39

Table 3.3 : Average and standard deviation calculations of OD_{600} values of mutant in absence and presence of 0.5mM and 1mM hydrogen peroxide.

	Control		**0.5mM H_2O_2**		**1mM H_2O_2**	
Time (h)	**AVG**	**STD**	**AVG**	**STD**	**AVG**	**STD**
0	-	-	0.32	0.00	0.30	0.00
1.5	0.42	0.01	0.35	0.01	0.32	0.00
3	0.84	0.01	0.46	0.00	0.36	0.00
4.5	1.55	0.02	0.71	0.02	0.44	0.00
6	2.20	0.04	1.04	0.00	0.57	0.00
8	3.81	0.08	1.70	0.01	0.78	0.02
9.5	4.88	0.06	3.20	0.04	1.27	0.03
11	6.02	0.16	4.51	0.33	2.10	0.23
12.5	6.19	0.07	5.26	0.10	3.24	0.01
24	7.19	0.08	7.06	0.35	7.88	0.18
27.5	6.41	0.28	8.16	0.21	9.07	0.32
30	7.80	0.22	8.12	0.29	10.04	0.51
48	8.86	0.47	8.56	0.06	9.79	0.15

Table 3.4 : Natural logarithm (ln) calculations of OD_{600} values of wild type and mutant in the absence and presence of 5mM and 1mM hydrogen peroxide.

	Control		**0.5mM H_2O_2**		**1mM H_2O_2**	
	AVG	**STD**	**AVG**	**STD**	**AVG**	**STD**
0	-1.17	-1.19	-1.18	-	-1.14	-1.20
1.5	-0.83	-1.18	-1.20	-0.87	-1.06	-1.15
3	-0.14	-1.01	-1.11	-0.18	-0.77	-1.02
4.5	0.46	-0.95	-1.04	0.44	-0.34	-0.82
6	0.85	-0.83	-1.03	0.79	0.04	-0.57
8	1.46	-0.62	-0.95	1.34	0.53	-0.24
9.5	1.74	-0.22	-1.02	1.59	1.16	0.24
11	1.78	0.13	-1.02	1.79	1.51	0.74
12.5	1.78	0.57	-1.04	1.82	1.66	1.17
24	1.85	1.72	1.34	1.97	1.95	2.06
27.5	1.96	1.77	1.86	1.86	2.10	2.20
30	1.92	1.77	1.85	2.05	2.09	2.31
48	1.99	1.83	2.07	2.18	2.15	2.28

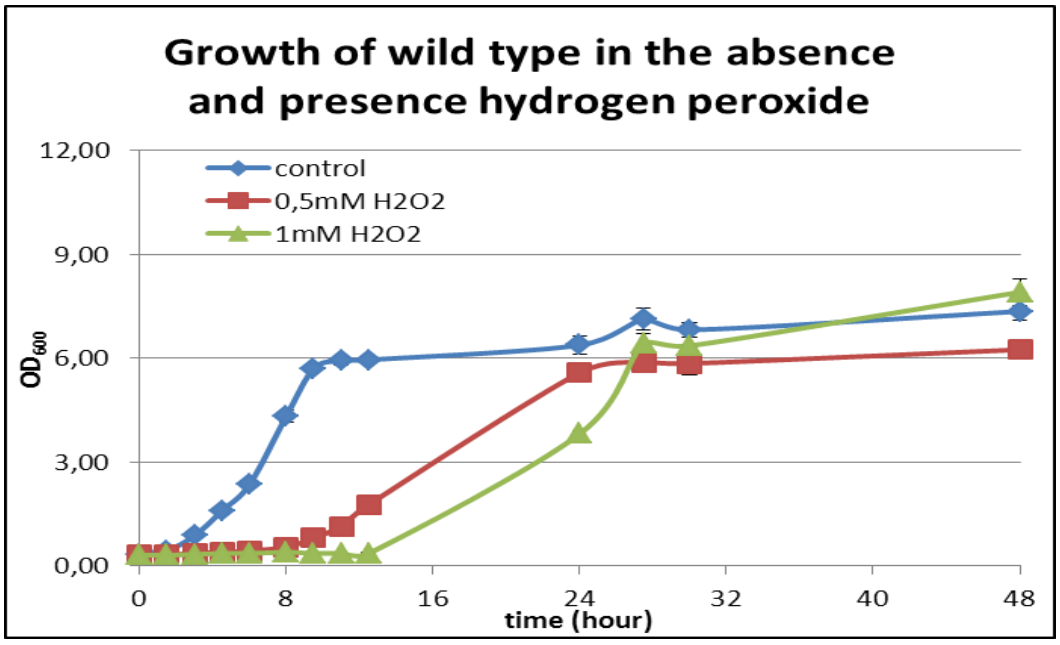

Figure 3.14 : OD_{600} measurements of wild type in the absence and presence 0.5 and 1 mM of H_2O_2 (48h under shaking condition).

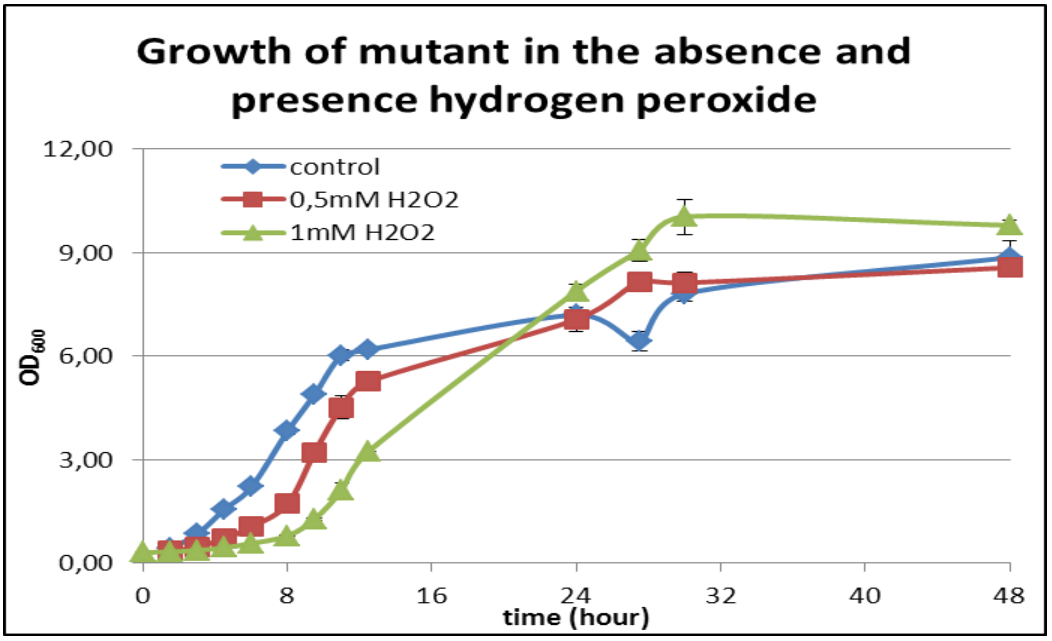

Figure 3.15 : OD_{600} measurements of mutant absence and presence 0.5 and 1 mM of stress condition (48h under shaking condition).

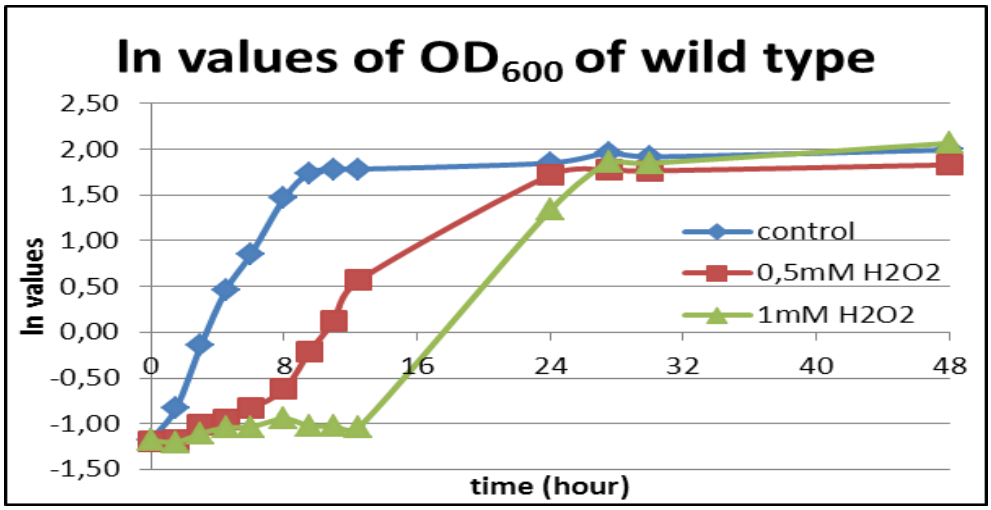

Figure 3.16 : ln values of OD_{600} values of wild type for growth curve.

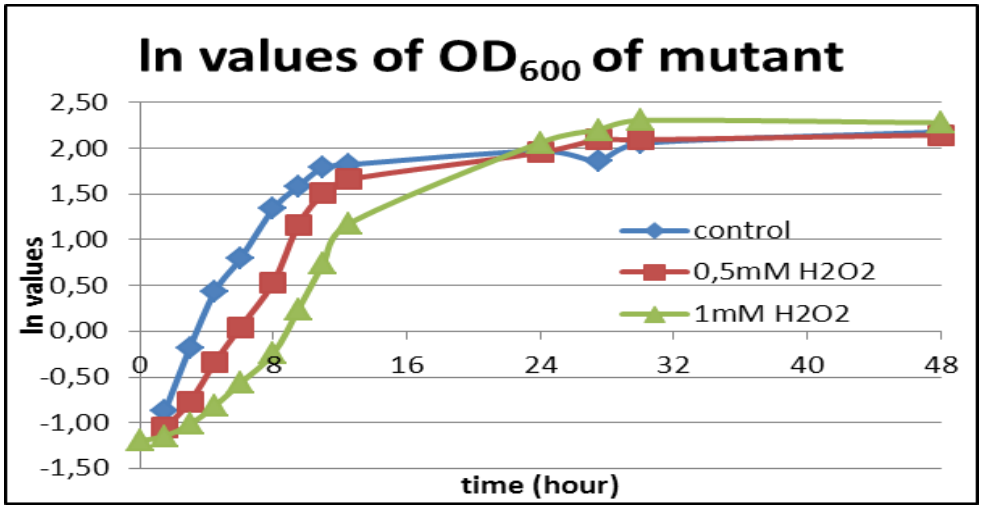

Figure 3.17 : ln values of OD_{600} values of mutant for growth curve

Table 3.5 : μ (h^{-1}) of wild type and mutant cells in the presence and absence of stress condition (0.5 mM hydrogen peroxide). μ stress/μ control was calculated to compare the growth rate of control condition to that of stress condition.

Strain	μ (μ^{-1}) control	μ (μ^{-1}) stress/μ (μ^{-1}) control
w/t control	0.36	0.93
w/t stress	0.34	
H7 control	0.37	0.94
H7 stress	0.34	

HPLC analysis gave information of ethanol, acetate, glycerol and maltose production and residual glucose amounts. During 48 hours of batch cultivation, samples were collected for HPLC analysis. Standard curve for the mentioned metabolites are shown in the Figure 3.18.

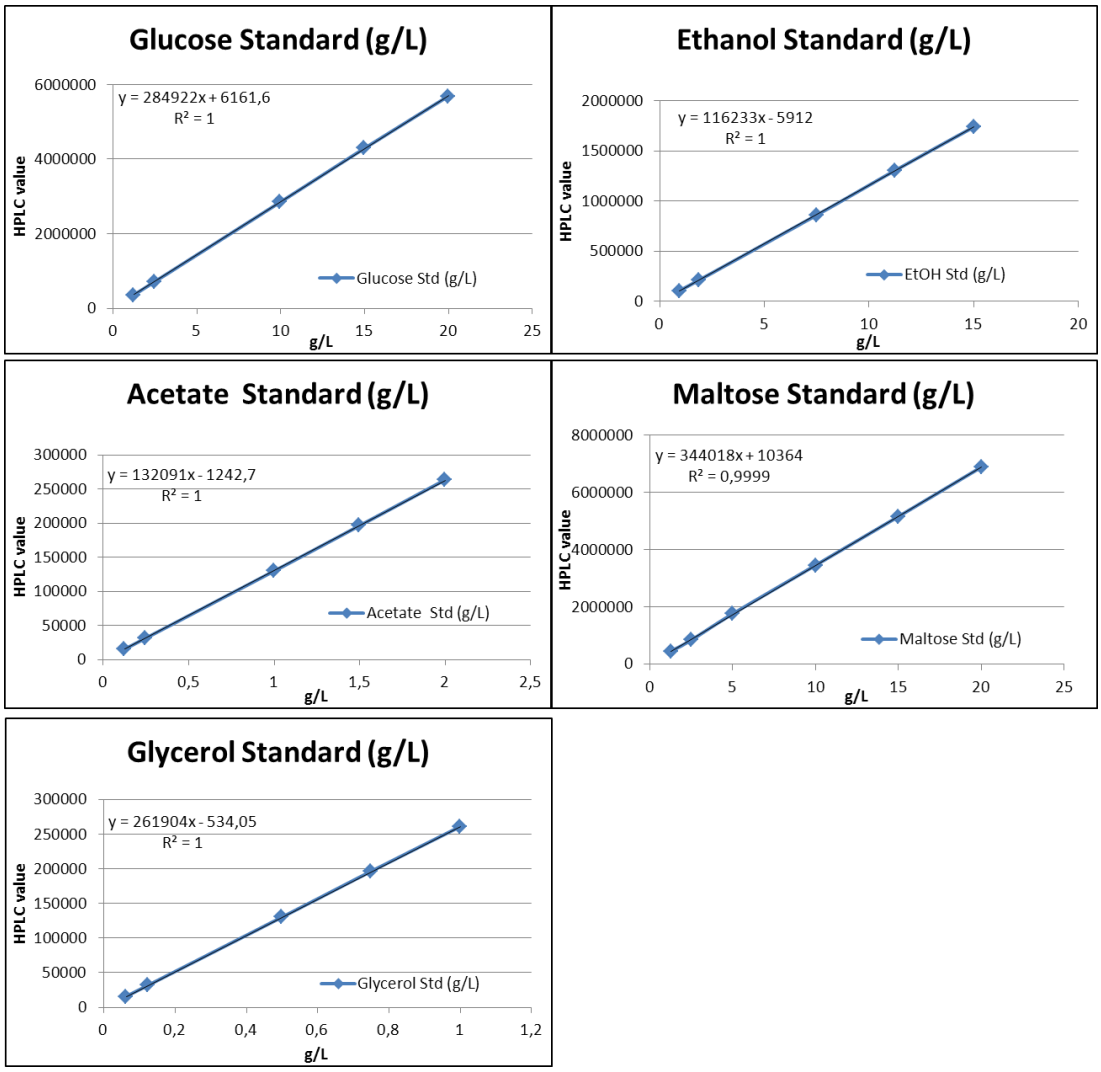

Figure 3.18 : HPLC standards for glucose, glycerol, acetate, ethanol and maltose. Graphics of metabolites' concentrations. Equations and R^2 values were demonstrated.

Mutant 'H7'consumed glucose faster than mutant in control conditions (Figure 3.19). However, when hydrogen peroxide stress was applied to wild type and mutant, wild type consumed glucose later than mutant.

