

SYSTEMS BIOLOGY APPROACH FOR QUANTITATIVE ANALYSIS OF
GLUTATHIONE METABOLISM IN NON-CONVENTIONAL YEASTS



by
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SYSTEMS BIOLOGY APPROACH FOR QUANTITATIVE ANALYSIS OF
GLUTATHIONE METABOLISM IN NON-CONVENTIONAL YEASTS

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To my family...



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ABSTRACT

SYSTEMS BIOLOGY APPROACH FOR QUANTITATIVE ANALYSIS OF GLUTATHIONE METABOLISM IN NON-CONVENTIONAL YEASTS

Non-conventional yeasts (NCY) attract increasing attention in industrial biotechnology context both in applications and fundamental understanding of the metabolism as they are typically richer in their molecular portfolio in comparison to conventional yeasts. Nevertheless, studies on the response of NCYs to different environmental stresses are in their infancy. In particular, oxidative and/or reductive stress typically arise as an inevitable part of the yeast's aerobic style. Yeast cells respond to oxidative stress via enzymatic (SOD, catalase, peroxidase reactions) and/or metabolic (glutathione or uric acid production pathways) through multi-omics and complex responses. Response to reductive stress, in turn, may result from increased antioxidant levels. Both typically disturb the intracellular redox balance. Departing from the question of how non-conventional yeasts respond to oxidative stress, this thesis focuses on the quantitative investigation of intracellular amino acids and glutathione levels in the NCYs. Following the systems biology approach, the generated data on fermentation profiles, fluxome, and targeted metabolome, as well as transcriptome data from literature, are collectively taken into account in response to both oxidative and reductive stresses for conventional and the non-conventional yeasts.

There was a discrepancy between organisms at low concentrations of amino acids. Furthermore, the amino acid levels are lower in the 150L scale by a factor of 3-5 for both yeasts. Oxidative stress induces a different short-term response in amino acid levels in both yeasts cultivated in a chemostat. The glutathione levels increased with 5 mM of H₂O₂-induced oxidative stress at the chemostat in *S. cerevisiae*. The results obtained in this study shed light on the different responses of these yeasts to hydrogen peroxide-induced oxidative stress. Comparing batch to batch, sample to sample, and injection to injection variances, it can be concluded that the targeted quantitative metabolomics approach was successfully applied in this thesis. Finally, yeast strains overproducing GSH are obtained via the classical UV-mutagenesis approach. These strains have up to a 50 percent increase in GSH content compared to the wild-type strain.

ÖZET

KONVANSİYONEL OLMAYAN MAYALARDA GLUTATYON METABOLİZMASININ KANTİTATİF ANALİZİ İÇİN SİSTEM BİYOLOJİSİ YAKLAŞIMI

Geleneksel olmayan mayalar (KOM), geleneksel mayalara kıyasla moleküler portföylerinde tipik olarak daha zengin olduklarından, hem uygulamalarda hem de metabolizmanın temel anlayışında endüstriyel biyoteknoloji bağlamında artan ilgi görmektedir. Bununla birlikte, KOM'ların farklı çevresel streslere tepkisi üzerine çalışmalar emekleme aşamasındadır. Özellikle oksidatif ve/veya indirgeyici stres tipik olarak mayanın aerobik hayat döngüsünün kaçınılmaz bir parçası olarak ortaya çıkar. Maya hücreleri oksidatif strese enzimatik (SOD, katalaz, peroksidaz reaksiyonları) ve/veya metabolik (glutatyon veya ürik asit üretim yolları) multi-omik ve karmaşık tepkiler yoluyla yanıt verir. İndirgeyici strese tepki, artan antioksidan seviyelerinden kaynaklanabilir. Her iki stres de tipik olarak hücre içi redoks dengesini bozar. Konvansiyonel olmayan mayaların oksidatif strese nasıl tepki verdiği sorusundan yola çıkarak, bu tez KOM'larda hücre içi amino asitlerin ve glutatyon seviyelerinin nicel araştırmasına odaklanmıştır. Sistem biyolojisi yaklaşımını takiben, literatürden alınan transkriptom verilerinin yanı sıra fermantasyon profilleri, fluksom ve hedeflenen metabolom hakkında oluşturulan veriler, geleneksel ve geleneksel olmayan mayalar için hem oksidatif hem de indirgeyici streslere yanıt olarak toplu olarak dikkate alınmıştır.

Bu çalışma ile düşük amino asit konsantrasyonlarında organizmalar arasında bir tutarsızlık olduğu saptanmıştır. Ayrıca, her iki maya için de amino asit seviyeleri 150L ölçeğinde 3L ölçeğine göre 3-5 kat daha düşüktür. Oksidatif stres, her iki mayada da kısa vadede amino asit seviyelerinde farklı tepkiye neden olmuştur. *S. cerevisiae*'da kemostatta 5 mM H₂O₂ ile indüklenen oksidatif stres ile glutatyon seviyeleri artmıştır. Bu tezde, partiden partiye, numuneden numuneye ve enjeksiyondan enjeksiyona varyansları karşılaştırarak, hedeflenen kantitatif metabolomik yaklaşımın başarıyla uygulandığı sonucuna varılabilir. Son olarak, yüksek GSH üreten maya türleri, klasik UV-mutajenez yaklaşımıyla elde edilmiştir. Bu suşlar, doğal suşa kıyasla GSH içeriğinde yüzde 50'ye varan bir artışa sahiptir. Bu tezde elde edilen sonuçlar, bu mayaların oksidatif veya redüktif strese karşı farklı tepkilerine ışık tutmaktadır.

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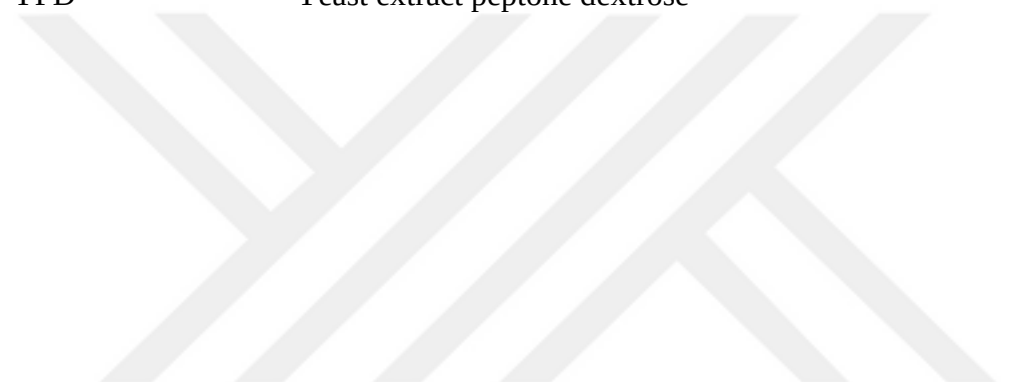
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LIST OF SYMBOLS/ABBREVIATIONS

$^{\circ}\text{C}$	Degree Celcius
μ	Specific growth rate
μL	Microliter
CO_2	Carbon dioxide
h	Hour
mM	Milimolar
m_s	Maintenance energy
O_2	Oxygen
q_i	Biomass specific rates
S	Stoichiometric matrix
v	Reaction rate
V	Working volume
γ	Degree of reduction
6PG	Phosphogluconate
COBRA	Constraint-based reconstruction and analysis
Cys	Cysteine
D	Dilution rate
DTT	Dithiothreitol
F	Flow rate
FBA	Flux balance analysis
G6P	Glucose 6-phosphate
GC-MS	Gas chromatography-mass spectrometry
GCS	γ -glutamyl-L-cysteine synthetase
Glu	Glutamate
Gly	Glycine
GMO	Genetically modified organism
GP	Glutathione peroxidase
GR	Glutathione reductase
GSH	Reduced glutathione
GSM	Genome scale metabolic model

GSSG	Oxidized glutathione
H ₂ O ₂	Hydrogen peroxide
HPLC	High performance liquid chromatography
NADP ⁺ /NADPH	Nicotinamide adenine dinucleotide phosphate
NCY	Non-conventional yeasts
OD	Optical density
PP	Pentose phosphate
ROS	Reactive oxygen species
Y	Yield
YPD	Yeast extract peptone dextrose



1. INTRODUCTION

1.1. CONVENTIONAL AND NON-CONVENTIONAL YEASTS

Baker's yeast, *Saccharomyces cerevisiae*, has been appearing in various areas for centuries, from bread making and different beverage production to the production of biobased materials, biofuels, and also to food supplements or feed additives. *S. cerevisiae*, a model organism for eukaryotic systems, is also a workhorse in industrial biotechnology, and as such, it is considered a conventional yeast. Besides *Saccharomyces*, several yeasts are available, called non-conventional yeasts (NCY). Important non-conventional yeasts can be listed as *Yarrowia lipolytica*, *Candida* species, methylotrophic yeasts; *Pichia pastoris*, *Pichia angusta* (*Hanseluna polymorpha*), *Pichia methanolica* (*Pichia pinus*); *Kluyveromyces lactis*. Non-conventional yeasts draw attention in the field of industrial biotechnology due to their ability to use different substrates, their post-transcriptional modification capabilities, and their tendency to produce and secrete recombinant proteins. Recent studies have shown that non-conventional yeasts have high potency to produce valuable biotechnological products from various recombinant proteins, several enzymes to the vaccine. With an increasing number of post-genomic studies and available dedicated molecular biology toolboxes, the use of non-conventional yeasts in the industry is increasing.

Pichia pastoris (*Komagataella pastoris*), the most well-known methylotrophic yeast, has the characteristics of being an essential host for recombinant protein production and reaching high cell concentrations. There is little study about the impacts of environmental stresses on the physiology of *P. pastoris*. In a chemostat-based study, Dragosits and colleagues described the effect of temperature on the proteome of recombinant *P. pastoris* [1]. Especially there was little to no study about the effects of oxygen availability, one of the most common problems in the industry. Oxygen also significantly affects cellular redox reactions that play a role in protein folding reactions within the cells as well as the growth and protein production of cells. Baumann et al., 2010 focused on understanding the physiological adaptation of *P. pastoris* to oxygen availability using a multi-level study that included transcriptomic, proteomic, and metabolic flux analyses, as well as unraveling potentially distinct features of such adaptation as described for *S. cerevisiae* [2].

Yarrowia lipolytica is a strictly aerobic, non-pathogenic yeast and has the capability of degrading various substrates (fats, oils, n-alkanes). Although *Y. lipolytica* is known as one of the most important lipase producers, it is used for several industrially important products such as organic acids, single cell oil, and γ -decalactone. It has a high secretion capability. Due to the strict aerobicity, it can grow high-glucose-containing mediums without affecting the respiration rate [3]. Biryukova et al. [4] investigated the adaptive response of the *Y. lipolytica* to oxidative stress caused by the oxidants hydrogen peroxide, menadione, and juglone. The adaptation of yeast cells to such oxidants was related to an increase in the activity of the principal enzymes involved in cell defense against oxidative stress, including catalase, superoxide dismutase, glucose-6-phosphate dehydrogenase, and glutathione reductase.

1.2. STRESS

Biological or physical unforeseen circumstances faced by the cell are called stress. Cells aim to maintain cellular homeostasis with a network of complex mechanisms against stresses. These mechanisms have been evaluated in various areas, from physiology to different -omic levels. Baker's yeast has also been a model organism in stress studies due to the availability of genome sequence and its simple cellular organization [5]. The stresses that yeasts are exposed to in both industrial context and/or changing environmental conditions can be classified as limitations/starvations, osmolarity, oxidative/reductive agents, pH, and heat shock.

1.2.1. Oxidative Stress

The cell initially evolved in a reducing environment. An increasing level of oxygen in the atmosphere has given the potential to the organisms for an efficient energy generation process of respiration and also the capability of producing intermediates responsible for maintaining cellular homeostasis. Also, it means that exposure to oxygen has possible damage to aerobic cells. Defense mechanisms have been developed to counter oxidative stress and the accumulation of reactive oxygen species (ROS) in all organisms. As a result, cells have evolved to survive both oxidative and reductive conditions. Maintaining redox

balance is essential for cellular homeostasis. Aerobic life induces oxidative stress, defined as high exposure to reactive oxygen species such as peroxides, alkyl hydroperoxides, and peroxide anion, as it leads to the production of ROS as an inevitable result of the physiological metabolism of all aerobically growing (micro)organisms. The type and content of the ROS define both beneficial and toxic effects on the cell. While cell proliferation and differentiation processes require low levels of ROS, on the other hand, ROS can damage cellular homeostasis and can be driven to cell aging and death. Induction of signaling pathways needs low levels of ROS; besides, it can be toxic when it reaches high levels. In the physiological state, the cells maintain their ROS levels with dynamic equilibrium among ROS generation and elimination processes. Cells have different response and protection mechanisms against ROS (Figure 1.1.). Responses to oxidative stress are usually on cell survival or cell death mechanisms. Cell survival mechanisms involve antioxidant enzyme mechanisms (superoxide dismutase, catalase, peroxidase reactions) [6], glutaredoxin/thioredoxin systems, metabolic products (glutathione, used in almost all eukaryotes), upregulation of various transcription factors (Yap1p, Skn7p, Msn2/4p) [7]. The dose of stress affects to activation of stress response mechanisms, which are vital under stress conditions. The first biological response aims to maintain cellular redox homeostasis. ROS can damage cells from various aspects ranging from initiating lipid peroxidation, aggregation, and fragmentation of proteins at the proteome level and amino acid modifications at the amino acid level to DNA damage depending on the ROS.

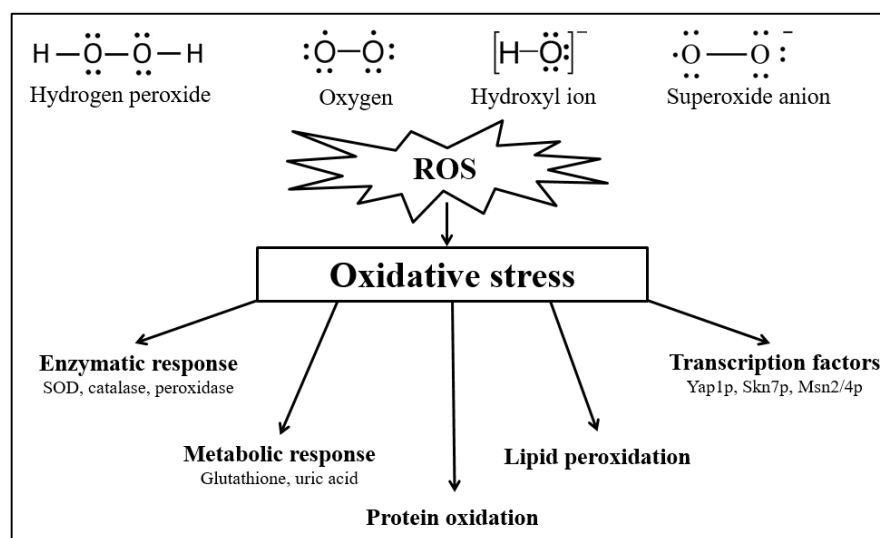


Figure 1.1. Responses of yeast on oxidative stress

There are two factors to produce ROS: cellular metabolic processes and environmental factors. Intracellular generation of ROS can be caused by the availability of carbon sources (glucose, glycerol, ethanol) and culture conditions (growth phase of culture) in the case of yeast *S. cerevisiae*. In addition to this, different kinds of environmental stressors can generate oxidative stress in yeasts ranging from peroxides (e.g., hydrogen peroxide, cumene hydroperoxide), toxins (e.g., citrinin), drugs (e.g., Epolauridine) to metals (e.g., zinc, arsenite). Hydrogen peroxide (H_2O_2) is the most widely oxidative stress inducer in yeast cells and has a high diffusibility [8,9]. Its low reactivity and high diffusibility allow it to enter all cellular compartments and react with any reactive molecule. The hydrophobic organic peroxide derivatives, tert-butyl hydroperoxide, cumene hydroperoxide, linolenic, and linoleic acid hydroperoxide, have a higher probability of reacting with membrane components. The toxicity level of oxidative stress inducers varies according to the type of inducer and yeast species. While lipid hydroperoxides are toxic effects for *S.cerevisiae* in the range of 0.05 mM, this toxicity level increases up to 5 mM for H_2O_2 . Menadione and paraquat are the redox cycling chemicals used to use generating O_2 in aerobically growing cells. Diamide and diethylmaleate are compounds generating oxidative stress indirectly [10].