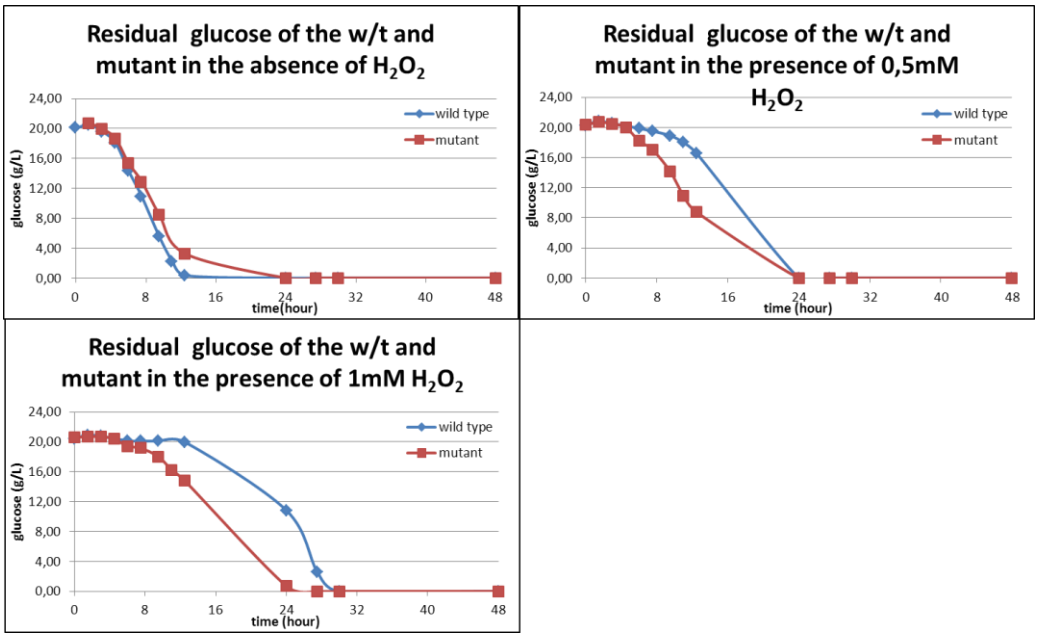

Figure 3.19 : Residual glucose concentration (g/L) versus time (hour).

Metabolite production can be observed in Figure on graphic 3.20 and 3.21. Stress factor affects glycerol production negatively for each strain. Wild type produced more glycerol than the mutant in each cultivation condition. Acetate results are slightly complicated for both wild type and the mutant. Wild type produced more acetate than the mutant (Figure 3.20). Wild type continued to produce acetate even during exponential phase but, mutant halted acetate production in the middle of exponential growth phase. Ethanol amount produced by wild type and mutant was close at the end of the 48 h for each condition, only mutant produced less ethanol in the presence of 1mM hydrogen peroxide. Additionally, stress condition delayed the ethanol production of wild type. Maltose production was normally low for the wild type. However mutant produced maltose during exponential phase at stationary phase.Considering metabolites, acetate and maltose production was different for wild type and mutant (Figure3.21).

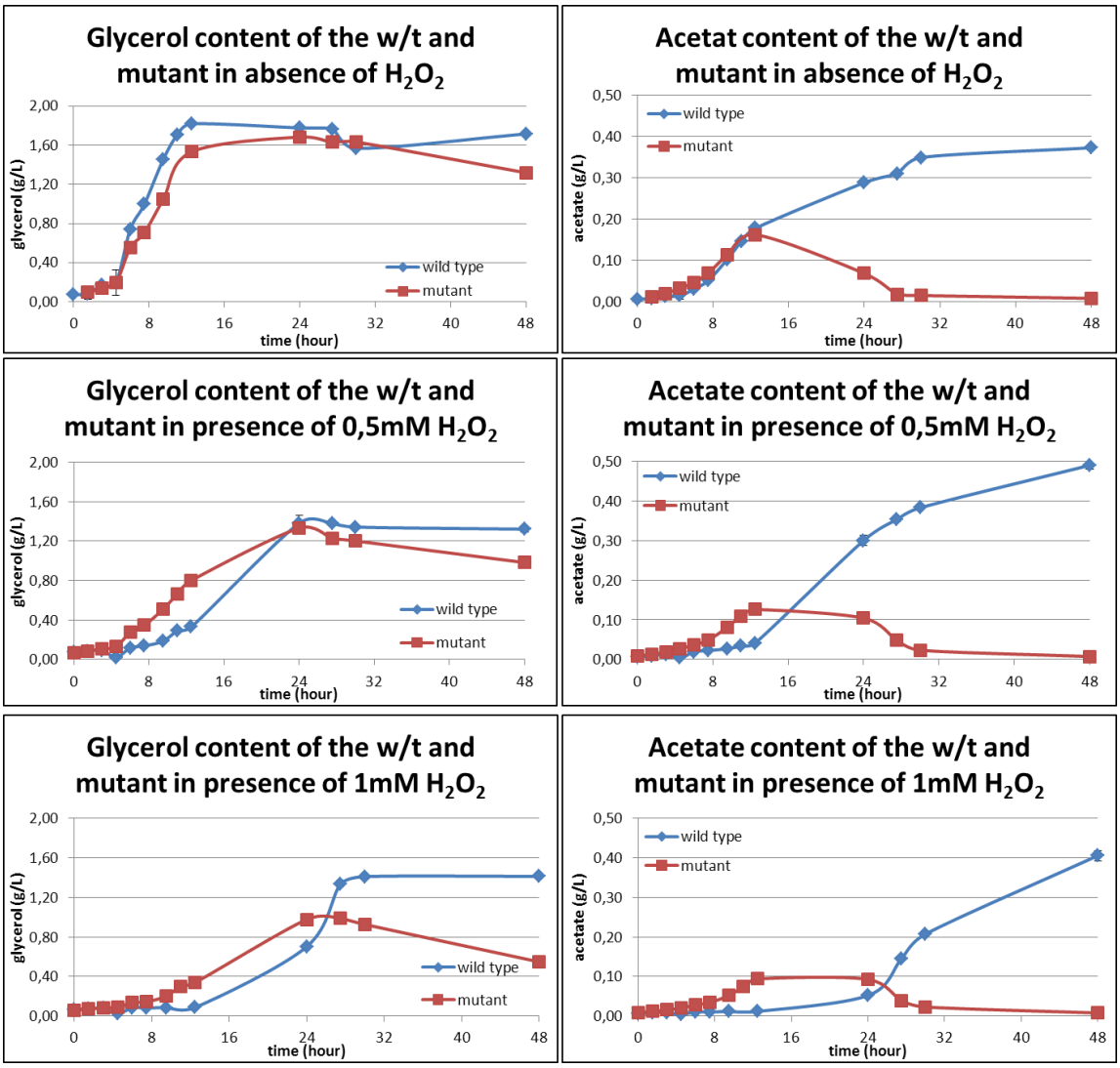

Figure 3.20 : Glycerol and acetate production (g/L) of both absence and presence of 0.5 and 1mM H_2O_2.

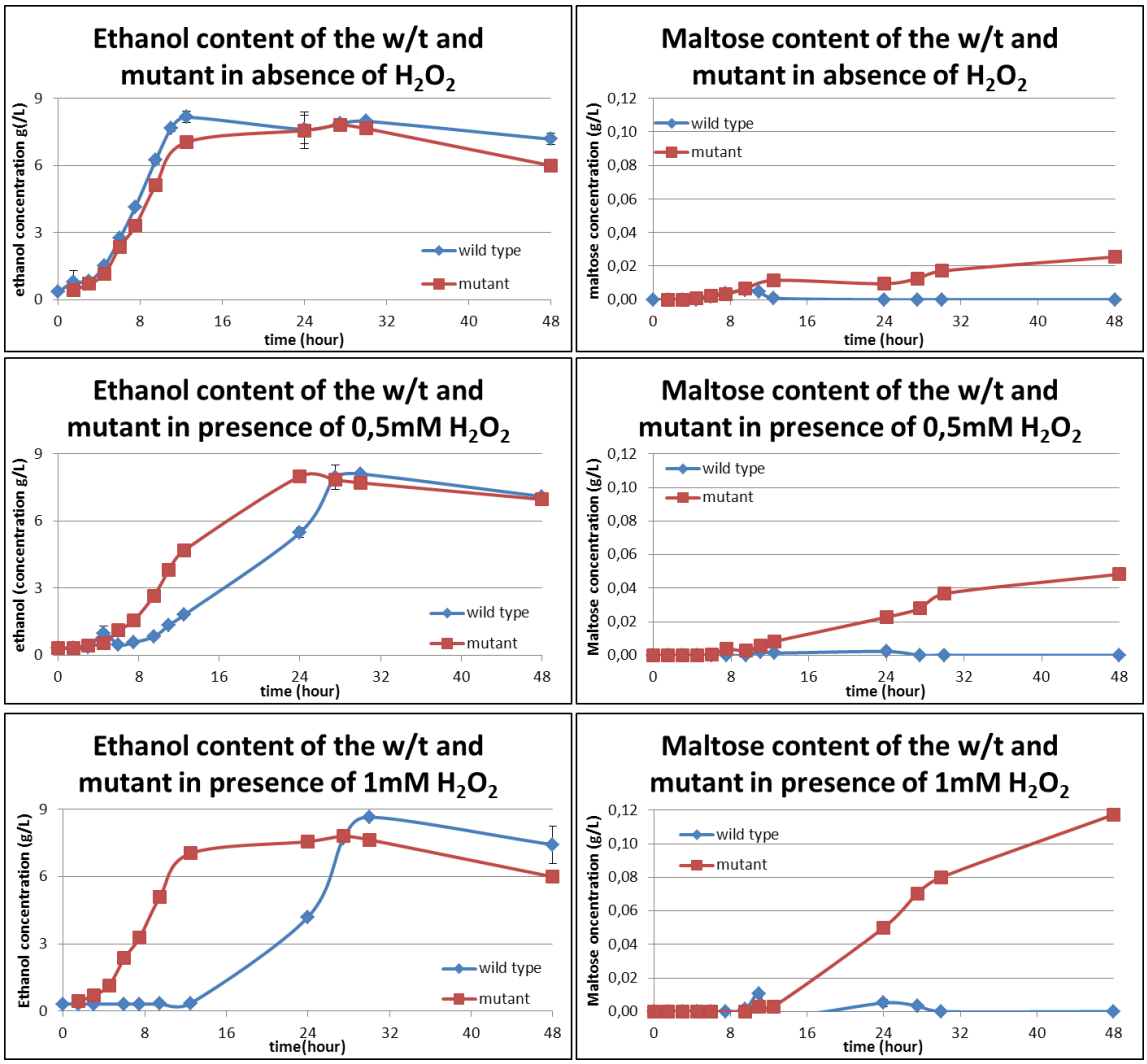

Figure 3.21 : Ethanol and maltose production (g/L) of both absence and presence of 0.5 and 1mM H_2O_2.

As figures 3.22 - 3.27 indicate, ethanol production was parelle with growth for each strain and condition. At the end of the cultivation, residual glucose results showed that strains go to stationary phase. Acetate, maltose and glycerol production amounts were not significant.

Table 3.6 : Average and standard deviations of glucose, glycerol, acetate, ethanol and maltose concentrations (g/l)of wild type in control condition.t (h)

	Glucose (g/l)		Glycerol (g/l)		Acetate (g/l)		Ethanol (g/l)		Maltose (g/l)	
t (h)	**AVG**	**STD**	**AVG**	**STD**	**STD**	**STD**	**AVG**	**STD**	**AVG**	**STD**
0	20.09	0.04	0.08	0.00	0.01	0.00	0.35	0.00	0.00	0.00
1.5	20.47	0.09	0.08	0.05	0.01	0.00	0.78	0.52	0.00	0.00
3	19.60	0.35	0.17	0.00	0.01	0.00	0.81	0.01	0.00	0.00
4.5	18.07	0.16	0.20	0.13	0.01	0.01	1.51	0.15	0.00	0.00
6	14.32	0.05	0.74	0.01	0.03	0.00	2.74	0.02	0.00	0.00
8	10.90	0.08	1.00	0.00	0.05	0.00	4.13	0.00	0.00	0.00
9.5	5.55	0.03	1.45	0.01	0.10	0.00	6.23	0.16	0.01	0.00
11	2.23	0.02	1.71	0.01	0.15	0.00	7.65	0.11	0.00	0.00
12.5	0.35	0.01	1.82	0.02	0.18	0.00	8.15	0.25	0.00	0.00
24	0.00	0.00	1.78	0.03	0.29	0.00	7.61	0.64	0.00	0.00
27.5	0.00	0.00	1.76	0.01	0.31	0.00	7.88	0.07	0.00	0.00
30	0.00	0.00	1.57	0.00	0.35	0.00	7.98	0.00	0.00	0.00
48	0.00	0.00	1.71	0.02	0.37	0.00	7.17	0.26	0.00	0.00

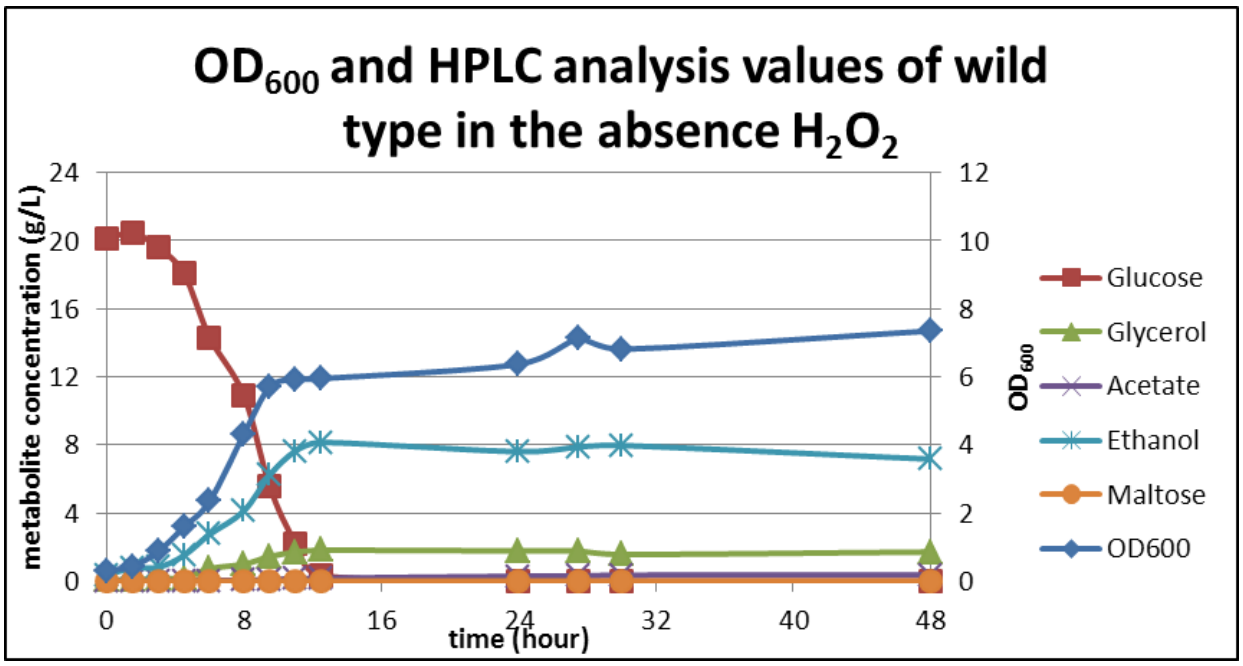

Figure 3.22 : OD_{600} measurement and metabolite concentrations of wild type, in the absence stress factor.