At the transcriptome level, oxidative stress response studies for *Saccharomyces cerevisiae* are conducted using either direct induction methods such as peroxide treatment or indirect induction methods such as drug or toxin treatment. The common mechanisms involved in oxidative stress response were found to be carbon metabolism, oxidation-reduction processes, and glutathione metabolic process via the collecting, analyzing, aggregating, and performing functional analysis of available transcriptome data from the response of yeast cells to various oxidative stresses using bioinformatic tools [9].

Growth phases affect the tolerance of yeasts to environmental stresses. Jamieson, 1992 and Werner-Washburne et al., 1993 showed that tolerance of exponentially growing cells to oxidative and thermal stress was lower than the cells in the stationary phase. The main reasons for this are thought to be the increasing number of mitochondria in the stationary phase cultures that are grown with glucose and the increased expression of some genes encoding stress shock proteins [11,12].

Focusing on conserved responses to oxidative stress across species, it can be seen that long-term transcriptional responses are the most prominent of these conserved responses [13–15].

Upregulation of superoxide dismutase, catalases, and glutathione/glutaredoxin systems are the coordinated transcriptional responses of microorganisms such as *Escherichia coli* and *Saccharomyces cerevisiae* to scavenge ROS [16,17]. In the same manner, mammalian cells use a long-term antioxidant response to detoxify ROS and, hingeing on the severity of stress, initiate either cell survival programs or cell death programs [18]. Upon exposure to ROS, cell survival is maintained with the basal expression of the aforementioned antioxidant enzymes and non-enzymatic antioxidants such as reduced glutathione until transcriptional regulatory defense mechanisms begin to work. Recent studies attract attention to glutathione peroxidase as one of the key short-term survival mechanisms. Sudden oxidative stress leads to detoxifying ROS with glutathione-based mechanisms and concomitant oxidation of NADPH. The decreased NADPH requires the rapid replenishing of NADPH pools. Glucose 6-phosphate (G6P) dehydrogenase and phosphogluconate (6PG) dehydrogenase in the oxidative branch of the pentose phosphate (PP) pathway catalysis are the main replenishing reactions in bacteria, *S. cerevisiae*, and most mammalian cells. Under oxidative stress, all cells increase the rate of NADP^+ to NADPH reduction, primarily by redirecting the glycolytic flux to the PP pathway [13,18,19].

Ralser et al., 2007-2009 reported that the short-term oxidative stress response in *S. cerevisiae* could be predominantly dependent on blockage of lower glycolysis [13,20]. Christodoulou et al., 2019 focused on the characterization of the short-term metabolic response of three species, *E. coli*, *S. cerevisiae*, and human dermal fibroblasts, to understand which mechanisms are conserved across kingdoms. There is a conserved metabolome among three species found by multivariate and timing analysis. It appears to inhibit the oxidative pentose phosphate pathway by the redox cofactor NADPH during normal growth in all three species while alleviating this inhibition to increase the pathway for detoxification of ROS during stress, with the only exception of *S. cerevisiae* when under high levels of oxidative stress exposure [18].

The eukaryotic model organism, *S. cerevisiae*, which has the simplicity of a single cell, can be viewed as a powerful tool for understanding complex biological responses by facilitating responses with the help of genetic, biochemical, and physicochemical, cell biological, and genome-wide experimental approaches. Deciphering oxidative stress response mechanisms is not only critical for neurodegenerative, cardiovascular diseases, cancer, or cell aging processes but also important for taking new steps to increase fermentation efficiency in many

different areas using microorganisms as a workhorse in industrial biotechnology [10]. Yeasts, which have an essential share in industrial biotechnology, are used in different fields, from bread yeast and single-cell protein production to bulk and fine chemicals production. In an industrial context, yeasts are exposed to stress as their environmental conditions change as well as being exposed to various stresses such as heat, nutrients, pH, osmotic and oxidative. Since oxidative stress is an inevitable part of the aerobic life cycles of yeasts, it has a significant share in terms of the evaluation of stress responses on yeasts. The oxidative stress response in yeast has been ascertained with several aspects of finding the toxicity level of stress agents [11,21,22], determining the cellular mechanisms [23,24], and identifying stress-related genes with mutant strains [25–27], and understanding the main mechanisms. Although there are various studies on the eukaryotic model organism *S. cerevisiae* in different aspects of oxidative stress, there is little to no study for non-conventional yeasts *P. pastoris* [22].

Recent studies of the methylotrophic yeast *P. pastoris* have focused on its response to ROS induced by methanol, which is used as a substrate for recombinant protein production. This study claimed that oxidative stress response was induced with methanol in *P. pastoris*. The heterologous protein production increased with the new mutant cell was designed with overexpression of genes related to an antioxidant mechanism against ROS [28]. These studies show that understanding the oxidative stress response mechanism is of great importance in terms of taking new steps to increase fermentation efficiency in many different areas, from recombinant protein production to fine chemicals.

Microorganisms require an abundant supply of amino acids to maintain their driving force for proliferation. The role of amino acids in the cell is not limited to their primary role as the primary building block of protein. It also plays a variety of other roles in the cell, including energy generation, nucleoside synthesis, homeostasis of cellular redox, and stabilizing cell osmolarity and pathogenesis. Amino acids and their derivatives, the most abundant compounds of all detected metabolites, are another primary target in oxidative stress, and their associated amino acid metabolism can be easily modified [29].

1.2.2. Reductive Stress

Generation of excess reactive oxygen species can be called oxidative stress; conversely, reductive stress can be described as an increased level of reducing equivalents in the form of redox couples (GSH/GSSG, NAD(P)H/NAD(P)⁺) [30]. Although studies on understanding the oxidative stress mechanism and response have attracted more attention than reductive stress studies, the effect of reductive stress also maintains its importance in terms of intracellular redox balance. Exposure to reductants can cause reductive stress; the superoxide anion is essentially a reductant. Under certain conditions, redox enzymes such as mitochondrial glutathione reductase and thioredoxin reductase can donate electrons to O₂ instead of their standard substrate, increasing the production of ROS. Reductive stress can be promoted using dithiothreitol (DTT) (experimentally), which is a potent reducing agent [24,31]. DTT can diffuse from membranes and prohibits disulfide bond formation. For instance, the folding and transport of vacuolar carboxypeptidase Y(CPY) from the endoplasmic reticulum is reversibly blocked by DTT. Moreover, DTT-induced reductive stress causes an accumulation of misfolded proteins in the endoplasmic reticulum. The study of Gasch & Spellmann (2000) showed that the transcriptional response of yeast cells to DTT-induced reductive stress was remarkably different from the general environmental stress response [24]. This study allows us to predict that different targets will be affected by DTT-induced reductive stress than many other types of stress. Amino acid biosynthesis and small metabolite excretion generate an excess of cytoplasmic NADH [32,33], which can be reoxidized under aerobic conditions by feeding into the mitochondrial respiratory chain.

Redox couples, NAD(H), NADP(H), and GSH/GSSG are essential regulators of cell metabolism. The role of glycolysis and the TCA cycle is the production of NADH that supplies electrons to mitochondrial oxidative phosphorylation and ATP. The role of NADP⁺ is to support PPP to generate NADPH which is essential for the reductive biosynthesis of nucleotides, amino acids, and lipids. GSH is a linker metabolite between amino acid metabolism and the redox state of cells. Therefore, the changes in the ratio of these three redox couples can be a reason for reductive stress and affect cell metabolism and vice versa [34].

1.2.3. Antioxidant Defenses and Redoxins

The antioxidant defense mechanisms and thiol redox pathways have a crucial role in maintaining the redox state of the cells. Antioxidant defense mechanisms are based on catalyzing ROS reduction by electron transfer. On the other hand, thiol redox mechanisms aim to maintain an acceptable thiol redox balance in the various cellular subcompartments.

1.2.3.1. Antioxidants

Superoxide dismutases (SOD), catalyzing the conversion of O_2^- to hydrogen peroxide to O_2 and catalases, converting hydrogen peroxide to molecular oxygen and water are important antioxidant defense mechanisms of virtually all aerobic organisms. In *S. cerevisiae*, Sod1 is a cytosolic Cu, Zn-superoxide dismutase in the mitochondrial inter-membrane space and other cell compartments, while Sod2 is an Mn-superoxide dismutase that exists in the mitochondrial matrix. There are various studies about the physiological roles of SODs in protecting against oxidative damage. These studies showed that although SODs have an important role in the redox balance of cells, they are not essential for aerobic life and that each SOD has a specific purpose.

S. cerevisiae contains both cytosolic (Ctt1) and peroxisomal (Cta1) catalases. The studies about understanding the physiological role of catalases in *S. cerevisiae* demonstrated that catalases play an important role in antioxidant defense during the stationary phase or when other antioxidant defense mechanisms are compromised [10].

P. pastoris has one glutathione peroxidase gene (PpGPX1). Due to the methylotrophic characteristic of the *P. pastoris*, the peroxisomal antioxidant enzymes; glutathione peroxidase (Pmp20) and catalase (Cta1) are used to scavenge ROS. There is no homolog of cytosolic catalase (CTT1) on the genome of *P. pastoris*. It was claimed that glutathione has two key roles in yeast methanol metabolism: protection against ROS and detoxification and oxidation of formaldehyde (HCHO). Glr1-catalyzed regeneration of GSH was suggested to play a key role in detoxifying formaldehyde during methanol metabolism [35].

1.2.3.2. Thiol redox mechanisms and peroxidases

Glutathione and the small proteins; thioredoxin and glutaredoxins are important thiol redox pathways in yeasts. Thioredoxin is a small protein having two redox-active cysteines inside

the conserved active site pattern Trp-Cys-Gly-Pro-Cys, which are oxidized to an intramolecular disulfide in a thiol-disulfide exchange reaction with a disulfide bound substrate. Thioredoxin does not have a direct role in reduction of ROS and its contribution to ROS detoxification is mostly accomplished through the action of target scavenging enzymes. *S. cerevisiae* has a cytosolic thioredoxin system composed of two redundant isoforms, Trx1 and Trx2, a thioredoxin reductase Trr1 [36], and a mitochondrial system composed of Trx3 and Trr2 [37]. Thioredoxin peroxidases catalyze the reduction of hydrogen peroxide and alkyl hydroperoxides to water and the corresponding alcohols and glutathione peroxidases, catalyzing the reduction of hydrogen peroxide organic peroxides to water and corresponding alcohol, at the expense of GSH are other important antioxidant mechanisms in yeasts.

Glutaredoxins are small proteins catalyzing thioltransferase activity using glutathione as a cofactor. In *S. cerevisiae*, there are three monothiol enzymes, Grx3, Grx4, and Grx5, and two dithiol glutaredoxins.

The GSH and thioredoxin pathways can be regarded as electron flow systems because there is a gradient in redox potentials along the channels, enabling electrons to pass from one molecule to the next. Finally, both routes need glucose as an electron source, oxidizing in the pentose phosphate pathway to recycle NADPH. Because of its extremely low redox potential of -315 mV, NADPH is the major hydrogen donor for thiol redox systems.

1.3. GLUTATHIONE

The well-known natural antioxidant glutathione (γ -glutamyl-cysteinyl-glycine; GSH) is a tripeptide formed by combining L- glutamate, L- cysteine, and glycine (Figure 1.2.). Its two carboxyl and amine groups allow the dissolution of GSH in aqueous solutions and polar solvents, and a thiol group contributes to its biological activity. Because of its antioxidant property caused by the thiol group of the cysteine moiety, glutathione is known as an important cellular redox buffer for maintaining the reducing thiol-disulfide balance.

The prominent role of glutathione is to maintain the redox potential in the cell against external stimuli such as nutritional and environmental stresses. GSH has an essential role in gene regulation and protection mechanisms of eukaryotes and most prokaryotes as an

antioxidant. It is responsible for protecting DNA, proteins, and other essential biomolecules against external perturbations such as oxidative damage, osmotic shock, pH stress, or toxic heavy metals.

Glutathione is an extremely potent antioxidant. Using GSH as a cofactor, glutathione peroxidase can decompose hydrogen peroxide and lipid peroxide produced during aerobic metabolism to water, less toxic hydroxy fatty acids, and oxygen. The enzyme glutathione peroxidase (GP) can be found in the cytosol, mitochondria, and peroxisomes. GSH is oxidized to GSSG in reactions catalyzed by glutathione peroxidase. Glutathione reductase (GR) uses NADPH as a cofactor to reduce GSSG back to GSH, which is primarily produced in the oxidative part of the pentose phosphate pathway by glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase. Glutathione reductase is an important enzyme for recycling GSSG to GSH evolutionarily conserved, with a high similarity between three-dimensional structures from *E. coli*, *S. cerevisiae*, and humans [38,39].

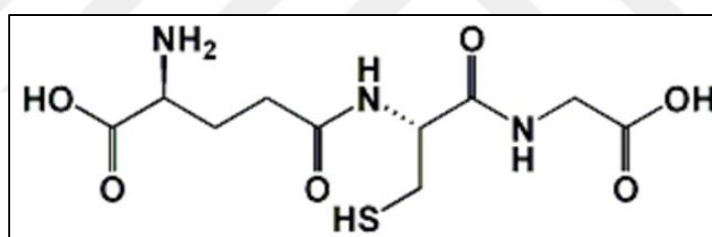


Figure 1.2. Chemical structure of glutathione [40]

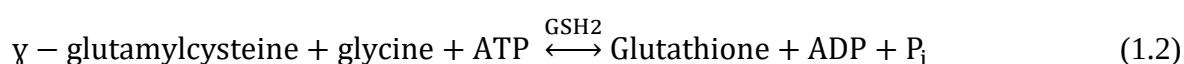
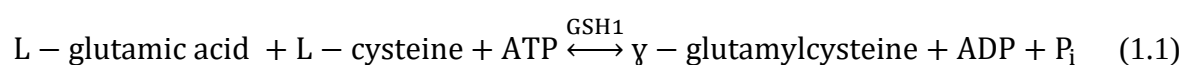
Glutathione is kept in cells in three different forms: reduced glutathione (GSH), oxidized glutathione (GSSG), and mixed disulfides. Glutathione is found in reduced form in over 90 percent of microbial, plant, and mammalian cells, while the oxidized form accounts for only 10 percent of it. Cytoplasm and mitochondrial membrane are the locations of both forms of glutathione. The redox potential was provided by the ratio of the glutathione to its disulfide-bonded dimeric form (GSSG). Its redox potential is -240 mV at pH 7.0 and 25°C. The ratio of reduced to the oxidized form of glutathione hinges importantly on enzymatic mechanisms responsible for glutathione synthesis and degradation [41].

Glutathione has a wide range of application areas, from the food, beverage, and feed industry to the healthcare/pharmaceutical and cosmetic industry. GSH has been used in the beverage

industry to stabilize the taste of beer and wine with its antioxidant capability [42–44]. It can also be involved in the food industry as a dough-modifier, as a tasting agent [44], or as a packaging film ingredient due to its protection from oxidation [45]. In addition, GSH has potential as a feed supplement in the feed industry [46], as well as a functional food and a dietary supplement for humans in the pharmaceutical industry [47,48]. Another area of use is as a skin whitening agent [49] and a skin cream for anti-aging [33] in the cosmetic industry.