Table 3.7 : Average and standard deviations of glucose, glycerol, acetate, ethanol and maltose concentrations of wild type in 0.5mM hydrogen peroxide treatment.

	Glucose (g/l)		Glycerol (g/l)		Acetate (g/l)		Ethanol (g/l)		Maltose (g/l)	
t(h)	**AVG**	**STD**	**AVG**	**STD**	**STD**	**STD**	**AVG**	**STD**	**AVG**	**STD**
0	20.23	0.03	0.07	0.00	0.01	0.00	0.32	0.00	0.00	20.23
1.5	20.81	0.04	0.08	0.00	0.01	0.00	0.33	0.00	0.00	20.81
3	20.51	0.46	0.09	0.00	0.01	0.00	0.35	0.00	0.00	20.51
4.5	2.49	1.27	0.02	0.01	0.00	0.00	0.98	0.33	0.00	2.49
6	19.86	0.17	0.12	0.00	0.02	0.00	0.47	0.00	0.00	19.86
8	19.50	0.11	0.14	0.00	0.02	0.00	0.58	0.02	0.00	19.50
9.5	18.90	0.11	0.19	0.00	0.03	0.00	0.83	0.04	0.00	18.90
11	17.99	0.72	0.29	0.01	0.03	0.00	1.34	0.05	0.00	17.99
12.5	16.51	0.00	0.33	0.00	0.04	0.00	1.80	0.01	0.00	16.51
24	0.00	0.00	1.38	0.08	0.30	0.01	7.18	1.81	0.00	0.00
27.5	0.00	0.00	1.38	0.02	0.35	0.01	7.58	0.74	0.00	0.00
30	0.00	0.00	1.58	0.34	0.38	0.00	8.11	0.05	0.00	0.00
48	0.00	0.00	1.32	0.03	0.49	0.01	7.10	0.01	0.00	0.00

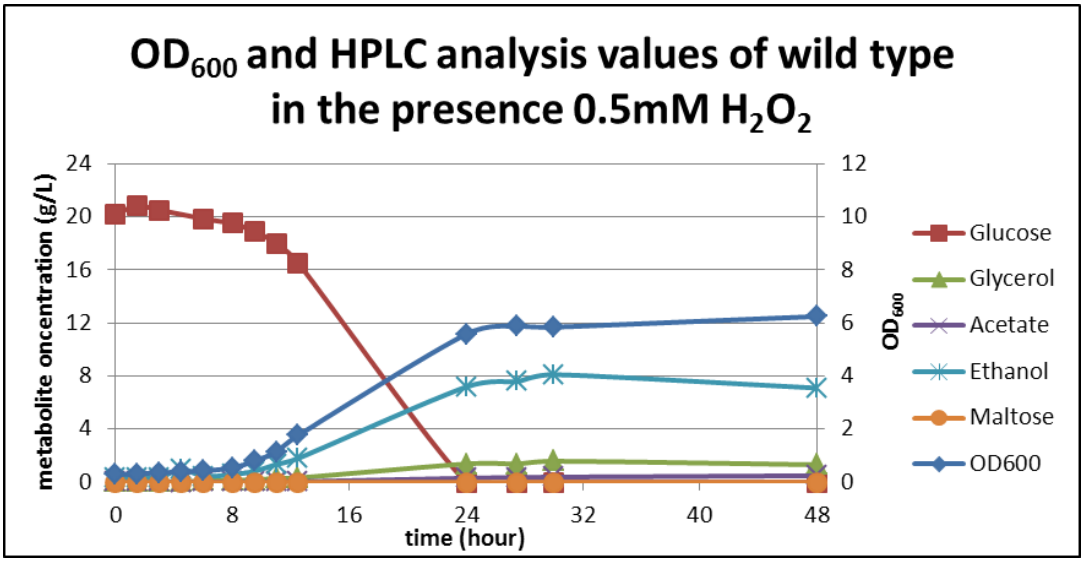

Figure 3.23 : OD_{600} measurement and metabolite concentrations of wild type, in the presence of 0.5mM H_2O_2.

Table 3.8 : Average and standard deviations of glucose, glycerol, acetate, ethanol and maltose concentrations of wild type in 1mM hydrogen peroxide treatment.

	Glucose (g/l)		Glycerol (g/l)		Acetate(g/l)		Ethanol (g/l)		Maltose (g/l)	
t (h)	**AVG**	**STD**	**AVG**	**STD**	**STD**	**STD**	**AVG**	**STD**	**AVG**	**STD**
0	20.38	0.12	0.07	0.00	0.01	0.00	0.31	0.00	0.00	0.00
1.5	20.92	0.07	0.08	0.00	0.01	0.00	0.31	0.00	0.00	0.00
3	20.75	0.02	0.08	0.00	0.01	0.00	0.32	0.00	0.00	0.00
4.5	7.36	0.57	0.03	0.01	0.00	0.00	-	-	0.00	0.00
6	20.16	0.05	0.08	0.00	0.01	0.00	0.32	0.00	0.00	0.00
8	20.10	0.10	0.08	0.00	0.01	0.00	0.32	0.01	0.00	0.00
9.5	20.11	0.13	0.09	0.00	0.01	0.00	0.32	0.01	0.00	0.00
11	5.32	0.02	1.33	0.00	-	-	-	-	0.01	0.00
12.5	19.97	0.01	0.09	0.00	0.01	0.00	0.35	0.00	0.00	0.00
24	10.84	0.02	0.70	0.00	0.05	0.00	4.20	0.01	0.01	0.00
27.5	2.62	0.01	1.33	0.00	0.14	0.00	7.72	0.00	0.00	0.00
30	0.01	0.00	1.41	0.01	0.21	0.00	8.66	0.09	0.00	0.00
48	0.00	0.00	1.41	0.01	0.41	0.01	7.42	0.83	0.00	0.00

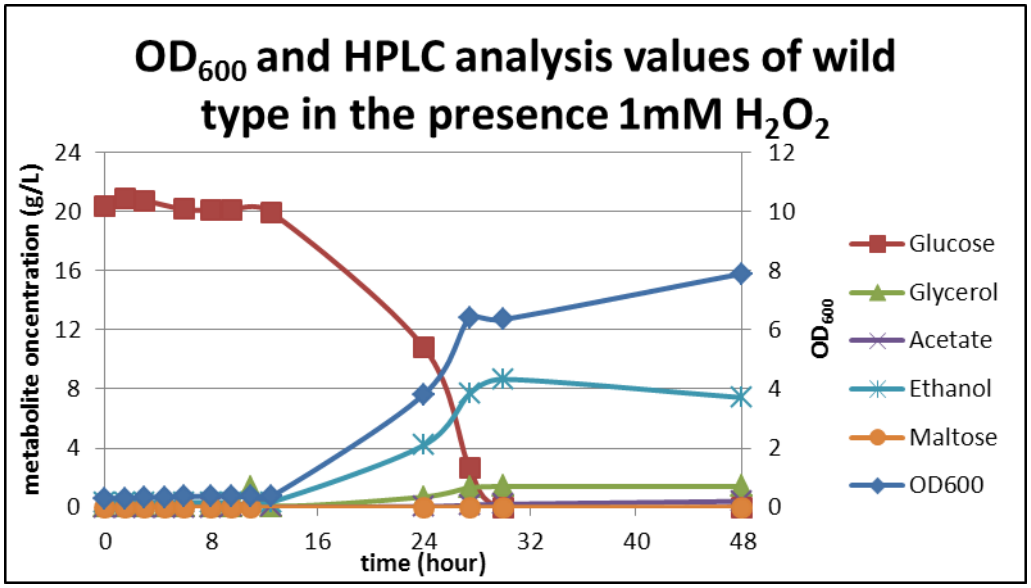

Figure 3.24 : OD_{600} measurement and metabolite concentrations of wild type, in the presence of 1mM H_2O_2.

Table 3.9 : Glucose, glycerol, acetate, ethanol and maltose concentrations of H7 in the absence of hydrogen peroxide.

	Glucose (g/l)		Glycerol (g/l)		Acetate (g/l)		Ethanol (g/l)		Maltose (g/l)	
t (h)	**AVG**	**STD**	**AVG**	**STD**	**STD**	**STD**	**AVG**	**STD**	**AVG**	**STD**
1.5	20.72	0.09	0.10	0.00	0.01	0.00	0.44	0.00	0.00	0.00
3	19.97	0.02	0.14	0.00	0.02	0.00	0.70	0.00	0.00	0.00
4.5	18.65	0.02	0.20	0.00	0.03	0.00	1.15	0.02	0.00	0.00
6	15.37	0.02	0.55	0.00	0.05	0.00	2.36	0.03	0.00	0.00
8	12.90	0.03	0.70	0.00	0.07	0.00	3.29	0.12	0.00	0.00
9.5	8.48	0.04	1.04	0.00	0.11	0.00	5.11	0.04	0.01	0.00
11	19.96	0.02	0.09	0.00	-	-	-	-	0.00	0.00
12.5	3.24	0.01	1.53	0.00	0.16	0.00	7.04	0.15	0.01	0.00
24	0.00	0.00	1.68	0.03	0.07	0.00	7.57	0.80	0.01	0.00
27.5	0.00	0.00	1.63	0.00	0.02	0.00	7.81	0.02	0.01	0.00
30	0.00	0.00	1.63	0.00	0.02	0.00	7.65	0.05	0.02	0.00
48	0.00	0.00	1.32	0.29	0.01	0.00	5.99	0.09	0.03	0.01

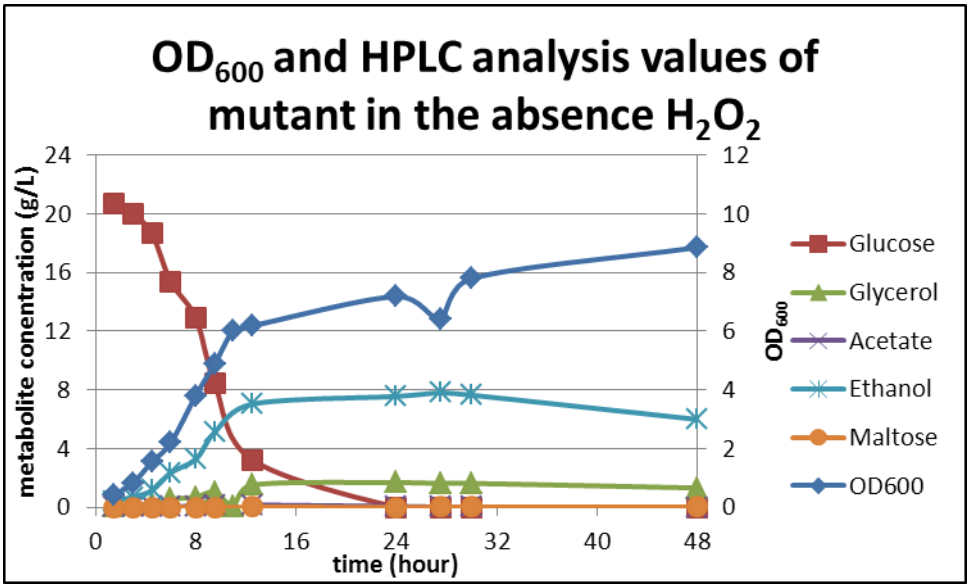

Figure 3.25 : OD_{600} measurement and metabolite concentrations of mutant, in the absence of stress factor.

Table 3.10 : Glucose, glycerol, acetate, ethanol and maltose concentrations of H7 in 0.5mM hydrogen peroxide treatment.

	Glucose (g/l)		Glycerol (g/l)		Acetate (g/l)		Ethanol (g/l)		Maltose (g/l)	
t (h)	**AVG**	**STD**	**AVG**	**STD**	**STD**	**STD**	**AVG**	**STD**	**AVG**	**STD**
0	20.33	0.05	0.07	0.00	0.01	0.00	0.31	0.00	0.00	0.00
1.5	20.69	0.04	0.09	0.00	0.01	0.00	0.34	0.00	0.00	0.00
3	20.44	0.05	0.11	0.00	0.02	0.00	0.42	0.00	0.00	0.00
4.5	20.01	0.11	0.18	0.00	0.03	0.00	0.56	0.00	0.00	0.00
6	18.19	0.05	0.30	0.00	0.04	0.00	1.12	0.05	0.00	0.00
8	17.04	0.05	0.40	0.00	0.05	0.00	1.56	0.06	0.00	0.00
9.5	14.16	0.06	0.56	0.00	0.08	0.00	2.67	0.07	0.00	0.00
11	10.93	0.09	0.71	0.01	0.11	0.00	3.80	0.15	0.01	0.00
12.5	8.72	0.07	0.97	0.01	0.13	0.00	4.69	0.10	0.01	0.00
24	0.00	0.00	1.28	0.05	0.10	0.00	7.15	1.46	0.02	0.00
27.5	0.00	0.00	0.94	0.00	0.05	0.00	7.84	0.03	0.03	0.00
30	0.00	0.00	0.85	0.48	0.02	0.01	7.71	0.05	0.04	0.00
48	0.00	0.00	0.65	0.00	0.01	0.00	6.97	0.07	0.05	0.00

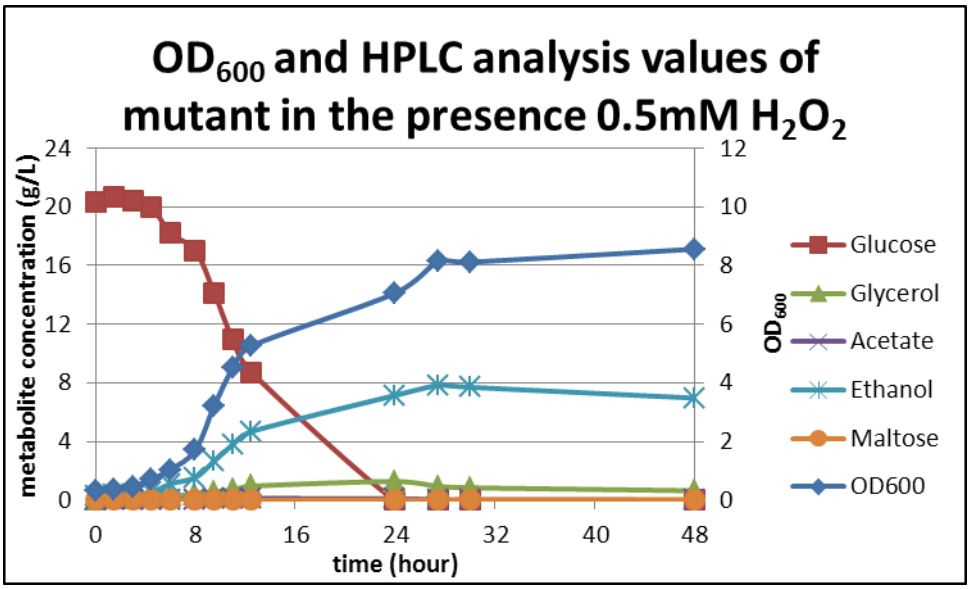

Figure 3.26 : OD_{600} measurement and metabolite concentrations of H7, upon 0.5mM of stress treatment.

Table 3.11 : Glucose, glycerol, acetate, ethanol and maltose concentrations of H7 in the presence of 1mM hydrogen peroxide stress.