1.3.1. Glutathione Synthesis, Degradation, and Regulation Mechanisms

Glutathione biosynthesis relies on the sequential, ATP-dependent action of two enzymes: (1) γ -glutamyl-L-cysteine synthetase (GCS), which is also known as glutamate-cysteine ligase and (2) glutathione synthetase (GSH2) in the cytosol (Figure 1.3.). The first step is the formation of γ -glutamyl-L-cysteine from L-cysteine and L-glutamic acid via GSH1 (EC 6.3.2.2) in eukaryotes and GshA in prokaryotes. This step is also a rate-limiting step due to feedback inhibition of GSH1 via glutathione. GSH inhibits the GSH1 enzymes by binding the active site of the glutamate-binding side and reacting with the thiol group of GSH. GCS is found as a single polypeptide form in yeast and bacteria. The second step is glycine addition to the C- terminal of γ -glutamyl-L-cysteine via GSH2 (EC 6.3.2.3) in eukaryotes and GshB in prokaryotes to obtain GSH. Recently, in bacteria, a single bifunctional enzyme -glutamylcysteine synthetases/glutathione synthetase (GshF) capable of GSH synthesis was discovered[50,51]. There is no feedback inhibition of GSH on GshF.



In yeast and bacteria lacking GCSs, a natural compensatory pathway for GSH synthesis was discovered. The first enzyme in the proline biosynthetic pathway is -glutamyl kinase (PRO1 in eukaryotes and proB in prokaryotes). The enzyme is responsible for the formation of -glutamyl phosphate. If this compound accumulates, it can non-enzymatically react with cysteine to form γ -glutamylcysteine, which is then catalyzed by glutathione synthetase to form GSH from γ -glutamylcysteine, and glycine [41,52].

Glutathione homeostasis has been established the balancing its synthesis and degradation. γ -glutamyltranspeptidase degrades GSH by transferring the γ -glutamyl moiety to acceptor substrates such as water (hydrolysis) or amino acids and peptides [53]. γ -glutamyltranspeptidase catalyzes the cleavage of GSH to glutamate and cysteinyl-glycine during the hydrolysis reaction. The enzyme γ -glutamyltranspeptidase is found in both prokaryotes and eukaryotes [54]. GSH, GSSG, and other γ -glutamyl substrates can be cleaved by γ -glutamyltranspeptidases (e.g., GSH-S conjugates).

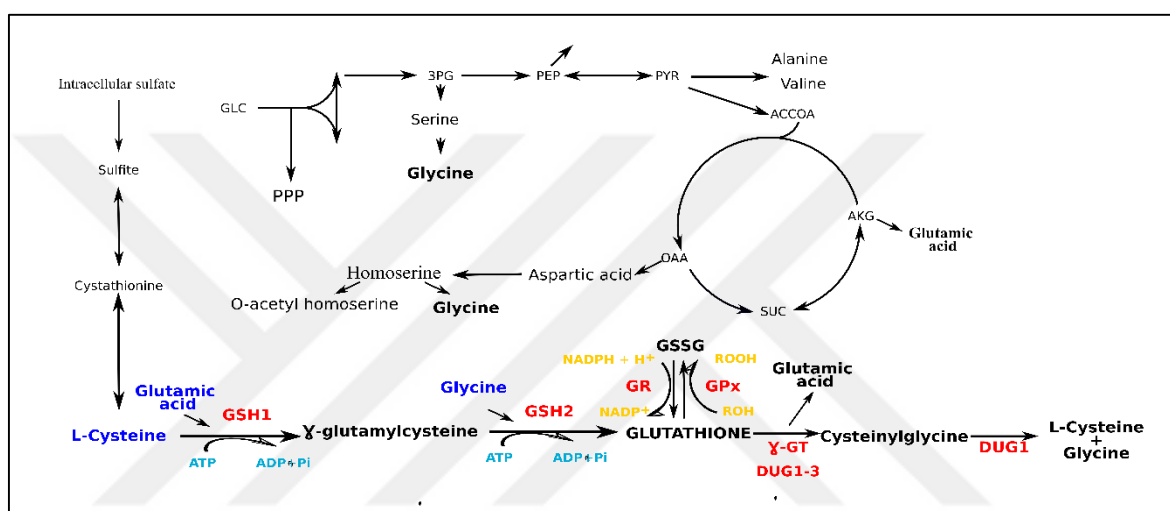


Figure 1.3. Glutathione biosynthesis and degradation in yeast adapted from [55]

Studies in *S. cerevisiae* showed that GSH homeostasis inside the cell was effectively controlled by glutathione degradation. γ -glutamyl transpeptidase (Cis2/Ecm38) and Dug (defective in the utilization of glutathione)-a pathway that contains cysteinylglycine peptidase (EC:3.4.13.-) are responsible for the degradation of GSH in the yeasts [56].

1.3.1.1. Glutathione redox balance and the regulation of GSH synthesis

The ratio of reduced to oxidized glutathione is defined as glutathione redox balance. This redox balance is a key indicator of cellular oxidative stress. A decrease in the GSH/GSSG ratio in cells causes significant oxidative stress damage. Increased GSH/GSSG ratio and intracellular GSH content are toxic due to non-selective glutathionylation of proteins, which causes metabolic processes to be disrupted [57]. Overexpression of the high-affinity glutathione transporter Hgt1/Opt1 in *S. cerevisiae* led to an increase in intracellular GSH levels several-fold but this increased sensitivity to exogenous glutathione of this strain

compared to the parental strain. GSH toxicity occurs as a result of GSH accumulation in this strain. GSH toxicity was also found in a *glr1Δ* strain as an altered redox balance [58].

There are three different mechanisms to regulate glutathione biosynthesis reactions: transcriptional, post-translational, and substrate-level regulations. Yap1 and Met4 are responsible for the regulation of sulfur assimilatory pathways and also the regulation of the *GSH1* gene. In yeast, Yap1 has a role in oxidative stress tolerance, and cellular localization regulates its transcriptional activity. Yap1 forms an intramolecular disulfide bond under oxidative stress. In *S. cerevisiae*, Yap1 is also responsible for the regulation of *GSH2* [59]. The transcriptional factor Met4 has an indirect role in glutathione biosynthesis via the regulation of sulfur-containing amino acid cysteine. GSH, together with methionine, homocysteine, and cysteine, can be used as the sole sulfur source by *S. cerevisiae*. Under nitrogen limitation, GSH is stored at the central vacuole in yeast cells [60].

Increasing intracellular GSH concentrations led to post-translational regulation of GSH biosynthesis with the feedback inhibition of *GSH1* activity. These post-translational regulations are essential for maintaining proper redox balance with controlling GSH biosynthesis in yeast. High intracellular GSH concentrations prevent further accumulation of GSH via feedback inhibition of *GSH1* gene expression and enzymatic activity of Gsh1. At the same time, these regulatory mechanisms ensure that GSH biosynthesis is accelerated when its intracellular concentration falls below the physiological level.

The third regulation mechanism of glutathione biosynthesis is substrate-level regulation. There are various studies about supplementation of especially precursor amino acids of glutathione in the yeast culture media. Wen et al., 2006 showed that cysteine is the major limiting compound for glutathione biosynthesis [61]. Also, the study by Wang et al., 2007 and Liang et al., 2008 supported that addition of cysteine into the culture media, intracellular GSH concentration significantly increased [62,63].

Glutathione-S-transferase also has a role in glutathione homeostasis in *S.cerevisiae*. GSH participates in protein redox signaling. Cysteine residues in proteins can be oxidized under oxidative stress conditions, resulting in the formation of sulfenic acid. Sulfenic acid residues can react with GSH to form glutathionylated proteins. Glutathionylated residues in protein frames can be reduced to cysteine by glutaredoxins. Glutaredoxins are reversible thiol-disulfide exchange enzymes that catalyze reversible thiol-disulfide exchange reactions

between protein thiols and the abundant thiol pool GSSG/GSH. Glutaredoxins, in fact, protect protein thiols from irreversible oxidation. They regulate protein activities under various conditions and play an important role in cell signaling and redox homeostasis [64]. Furthermore, this mechanism prevents GSH loss under oxidative conditions, resulting in GSSG accumulation. Increased intracellular GSSG content may alter the redox equilibrium [65]. A decrease in intracellular GSSG content can help to stabilize the redox equilibrium. When GSSG reacts with a protein's sulfhydryl group (Protein-SH), a mixed disulfide is formed GSSG is either reduced to GSH or actively transported out of the cell.

1.3.2. Production of GSH

Studies focus on extracting glutathione from various sources, such as animal and plant extracts. Production cost increased due to the limited raw material and considerably low amount of GSH in the raw materials. Thus, alternative methods had to be developed. There are three methods known to produce glutathione: chemical synthesis, enzymatic synthesis, and microbial synthesis.

1.3.2.1. Chemical Synthesis

In 1935, GSH was chemically synthesized from N-carbobenzoxycystine and glycine ethyl ester [66]. In the 1950's, the chemical synthesis process was commercialized, but the product of this process was a racemic mixture of D- and L- isomers of GSH. It was necessary to separate the physiologically active L- form from the mixture of D-and L-. After Bloch's study that showed the biosynthesis of GSH in an isolated liver and described the biosynthetic pathway for GSH [67], enzymatic and fermentative routes have begun to attract attention to produce it [68].

1.3.2.2. Enzymatic Synthesis

L-glutamic acid, L-cysteine and glycine, ATP, enzymes (GSH1 and GSH2), appropriate pH, and cofactors are necessary to produce GSH enzymatically. For large-scale production, ATP-requiring reactions present challenges in scale-up as there is no efficient ATP-regenerating system [68,69].

1.3.2.3. *In Vivo Microbial Synthesis*

The high cost of three precursor amino acids motivates the work on the production of GSH by fermentation using the entire metabolism instead of the enzymatic route. Although there are various studies to produce GSH from bacteria, industrial production is mainly based on fermentation with *Saccharomyces cerevisiae* and *Candida utilis* since these yeasts are fast-growing organisms accumulating high intracellular glutathione concentrations, and can be grown to high cell densities on low-cost substrates, and are relatively simple to handle on a large scale, without endotoxic reactions in humans, unlike bacteria [57,68]. Glutathione concentrations in wild-type *S. cerevisiae* strains have been reported to range between 0.1 and 1.0 percent of dry cell weight, depending on growth conditions and strain characteristics. Several strategies for increasing glutathione accumulation in yeast have been asserted. The use of producer strains with increased glutathione biosynthetic capacity, obtained either through random mutagenesis or through genetic engineering techniques, in conjunction with the addition of precursor amino acids, particularly cysteine, to the cultivation media, has been proposed as the main strategy for increasing glutathione production [44,62,69].

The selection of a suitable production strain is the essential parameter for glutathione biosynthesis. Generally, strains having the capability of accumulating of high intracellular glutathione concentrations draw attention to further optimization studies. Evolutionary or metabolic engineering is the most preferred technology for strain optimization. There are various reported studies to increase glutathione synthesis with the overexpression of both *GSH1* and *GSH2* genes or separately. However, intracellular glutathione concentrations increased approximately two-fold due to the feedback inhibition mechanism of glutathione [57,70]. Physical or chemical mutagenesis is another strategy to isolate glutathione overproducers in the literature.

Under lab-scale bioreactor conditions, adaptive evolutionary engineering of the *S. cerevisiae* strain with acrolein as a selection agent led to an intracellular glutathione concentration of 5.9 percent [71]. Genetic engineering of non-conventional yeast *Pichia pastoris* and *Hansenula polymorpha* resulted in high cell densities and intracellular glutathione contents [72,73].

Nevertheless, the use of such genetically modified organisms (GMOs) for bulk glutathione production is limited for food applications and is subject to mandatory declaration

(2001/18/EG) in the European Union. Generally, as strain properties are more adapted to high glutathione productivity in a given fermentation process, it becomes more difficult to maintain the stability of these genetically modified strains under large-scale conditions [44].

Table 1.1. Biomass and glutathione concentrations of different yeast species

Strain	Strategy	Biomass (gL ⁻¹)	Glutathione (percent)	Ref.
<i>S. cerevisiae</i> T65	Precursors: Cys, Glu, Gly, Ser	133.0	1.4	[74]
<i>S. cerevisiae</i> G-14	RQ, precursors: Cys, Glu, Gly	132.0	1.5	[62]
<i>S. cerevisiae</i> T65	Precursors: Cys, Glu, Gly	133.3	1.6	[75]
<i>C. utilis</i> WSH 02-08	Precursors: Cys, Glu, ATP addition, coupled with H ₂ O ₂ stresses	112.3	2.2	[76]
<i>C. utilis</i> WSH 02-08	Precursors: Cys, Glu, ATP addition, SDS addition	101.0	2.5	[77]
<i>S. cerevisiae</i> GB Italy	Cell permeabilization via detergents, precursors: Cys, Gly	100	3.2	[78]
<i>H. polymorpha</i> Moxp-GSH2	GMO strain	72.0	3.2	[73]
<i>S. cerevisiae</i> KY6186	Feedforward/ Feedback control	63.8	3.7	[79]
<i>S. bayanus</i> Sa-00645	Precursor: Cysteine ethyl ester	21.9	3.9	[80]
<i>P. pastoris</i> D-18	GMO, precursors: Cys, Glu, Gly	98.2	4.2	[72]

A significantly higher amount of glutathione can be produced by recombinant bacteria in a shorter cultivation time. However, due to the endotoxin risks, this is not desired, the selection

and optimization of glutathione producer strain require considerable attention for further application areas of the product. The selection of yeast strains as glutathione producers gains attention, especially for food applications of glutathione [44]. There are reports on glutathione production from different yeast strains are shown in Table 1.1. As a result, to express bifunctional glutathione synthetase, *gshF* (that avoids inhibiting the GCS with a product), genes in various yeast species were studied from different groups. Although the *GshF* genes were taken from each of *L. monocytogenes*, *S. agalactiae*, and *L. plantarum* and cloned and expressed in methylotrophic yeast *P.pastoris*, only *GshF* from *L. monocytogenes* showed significant protein expression and catalytic activity, resulting in a decrease in the accumulation of the intermediate metabolite γ -glutamylcysteine [81]. Another study on bifunctional glutathione synthetase was based on the genes from *S. thermophilus* and integrating them into the ribosomal DNA of *S. cerevisiae* at a high copy number; thus, they showed that the recombinant strain accumulated threefold more glutathione than the reference strain [82].

The composition of the medium is critical for achieving high cell densities and intracellular glutathione accumulation. Cha et al., 2004 used the design of experiment approaches [83], and Zhang et al., 2007 applied alternative substrates to optimize production conditions [84]. Recently, a chemically defined medium based on the elemental composition of yeast that was suitable for high cell density cultivation as well as high intracellular glutathione concentrations was developed in the study by Schmacht et al., 2017 [69]. The use of ammonium sulfate, which acts as both a nitrogen and sulfur source, was beneficial for both biomass and glutathione production instead of using yeast extract as a complex ingredient.

The single-shot addition of cysteine, glycine, and glutamate as precursors for glutathione synthesis was the most popular strategy for increasing glutathione production. Other inducing amino acid combinations, such as methionine, serine, or cysteine ethyl ester, were also used. Cysteine was found to be an essential amino acid for glutathione production [85,86]. Alfafara et al., 1992b discovered that single-shot addition of L-cysteine was better than continuous addition with constant L-cysteine concentration to increase glutathione production without inhibiting growth [87]. As a result, the mass balance-based feeding strategy that is based on cysteine addition to a culture in the glutathione production phase in a single shot increased the production rate approximately two-fold.

A suitable cultivation strategy is another important requirement for maximum intracellular glutathione content. The fed-batch mode for high cell densities is the most common strategy for glutathione production on an industrial scale, but alternatives such as chemostat or repeated fed-batch approaches are also being researched. Feedforward control based on the carbon source feeding profile and feedback control based on the ethanol concentration is the dominant strategies for the production of glutathione without by-product formation [44].

1.4. SYSTEM BIOLOGY

System biology is an area of biology that has recently drawn attention and has found various application areas for understanding biological systems [88,89]. The systems biology approach aims to start from the initial observation (from experiments) and then building-related hypothesis, if possible, setting up a model of the hypothesis after doing the necessary experiments, taking multiple omics data, and analyzing these data using different databases, mathematical models, and bioinformatics techniques to verify the hypothesis and/or to produce new hypotheses [90] and the knowledge generated about the system continues in a loop as in Figure 1.4.