	Glucose (g/l)		Glycerol (g/l)		Acetate (g/l)		Ethanol (g/l)		Maltose (g/l)	
t (h)	**AVG**	**STD**	**AVG**	**STD**	**STD**	**STD**	**AVG**	**STD**	**AVG**	**STD**
0	20.63	0.22	0.06	0.00	0.01	0.00	0.29	0.01	0.00	0.00
1.5	20.67	0.00	0.07	0.00	0.01	0.00	0.29	0.00	0.00	0.00
3	20.71	0.10	0.08	0.00	0.02	0.00	0.30	0.01	0.00	0.00
4.5	20.37	0.31	0.09	0.00	0.02	0.00	0.34	0.00	0.00	0.00
6	19.42	0.02	0.14	0.02	0.03	0.00	0.51	0.02	0.00	0.00
8	19.16	0.22	0.15	0.00	0.03	0.00	0.58	0.01	0.00	0.00
9.5	18.02	0.05	0.20	0.00	0.05	0.00	1.02	0.00	0.00	0.00
11	16.25	0.18	0.30	0.01	0.07	0.00	1.75	0.12	0.00	0.00
12.5	14.78	0.12	0.34	0.00	0.09	0.00	2.19	0.05	0.00	0.00
24	0.70	0.00	0.98	0.00	0.09	0.00	7.42	0.25	0.05	0.00
27.5	0.00	0.00	0.99	0.00	0.04	0.00	7.85	0.01	0.07	0.00
30	0.00	0.00	0.93	0.00	0.02	0.00	7.67	0.28	0.08	0.00
48	0.00	0.00	0.55	0.00	0.01	0.00	6.52	0.08	0.12	0.00

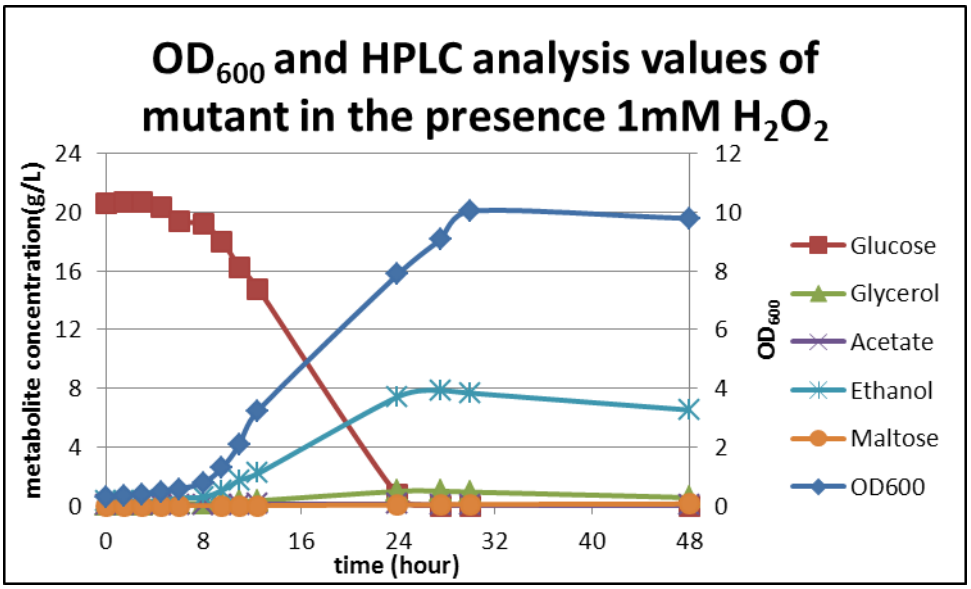

Figure 3.27 : OD_{600} measurement and metabolite concentrations of H7, upon 1mM H_2O_2 stress application.

Cell dry weight measurement was carried out three times for each sampling. Cell dry weight results of H7 was more than that of wild type in each condition (Figure3.28).

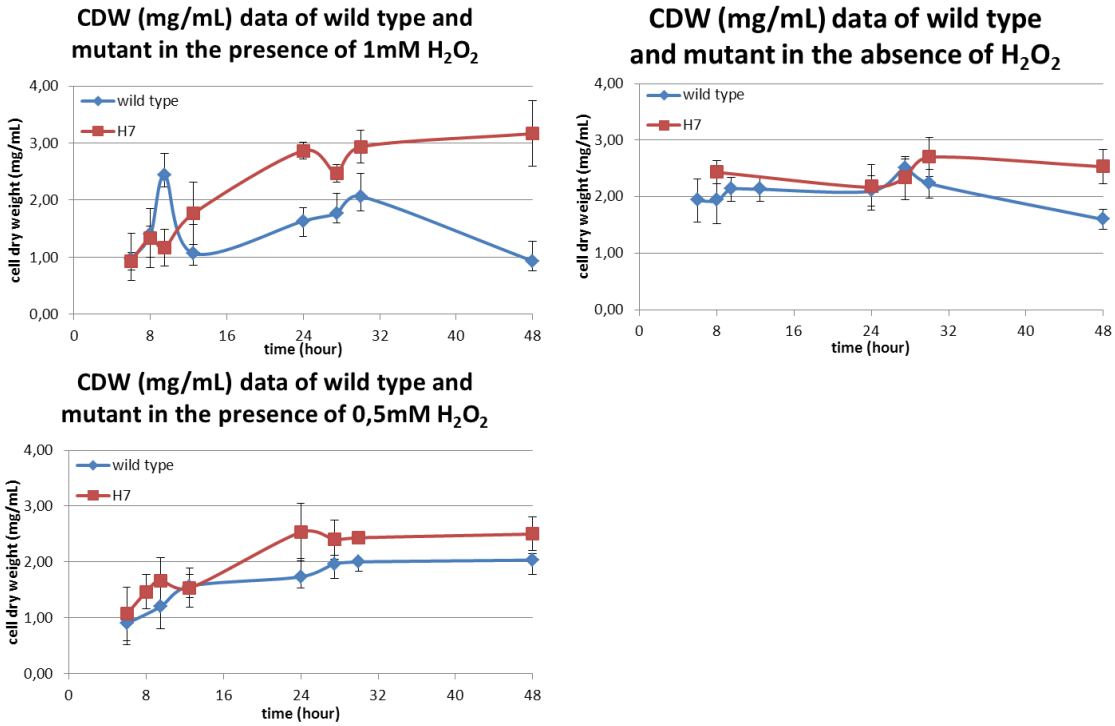

Figure 3.28 : Cell Dry Weight data given size in mg/ml on graphic.

3.2.3 Trehalose and glycogen content determination by enzymatic reaction

To investigate trehalose and glycogen production of wild type and oxidative stress resistance mutant 'H7', samples were collected during their batch cultivation. Furthermore, samples were collected also for cell dry weight and optical density was measured to observe growth of cultures. As a result, high trehalose and glycogen content of mutant was very significant. However, for H7 the highest amounts of trehalose and glycogen were observed at 17.5 hour of incubation. Presence of 0.5 mM hydrogen peroxide caused more increase in of these disaccharide and polysaccharide in mutant type. With stationary phase, amounts of the trehalose and glycogen started to decrease (Figure3.30 and 3.31). Already hydrogen peroxide triggers trehalose and glycogen production (Sillje *et al.*, 1999), oxidative stress resistant strain produced these reserve carbohydrates in normal condition. It means that H7 was a trehalose and glycogen producer even in non-stress condition (Parrou and François, 1997).

Trehalose-glycogen content of the samples were given by correlating them to their corresponding CDW/ ml results. Cell dry weight measurement was carried out three times for each sampling. CDW increase was very fast in exponential phase for each strain and condition. CDW increases become slow with diauxic shift. Because of low hydrogen peroxide concentration, there are no differences of wild type's CDW in the presence and absence of hydrogen peroxide. However, at the end of the 30th hour, cell dry weight of the mutant strain was higher than wild type (does not matter of absence or presence of hydrogen peroxide.

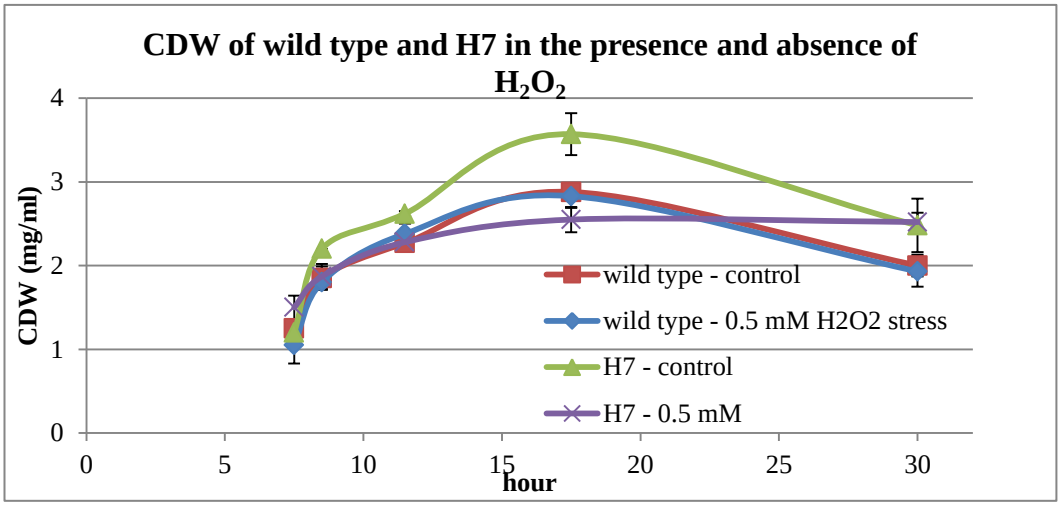

Figure 3.29 : Cell dry weight data of wild type and H7 (with and without hydrogen peroxide application).

While glycogen production increased slightly with stationary phase in w/t, it decreased in mutant strain (Figure 3.31).

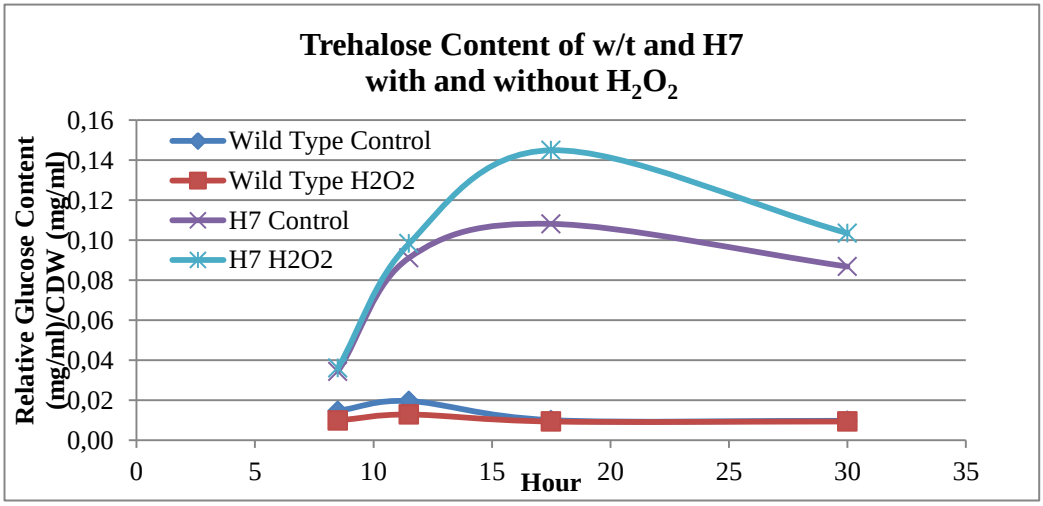

Figure 3.30 : Trehalose production of w/t and H7 both in the presence and absence of H_2O_2.

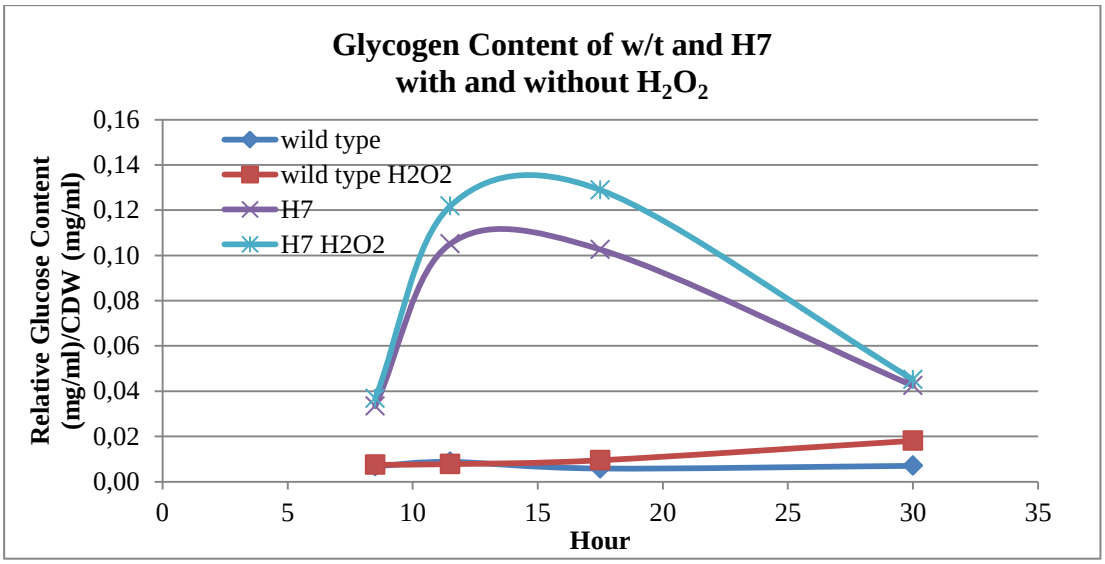

Figure 3.31 : Effects of oxidative stress on glycogen levels in wild type and oxidative stress resistant mutant.

3.3 Expression level determination of *OCA1*, *MSN2* and *MSN4* genes for wild type and oxidative stress-resistant mutant by quantitative PCR

3.3.1 Determination of *OCA1* expression level by qPCR analysis

Determination of expression level of *OCA1* was carried out by real-time PCR. Firstly total RNA was extracted from the w/t and H7 strains in 0.5mM H_2O_2 stress treated and untreated continuous and pulse conditions. When strains reached to 3 OD_{600}, RNA extraction was carried out. cDNA was synthesized and these products used as template for qPCR. Extracted total RNA and cDNA amounts were measured with Nanodrop 2000 (Table 3.12). Experiment carried out three times and their $2^{-\Delta\Delta CT}$ values was calculated. The expression difference was expressed as fold change values from the ratio of control wild type results.

Real time PCR was performed by using the cDNA. The error, efficiency and slope values are shown in Table 3.13 for successive qPCR set .

Table 3.12 : RNA concentration of samples' RNA and cDNA (260/280 rates are given). cDNA concentration were measured upon 1:10 dilution.

	RNA (ng/µl)	**RNA 260/280**	**cDNA (ng/µl)**	**cDNA 260/280**
Wild Type control	332.60	2.16	204.9	1.84
Wild Type with H_2O_2	231.20	2.15	201.2	1.84
H7 control	96.70	2.13	207.3	1.84
H7 with H_2O_2	101.30	2.11	218.6	1.84

Table 3.13 : Error, efficiency and slope of *ACT1* and *OCA1* for each experiment set.

	1st set of experiment		**2nd set of experiment**		**3th set of experiment**	
	ACT1	***OCA1***	***ACT1***	***OCA1***	***ACT1***	***OCA1***
Error	0.06	0.05	0.05	0.15	0.05	0.04
Efficiency	1.94	1.88	1.93	1.92	1.93	1.89
Slope	-3.48	-3.655	-3.49	-3.54	-3.49	-3.61

Table 3.14 : CP, calculated ΔC_T and $2^{-\Delta\Delta CT}$ values of first experiment set.