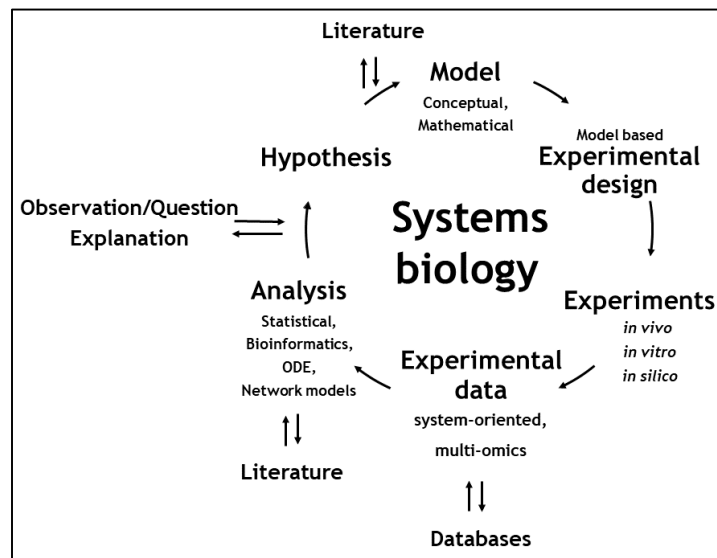


Figure 1.4. Systems biology approach [91]

Systems biology considers the entire system as a whole instead of focusing to individual parts. In other words, the aim of systems biology is a quantitative understanding of the

mechanisms of biological systems using multiple-omics approaches and mathematical models. There are two kinds of approaches to understanding mechanisms from the point of systems biology. The first approach, called bottom-up, can be defined as using small parts of systems and detailed biochemical knowledge to define the entire system. The second approach, called top-down, intends to make new deductions from large data sets such as from genome, which is complete genetic information, the proteome is whole proteins; transcriptome, which is all mRNAs; and metabolome, which is all metabolites (small molecules) [92]. In particular, the study of mathematical models together with experimental work is indispensable for understanding the system, producing new hypotheses, and for experimental design [93].

1.4.1. -Omes

The genome contains all sequences in the genetic material of a cell, including non-coding regions. Whole-genome sequencing studies aroused curiosity about the functions of cells. Developed technologies showed that the genome sequence of genes does not only enough to describe the functions of individual genes, instead, it requires evaluating a wide range of genes, called functional genomics. High throughput analysis techniques and bioinformatics have a crucial role in identifying a wide range of components that require -omic techniques (Figure 1.5.).

Transcriptome consists of the whole mRNA of the cell, proteome refers to the whole proteins present in the cell, metabolome includes all low molecular weight molecules, metabolites associated with the cell metabolism, fluxome refers to all fluxes responsible for metabolic networks of cells, and interactome consists of all interactions among various components of the cell.

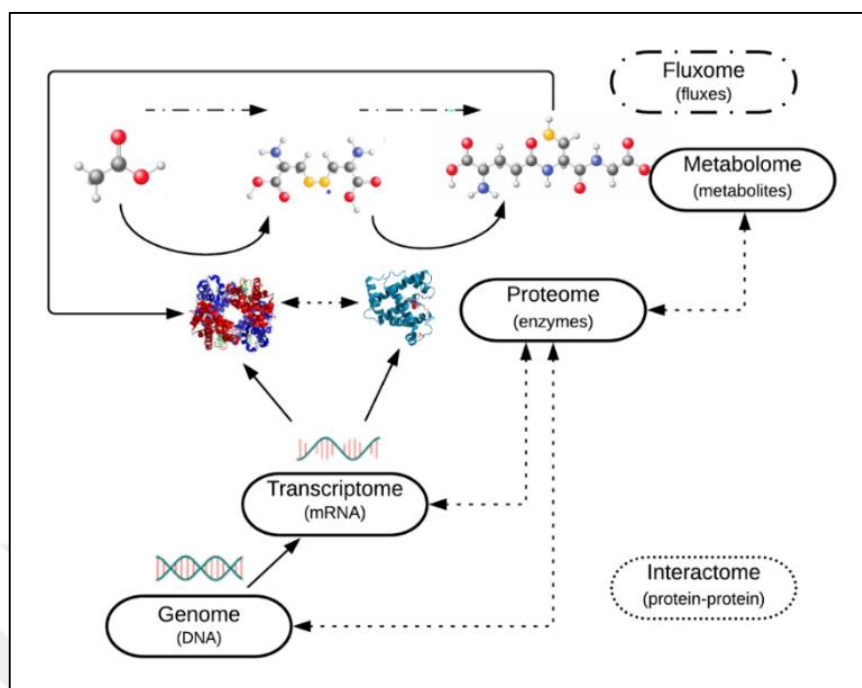


Figure 1.5. Schematic overview of the cells on different-omes [94]

1.4.1.1. *Metabolome*

All the synthesis processes of the building blocks (anabolic reactions) and the degradation processes (catabolic reactions) to obtain energy for these purposes is called metabolism. The main components of the (bio)reactions taking place in metabolism are called metabolites. The interpretation of these biochemical reactions and metabolites is required to overcome the complexity of cells.

Key among post-genomic studies to understand metabolism, metabolome focuses on the detection, identification, and quantification of low-molecular-weight molecules, metabolites, produced or modified by cells. Metabolome covers a wide variety of chemically different compounds from carbohydrates to amino and non-amino organic acids, alcohols and ketones, lipids, and complex molecules by the cells. These metabolites have various important roles among different metabolic pathways as an intermediate of reactions. Indeed, metabolites can be thought of as a snapshot of the physiological state of the cell. From this perspective, metabolome studies gain importance to investigate the response of cells against environmental or genetic changes. Metabolites have limited half-life as they are being continuously taken up, produced, broken down, and released out by the cell.

Endometabolites, present inside the cell, should be extracted using appropriate and reliable methods to analyze, and exometabolites, secreted by the cells or present in the culture media, can be obtained after more practical and common methods such as centrifugation[95]. This limited half-time of metabolites necessitates rapid sampling and extraction methods and diverse chemical natures of those metabolites require different separation and detection/identification technologies. The following terms are conceptually important, to better describe the scope of metabolome analysis:

- Metabolic fingerprinting focuses on the global fingerprint of metabolites using high-throughput technologies (NMR or MS spectra) without focusing on any specific metabolite.
- Metabolic footprinting aims to analyze the extracellular metabolites of (micro)organisms.
- Metabolite profiling aims to analyze and (semi-)/quantify metabolites as members of specific classes such as lipids and carbohydrates.
- Targeted metabolite analysis aims to quantitatively analyze the metabolites in the specified branch of metabolism.

The experimental setup has an important role in systems biology studies to obtain a well-defined characterization of the physiological state

1.4.1.2. *Sample preparation*

A universal sample preparation method for metabolome study does not exist due to both the necessity of the cell-specific methods and the chemical diversity of metabolites. The basic steps of the sample preparation is presented in Figure 1.6.

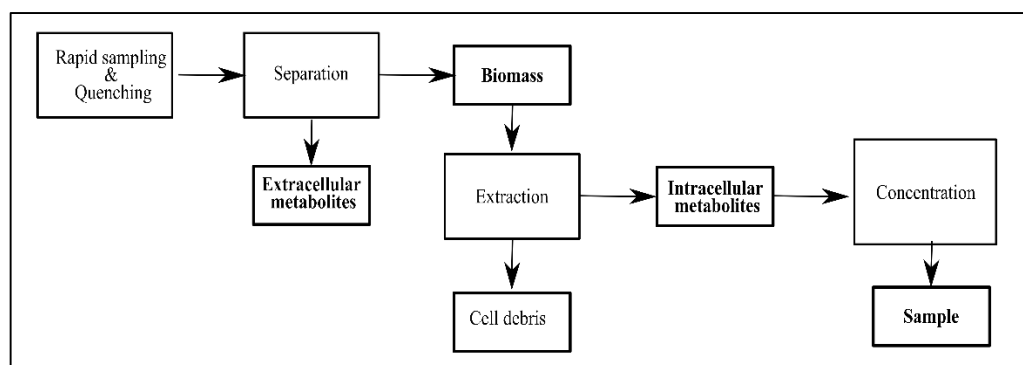


Figure 1.6. A general overview of sample preparation for metabolome studies [94][96]

1.4.1.3. *Rapid sampling*

The turnover time of the metabolites is the ratio between the intracellular concentration and the total influx. The fast turnover times of intracellular metabolites require methods for sampling that should be as fast as possible. While the turnover time of metabolites in central carbon metabolism is very short, such as 2-20 seconds, the evaluation of intracellular concentrations of these intermediates requires sub-second quenching methods, the turnover time of amino acids is relatively longer, up to 50-3000 seconds [97] (Table 1.2.).

Table 1.2. Turnover times of intracellular metabolites during cold methanol quenching in *S. cerevisiae* under aerobic glucose-limited conditions ([97])

Metabolite	Estimated turnover times(s)
Pyruvate	2
FBP	3
Oxoglutarate	5
PEP	6
2PG+3PG	7
Succinate	20
Tyrosine	57
Glycine	247
Lysine	619
Glutamic acid	1112
Alanine	3268

There are different fast sampling devices and techniques for both Erlenmeyer flask cultivation and bioreactors that are only used for fast sampling also include steps such as quenching and extraction [98,99].

1.4.1.4. *Quenching*

Catching up the desired metabolic state of the cells requires instantaneously quenching enzymatic activities of biochemical reactions using ideal solutions. The quenching process can be carried out with aqueous solutions with remarkable temperature change (freezing

(below -20 ° C degrees) or boiling) or severe pH change (very acidic or very basic) to stop enzymatic reactions as soon as possible without intracellular metabolite leakage to the extracellular environment (Table 1.3.). The turnover time of extracellular metabolites is higher than the turnover time of intracellular metabolites since enzymes are at very low concentrations outside the cell and additionally dilute in the extracellular environment [100]. Due to the distinctive structures and compositions of the cell envelope of different microorganisms and the necessity of preventing intracellular metabolite leakage, there is no common quenching method that can be used for each microorganism. Yeasts have cell envelopes containing large amounts of proteins and polysaccharides and relatively low lipids and chitin.

Table 1.3. Common quenching methods for yeasts

Quenching method	Yeast	Reference
Cold-methanol (60 percent v/v) solution	<i>S. cerevisiae</i> , <i>P. pastoris</i>	[101], [102]
Pure methanol at -40 °C	<i>S. cerevisiae</i> , <i>P. pastoris</i>	[97]
Cold glycerol–saline solution	<i>S. cerevisiae</i>	[103]
60 percent methanol, 0.11 M ammonium bicarbonate	<i>P. pastoris</i>	[102]
60 percent(v/v) buffered methanol (-58°C, 10 mM HEPES)	<i>S. cerevisiae</i> , <i>P. pastoris</i>	[104]

1.4.1.5. Extraction

Extraction protocols aim to obtain metabolites from the intracellular space via increasing cell envelope permeability with extracting agents. The extraction methods can be categorized as chemical methods ((acidic(perchloric acid) or alkaline treatments), boiling ethanol, hot water, cold methanol), mechanical methods (microwave, ultrasonic, pressurized liquid extraction, and supercritical fluid extraction), physical methods (freezing-thawing, heating, osmotic shock) and also combinations of these. It is essential that the extraction

method is fast and reliable and that it can extract as wide a range of metabolites as possible (from polar to non-polar).

1.4.1.6. Concentration

Relatively low concentrations of intracellular metabolites require concentrating the samples to allow detection after extraction protocols. The concentration steps can be performed with e.g. vacuum dry evaporation systems, concentrators with inert gas, rotary evaporators, and freeze-dryers (lyophilization).

1.4.1.7. Analytical methods

Quantitative metabolome studies require the detection and quantification of a wide range of chemical diversity in small amounts of samples. The most widely used techniques for large scale analysis of metabolome are nuclear magnetic resonance (NMR) and mass spectrometry (MS) based technologies (liquid chromatography (LC-MS), gas chromatography (GC-MS)), and (ultra)/high-pressure liquid chromatography (UPLC/HPLC) with UV or fluorescent detectors. The analysis of metabolites is case-specific due to the complex nature of tissue/organism and the chemical diversity of metabolites. Additionally, there are also benefits and drawbacks of each technology. For instance, NMR is a non-invasive method and a substantial method for metabolic fingerprinting, but it has lower sensitivity and is not suitable for quantification of metabolites. Mass spectrometry, especially LC-MS/MS and GC-MS, is the most preferred technique to detect metabolites with high sensitivity (down to nanomolar concentrations), selectivity, and robustness. Also, MS-based analysis allows for the detection of thousands of metabolites and the identification of hundreds of organic molecules. A comprehensive metabolome study requires the use of multiple technologies, as there is unfortunately no method that can identify and quantify all metabolites of an organism in a single run [94].

1.5. SHORT TERM STIMULUS-RESPONSE EXPERIMENTS

Much effort has been spent in the past to investigate the gene sequences of organisms (genomics), gene transcription (transcriptomics), and the translation (proteomics) of coded information. The metabolome (metabolomics) has so far played a minor role in the field of

'omics' research. Response to oxidative stress by yeast cells is typically complex, tightly regulated, and involves several -omic layers following a time hierarchy. Dedicated experiments exploiting this time hierarchy would ultimately allow isolating a single-omic response to external stimuli. Such an approach has been adopted in stimulus-response experiments focusing on monitoring the system response upon external perturbation of a well-defined metabolic state (e.g., steady-state in a chemostat) within a couple of minutes [105]. The resulting data can be used to construct and parametrize a dynamic model for metabolic reaction networks [106–108].

Stimulus-response experiments require sample frequencies typically on a subsecond time scale on the short-term dynamics of enzymatic reactions. The relaxation times after stimulation for various cellular responses on a time scale were ranked by Roels in Figure 1.7. [109]. Mass action law kinetics clearly shows the fastest response, followed by allosteric processes that control enzyme activity. Changes at the genetic or proteomic levels are more gradual, and enzyme concentrations can be assumed to remain constant for a few minutes after stimulation. With this in mind, there is no effect on the genetic or proteomic properties of metabolism within a couple of minutes observation window on the stimulus-response experiments. As a result, the recorded response will only involve information regarding enzyme kinetics, excluding the effects of gene regulation, protein inactivation, and other factors [110].

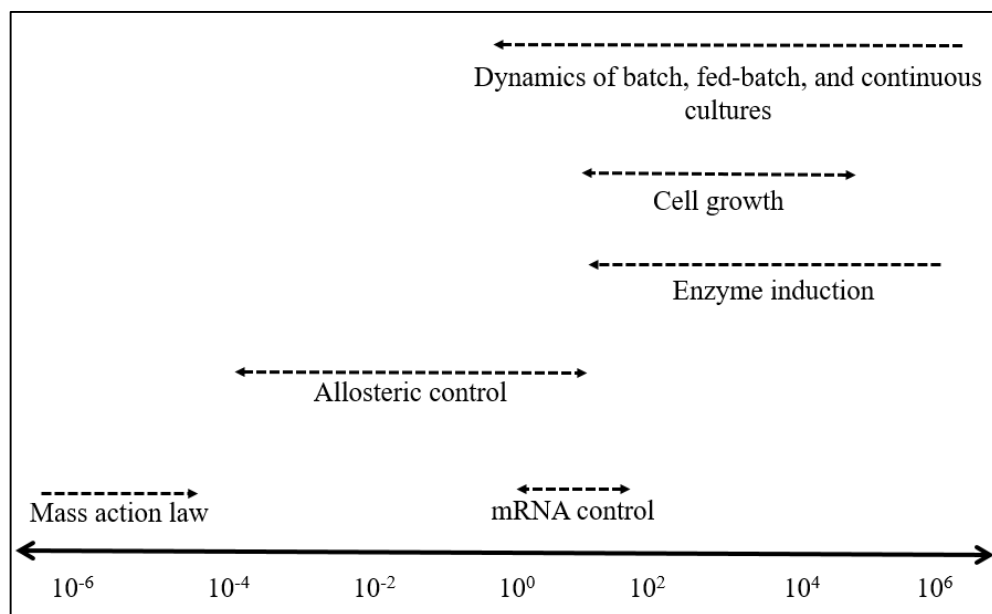


Figure 1.7. Characteristic times of adaptational mechanisms in organisms (adapted from Roels et al., 1983) [109,110]

A typical short-term stimulus-response experiment involves taking "snapshots of the metabolism" upon external perturbation by rapidly quenching the metabolism to stop all enzymatic activities, separating cells from the fermentation medium, and extracting and quantification of intracellular metabolites. Focusing on the within-minutes response of the cells and assuming that changes in enzyme concentration will be negligible, the response can be attributed to metabolic level and the kinetic interactions [111,112].