Set1	**CP (*ACT1*)**	**CP (*OCA1*)**	**ΔC_T(CP Target-CP Control)**	**$2^{-\Delta\Delta CT}$**	**$2^{-\Delta\Delta CT}$as fold of wild type**
Wild Type control	19.89	20.86	0.97	0.5105	1.00
Wild Type with H_2O_2	21.61	22.02	0.41	0.7526	1.47
H7 control	19.53	20.95	1.42	0.3737	0.73
H7 with H_2O_2	20.60	21.11	0.51	0.7022	1.38

Table 3.15 : CP, calculated ΔC_T and $2^{-\Delta\Delta CT}$ values of second experiment set.

Set2	**CP (*ACT1*)**	**CP (*OCA1*)**	**ΔC_T(CP Target-CP Control)**	**$2^{-\Delta\Delta CT}$**	**$2^{-\Delta\Delta CT}$as fold of wild type**
Wild Type control	19.89	20.82	0.93	0.5249	1.00
Wild Type with H_2O_2	21.61	22.87	1.26	0.4175	0.80
H7 control	19.53	21.54	2.01	0.2483	0.47
H7 with H_2O_2	20.60	21.20	0.60	0.6598	1.26

Table 3.16 : CP, calculated ΔC_T and $2^{-\Delta\Delta CT}$ values of third experiment set.

Set3	**CP (*ACT1*)**	**CP (*OCA1*)**	**ΔC_T(CP Target-CP Control)**	**$2^{-\Delta\Delta CT}$**	**$2^{-\Delta\Delta CT}$as fold of wild type**
Wild Type control	23.71	20.94	1.05	0.4830	1.00
Wild Type with H_2O_2	25.76	22.79	1.18	0.4414	0.91
H7 control	22.28	21.52	1.99	0.2517	0.52
H7 with H2O2	23.69	21.12	0.52	0.6974	1.44

The results of the set 1, set 2 and set 3 were collected on the Table 3.17. Their average CP values and their corresponding standard deviation are shown on the same table.

Table 3.17 : Average value was taken for $2^{-\Delta\Delta CT}$ and the expression level vas calculated as fold of wild type. Results from continuous hydrogen peroxide application to strain.

	Set 1	Set 2	Set 3	AVG	ST DEV
Wild Type control	1.00	1.00	1.00	1.00	0.00
Wild Type with H_2O_2	1.21	0.84	1.30	1.12	0.24
H7 control	0.73	0.47	2.14	1.78	0.34
H7 with H_2O_2	1.95	1.36	1.82	1.71	0.31

As a result, *OCA1* expression of wild type was very close with both presence and absence hydrogen peroxide. *OCA1* expression of mutant strain was 1.7 fold of wild type upon continuous H_2O_2 stress application. For each strain, there was no significant difference of *OCA1* expression between presence and absence of 0.5 mM hydrogen peroxide (Figure 3.32).

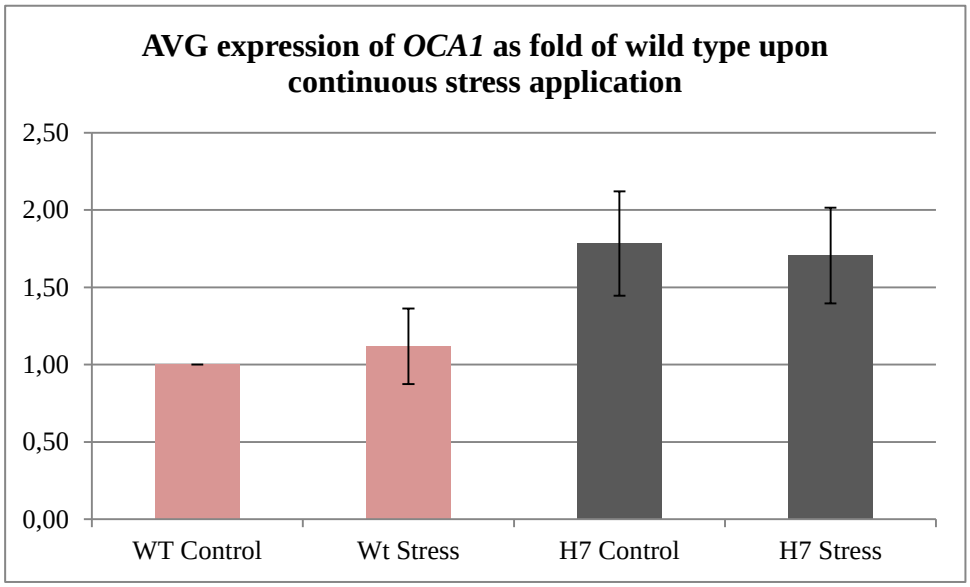

Figure 3.32 : Graphical representation of $2^{-\Delta\Delta CT}$ as fold of wild type values. Graphic presents of average value of $2^{-\Delta\Delta CT}$as fold of wild type.

In the second part of this experiment, 1 mM pulse H_2O_2 stress was applied to strains to observe expression level of *OCA1*.Cultures were grown to $OD_{600}$of 1, and they were incubated in the presence of 1 mM H_2O_2 for 90 min. RNA purification was applied before and after pulse stress application (Table 3.9). Error, efficiency and slope values for real-time was shown on the table 2.10. For analysis of these results,$2^{-\Delta\Delta CT}$ method was used. CP, $\Delta C_T$and $2^{-\Delta\Delta CT}$ calculations for each set of experiment were given table below. The results of the set1, set2 and set3 were collected on the Table 3.22. Their average CP values and their corresponding standard deviation were shown at the same table.

Table 3.18 : RNA and cDNA concentrations of strains (260/280 rates are given). cDNA concentration was measured upon 1:10 dilution.

	RNA (ng/µl)	**RNA 260/280**	**cDNA (ng/µl)**	**cDNA 260/280**
Wild Type control	53.3	2.10	442.8	1.81
Wild Type with H_2O_2	41.4	2.07	396.0	1.81
H7 control	93.2	2.14	409.5	1.81
H7 with H_2O_2	35.8	2.06	377.4	1.81

Table 3.19 : Error, efficiency and slope of *ACT1* and *OCA1* for each experiment set.

	1st set of experiment		**2nd set of experiment**		**3th set of experiment**	
	ACT1	***OCA1***	***ACT1***	***OCA1***	***ACT1***	***OCA1***
Error	0.06	0.05	0.05	0.15	0.05	0.04
Efficiency	1.94	1.88	1.93	1.92	1.93	1.89
Slope	-3.48	-3.655	-3.49	-3.54	-3.49	-3.61

Table 3.20 : CP, calculated ΔC_T and $2^{-\Delta\Delta CT}$ values of first experiment set.

Set1	**CP (*ACT1*)**	**CP (*OCA1*)**	**ΔC_T(CP Target-CP Control)**	**$2^{-\Delta\Delta CT}$**	**$2^{-\Delta\Delta CT}$as fold of wild type**
Wild Type control	26.21	32.28	6.07	0.0149	1.0000
Wild Type with H_2O_2	24.56	31.91	7.35	0.0061	0.4118
H7 control	22.83	29.35	6.52	0.0109	0.7320
H7 with H_2O_2	24.59	31.23	6.64	0.0100	0.6736

Table 3.21 : CP, calculated ΔC_T and $2^{-\Delta\Delta CT}$ values of second experiment set.

Set2	**CP (*ACT1*)**	**CP (*OCA1*)**	**ΔC_T(CP Target-CP Control)**	**$2^{-\Delta\Delta CT}$**	**$2^{-\Delta\Delta CT}$as fold of wild type**
Wild Type control	26.21	32.24	6.03	0.0153	1.0000
Wild Type with H_2O_2	24.56	31.74	7.18	0.0069	0.4506
H7 control	22.83	28.86	6.03	0.0153	1.0000
H7 with H_2O_2	24.59	30.61	6.02	0.0154	1.0070

Table 3.22 : CP, calculated ΔC_T and $2^{-\Delta\Delta CT}$ values of third experiment set.

Set3	**CP (*ACT1*)**	**CP (*OCA1*)**	**ΔC_T(CP Target-CP Control)**	**$2^{-\Delta\Delta CT}$**	**$2^{-\Delta\Delta CT}$as fold of wild type**
Wild Type control	26.21	32.33	6.12	0.0144	1.0000
Wild Type with H_2O_2	24.56	31.65	7.09	0.0073	0.5105
H7 control	22.83	29.13	6.30	0.0127	0.8827
H7 with H2O2	24.59	30.63	6.04	0.0152	1.0570

Table 3.23 : Average value was taken of $2^{-\Delta\Delta CT}$ as fold of wild type and their standard deviation was calculated. Results from continuous hydrogen peroxide application to strain.

	Set 1	Set 2	Set 3	AVG	ST DEV
Wild Type control	1.00	1.00	1.00	1.00	0.00
Wild Type with H_2O_2	0.41	0.45	0.51	0.46	0.05
H7 control	0.73	1.00	0.88	0.87	0.13
H7 with H_2O_2	0.67	1.01	1.06	0.91	0.21

As a result, it was found that *OCA1* expression of wild type was close to that of H7 mutant in the control condition. However, *OCA1* expression of H7 was 2 fold to of wild type upon pulse H_2O_2 treatment. However, first part of this experiment showed that for each strain there was no significant difference of *OCA1*expression both in the presence and absence of continuous 0.5mM hydrogen peroxide conditions (Figure 2.33).

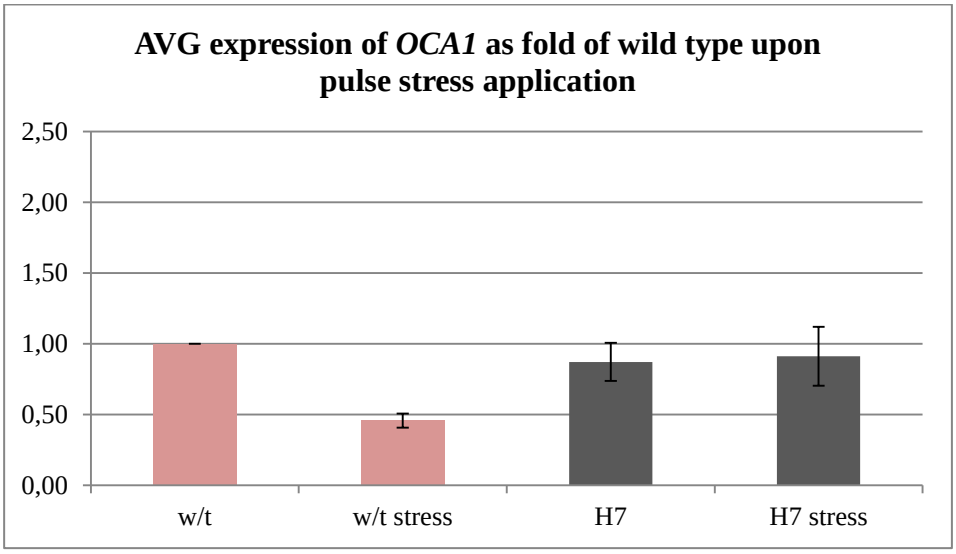

Figure 3.33 : Graphical representation of $2^{-\Delta\Delta CT}$ as fold of wild type values. Graphic presents of average value of $2^{-\Delta\Delta CT}$ as fold of wild type.

3.3.2 Determination of *MSN2* expression level by qPCR analysis

To investigate *MSN2* expression level of wild type and mutant, real-time PCR was used. For this purpose, strains were grown on minimal medium in the absence and presence of stress application. 0.5mM hydrogen peroxide was treated continuously to strains as stress condition. After RNA isolation and cDNA synthesis, real-time PCR was carried out. Results were analyzed with $2^{-\Delta\Delta C}$ method. RNA and cDNA

measurments are shown in Table 3.24.Error, efficiency and slope values of this experiment, calculations of $2^{-\Delta\Delta CT}$, average and standard deviation ware given on theTables 3.25- 3.27 respectively.

Table 3.24 : RNA and cDNA measurements of strains were given (260/280 rates are given). cDNA concentration were measured upon 1:10 dilution.

	RNA (ng/µl)	**RNA 260/280**	**cDNA (ng/µl)**	**cDNA 260/280**
Wild Type control	53.3	2.10	223.0	1.82
Wild Type with H_2O_2	41.4	2.07	224.8	1.81
H7 control	93.2	2.14	286.8	1.83
H7 with H_2O_2	35.8	2.06	215.4	1.84

Table 3.25 : Error, efficiency and slope of *ACT1* and *MSN2* for each experiment set.

	1st set of experiment		**2nd set of experiment**	
	ACT1	***MSN2***	***ACT1***	***MSN2***
Error	0.01	0.01	0.06	0.05
Efficiency	2.01	2.00	1.94	1.99
Slope	-3.31	-3.33	-3.49	-3.55

Table 3.26 : CP, calculated ΔC_T and $2^{-\Delta\Delta CT}$ values of first experiment set.

Set1	**CP (*ACT1*)**	**CP (*MSN2*)**	**ΔC_T(CP Target-CP Control)**	**$2^{-\Delta\Delta CT}$**	**$2^{-\Delta\Delta CT}$as fold of wild type**
Wild Type control	18.54	22.56	4.02	0.06	1.00
Wild Type with H_2O_2	20.92	25.51	4.59	0.04	1.67
H7 control	18.20	22.97	4.77	0.04	0.59
H7 with H_2O_2	20.92	22.50	4.54	0.04	1.70

Table 3.27 : CP, calculated ΔC_T and $2^{-\Delta\Delta CT}$ values of second experiment set.

Set2	**CP (*ACT1*)**	**CP (*OCA1*)**	**ΔC_T(CP Target-CP Control)**	**$2^{-\Delta\Delta CT}$**	**$2^{-\Delta\Delta CT}$as fold of wild type**
Wild Type control	18.18	22.53	4.35	0.05	1.00
Wild Type with H_2O_2	20.88	25.42	4.54	0.04	0.88
H7 control	18.17	23.20	5.03	0.03	0.62
H7 with H_2O_2	17.98	22.48	4.50	0.04	0.90

Table 3.28 : Average value was taken of $2^{-\Delta\Delta CT}$ as fold of wild type and their standard deviation was calculated. Results from continuous hydrogen peroxide application to strain.

	Set 1	**Set 2**	**AVG**	**ST DEV**
Wild Type control	1.00	1.00	1.00	0.00
Wild Type with H_2O_2	0.67	0.88	0.78	0.14
H7 control	0.59	0.62	0.61	0.02
H7 with H_2O_2	1.70	0.90	0.80	0.14

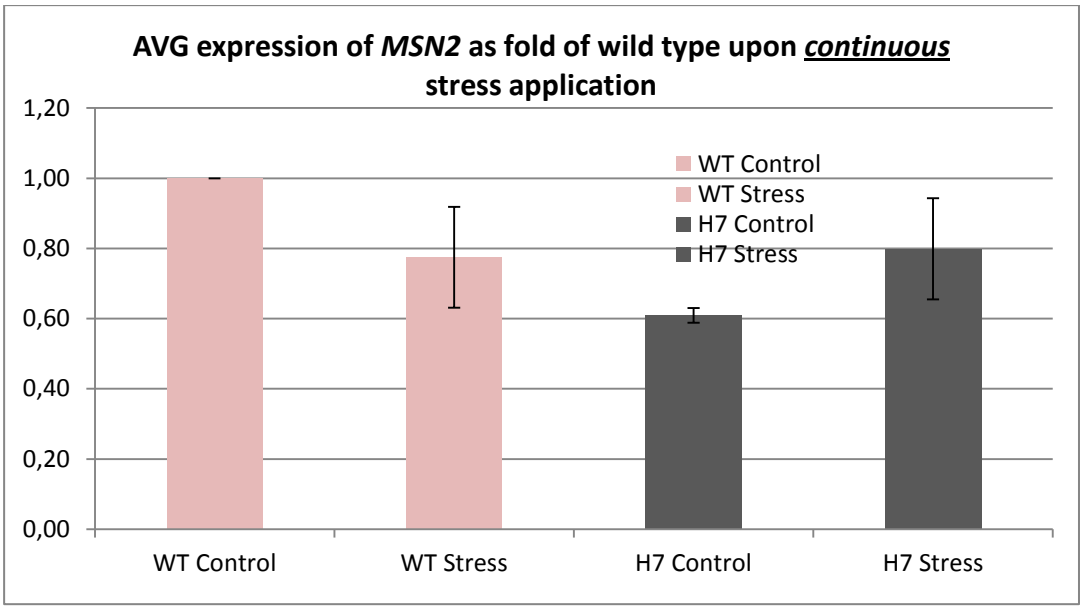

Figure 3.34 : Graphical representation of $2^{-\Delta\Delta CT}$ as fold of wild type values. Graphic represents average value of $2^{-\Delta\Delta CT}$ as fold of wild type.

As a result, *MSN2* was down regulated for H7 when compared with wild type in the control conditions. It was unexpected to see that H_2O_2 stress application decreased *MSN2* expression for both wild type and H7 (Figure 3.34).

3.3.3 Determination of *MSN4* expression level by qPCR analysis

Expression level determination via qPCR of *MSN4* was carried out. Same RNA and cDNA with RNA and cDNA was used for *MSN4* expression level determination. The eaction error, efficiency and slope values are given on Table 3.29.

Table 3.29 : Error, efficiency and slope of *ACT1* and *MSN4* for each experiment set.

	1st set of experiment		**2nd set of experiment**	
	ACT1	***MSN4***	***ACT1***	***MSN4***
Error	0.01	0.08	0.06	0.00
Efficiency	2.01	1.99	1.94	2.01
Slope	-3.31	-3.35	-3.49	-3.30

Table 3.30 : CP, calculated ΔC_T and $2^{-\Delta\Delta CT}$ values of first experiment set of *MSN4*.

Set1	CP (*ACT1*)	CP (*MSN4*)	ΔC_T(CP Target-CP Control)	$2^{-\Delta\Delta CT}$	$2^{-\Delta\Delta CT}$ as fold of wild type
Wild Type control	18.54	20.47	1.93	0.26	1.00
Wild Type with H_2O_2	20.92	23.23	2.31	0.20	0.77
H7 control	18.20	21.07	2.87	0.14	0.52
H7 with H_2O_2	20.92	21.30	3.34	0.10	0.38

Table 3.31 : CP, calculated ΔC_T and $2^{-\Delta\Delta CT}$ values of second experiment set of *MSN4*.

Set2	CP (*ACT1*)	CP (*OCA1*)	ΔC_T(CP Target-CP Control)	$2^{-\Delta\Delta CT}$	$2^{-\Delta\Delta CT}$ as fold of wild type
Wild Type control	18.18	22.56	1.02	0.25	1.00
Wild Type with H_2O_2	20.88	23.35	2.43	0.19	0.75
H7 control	18.17	21.60	3.40	0.09	1.38
H7 with H_2O_2	17.98	21.44	3.48	0.09	1.36

Table 3.32 : Average value was taken of $2^{-\Delta\Delta CT}$ as fold of wild type and their standard deviation was calculated for *MSN4* gene expression.

	Set 1	Set 2	AVG	ST DEV
Wild Type control	1.00	1.00	1.00	0.00
Wild Type with H_2O_2	0.77	0.75	0.76	0.01
H7 control	0.52	0.45	0.45	0.10
H7 with H_2O_2	1.38	0.36	0.76	0.0.1

As a result, *MSN4* was down-regulated for H7 when compared with wild type. Moreover, H_2O_2 presence reduced expression of *MSN4* for both wild type and oxidative stress resistant mutant (Figure 3.35).

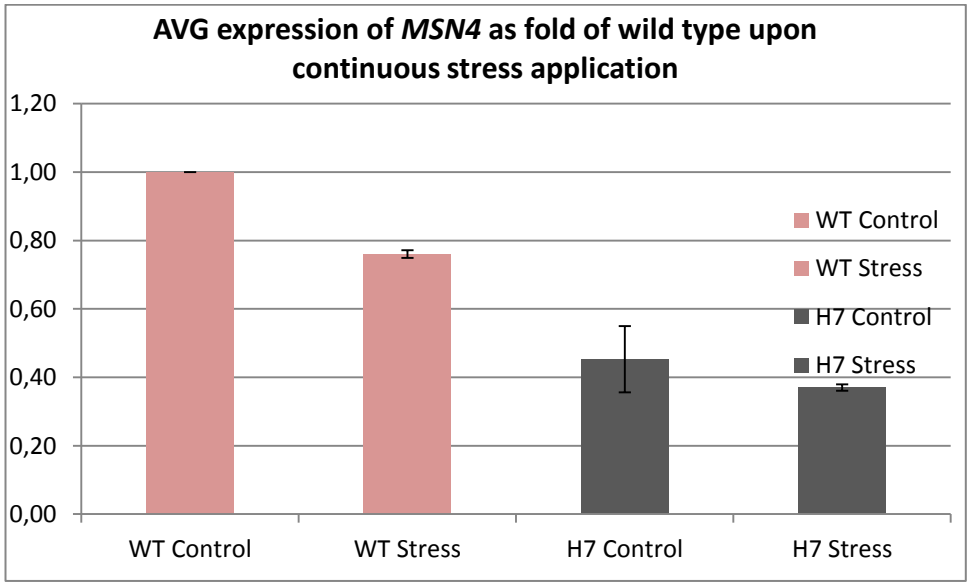

Figure 3.35 : Graphical representation of $2^{-\Delta\Delta CT}$ as fold of wild type values. Graphic represents average value of $2^{-\Delta\Delta CT}$ as fold of wild type.

3.4 Microarray Analysis of wild type and oxidative stress resistant mutant

Microarray analysis was carried out by using kits and microarrays from Agilent Microarray technology. RNA purification was realized from wild type and H7 samples at their exponential phase (OD_{600} ~1). Wild type and H7 were grown in parallel for 4 and 3 times respectively in order to have biological replicates. OD_{600} values at which RNA purification was applied are shown in the Table 3.33. RNA amount and quality was measured by NanoDrop and BioAnalyser, respectively. Nanodrop gave information about RNA concentration, protein/DNA and ethanol etc. contamination. Bioanalyser also gave RNA concentration and integrity of RNA information (RIN value). Samples corresponding RNA concentration and their RIN values are given respectively on Table 3.34 and 3.35.

Table 3.33 : Initial and just before purification OD_{600} values of cultures.

Strain	**Initial OD_{600} of the cultures**	**OD_{600} of the cultures just before the RNA extraction**	
		Culture 1	**Culture 2**
Wild type	0.12	1.16	1.08
Mutant	0.10	0.96	0.97

Table 3.34 : Nanodrop measurements of RNA of strains.

Strain	**ng/µl of the cultures**	
	Culture 1	**Culture 2**
Wild type	625	455
Mutant	9.2	9.3

Table 3.35 : RNA integrity number of RNA of strains.

Strain	**RIN of the cultures**	
	Culture 1	**Culture 2**
Wild type	9.9	10.0
Mutant	9.2	9.3

According to microarray results, two-fold change or more changes were accepted as significant when expression level of wild type and H7 were considered. There are 400 downregulated and 675 upregulated genes for mutant when compared to wild type with a *p* value lower than 0.05. However, it was interesting that *GPH1*, *PGM2*, *TSL1*, *HXK1*, *HSP12*, *CYC7*, *RTN2*, *BDH2* and *YNL194C* were upregulated with 50 fold in H7 compared to wild type. Most important upregulation was observed in *CYC7*. Its expression level was 200 fold to that of wild type. It encodes cytochrome c isoform 2. It has a role on electron transport chain and its expression level increases

under hypoxic conditions (Montgomery D. L.,1980). Some important upregulated genes and functions are given on the Table 3.36. Additionally, genes which have 5 fold increase in mutant are indicated with information on general name, systematic name and role of genes as a Table in Appendix. These gene products were generally related to carbohydrate and energy metabolism. Expression of trehalose and glycogen anabolism genes were very significant. Moreover, expression of *GPH1*,which has a has a role on glycogen catabolism, was also high. These expression level results were in parallel with the high trehalose and glycogen production characteristics of the mutant, H7. Besides, maltose concentration that was produced by mutant was observed higher than wild type by HPLC measurement. The increase in expression level of glycogen catabolism genes supported this result.

Table 3.36 : Genes whose expression was 50 fold higher or equal to that of wild type.

Gene Name	Molecular Function
GPH1	Glycogen phosphorilase activity
BDH2	Diacetyl reductase ((R)-acetoin forming activity) activity
TSL1	Trehalose-phosphatase activity; alpha, alpha-trehalose-phosphate synthase activity
PGM2	Phosphoglucomutase activity Intramolecular transferase activity,phosphotransferases
HXK1	Hexokinase activity

Intracellular metabolism of glucose starts with phosphorylation of glucose at C6. Glucose catabolism, glucose storage as trehalose and glycogen begin with this chemical reaction. Three enzymes, hexokinases Hxk1p, Hxk2p, and glucokinase Glk1p catalyze this step. *HXK1, HXK2* and *GLK1* provide uptake of glucose (Bisson L. F., 1983). *HXK1* and *HXK2* are isoenzymes, but *HXK2* is dominant hexokinase enzyme (Walsh R. B., 1983). *HXK2* is expressed when cell grown on fermentable medium, but cells went on non-fermentable carbon source *HXK2* is repressed and *HXK1*and *GLK1* are de-repressed (Lobo Z., 1977). *HXK2* is 50-fold upregulated in the mutant. This can be explained by stress resistant phenotype of the mutant, H7. Therefore, the individual can have a metabolism to protect itself from any stress condition. One of the most important upregulation is *TDH1*. *TDH1*, *TDH2* and *TDH3* are catalyzed reaction of glyceraldehyde-3-phosphate to 1,3 bis-phosphoglycerate (McAlister, 1985). Interestingly, *TDH1* expression is observed in stationary phase (Delgado M. L., 2001). *HXK1* and *TDH1* are normally upregulated under limited

glucose condition and both of these genes are highly expressed in H7. The high expression characteristics of H7 for *HXK1* and *TDH1* genes can be evolved during the long and stresful selection and evolution procedure to survive in any condition, primarily to adaptation to starvation condition and utilizing non-fermentable carbon source.

PGM2 is also one of the highest upregulated genes. Their upregulation can be observed on images of trehalose-glycogen biosynthesis and carbon metabolism pathways (Figure 3.36, 3.37, 3.38). *PGM1* and *PGM2* catalyze from glucose-1-phosphate and glucose-6-phosphate (Dey N. B., 1994). They encode phosphoglucomutase. *S. cerevisiae* has two isoform of phosphoglucomutase, major isoform is Pgm2p and minor isoform is Pgm1p. *PGM2* expression of mutant is 50 fold higher than that of wild type. Previous studies (Lee K. S., 2011) showed that overexpression of *PGM2* incerased the fermentative growth (Lee K.S, 2011). This phenotype is observed for oxidative stress resistant mutant when HPLC results were considered. All of genes worked on glycogen and trehalose metabolism had high expression levels. These results are corrected enzymatic trehalose and glycogen determination experiment results. Other than this, previous studied that shows that lack of *PGM2* decreases iron resistance of strain (Mulet J. M., 2004). Additionally, impotant, *PGM2* (phosphoglucomutase) expression is regulated by *YAP1*. Oxidative stress resistant mutant used in study is sensitive to iron as results of spot assay made by treating strain with iron to strain. In the light of these informations, relation of iron resistance and effects of *PGM2* was proved. Besides, *YAP1* deletion strain show high sensitivity to iron (Figure 3.11).

Another interesting point is degradation of glycogen and trehalose. As HPLC data gave information about maltose production, while wild type did not produce maltose, mutant produce maltosed continously. Additionally, nearly all trehalose and glycogen degradation genes expression increased for H7 on microarray data. These results overlap. Moreover, *IGD1* expression was upregulated in H7. This gene product inhibits *GDB1* (Figure 3.36). Maybe, *IGD1* protected glycogen structure without degradation to maltose in cell which provided much more time for survival. As a result, both gene expression levels could be increased, but H7 resistance can be canalized to survive by utilizing carbon source. This increase in carbon utilisation could be seen also in glycolysis and TCA.

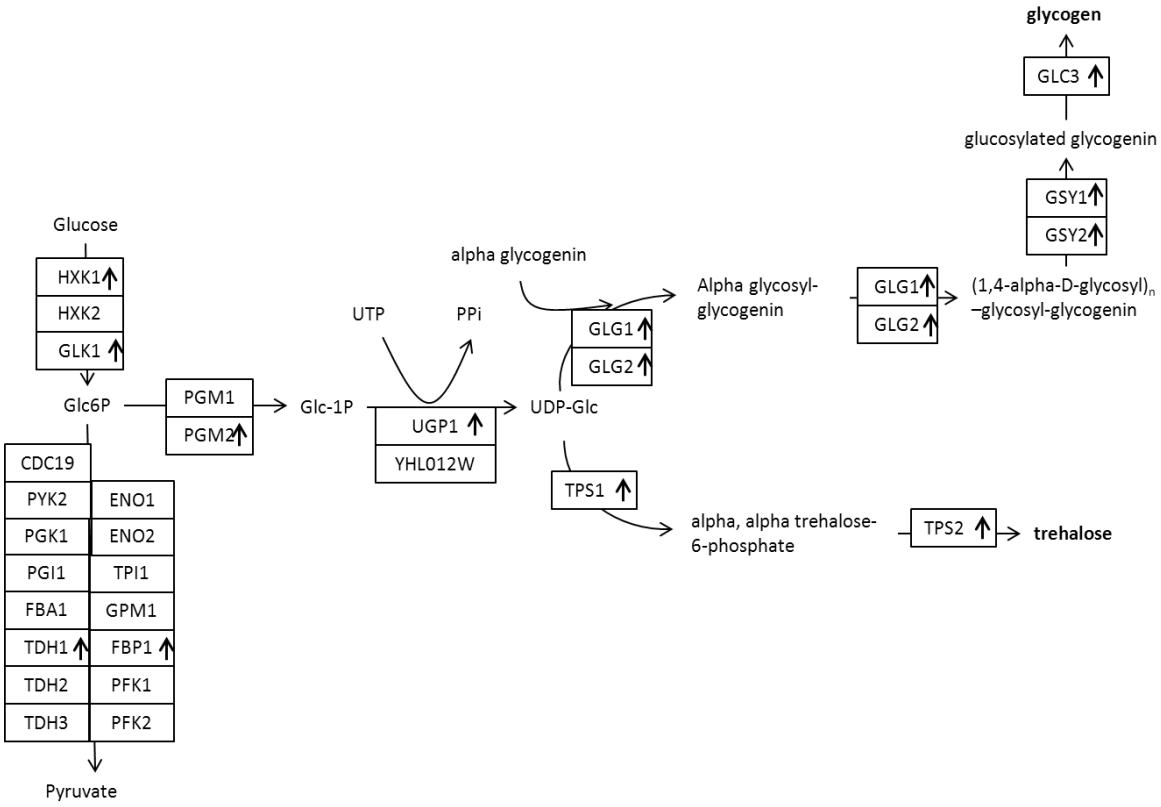

Figure 3.36 : Trehalose and glycogen biosynthesis pathway of *S. cerevisiae*. Up-arrows show upregulated genes.