As a result, the obtained data or metabolic models are only valid for the conditions chosen. The experimental scenario chosen may limit the prediction of metabolic behavior under different conditions. For example, metabolism in a wild-type organism does not always have the same properties as metabolism in a recombinant production organism in a production process. Consequently, if the metabolism of a production strain under production conditions must be studied, the experimental conditions for the stimulus-response experiment should be as similar to the production process as possible [110].

1.6. CULTIVATION MODES

1.6.1. Batch Cultivation

Batch system is defined as closed system, whereby all the substrates are added into the reactor and at the end, products and unused substrates are collected [113]. At constant volume, the change of biomass is expressed as:

$$\frac{dX}{dt} = (\mu - k_d) \cdot X \quad (1.3)$$

where k_d is the specific degradation constant; μ is the specific growth rate and x is the biomass concentration. In case, the product is growth associated (e.g. enzymes), the substrate consumption is:

$$\frac{dS}{dt} = -\left(\frac{\mu}{Y_{XS}} + m_s\right) \cdot X \quad (1.4)$$

with m_s is the amount of substrate consumed for maintenance. In case, the product formation is not directly coupled with growth, the substrate consumption rate is:

$$\frac{dS}{dt} = - \left(\frac{\mu}{Y_{XS}} + \frac{q_p}{Y_{PS}} + m_s \right) \cdot X \quad (1.5)$$

1.6.2. Fed-batch Cultivation

During the fed-batch process, system is fed (typically with fresh substrate) continuously or intermittently. This operation mode is typically used to prevent substrate inhibition and toxic products/by-products [113]. The change of biomass can be expressed as:

$$\frac{dX}{dt} = D \cdot x_i + (\mu - k_d - D) \cdot X \quad (1.6)$$

with $D=F/V$ being the dilution rate (h^{-1}). In case the product is formed but not directly coupled with energy metabolism, the rate of consumed substrate is defined as:

$$\frac{dS}{dt} = D \cdot (S_i - S) - \left(\frac{\mu}{Y_{XS}} + \frac{q_p}{Y_{PS}} + m_s \right) \cdot X \quad (1.7)$$

1.6.3. Continuous Cultivation

Continuous operation mode is the easiest and most feasible method for keeping cells in constant conditions considering both metabolic state and physicochemical conditions. Continuous mode is based on the continuous flow of fresh medium at a constant rate, while the culture liquid comes out at the same rate and becomes a steady-state under constant environmental conditions. These cultures are a preferred strategy for understanding the response of cells to both external stimuli (environmental stresses) and internal traits. Since chemostat cultivation is under steady-state operating conditions, it allows us to observe the effect of only one parameter and obtain robust and reliable data by keeping all other parameters constant. During continuous processes, there are inputs and outputs to the system [113,114]. At steady state, cell growth can be expressed as:

$$F_{in} \cdot x_i - F_{out} \cdot X + \mu \cdot X \cdot V = 0 \quad (1.8)$$

In case of sterile feed ($x_i = 0$),

$$\mu = \frac{F_{out}}{V} = D \quad (1.9)$$

In case, non-growth associated product formation, substrate consumption is expressed as:

$$F_{in} \cdot S_i - F_{out} \cdot S - \left(\frac{\mu}{Y_{XS}} + \frac{q_P}{Y_{PS}} + m_s \right) \cdot X \cdot V = 0 \quad (1.10)$$

These cultures are important not only for kinetic and metabolic characterization but also in providing quantitative information at the whole-cell level [114].

1.7. MODELLING FOR UNDERSTANDING METABOLISM

The abstracting of reality, preferably in a simplified form, is the heart of modeling. Models should be built or kept as simple as possible. They provide improved understanding and allow prediction, scenario evaluation, assumption checking, and occasionally optimizing of the underlying mechanisms of biological processes [92,115]. Though there are numerous types of models, e.g., verbal, graphical, business, physical, abstract, and probabilistic models throughout this thesis, deterministic mathematical models are the focus.

While models can be the end result of a given study, these can as well be an intermediate tool for the in-depth study of a given (bio)system. As such, models and experiments should be considered integral and closely interacting tools to grasp the true understanding of the (nearly-)exact mechanisms and to gain new perspectives on biological systems.

Defining the purpose or the scope of the model is the first key step in model building. Within an industrial biotechnology setting, models can have various purposes, e.g., finding targets for metabolic engineering to enhance the desired product, decrease by-products, reconcile experimental, inherently noisy data, understanding dynamics of processes at the cellular level, to control bioprocesses at a process level. When used at a process level, kinetic models are used to understand the dynamics of fermentation in terms of the rates of growth, substrate consumption, and product formation rates, which, in turn, can be calculated via measuring the extracellular metabolites. These can typically be used to predict fermentation behavior under different external conditions as well as for scale-up studies [116]. When coupled with mass balances, kinetic models consist of a set of ordinary differential equations, yet they are limited in size; typically, a handful of state variables are used. In a kinetic model, if biomass

composition varies with environmental changes, it is further specified as a structured kinetic model. The unstructured kinetic model, in contrast, is based on the “assumption that total biomass concentration is a single component. Studies showed that unstructured models could define the observed behavior during exponential and pseudo-steady states with good approximation. These models give valuable information to design bioprocesses and identify key kinetic parameters. These models require rich data. The actual process can be indicated with these models. These models only represent conditions under the operating process [117,118].

In particular black-box models, cell metabolism is expressed as a function of the net influx and efflux of the reactants to products by one single macromolecule equation (e.g., biomass formation) (Figure 1.8.). The black box model does not require biochemical information and relies on the measurement of the inflow and outflow of the substrates and products [119].

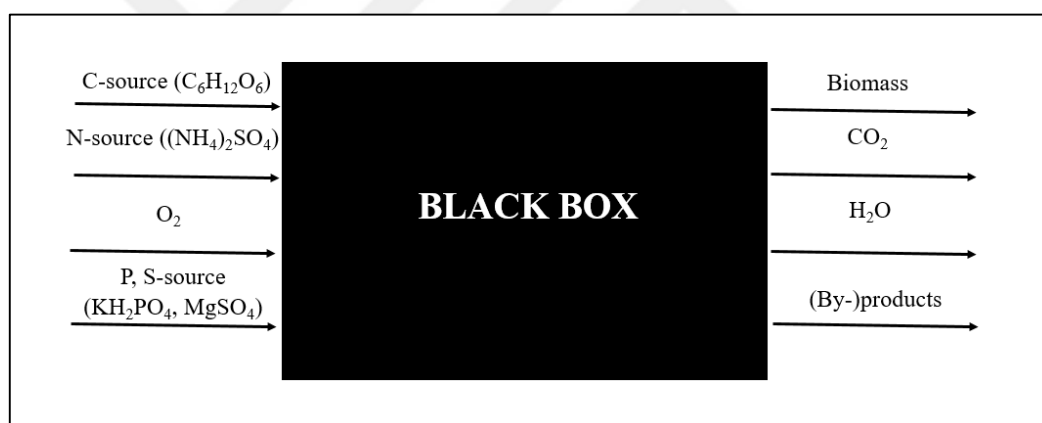


Figure 1.8. A black-box description of microbial fermentations

In this thesis, steady state, flux modeling be the primary focus. These models consist of a list of biochemical reactions with a known stoichiometry of nearly-all metabolic reactions. These models can be used to simulate the growth and product formation using flux balance analysis, predict the effect of knock-out studies as well as determine the flux distribution within the cell. These models, as opposed to kinetic models, consist of a set of linear algebraic relations and contain a large number (around 1000) of state variables.

The size of the model depends not only on the scope of the model but also on the available information related to the subject. Two alternative approaches are present to discriminate the

"resolution" of models for biological systems: black box and (light)grey-box models. Black box models rely on the overall balance between inputs and outputs of the system without considering the transformations that occur inside the cell. These are limited to extracellular information. On the other hand, (light)grey models take into account known reactions involved in the metabolism of the cell [119]. In a sense, the "color shade" of the model depends on the available biological information. For this, white models would ultimately represent the systems where the biological details are perfectly available.

1.7.1. Flux Balance Analysis

Steady-state models, also known as genome-scale metabolic models, comprise the known biochemical reactions for a microorganism. Flux balance analysis (FBA) focuses on calculating the flow of metabolites in the metabolic network based on constraints. Flux balance analysis requires that the stoichiometric coefficients of each metabolic reaction be defined as a stoichiometric matrix (S) with a size of $m \times n$, the rows of which contain each metabolite and the columns representing their reactions, and the vector (v) with a size of n in which the flux through each reaction in the network.

The mass balance equation for each metabolite (x) can be expressed using a matrix notation as follow:

$$\frac{dx}{dt} = S \cdot v \quad (1.11)$$

where S is the stoichiometric matrix and v . At a steady state, the equation can be written as [120]

$$S \cdot v = 0 \quad (1.12)$$

The stoichiometric coefficient of each metabolite is the intersection of the respective column and row, and if the coefficient is a negative value, this metabolite is consumed in the reaction; if it is positive, that metabolite is produced at the end of the reaction.

The system is generally under-determined because the number of reactions (variables) is substantially more than the number of metabolites (constraints) in metabolic models; thus, there is no unique solution for the system of linear equations [120]. The solution space can

be constrained with the stoichiometric coefficients of S , thermodynamic constraints and upper/lower limits of fluxes, and organism-specific constraints. Flux Balance Analysis is based on the assumption that the system will attain a steady-state while maximizing or minimizing an objective function, and it represents the flux distribution of the metabolism within a constrained solution space (Figure 1.9.). After defining the objective function, linear programming is used to solve the model and identify flux distributions within a constrained solution space. Although a metabolic objective function is usually a biomass reaction in genome-scale metabolic models, maximization of ATP production and target metabolite production can also be defined as objective function [121,122].

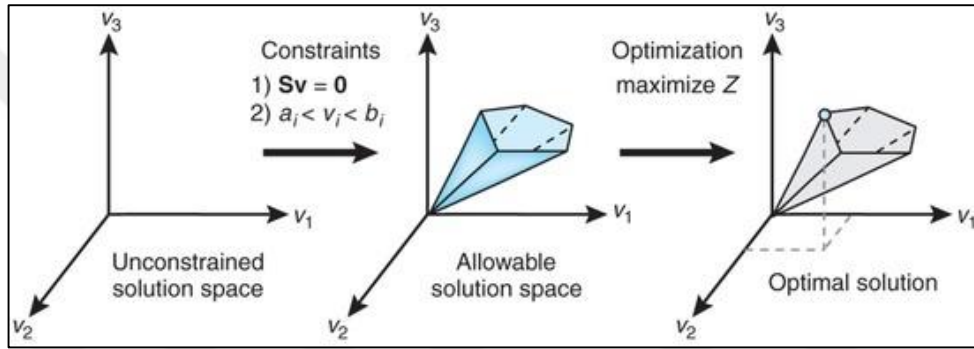


Figure 1.9. The basis of constraint-based modeling. The main components of constraint-based metabolic models include stoichiometric matrices and flux constraints.

The mathematical representation of objective function (Z) is in the form of Equation 3,

$$Z = c^T \cdot v \quad (1.13)$$

where c denotes the coefficients of each reaction that contributes to the objective function.

Due to the availability of several optimum solutions (alternate optima) with the same objective value, the flux distribution calculated by FBA is really just one solution that ensures the maximum objective. Flux variability analysis (FVA) determines the minimum and maximum values of all reaction rates at the optimal value of the objective function value [123].

1.8. SCALE UP STRATEGY FOR FERMENTATION SYSTEMS

Current biotechnological applications need a vast quantity of low-cost, high-quality products. Transfer of laboratory-scale processes to industrial scale is required to produce high-quality products by the industry. Environmental circumstances have a major influence on cell physiology and, in many cases, product yields and quality. As a result, the culture milieu should ideally stay consistent across all scales during process development. Pilot- and industrial-scale production will have different conditions from laboratory scale. The scale-up strategies for empirical approaches are based on the similarity principle and dimensional analysis via keeping particular parameters constant during the scale-up (e.g., mixing time, power input, oxygen transfer coefficient (k_{La}), impeller tip speed, etc.). However, with the increasing scale from laboratory to large scale in the change in geometric and physical conditions, heterogeneities in pH, dissolved oxygen, substrate, temperature, and shear stress in the different regions of bioreactors will result [124–126]. The flow field conditions and physiological responses of microbial cells are two important standpoints for successful scaling up.

The scale-up in fermentation aims to produce a higher amount of products while simultaneously increasing or at least maintaining specific yields and product quality. The changes in geometric and physical conditions can cause decreased process consistency and batch-to-batch variations due to less favorable mixing behavior and impaired physiological reaction conditions [127].

The scale-up can be divided at four stages: shake flask level, laboratory fermenter level (3–20 kg), pilot fermenter level (50–5000 kg) and production level (25–100 tonnes) relating with type of processes [128].

1.8.1. Problems with Scale-up

The main problem with the increasing vessel sizes is the reduced mixing quality. The substrate and oxygen show opposite gradients due to the design of the conventional fermenters where substrate feed is added from the top and aeration from bottom. The larger fermenters cause larger depletion zones of substrate and oxygen due to longer distances.

Moreover, the amount and time of culture broth that needs to be mixed increases with increasing volume. In large volumes, stronger hydraulic pressure is added to the factors affecting the oxygen transfer rate [125].

1.9. STRAIN IMPROVEMENT BY CLASSICAL TECHNIQUES

Strain development is critical to the commercialization of microbial fermentation systems by means of increasing productivity or the ability to use cheaper substrates. Rational screening and genetic engineering have recently attracted attention; random mutagenesis is still cost-effective and frequently used for short-term strain improvement [129,130]. Classical mutagenesis techniques result in random changes in the genetic material of the microorganisms to enhance the desired traits. The general workflow of the classical mutagenesis can be categorized into three steps: 1) exposure of the native strain to the mutagen, 2) colony screening of the surviving population and cultivation of colonies at down to microscale (e.g., 96-well plate) and 3) screening of target product with relevant assays [130]. Mutagenesis can be improved in terms of mutagen type and dose, mutagen specificity effects can be considered, and mutagenesis itself can be intensified or directed to produce the highest frequency of desired mutant types among the isolates to be screened [129,130]

Mutagenic treatments can be categorized as physical, e.g., gamma, ultraviolet (UV), and X-irradiation; and chemical, e.g., ethyl methane sulphonate (EMS), methylmethane sulphonate (MMS), and nitrosomethyl guanidine (NTG). Far ultraviolet is the most user-friendly of all mutagens, and it appears to cause practically all known forms of DNA damage [129,130].

With the advances in molecular biology techniques, recombinant DNA technology is of great interest in the targeted improving microbial strains of industrial microorganisms. The recombination techniques have been used to improve the substrate uptake capability, increase the tolerance to end-product, and enhance product yield for microbial strain improvement [130]. Rational metabolic and cellular engineering, adaptive evolutionary engineering, and also combined analysis of large amounts of omics data with *in silico* modelling approaches are different perspectives for strain improvement [131]. Nevertheless, a primary reason why classical techniques are still the preferred is that they do not require prior knowledge about the biochemical route, transportation, and regulation mechanisms of

metabolite. Moreover, with the advances in the sensitivity and automation of analytical instruments, system variability decreases, while sensitivity and precision increase [132].



2. AIM OF THE THESIS

This study aimed to further our understanding of the responses of non-conventional yeasts to oxidative stress. This aim was achieved through stimulus-response technology and a systems biology approach using tools of phenome (fermentation profiles), targeted metabolome, and fluxome. The challenges of this study were chemostat cultivation of different yeasts at the same dilution rate, catching metabolome dynamics, and measuring low concentrations of intracellular metabolites. An integral part of this probe is oxidative stress responses, one of which is glutathione. Following this, it was aimed to improve a wild-type strain of non-conventional yeast *Y. lipolytica* to produce higher GSH by classical UV-mutagenesis.

3. MATERIALS

3.1. STRAINS

Saccharomyces cerevisiae CEN.PK117-3D,

Pichia pastoris ATCC 28485,

Yarrowia lipolytica ATCC 20460.

3.2. CHEMICALS

4-aminobenzoic acid (Sigma-Aldrich), Acetonitrile (HPLC-grade), Ammonium sulfate (Merck), Antifoam Y-30 emulsion (Sigma Aldrich), Bacteriological agar (Sigma-Aldrich), Boric acid (Sigma-Aldrich), Ca-D-pantothenate, Calcium chloride dihydrate (Sigma-Aldrich), Cobalt chloride hexahydrate, Copper(II)sulfate pentahydrate, D(+)-Glucose monohydrate (Sigma-Aldrich), D-biotin(Sigma-Aldrich), Diethyl ethoxymethylenemalonate (Sigma-Aldrich), Dinitrosalicylic acid (Sigma-Aldrich), Distilled water, Dithiothreitol (DTT (Sigma Aldrich)), EDTA disodium salt (Merck), Ethanol (HPLC grade), Glycerol (Sigma-Aldrich), Hydrogen peroxide (30 percent) (Sigma-Aldrich), Iron sulfate heptahydrate(Sigma-Aldrich), Magnesium sulfate heptahydrate (Sigma-Aldrich), Manganese (II) chloride dihydrate, Methanol (HPLC grade), Millipore water, Myo-inositol, Nicotinic acid, Orthophosphoric acid (Isolab), Oxidized glutathione (Bioshop), Peptone Bacteriological (HiMedia Laboratories), Potassium iodide (Sigma-Aldrich), Potassium phosphate monobasic, Pyridoxal hydrochloride, Reduced Glutathione (Bioshop), Sodium azide (Sigma-Aldrich), Sodium hydroxide (Sigma-Aldrich), Sodium molybdate dihydrate, Sulfuric acid (Honeywell Riedel-de-Haën),Thiamine hydrochloride, Yeast extract (Sigma Aldrich), Zinc sulfate heptahydrate.

3.3. DEVICES

Air compressor, Analytical balances (Mettler Toledo), Autoclave (Daihan Scientific, Wisd Laboratory Instruments), Bioreactor Minifors 2 (INFORS HT) with pH probe (Hamilton and DO probe (Hamilton) and peristaltic pumps, Centrifuges (Eppendorf 5424 and Gyrozen 1580R), Freeze-dryer (Labconco), Fume hood (GreenLab), HPLC system with UV and Refractive index detectors (Shimadzu Nexera XR), Incubator (Mettmert), Laminar flow cabinet (GreenLab), BlueInOneFerm gas analyzer (Blue Sens), Peristaltic pump (Watson Marlow), pH meter (Mettler Toledo), Refrigerator (Arçelik), Rotary evaporator (Heidolph), Shaker incubator (Innova 44, New Brunswick Scientific), Spectrometer (Genesys 10S UV-VIS, Thermo Scientific), Steam generator (Alba Makina), Chiller (Gazi), Boiler, Thermostable water bath (Grant SUB Aqua 12 Plus), Vacuum pump, Vortex (Scilogex MX-S), Water-cooled cooling circulator (Kendal), Ultrapure water system (Milli-Q, Millipore), Ultra low-temperature freezer (Forma 88000, Thermo Scientific), Microplate reader (Varioskan LUX, Thermo Scientific).

3.4. CONSUMABLES

Centrifuge tubes (15, 50 mL), Cryotubes, Cuvette, Erlenmeyer flasks, Glass bottles, Membrane filters (0.22 μ m), Microcentrifuge tubes (1.5, 2 mL), Micropipettes and tips, Petri dishes, Plastic inoculation loops, Sterile & nonsterile syringe filters (0.22 μ m), Sterile air/gas filters (0.22 μ m), Syringes (5, 10, 50 mL), Vacuum filtration set, Vials and vial caps, IC-Pak Ion Exclusion Column (7.8x300mm, Waters), XBridge C18 column (5 μ m 4.6x150mm, Waters), Quantification kit for oxidized and reduced glutathione (Sigma Aldrich 38185).

3.5. SOFTWARE

MATLAB Version: 9.9.0.81467703 (R2020b) License Number: 968398, COBRA Toolbox v3.0 [133].

4. METHODS

4.1. STRAINS AND PRECULTURE CONDITIONS

Saccharomyces cerevisiae CEN.PK113-7D, *Pichia pastoris* ATCC 28485, and *Yarrowia lipolytica* ATCC 20460 were kept in 30 percent glycerol at -80°C prior to use in this study. Yeasts cells grow to a stationary phase in 250 mL Erlenmeyer flasks with 50 mL of double Mineral medium at 180 rpm and 30°C [134]. The composition of the double mineral medium was as follows (per liter) 10 g glucose, 10 g ammonium sulfate, 6 g potassium phosphate monobasic, 1 g magnesium sulfate heptahydrate, 2 mL of 1000X vitamin solution, and 20 mL of 100X trace elements. Glucose solution was separately sterilized at 121°C for 3 min. 1000X vitamin solution and 100X trace elements solution were sterilized with syringe filters.

4.2. OXIDATIVE AND REDUCTIVE STRESS DOSAGE RESPONSE

4.2.1. Hydrogen Peroxide Plate Sensitivity Assays

Yeasts were cultivated on YPD medium until they reached OD₆₀₀ 0.6. 10 µL of each sample was spread onto the YPD agar plate containing 1, 2, 5, and 10 mM of H₂O₂. Plates were incubated at 30°C for 3 days.

4.2.2. Dithiothreitol Plate Sensitivity Assays

Cells were cultivated in a YPD medium until they reached OD₆₀₀ 0.3. Then, 10 µL of each sample was spread onto the YPD agar plate containing 0, 1, 2, 5, 10, 20, and 40 mM of dithiothreitol (DTT). Plates were incubated at 30 °C for 3 days.

4.3. CHEMOSTAT CULTIVATIONS

Cells were aerobically cultivated in a double mineral medium at a dilution rate of 0.1 h⁻¹ in 4 L bioreactors (Minifors 2, Infors HT, Switzerland) with a working volume of 1.5 L

supplemented, 200 $\mu\text{L L}^{-1}$ Antifoam Y-30 emulsion (Sigma Aldrich). Chemostat cultivation was preceded by a batch phase performed on a medium identical to the feed medium. Following the completion of the batch phase, medium feeding began, as evidenced by a sharp decrease in the off-gas compositions of carbon dioxide and oxygen. The culture temperature was kept at 30°C, and pH was controlled at 5.5 using 3 M orthophosphoric acid and 3 M sodium hydroxide. Yeasts were sparged with sterile air with a 0.75 L h^{-1} flow rate and stirred initially at 500 rpm. DO level was maintained above 20 percent via cascade control of agitation speed. The fractions of carbon dioxide and oxygen were followed with a BlueInOneFerm gas analyzer (BlueSens, GmbH, Herten, Germany). An added amount of acid, base, and antifoam was monitored during the operation.

Chemostat cultures were assumed to be steady-state when, after 5 volume changes, the culture biomass production, carbon dioxide, and oxygen compositions of off-gas readings did not change more than 5 percent. Furthermore, glucose-limitation was confirmed by measuring the residual glucose concentration under 0.9 g L^{-1} at a steady state. Stress pulses to the bioreactor were directly injected into the bioreactor with a sterile syringe. The feed pump and outflow pump were stopped at the start of a pulse experiment and restarted 5 min after each pulse. The workflow of perturbation experiments was shown in Figure 4.1.

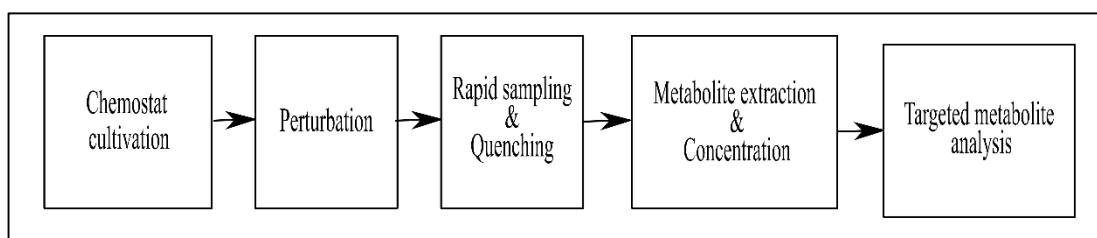


Figure 4.1. The workflow of perturbation experiments performed in this thesis

4.4. HYDROGEN PEROXIDE PERTURBATION (OXIDATIVE STRESS)

Before hydrogen peroxide (H_2O_2) perturbation, 10 mL of culture broth was rapidly sampled as a reference state. Then 1 mM, 5 mM, and 40 mM (final concentration) of H_2O_2 was pulsed with an aseptic syringe to the reactor after 5 residence time. To observe the response of yeasts, broth samples were rapidly withdrawn and quenched 1, 3, and 5 min after exposure to H_2O_2 , and metabolites were analyzed after extraction.

4.5. DITHIOTHREITOL PERTURBATION (REDUCTIVE STRESS)

Before dithiothreitol (DTT) perturbation, 10 mL of culture broth was rapidly sampled as a reference state. Then 20 mM (final concentration) of DTT was pulsed with an aseptic syringe to the reactor after 5 residence time. To observe the response of yeasts, broth samples were rapidly withdrawn and quenched 1, 3, and 5 min after exposure to DTT, and metabolites were analyzed after extraction.

4.6. SAMPLING AND QUENCHING FOR INTRACELLULAR METABOLITES

10 mL of broth samples were rapidly withdrawn and quenched into 35 mL pure methanol at $<-40^{\circ}\text{C}$, quickly vortexed, and placed back in the -40°C . To remove the extracellular medium, samples were centrifuged at 5000 g for 5 min, at -12°C (1580R, Gyrozen, Korea). Supernatants were stored at -80°C for the determination of extracellular metabolites [106].

4.7. EXTRACTION OF INTRACELLULAR METABOLITES

Firstly, 35 mL of 75 percent ethanol was heated in a water bath at 95°C for 5 min, and the boiling ethanol was poured on the cell pellet quickly, and the tubes were vortexed as fast as possible and put on the water bath for 3 min. Then, each tube was placed at -40°C , and samples were centrifuged at 7000 g for 10 min at 4°C (1580R, Gyrozen, Korea), and the extracts were stored at -80°C [98].

4.8. CONCENTRATING OF INTRACELLULAR EXTRACTS

The extracts were concentrated via a rotary evaporator (Heidolph), and then samples were dried using a freeze-dryer (Labconco). Before analysis, the extracts were resuspended on 500-1000 μL double-distilled water.

4.9. METABOLITE QUANTIFICATION

4.9.1. Organic Acids, Sugars, and Ethanol Analysis

Organic acids, ethanol, and sugars were analyzed by HPLC (LC20AD, Shimadzu, Kyoto, Japan) equipped with an RI detector and Aminex 87HPX-87H ion exclusion column (Biorad) maintained at 50°C using 5 mM sulfuric acid as a mobile as at 0.6 mLmin⁻¹.

Residual sugar concentration was followed with dinitrosalicylic acid (DNS) method [135].

4.9.2. Amino Acids Analysis by HPLC

Identification and quantification of amino acids were carried out by HPLC (LC20AD, Shimadzu, Kyoto, Japan) after diethyl ethoxymethylenemalonate (DEEMM) derivatization, equipped with a UV detector (280 nm) at 25°C using an XBridge C18 column (5µm 4.6x150 mm, Waters) eluted with two mobile phases: 25 mM sodium acetate buffer (pH 5.8) with 0.02 percent sodium azide and an 80:20 acetonitrile: methanol (4:1(v/v), at 0.8 mL min⁻¹ [136].

4.9.3. Glutathione Analysis

Identification and quantification of glutathione were carried out by HPLC (LC20AD, Shimadzu, Kyoto, Japan) equipped with a UV detector (210 nm) at 30°C using an XBridge C18 column (5µm 4.6x150 mm, Waters) eluted with 25 mM sodium phosphate monobasic (pH 3.5) at 0.3 mL min⁻¹[137].

Total glutathione (reduced and oxidized glutathione) was determined with a colorimetric assay kit (Quantification kit for oxidized and reduced glutathione (Sigma Aldrich 38185).

4.10. MEASUREMENT OF CELL DRY WEIGHT

Optical density was followed with UV- spectrometry (Thermo Scientific Genesys 10S UV-vis) at 600 nm. Dry weights of the cells were used to determine the biomass concentration. 10 mL of sample was taken in pre-weighted tubes and centrifuged at 10000 rpm, pellet samples were washed with distilled water or PBS solution, and then washed pellets were dried on tubes at 105°C for drying for at least 24 h.

4.11. SCALE-UP OF *S.CEREVISIAE* AND *P. PASTORIS*

S. cerevisiae and *P. pastoris* were aerobically grown in a 150 L bioreactor with a 100 L initial working volume. Fed-batch cultivation was preceded by a batch phase performed on a medium containing 180 gL⁻¹ glucose. A constant rate feeding strategy was applied to prevent by-product formation. Following the completion of the batch phase, medium feeding began, as evidenced by a sharp decrease in the off-gas compositions of carbon dioxide and oxygen. The culture temperature was kept at 30°C, and pH was controlled at 5.5 using 3 M orthophosphoric acid and 3 M sodium hydroxide. Yeasts were sparged with sterile air with a 100 Lh⁻¹ flow rate and stirred initially at 300 rpm. DO level was maintained above 20 percent via cascade control of agitation speed. The fractions of carbon dioxide and oxygen were followed with a BlueInOneFerm gas analyzer (BlueSens, GmbH, Herten, Germany). An added amount of acid, base, and antifoam were monitored during the operation. First of all, cells were cultivated at fed-batch mode to determine the mid-exponential phase of yeasts. Then, the oxidative stress was applied by adding a 5 mM final concentration of hydrogen peroxide (H₂O₂) was pulsed to the reactor at the mid-exponential phase. The sample was withdrawn and quenched before hydrogen peroxide perturbation to understand the effects of oxidative stress on the intracellular amino acid and glutathione levels.

4.12. ELEMENTAL BALANCES

Biomass-specific rates of growth (μ), glucose consumption ($-q_s$), oxygen consumption ($-q_{O_2}$), carbon dioxide production ($-q_{CO_2}$), were calculated from the steady-state mass balances. The cumulative carbon balance accounts for the substrate used and accumulation

of the carbon for biomass, carbon dioxide and by-products if any. In the same manner, cumulative electron balance accounts for the electrons from glucose, transferred to biomass and oxygen, each weighed by the corresponding degree of reduction. The biomass formula for *S. cerevisiae* was used as described in [138], and for *P. pastoris* was used as in [139]. The calculated average values of the specific rates were reconciled under the constraint of satisfying the elemental conservation relations [140].

Statistical significance in the change in intracellular metabolite levels was assessed using paired Student's *t*-test (prior and posterior to the oxidative stress) under the null hypothesis that there is no change ($p < 0.05$).