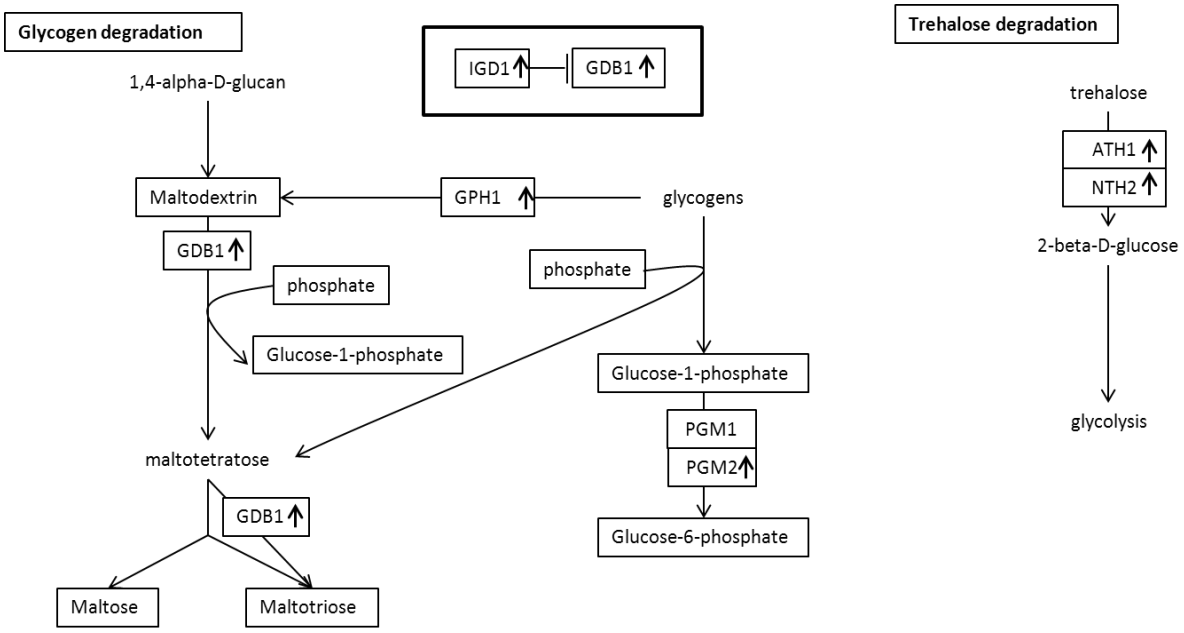

Figure 3.37 : Trehalose and glycogen degradation pathway of *S. cerevisiae*.

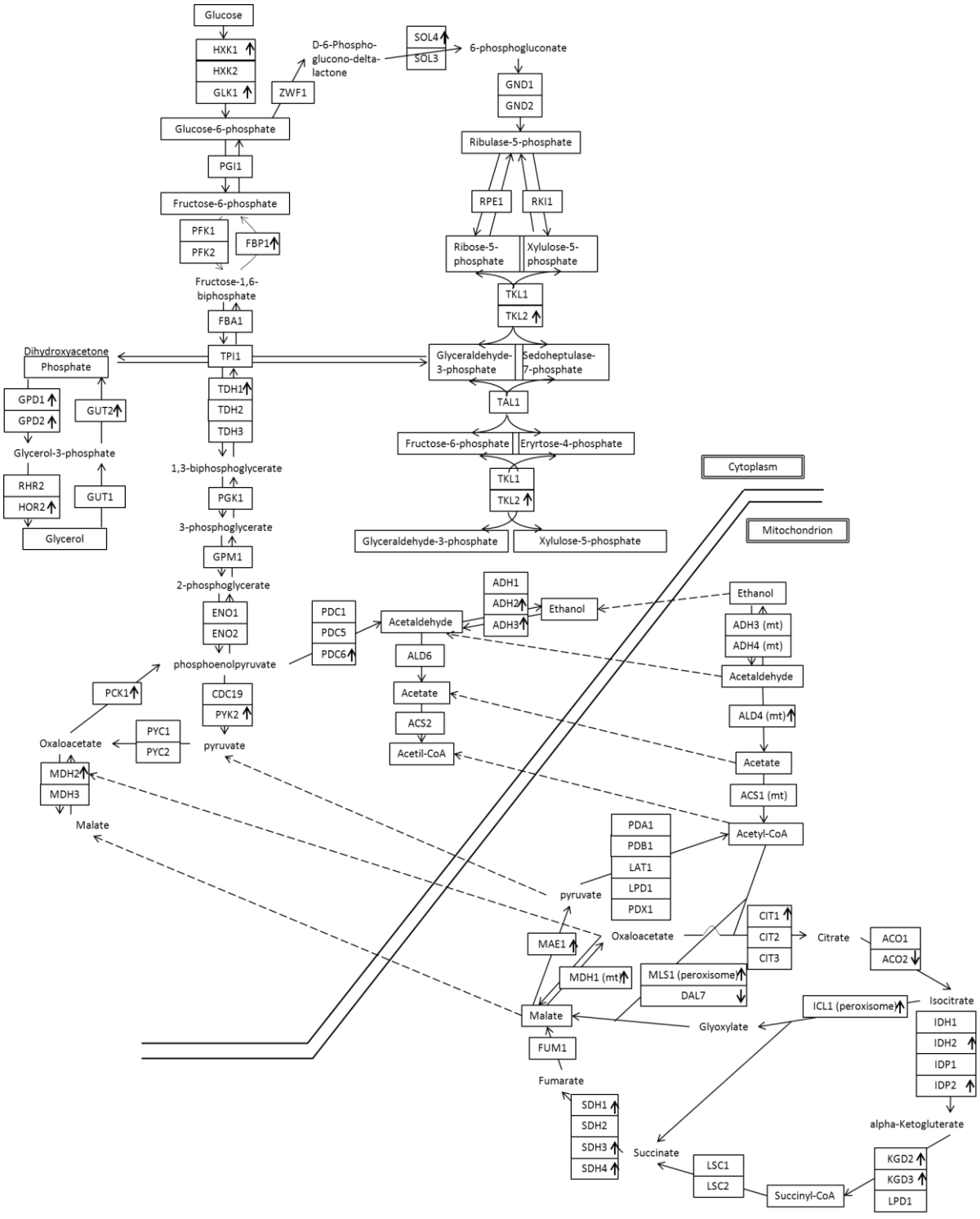

Figure 3.38 : Basic carbon metabolism of *S. cerevisiae* Up-arrows indicate upregulated genes and down-arrows remark down regulated genes in mutant.

4. CONCLUSIONS

In this study oxidative stress-resistant *Saccharomyces cerevisiae* mutant, H7, that was obtained by evolutionary engineering strategy, was investigated for its genetic, phenotypic, physiologic and transcriptomic characteristics. Within this scope, mutant, diploid and gene knock-out strains were tested by spot assay as phenotyping study. HPLC analysis and enzymatic trehalose and glycogen determination was carried out for physiological research. Finally, microarray and qPCR were performed within the context of transcriptomic analysis.

As spot assay results, hydrogen peroxide stress resistant mutant can survive and grow more than wild type in medium supplemented with acetate and ethanol as carbon sources instead of glucose. It indicated that mutant could consume this carbon source better than the wild type. Oxidative stress resistance could provide the mutant t ability of utilizing different carbon sources. The other spot assay where calcium supplemented medium was used supported other studies for the effect of calcium on oxidative stress. Moreover, it was shown that calcium may help oxidative stress resistant mutant to survive more than the wild type as mentioned in the literature Furthermore, another experiment carried out with diploid cells, gave a clue on semidominance of oxidative stress resistance of hydrogen peroxide resistant mutant (Popa C. V., 2010).

The most importance differences based on HPLC analysis results were maltose and acetate production profiles of wild type and H7. Interestingly, oxidative stress resistant mutant produced much more maltose than the wild type during stationary phase. Additionally, H7 accumulated trehalose and glycogen more than wild type. Nevertheless, trehalose and glycogen concentrations were decreasing during stationary phase for mutant. Glycogen decrease and maltose increase at the stationary phase pointed the glycogen catabolism in H7 cells. Besides, as results of microarray, glycogen catabolism genes expression levels were very high for the mutant. High expression level of glycogen catabolism genes might prove that glycogen was degraded to maltose in starvation condition at stationary phase.

Furthermore, acetate amount decreased during stationary phase. The mutant could survive on acetate supplemented solid minimal medium. These two experimental results showed that acetate catabolism of mutant might have been differentiated.

qPCR was performed for *OCA1*, *MSN2* and *MSN4*, and microarray was carried out to have all information of entire gene expression level of oxidative stress resistant mutant. *OCA1* is putative tyrosine kinase and in this study both in microarray and qPCR experiments; there was no significant increase of decrease in expression levels. *MSN2* and *MSN4* are general stress response genes and for these genes, there were also no significant expression level diffences between wild type and the mutant (Alic N., 2001).

According to microarray data, *CYC7*, produces 'cytochrome c isoform 2', expression level had a significant upregulation for mutant. *CYC7* occupy in mitochondrial intermembrane space and have a role that transfers electrons from ubiquinone-cytochrome c oxidoreductase to cytochrome c oxidase during respiration (Burke P. V., 1997; Downie J. A., 1977). On the other hand, trehalose and glycogen metabolism genes were significantly as remarkable.

In accordance with microarray results, genes of trehalose and glycogen biosynthesis metabolism were upregulated at high levels.

As a future remark, microarray results should be analyzed in more detail and genes/regulators of glycogen metabolism should be investigated carefully, using detection mutants and/or overexpression studies.

REFERENCES

Alic N, Higgins VJ, Pichova A, Breitenbach M, Dawes IW (2003) Lipid hydroperoxides activate the mitogen-activated protein kinase Mpk1p in Saccharomyces cerevisiae. Journal of Biological Chemistry 278:41849-41855.

Ames BN, Shigenaga MK, Hagen TM (1993) Oxidants, antioxidants, and the degenerative diseases of aging. Proceedings of the National Academy of Sciences of the United States of America 90:7915-7922.

Anderegg RJ, Betz R, Carr SA, Crabb JW, Duntze W (1988) Structure of *Saccharomyces cerevisiae* mating hormone A-factor - identification of S-farnesyl cysteıne as a structural component. Journal of Biological Chemistry 263:18236-18240.

Antunes F, Han D, Cadenas E (2002) Relative contributions of heart mitochondria glutathione peroxidase and catalase to H_2O_2 detoxification in in vivo conditions. Free Radical Biology and Medicine 33:1260-1267.

Arguelles JC (2000) Physiological roles of trehalose in bacteria and yeasts: a comparative analysis. Archives of Microbiology 174:217-224.

Astell CR, Ahlstromjonasson L, Smith M, Tatchell K, Nasmyth KA, Hall BD (1981) The sequence of the dnas coding for the mating-type loci of *Saccharomyces cerevisiae*. Cell 27:15-23.

Baudin A, Ozier-Kalogeropoulos O, Denouel A, Lacroute F, Cullin C. (1993) A simple and efficient method for direct gene deletion in Saccharomyces cerevisiae. Nucleic Acid Research 21:3329-3330

Bailey JE (1991) Toward a science of metabolic engineering. Science 252:1668-1675.

Bailey JE, Sburlati A, Hatzimanikatis V, Lee K, Renner WA, Tsai PS (1996) Inverse metabolic engineering: A strategy for directed genetic engineering of useful phenotypes. Biotechnology and Bioengineering 52:109-121.

Bienert GP, Schjoerring JK, Jahn TP (2006) Membrane transport of hydrogen peroxide. Biochimica Et Biophysica Acta-Biomembranes 1758:994-1003.

Bisson LF and Fraenkel DG (1983) Involvement of kinases in glucose and fructose uptake by *Saccharomyces cerevisiae.* Proceedings of the National Academy of Sciences of the United States of America 80(6):1730-4

Burke PV (1997) Effects of oxygen concentrarion on the expression of cytochrome c in Saccharomyces cerevisiae Journal of Biological Chemistry 272(23):14705-12

Cakar ZP, Turanli-Yildiz B, Alkim C, Yilmaz U (2012) Evolutionary engineering of Saccharomyces cerevisiae for improved industrially important properties. Federation of European Microbiological SocitesYeast Res 12:171-182.

Cramp DG (1967) New automated method for measuring glucose by glucose oxidase. Journal of Clinical Pathology 20:910-912.

Crowe JH, Crowe LM, Chapman D (1984) Preservation of membranes in anhydrobiotic organisms: the role of trehalose. Science 223:701-703.

de Polavieja G, Karathia H, Vilaprinyo E, Sorribas A, Alves R (2011) *Saccharomyces cerevisiae* as a Model Organism: A Comparative Study. Public Library of Science ONE 6:e16015.

Delgado ML (2001) The glyceraldehyde-3-phosphate dehydrogenase polypeptides encoded by Saccharomyces cerevisiae *TDH1*, *TDH2* and *TDH3* genes are also cell Wall proteins. Microbiology 147(Pt2):411-7

Dey NB (1994) The glycosylation of phosphoglucomutase is modulated by source and heat shock in *Saccharomyces cerevisiae*. J Biol Chem 269(43):27143-8

Downie JA (1977) Structural gene for yeast iso-2-cytochrome c. J Mol Biol 113(2):369-84

Frankenberg D, Frankenberg-Schwager M, Harbich R (1993) Mechanisms of oxygen radiosensitization in irradiated yeast. I. DNA double-strand breakage. Int J Radiat Biol 64:511-521.

Gershon H, Gershon D (2000) The budding yeast, *Saccharomyces cerevisiae*, as a model for aging research: a critical review. Mechanisms of Ageing and Development 120:1-22.

Gibson BR, Lawrence SJ, Boulton CA, Box WG, Graham NS, Linforth RST, Smart KA (2008) The oxidative stress response of a lager brewing yeast strain during industrial propagation and fermentation. Fems Yeast Research 8:574-585.

Gietz RD, Woods RA (2002) Transformation of yeast by lithium acetate/single-stranded carrier DNA/polyethylene glycol method. Methods Enzymol 350:87-96.

Glover JR, Lindquist S (1998) Hsp104, Hsp70, and Hsp40: A novel chaperone system that rescues previously aggregated proteins. Cell 94:73-82.

Goffeau A (1996) 1996: A vintage year for yeast and Yeast. Yeast 12:1603-1605.

Goffeau A, Barrell BG, Bussey H, Davis RW, Dujon B, Feldmann H, Galibert F, Hoheisel JD, Jacq C, Johnston M, Louis EJ, Mewes HW, Murakami Y, Philippsen P, Tettelin H, Oliver SG (1996) Life with 6000 genes. Science 274:546-&.

Halliwell B, Gutteridge JMC (2007) Free radicals in biology and medicine. Oxford ; New York: Oxford University Press.

Hartwell LH (1974) *Saccharomyces-cerevisiae* cell-cycle. Bacteriological Reviews 38:164-198.