4.12.1. Carbon and DoR Balances

$$6q_S + \mu + q_{CO_2} = 0 \quad (4.1)$$

Degree of reduction means that the number of equivalent electrons, available for oxygen (electron acceptor) upon combustion ($\gamma_{\text{glucose}}=24$, $\gamma_{\text{ethanol}}=12$, $\gamma_{\text{oxygen}}=-4$, $\gamma_{\text{glycerol}}=14$, $\gamma_{\text{acetate}}=11$ and $\gamma_{\text{biomass}}=4.2$).

$$\sum \gamma_i q_i = 0 \quad (4.2)$$

In the same manner, cumulative electron balance accounts for the electrons from glucose transferred to biomass and oxygen, each weighed by the corresponding degree of reduction (DoR).

$$24q_S + 4.2\mu - 4q_{O_2} = 0 \quad (4.3)$$

4.13. FLUX BALANCE ANALYSIS

A reconstructed genome-scale metabolic model for Yeast 8.5.0 was published in 2020 [141]. The model contains 2741 metabolites, 4058 reactions, and 1150 genes. Metabolic flux distributions were estimated through flux balance analysis [142]. The contextualized constrained metabolic model for *P. pastoris* iMT1026v3 was published in 2019 [143]. The model contains 1711 metabolites, 2246 reactions, and 1026 genes. Metabolic flux

distributions were estimated through flux balance analysis. In this thesis, Yeast 8.5.0 and *P. pastoris* iMT1026v3 were used for the FBA simulations of *S. cerevisiae* and *P. pastoris*, respectively.

COBRA Toolbox[133] was used in MATLAB with Gurobi9.5 [144] as the optimization solver for performing constraint-based metabolic flux analyses.

4.14. STRAIN IMPROVEMENT OF *YARROWIA LIPOLYTICA*

4.14.1. UV-Mutagenesis of *Yarrowia Lipolytica*

Exponentially growing *Y. lipolytica* cultures (OD600 = 0.7) were transferred to the sterile Petri dishes and exposed to UV irradiation for 30 to 40 minutes in a sterile cabinet. Then, the samples were kept at -20°C for 5 minutes and stored in the dark for 30 minutes to avoid light-induced DNA repair. Afterward, 10 µL of mutagenized cultures were spread on the YPD agar plate containing 10, 15, and 20 mM H₂O₂. Plates were incubated at 30°C for 3 days.

4.14.2. Cultivation of Mutant *Yarrowia Lipolytica*

Mutant strains of *Yarrowia lipolytica* were screened via growing in 2XVerduyn medium until they reached OD600 3.5. Different concentrations of H₂O₂ were pulsed, and after 3 hours, total glutathione levels were determined with a quantification kit for oxidized and reduced glutathione. The workflow of strain improvement process for *Y. lipolytica* was shown in Figure 4.2.

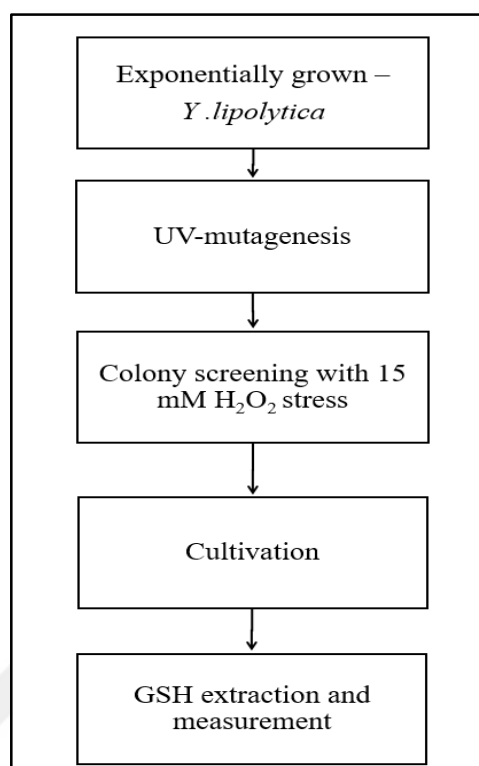


Figure 4.2. Workflow of the strain improvement process for *Y. lipolytica*

5. RESULTS AND DISCUSSION

Both *S. cerevisiae* and *P. pastoris* were subjected to oxidative and reductive stresses, and targeted metabolite analysis was carried out. First, stress dosage experiments were performed, and the results were presented as plate sensitivity assays. From these assays, 1, 5, and 40 mM H₂O₂ for oxidative stress and 20 mM DTT for reductive stress were decided to use subsequent perturbation experiments.

5.1. PLATE SENSITIVITY ASSAYS

5.1.1. Oxidative Stress

The patch/plate assays were used to examine the toxicity level of hydrogen peroxide on the yeast cells. For *P. pastoris* and *Y. lipolytica*, 10 mM of H₂O₂ was found to be the toxic concentration, while 5 mM of H₂O₂ is toxic for *S. cerevisiae*, (Figure 5.1.), similar to earlier studies [10].

5.1.1. Reductive Stress

Similarly, the patch/plate assays were used to determine the toxicity level of DTT on the yeast cells. For both yeasts, 40 mM was the toxic concentration for DTT (Figure 5.2.). In Trotter et al., 2002, the wild-type *S. cerevisiae* was extremely resistant, growing on plates containing DTT concentrations as high as 16 mM [31]. Therefore, 20 mM of DTT was selected in subsequent perturbation experiments.

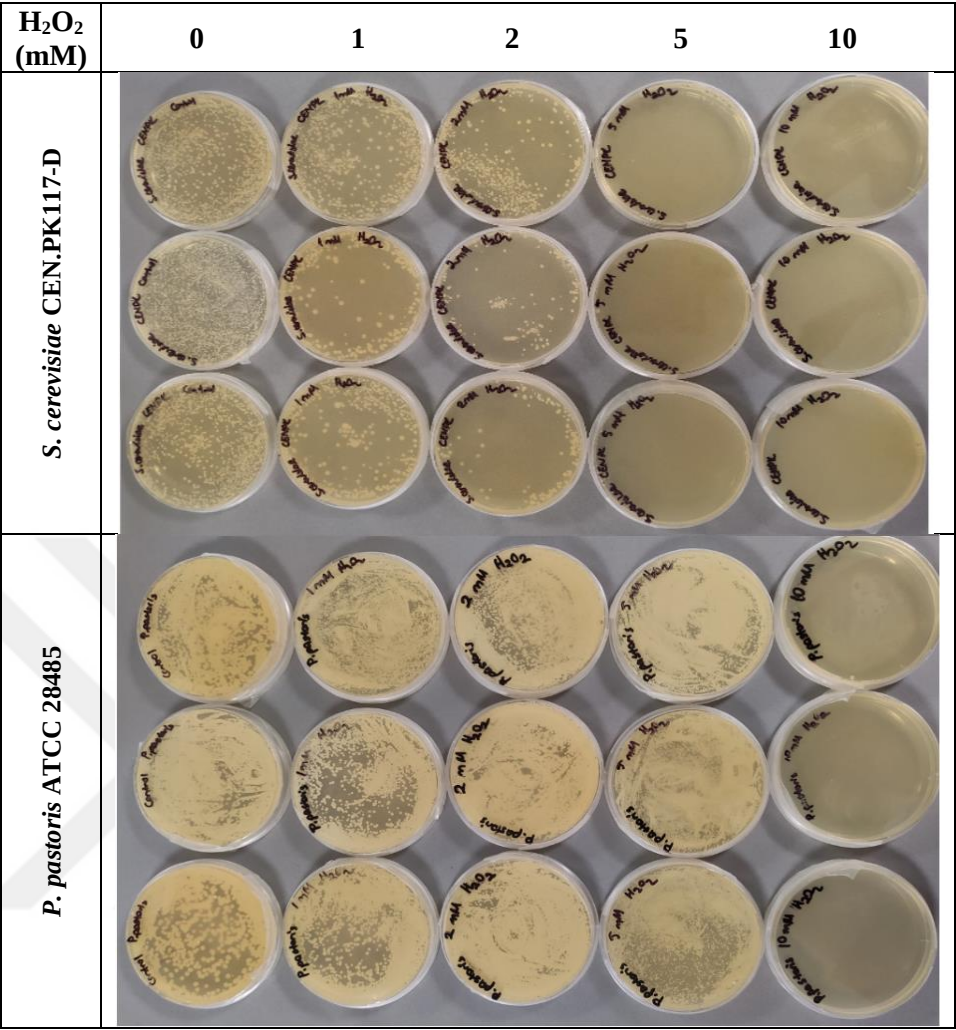


Figure 5.1. YPD agar plates containing 0, 1, 2, 5, and 10 mM of H₂O₂ after 3 day incubation

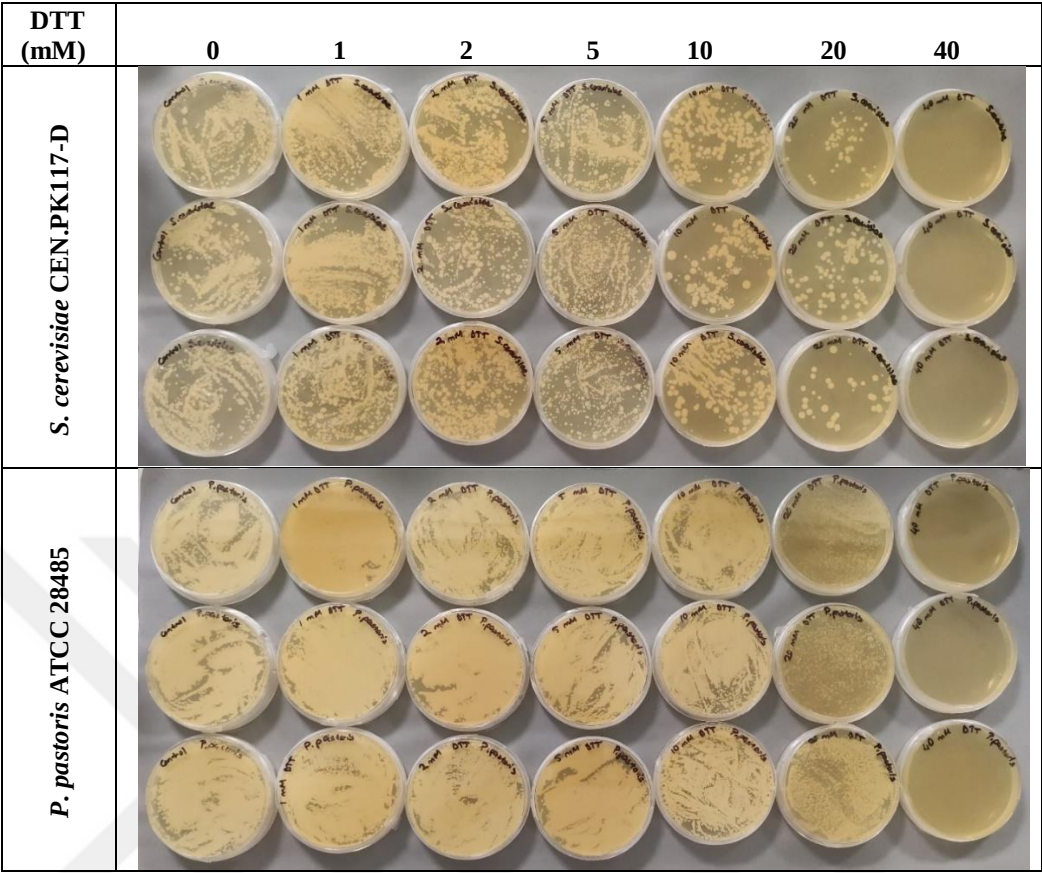


Figure 5.2. YPD agar plate containing 0, 1, 2, 5, 10, 20, and 40 mM of DTT after 3 day incubation

5.2. FERMENTATIONS

5.2.1. Chemostat Cultivation of *S. cerevisiae* and *P. pastoris* and 1 mM of Hydrogen Peroxide Perturbation

To understand the oxidative stress response, H₂O₂ perturbation experiments were used in this study. Starting with well-characterized steady-state cultures, is essential to stimulus-response experiments. With this aim, glucose-limited chemostats were carried out at a dilution rate of 0.1 h⁻¹ for both yeasts.

There was a significant difference between *P. pastoris* and *S. cerevisiae* in batch fermentation profiles. With an initial glucose concentration of 10 gL⁻¹, *P. pastoris* produces approximately 1.5 gL⁻¹ ethanol, while *S. cerevisiae* produces around 4 gL⁻¹. In line with

ethanol production, *S. cerevisiae* also produces 0.3-0.5 gL⁻¹ glycerol and acetate during batch culture, while there is no detectable by-product formation by *P. pastoris*. As Crabtree-positive yeast, the glucose yield of biomass for *S. cerevisiae* was nearly 4 times lower than *P. pastoris*. There has been a contradiction for *P. pastoris* related to its Crabtree status. While different studies reported that *P. pastoris* was Crabtree-negative yeast [145,146], production of low levels of ethanol (approximately 1.5 gL⁻¹ ethanol) was reported in the presence of excess glucose[147]. Due to its Crabtree-positive phenotype, *S. cerevisiae* reached steady-state nearly 20 hours after the start of fermentation in our studies.

The chemostats reached the steady-state after 5 residence times, confirmed by stable off-gas measurements and biomass concentrations. During chemostats, glucose was entirely used for biomass generation and carbon dioxide production by both yeasts. The steady-state prior to H₂O₂ perturbation was well-characterized with biomass concentrations and calculated biomass-specific uptake and secretion rates and carbon and degrees of reduction recoveries, presented in Table 5.1.

Table 5.1. Steady-state data in aerobic glucose-limited *S.cerevisiae* and *P.pastoris* chemostat cultures, D=0.1 h⁻¹. The q-rates are given in mmol DCW⁻¹ h⁻¹, and the recoveries in percentages

	<i>Measured (unbalanced) specific rates</i>					
	<i>- q_s</i>	<i>q_x</i>	<i>q_{CO₂}</i>	<i>- q_{O₂}</i>	C-recovery	DoR-recovery
<i>S. cerevisiae</i>	1.046±0.001	3.847±0.004	2.450±0.022	2.274±0.029	100±0.00	100±0.01
<i>P. pastoris</i>	0.848±0.010	3.713±0.019	1.633±0.081	1.438±0.167	104±0.01	104±0.04

Upon H₂O₂ perturbation to the steady-state, dissolved oxygen level reached 120-130 percent within seconds and returned to its pre-stress value within minutes (Figure 5.3A. and Figure 5.3B.). The biomass levels stayed constant in both yeasts within 2 hours upon stress, which seems to be in contrast to earlier work in batch where a 10-50 percent decrease in cell viability was reported after oxidative stress. This is possibly due to the difference in cultivation mode, as we continuously dilute (partially dead) cells in the chemostat system. Jamieson, 1992 and Werner-Washburne, et al., 1993 showed that the tolerance of

exponentially growing cells to oxidative stress was lower than the cells in the stationary phase [11,12]. They attributed this observation to (i) the increasing number of mitochondria in the stationary phase for the cultures grown on glucose and (ii) the increased expression of genes encoding stress shock proteins. In our study, the cells grow far below their maximum growth rate (similar to exponential growth), possibly making them more tolerant to oxidative stress.