Hasan R, Leroy C, Isnard AD, Labarre J, Boy-Marcotte E, Toledano MB (2002) The control of the yeast H2O2 response by the Msn2/4 transcription factors. Molecular Microbiology 45:233-241.

Hohmann S, Mager WH (2003) Yeast Stress Responses: Springer.

Hon T, Dodd A, Dirmeier R, Gorman N, Sinclair PR, Zhang L, Poyton RO (2003) A mechanism of oxygen sensing in yeast. Multiple oxygen-responsive steps in the heme biosynthetic pathway affect Hap1 activity. J Biol Chem 278:50771-50780.

Jamieson DJ (1998) Oxidative stress responses of the yeast *Saccharomyces cerevisiae*. Yeast 14:1511-1527.

Jennings DH (1993) Stress tolerance of fungi. New York: M. Dekker.

Karathia H, Vilaprinyo E, Sorribas A, Alves R (2011) *Saccharomyces cerevisiae* as a Model Organism: A Comparative Study. PLoS ONE 6.

Kawada Y, Khan M, Sharma AK, Ratnayake DB, Dobashi K, Asayama K, Moser HW, Contreras MA, Singh I (2004) Inhibition of peroxisomal functions due to oxidative imbalance induced by mistargeting of catalase to cytoplasm is restored by vitamin E treatment in skin fibroblasts from Zellweger syndrome-like patients. Mol Genet Metab 83:297-305.

Kurtz S, Rossi J, Petko L, Lindquist S (1986) An ancient developmental induction - heat-shock proteins induced in sporulation and oogenesis. Science 231:1154-1157.

Lee KS (2011) Improved galactose fermentation of Saccharomyces cerevisiae through inverse metabolic engineering. Biotechnol Bioeng 108(3):621-31

Lobo Z and Maitra PK (1977) Physiological role of glucose-phosphorylating enzymes in Saccharomyces cerevisiae. Arch Biochem Biophys 182(2):639-45

Martinez-Pastor MT, Marchler G, Schuller C, Marchler-Bauer A, Ruis H, Estruch F (1996) The *Saccharomyces cerevisiae* zinc finger proteins Msn2p and Msn4p are required for transcriptional induction through the stress response element (STRE). EMBO J 15:2227-2235.

McAlister L and Holland MJ (1985) Isolation and characterization of yeast strains carrying mutations in the glyceraldehyde-3-phosphate dehydrogenase genes. J Biol Chem 260(28):15013-8

Michels CVA (2002) Genetic Techniques for Biological Research: A Case Study Approach: J. Wiley.

Mitchel REJ, Morrison DP (1982) Heat-shock induction of ionızing-radiation resistance in *Saccharomyces cerevisiae*, and correlation with stationary growth phase. Radiation Research 90:284-291.

Monaro KA (2011) The Response to Heat Shock and Oxidative Stress in Saccharomyces cerevisiae.

Montgomery DL (1980) Isolation and sequence of the gene for iso-2-cytochrome c in *Saccharomyces cerevisiae.* Proc Natl AcadSci USA 77(1):541-5.

MoradasFerreira P, Costa V, Piper P, Mager W (1996) The molecular defences against reactive oxygen species in yeast. Molecular Microbiology 19:651-658.

Mulet JM (2004) The trehalose pathway and intracellular glucose phosphates as modulators of potassium transport and general cation homeostasis in yeast. Yeast 21(7): 569-82

Nadal E, Ammerer G, Posas F, (2011) Controlling gene expression in response to stress Vol:12: 833-845

Popa C-V, Dumitru I, Ruta LL, Danet AF, Farcasanu IC (2010) Exogenous oxidative stress induces Ca^{2+} release in the yeast Saccharomyces

Roth R, Halvorson HO (1969) Sporulation of yeast harvested during logarithmic growth. J Bacteriol 98:831-832.

Schaechter M (2009) Desk Encyclopedia of Microbiology: Academic Press.

Schmitt AP, McEntee K (1996) Msn2p, a zinc finger DNA-binding protein, is the transcriptional activator of the multistress response in *Saccharomyces cerevisiae*. Proc Natl Acad Sci U S A 93:5777-5782.

Schmittgen TD, Livak KJ (2008) Analyzing real-time PCR data by the comparative C(T) method. Nat Protoc 3:1101-1108.

Sillje HHW, Paalman JWG, ter Schure EG, Olsthoorn SQB, Verkleij AJ, Boonstra J, Verrips CT (1999) Function of trehalose and glycogen in cell cycle progression and cell viability in *Saccharomyces cerevisiae*. Journal of Bacteriology 181:396-400.

Stahl F (1996) Meiotic recombination in yeast: Coronation of the double-strand-break repair model. Cell 87:965-968.

Steels EL, Learmonth RP, Watson K (1994) Stress tolerance and membrane lipid unsaturation in *Saccharomyces cerevisiae* grown aerobically or anaerobically. Microbiology 140 (Pt 3):569-576.

Walsh RB (1983) Cloning of genes that complement yeast hexokinase and glucokinase mutants. J Bacteriol 154(2): 1002-4

Whitmarsh AJ, Davis RJ (1998) Structural organization of MAP-kinase signaling modules by scaffold proteins in yeast and mammals. Trends in Biochemical Sciences 23:481-485.

You KM, Rosenfield CL, and Knipple DC (2002) Ethanol Tolerance in the Yeast Saccharomyces cerevisiae Is Dependent on Cellular Oleic Acid Content. Applied and Environmental Microbiology p. 1499–1503.

APPENDICES

APPENDIX A: Tables of Microarray

APPENDIX A: Tables of Microarray

Table A.1 : Upregulated genes on mutant with 2 fold increased compared to wild type. Systematic name, gene name and average fold change of mutant are given.

	Systematic name	Gene name	Average Fold Change
Response to stress	YAL005C	*SSA1*	6.29
	YBR072W	*HSP26*	41.20
	YBR126C	*TPS1*	5.019
	YBR169C	*SSE2*	7.04
	YCR021C	*HSP30*	6.93
	YDL079C	*MRK1*	5.73
	YDL222C	*FMP45*	42.81
	YDR074W	*TPS2*	12.46
	YDR171W	*HSP42*	6.61
	YDR258C	*HSP78*	12.28
	YER103W	*SSA4*	8.74
	YFL014W	*HSP12*	96.76
	YGR088W	*CTT1*	39.51
	YGR243W	*FMP43*	13.08
	YIL101C	*XBP1*	10.99
	YLL026W	*HSP104*	6.87
	YML100W	*TSL1*	61.37
	YOL052C-A	*DDR2*	20.79
	YOR049C	*RSB1*	24.95
	YPL223C	*GRE1*	6.40
	YPL240C	*HSP82*	8.21
Glycogen biosynthetic process	YEL011W	*GLC3*	15.51
	YFR015C	*GSY1*	20.66
	YKL035W	*UGP1*	7.12
	YKR058W	*GLG1*	6.12
	YLR258W	*GSY2*	11.98
	YMR105C	*PGM2*	50.73
	YOR187C	*GAC1*	13.76
Carbonhydrate metabolic process	YBR018C	*GAL7*	16.03
	YBR299W	*MAL32*	9.97
	YCL040W	*GLK1*	6.51
	YDR516C	*EMI2*	16.23
	YEL011W	*GLC3*	15.51
	YFR053C	*HXK1*	61.73
	YGR043C	*NQM1*	9.44
	YGR194C	*XKS1*	6.11
	YGR248W	*SOL4*	28.11
	YGR292W	*MAL12*	10.63
	YIL099W	*SGA1*	7.78

	YLR377C	*FBP1*	10.00
	YMR085W		6.85
	YMR105C	*PGM2*	50.73
	YPR160W	*GPH1*	51.78
Trehalose biosynthetic process	YBR126C	*TPS1*	5.02
	YDR074W	*TPS2*	12.46
	YKL035W	*UGP1*	7.12
	YML100W	*TSL1*	61.37
	YMR105C	*PGM2*	50.73
Glucose 6-phosphate metabolic process	YLC040W	*GLK1*	6.51
	YDR516C	*EMI2*	16.23
	YMR105C	*PGM2*	50.73
Ethanol metabolic process	YLR251W	*SYM1*	8.99
	YMR303C	*ADH2*	14.45
	YOR374W	*ALD4*	7.76
Carbohydrate transport	YDR342C	*HXT7*	27.39
	YDR343C	*HXT6*	24.59
	YDR536W	*STL1*	18.73
	YGR289C	*MAL11*	7.43
	YHR096C	*HXT5*	17.08
	YLR081W	*GAL2*	6.80
Pentose-phosphate shunt	YBR117C	*TKL2*	20.79
	YGR043C	*NQM1*	9.44
	YGR248W	*SOL4*	28.11
	YGR256W	*GND2*	8.87
Protein refolding	YAL005C	*SSA1*	6.29
	YBR169C	*SSE2*	7.04
	YDR258C	*HSP78*	12.28
	YPL240C	*HSP82*	8.21
Energy reserve metabolic process	YER067W	*RGI1*	23.30
	YIL057C	*RGI2*	7.07
Maltose catabolic process, sucrose catabolic process	YBR299W	*MAL32*	9.97
	YGR292W	*MAL12*	10.63
Glycolysis	YCL040W	*GLK1*	6.51
	YDL021W	*GPM2*	13.85
	YDR516C	*EMI2*	16.23
	YFR053C	*HXK1*	61.73
	YOR347C	*PYK2*	5.71
Cellular response to oxidative stress	YDR453C	*TSA2*	7.15
	YFL014W	*HSP12*	96.76
	YJR096W		9.05
	YKL026C	*GPX1*	7.91
	YKL086W	*SRX1*	5.07
	YMR250W	*GAD1*	21.93
	YOR120W	*GCY1*	8.29
Protein unfolding	YDR258C	*HSP78*	12.28
	YLL026W	*HSP104*	6.87
Glucose import, mannose metabolic process	YCL040W	*GLK1*	6.51
	YFR053C	*HXK1*	61.73
Glycogen catabolic process	YIL099W	*SGA1*	7.78

	YPR160W	*GPH1*	51.78
Oxidation-reduction process	YAL061W	*BDH2*	56.64
	YDL085W	*NDE2*	6.23
	YDR453C	*TSA2*	7.15
	YGR088W	*CTT1*	39.51
	YGR256W	*GND2*	8.87
	YHL021C	*AIM17 FMP12*	10.44
	YIL111W	*COX5B*	6.49
	YJR096W		9.05
	YKL026C	*GPX1*	7.91
	YKL086W	*SRX1*	5.07
	YML054C	*CYB2*	7.62
	YMR169C	*ALD3*	31.96
	YMR303C	*ADH2*	14.45
	YOR120W	*GCY1*	8.29
	YOR374W	*ALD4*	7.76
	YPL017C	*IRC15*	6.69
Maltose metabolic process	YBR299W	*MAL32*	9.97
	YGR289C	*MAL11*	7.43
	YGR292W	*MAL12*	10.63
D-xylose catabolic process, arabinose catabolic process	YJR096W		9.05
	YOR120W	*GCY1*	8.29
Negative regulation of peptidase activity	YLR178C	*TFS1*	12.07
	YMR174C	*PAI3*	6.00
Cellular response to water deprivation	YGR088W	*CTT1*	39.51
	YJL144W		7.95
Hexose transport	YDR342C	*HXT7*	27.39
	YDR343C	*HXT6*	24.59
	YHR096C	*HXT5*	17.08
Pentose-phosphate shunt, oxidative	YGR248W	*SOL4*	28.11
	YGR256W	*GND2*	8.87
glycogen metabolic process	YER054C	*GIP2*	5.91
	YOR178C	*GAC1*	13.76
	YPR160W	*GPH1*	51.78
Ammonium transmembrane transporter activity	YCR010C	*ADY2*	11.89
	YNR002C	*ATO2/ FUN34*	6.03
Alditol:NADP+ 1-oxidoreductase activity	YJR096W		9.05
	YOR120W	*GCY1*	8.29
Transporter activity	YDR342C	*HXT7*	27.39
	YDR343C	*HXT6*	24.59
	YDR536W	*STL1*	18.73
	YGL104C	*VPS73*	5.26
	YGR289C	*MAL11*	7.43
	YHR096C	*HXT5*	17.08
	YLR081W	*GAL2*	6.80

Table A.2 : Downregulated genes on H7 compared to wild type with a 2 fold decrease. Systematic name, gene name and average fold change of H7 was shown.

	Systematic name	Gene name	Average Fold Change
ribosome biogenesis	YBR267W	*REI1*	6.08
	YER127W	*LCP5*	5.09
	YHR066W	*SSF1*	5.59
	YIL091C	*UTP25*	5.00
	YJL122W	*ALB1*	6.70
	YKL078W	*DHR2*	5.15
	YKR024C	*DBP7*	7.03
	YNL112W	*DBP2*	7.70
	YNL182C	*IPI3*	5.04
	YPL043W	*NOP4*	5.02
rRNA processing	YDR299W	*BFR2*	5.01
	YER127W	*LCP5*	5.09
	YGR159C	*NSR1*	7.19
	YIL091C	*UTP25*	5.00
	YKL078W	*DHR2*	5.15
	YKR024C	*DBP7*	7.03
	YNL112W	*DBP2*	7.70
	YNL182C	*IPI3*	5.04
	YPL043W	*NOP4*	5.02
ribosomal large subunit assaembly	YCR072C	*RSA4*	5.74
	YHR066W	*SSF1*	5.59
	YKR024C	*DBP7*	7.03
	YNL182C	*IPI3*	5.04
ribosomal large subunit biogenesis	YAL025C	*MAK16*	5.18
	YBR267W	*REI1*	6.08
	YJL122W	*ALB1*	6.70
conjugation with cellular fusion	YDL039C	*PRM7*	5.23
	YHR066W	*SSF1*	5.59
negative regulation of transcription termination, DNA-dependent, peptidyl-arginine methylation, protein methylation	YBR034C	*HMT1*	5.77
high-affinity zinc ion transport, zinc ion transmembrane transport	YGL255W	*ZRT1*	8.41

CURRICULUM VITAE

Name Surname:	Nazlı KOCAEFE
Place and Date of Birth:	Malatya/ 24.10.1987
Address:	126/11 Sk. Esen 85 Sitesi A/2 Blok D:3 Evka 3/ Bornova/ İzmir
E-Mail:	nazlikocaefe@gmail.com
B.Sc.:	Istanbul University/ Molecular Biology and Genetics
M.Sc.:	Istanbul Technical University/ Molecular Biology-Genetics and Biotechnology

PUBLICATIONS/PRESENTATIONS ON THE THESIS

- **Nazli Kocaefe**, Ulku Yilmaz, Ceren Alkim, Z. Petek Cakar, Physiological and Genetic Analyses of an Oxidative Stress-Resistant *Saccharomyces cerevisiae* Mutant. International Conference on Enzyme Science and Technology, ICEST **2011**, abstract book, 31 October – 4 November, Izmir, Türkiye.

- Can Holyavkin, Ceren Alkim, Ulku Yilmaz, Berrak G. Balaban, Gokhan Kucukgoze, **Nazli Kocaefe**, Sema Gunduz, Suleyman Akman, Z. Petek Cakar, Metal Content and *COT1*/*AFT1* Expression of Cobalt-Resistant *Saccharomyces cerevisiae* Mutant Obtained by Evolutionary Engineering. ICEST **2011**, abstract book, 31 October – 4 Novermber, İzmir, Türkiye.