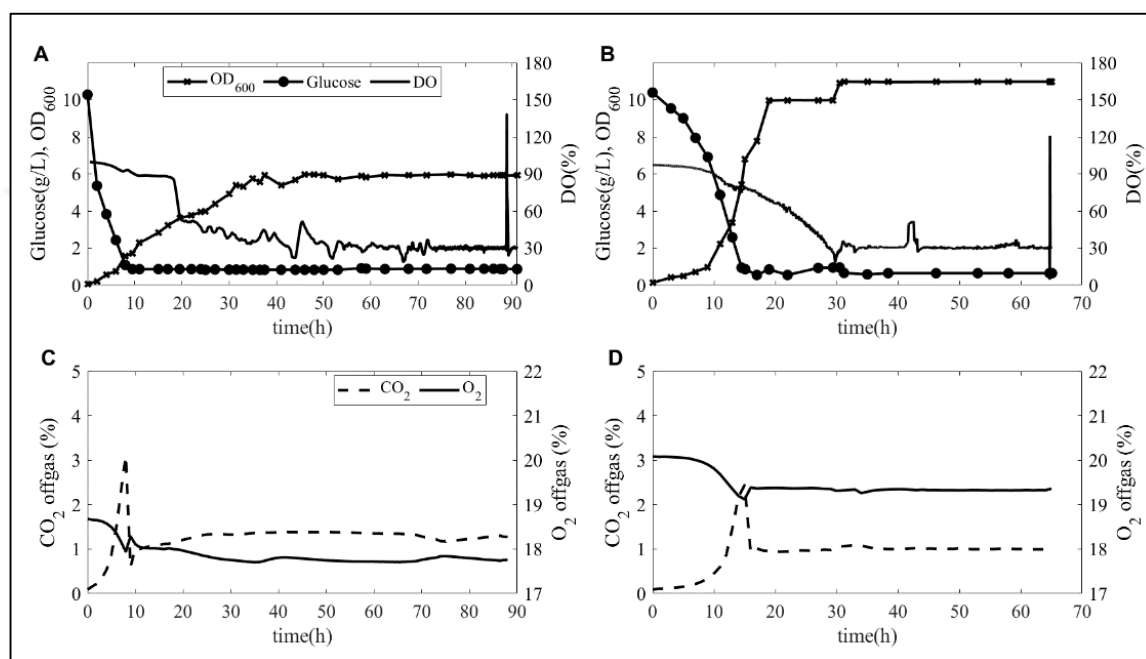


Figure 5.3. Physiological measurements of *S. cerevisiae* and *P. pastoris* during chemostat cultures for 1 mM hydrogen peroxide perturbation. Biomass accumulation, measured substrate concentration and dissolved oxygen level of (A) *S.cerevisiae* and (B) *P.pastoris*, and off-gas measurements of (C) *S.cerevisiae* and (D) *P.pastoris*

5.2.2. Amino Acid Profiling of *S.cerevisiae* and *P.pastoris* After 1 mM Oxidative Stress

The intracellular concentrations for 15 amino acids prior to and after H_2O_2 perturbation was measured with HPLC. To decide the sampling period, the turn-over times of intracellular amino acids are considered, and under similar cultivation conditions, these vary between 30-500 seconds [108]. Based on this, samples were collected after 300 seconds upon hydrogen peroxide perturbation. Each of these 15 amino acids' response is presented relative to its steady-state level (Figure 5.4.).

Different sets of amino acids for *S. cerevisiae* and *P. pastoris* displayed changes in a statistically significant manner ($p\text{-value} \leq 0.05$). Methionine, phenylalanine, and leucine decreased (up to 30 percent decrease), alanine, aspartic acid, and valine increased (up to 18 percent increase) for *P. pastoris*. Alanine, glutamate, and tryptophan decreased (up to 24 percent decrease), and asparagine and leucine (up to 97 percent) increased for *S. cerevisiae*.

In what follows, the amino acids were grouped for further discussion based on the metabolite at the start of the production pathway for the corresponding amino acid: α -ketoglutarate (α -KG) derived glutamic acid, lysine, proline, arginine; 3-phosphoglycerate (3-PG) derived glycine, serine, cysteine; pyruvate (PYR) derived alanine, valine, leucine; erythrose-4-phosphate (E4P) derived phenylalanine, tryptophan; oxaloacetate (OAA) derived methionine, asparagine, and aspartic acid. While the level of 3-PG-derived amino acid glycine increased by 9 percent for *S. cerevisiae*, it decreased by 12 percent for *P. pastoris*. Serine decreased by 18 percent for *P. pastoris*, yet there was a high variance for *S. cerevisiae*. The level of 3-PG was reported to slightly increase within a minute in *S. cerevisiae* upon 0.5 mM of H_2O_2 treatment. Lastly, cysteine levels were nearly constant, increasing only 4 percent for *S. cerevisiae* and decreasing 7 percent for *P. pastoris*.

α -KG-derived amino acids exhibited different trends for both yeasts: 12 percent decrease (glutamate) for *S. cerevisiae* and nearly constant for *P. pastoris*. In the study by Christodoulou et al., 2019, the continuous decrease of α -KG and glutamate levels was observed in minimal media growing *S. cerevisiae* upon 0.5 mM of H_2O_2 treatment, supporting the explanation for the decrease. Note that glutamate is the predominant amino acid in both yeasts [139] and the precursor amino acid for glutamine, proline, and arginine. For *P. pastoris*, a 7 percent decrease was observed for the arginine level. Our decreased arginine levels are consistent with Christodoulou et al., 2018 that when 1 mM of H_2O_2 stress was applied to the exponentially growing *E. coli*, the intracellular arginine level decreased by half. The lysine level was nearly unchanged for both yeasts. Proline decreased by 12 percent for *P. pastoris*. It has been shown in various studies that proline is a protective metabolite in *S. cerevisiae* against many stresses such as freezing, ethanol, oxidation, and osmotic pressure [148–150].

E4P-derived amino acids (phenylalanine and tryptophan) displayed different patterns for both yeasts. While a 30 percent decrease was observed for phenylalanine level, tryptophan

level was nearly constant (only a 2 percent decrease) for *P.pastoris*. For *S.cerevisiae*, a 24 percent decrease was observed for tryptophan level, but there was a high variance for phenylalanine measurements. Several studies report that cells direct their metabolism to the pentose phosphate pathway (PPP) to produce NADPH to maintain their redox balance upon oxidative stress[13,19]. Christodoulou et al., 2019 demonstrated that this mechanism conserved among kingdoms (from bacteria to mammalian cells) under low oxidative stress. PPP acts as NADPH supplier for reductive steps of anabolic reactions and as intermediates of aromatic amino acids (Phe, Trp, Tyr) and nucleic acid biosynthesis[18].

OAA-derived amino acids represented different trends a 30 percent decrease for methionine and constant asparagine for *P. pastoris*. Aspartic acid increased by 13 percent in *P. pastoris* while decreasing by 9 percent in *S. cerevisiae*. Christodoulou et al., 2018 reported that when 1 mM H₂O₂ stress was applied to the exponentially growing *E. coli*, the intracellular aspartate level increased 1.6 fold within 60 seconds.

A decreasing trend was observed for *S. cerevisiae* in PYR-derived amino acids (alanine and valine). There was a 14 percent decrease for alanine and a 6 percent decrease in valine levels in *S. cerevisiae*, yet an 18 percent increase for alanine and valine levels in *P. pastoris*. Interestingly, a 40 percent decrease in leucine was observed for *P. pastoris*. For *S. cerevisiae*, leucine level nearly reached two-fold (97 percent increase). In the study by Christodoulou 2019, for minimal-medium growing *S. cerevisiae*, pyruvate level slightly decreased during 60 sec after 0.5 mM H₂O₂ stress [18].

Several studies on the transcriptomic response of H₂O₂-induced oxidative stress in yeasts have shown that genes involved in environmental stress response, glutathione biosynthesis, and pentose phosphate pathway are induced [9,24,151]. Zhao et al., 2015 demonstrated that genes are responsible for carbohydrate metabolism, glycolysis and pyruvate metabolism, peroxisomal matrix, degradation of fatty acid, amino acid (alanine, aspartate, glutamate) metabolism differentially expressed between the hydrogen peroxide tolerant and the control strain of *S. cerevisiae*. *ASP1*, *ARG4*, *GLT1*, *ALT2*, *GAD1*, *GDH2*, *GDH3*, responsible for alanine, aspartate, and glutamate metabolism, are among the significantly differentially regulated genes between the H₂O₂-tolerant and control strain [152].

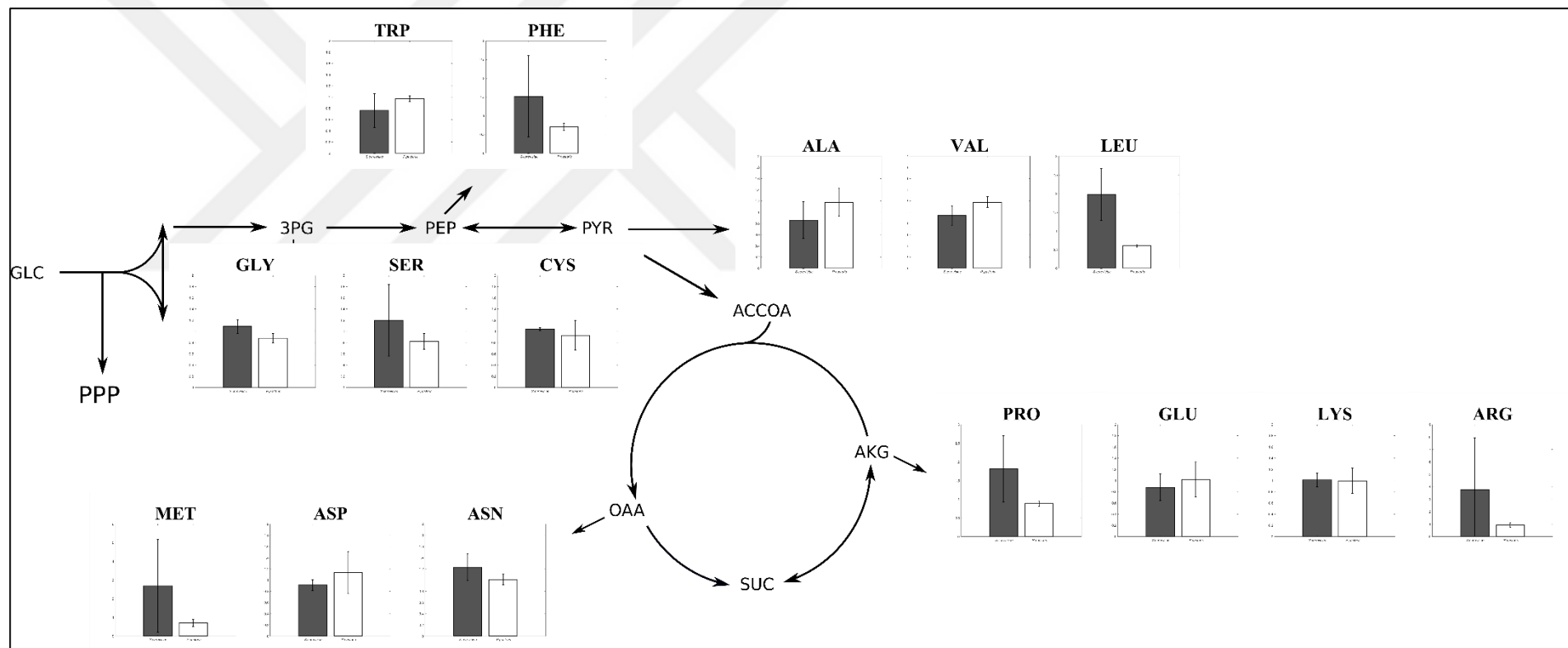


Figure 5.4. The response of each amino acid to oxidative stress is presented as a relative level to its steady-state concentration

Reduced and oxidized glutathione (glutathione disulfide (GSSG)) levels were shown in Table 5.2. H_2O_2 stress causes a rise in GSSG levels, and in turn, the redox state is maintained via reduction of GSSG back to GSH. The increase in the GSSG level can also be explained by glutaredoxin mechanisms to stabilize the redox equilibrium inside cells [65]. In our study, reduced glutathione (GSH) level increased for *P.pastoris* but decreased for *S.cerevisiae* upon oxidative stress. This might be due to either the use of alternative mechanisms to cope with oxidative stress or the differential dynamics of the GSH/GSSG for each yeast sample. The decrease of GSH in *S. cerevisiae* can additionally be explained by the use of glutathione as a cofactor of glutathione peroxidase, one of the key short-term survival mechanisms against oxidative stress in *S. cerevisiae*.

While *P. pastoris* enhanced total glutathione pool size, this was reversed for *S.cerevisiae*. The decrease in total glutathione pool size can be related to the NADPH regeneration because glutathione peroxidase-dependent and glutathione-based detoxification mechanisms of ROS require a continuous supply of NADPH. Similar to our study, the ratio of oxidized to reduced glutathione was shown to be increased in Christodoulou 2019 after 5 seconds, and was stable after 10 seconds upon 20 mM of H_2O_2 treatment for *S. cerevisiae* and *E. coli*. Also, they observed that the ratio of oxidized to reduced glutathione continuously increased upon 0.5 mM H_2O_2 treatment for *S. cerevisiae* growing in rich media.

Table 5.2. The response of reduced and oxidized glutathione for both yeasts. *The $\log_2(FC)$ of the reduced and oxidized glutathione response to oxidative stress is presented relative to its steady-state level

	GSH	GSSG	Pool size
<i>P.pastoris</i>	0.30	1.41	1.6
<i>S.cerevisiae</i>	-1.29	0.45	0.85

Among the precursor amino acids of glutathione, a 9 percent increase was observed for glycine in *S. cerevisiae*, while a 12 percent decrease for *P. pastoris*. Conversely, glutamate remains almost constant in *P. pastoris*, while 12 percent decreases in *S. cerevisiae*. While a 4 percent increase was observed for cysteine level in *S. cerevisiae*, a 7 percent decrease was

observed in *P. pastoris*. Finally, we found no direct correlation between precursor amino acids of glutathione for both yeasts.

In this study, oxidative stress agent H_2O_2 was applied as a single pulse to the yeast culture initially at a metabolic steady-state. Continuous operation mode (chemostat) is the easiest and most feasible method for keeping cells in constant conditions considering both metabolic state and physicochemical conditions. These cultures are a preferred strategy for understanding the response of cells to both external stimuli (environmental stresses) and internal traits. Since chemostat cultivation is under steady-state operating conditions, it allows us to observe the effect of only one parameter and obtain robust and reliable data by keeping all others constant. These cultures are important for kinetic and metabolic characterization as well as for providing quantitative information at the whole-cell level [114]. Furthermore, Christodoulou et al., 2019 and Christodoulou et al., 2018 applied hydrogen peroxide as a single pulse to understand the short-term response of oxidative stress in batch cultures; there are various studies applying oxidative stress via different exposure times ranging from minutes to hours [22,153]. To our knowledge, this study is the first in which oxidative stress is given to a chemostat as an H_2O_2 pulse, and (part of) the response of metabolism is quantitatively monitored.

In our study, oxidative stress induces a different short-term response in amino acid levels in both yeasts cultivated in a chemostat. Considering the changing amino acids after 1 mM of hydrogen peroxide-induced oxidative stress, methionine, serine, and phenylalanine significantly change most in *P.pastoris*, while alanine and tryptophan change most in *S.cerevisiae*. Leucine is the significantly changed amino acid for both yeasts upon stress.

To assess the oxidative stress response from a broader perspective, quantification of intermediates of central carbon metabolism, cofactors, and nucleotides can be analyzed with advanced MS-based platforms (GC-MS, LC-MS). Furthermore, stress can be applied as a step-change (as an alternative to pulse) to understand different aspects of the effects of oxidative stress. There are various experimental designs for oxidative stress response studies, e.g., applying stress agents with different concentrations via different exposure times ranging from minutes to hours [22,153].

5.2.3. Chemostat Cultivation of *S.cerevisiae* and *P.pastoris*, and 5 mM of Hydrogen Peroxide Perturbation

Similar 1 mM of perturbation case, glucose-limited chemostats were carried out, in duplicates, at a dilution rate of 0.1 h^{-1} for both yeasts (Table 5.3.). The oxidative stress level was selected as 5 mM of H_2O_2 for this study to see the effects of lethal (toxic) concentrations. Upon 5 mM of H_2O_2 induced-oxidative stress to the culture at a steady-state, the dissolved oxygen level reached 300 percent within seconds and returned to its pre-stress value within 5 minutes (Figure 5.5A. and Figure 5.5B.). Furthermore, cell concentration was slightly decreased for both yeasts within 1 hour upon stress applied. The decreasing cell concentration can be explained by the increasing ROS levels, and changes in the intracellular redox environment. Decreasing cell viability after exposure to hydrogen peroxide can lead to a decrease in cell concentration.

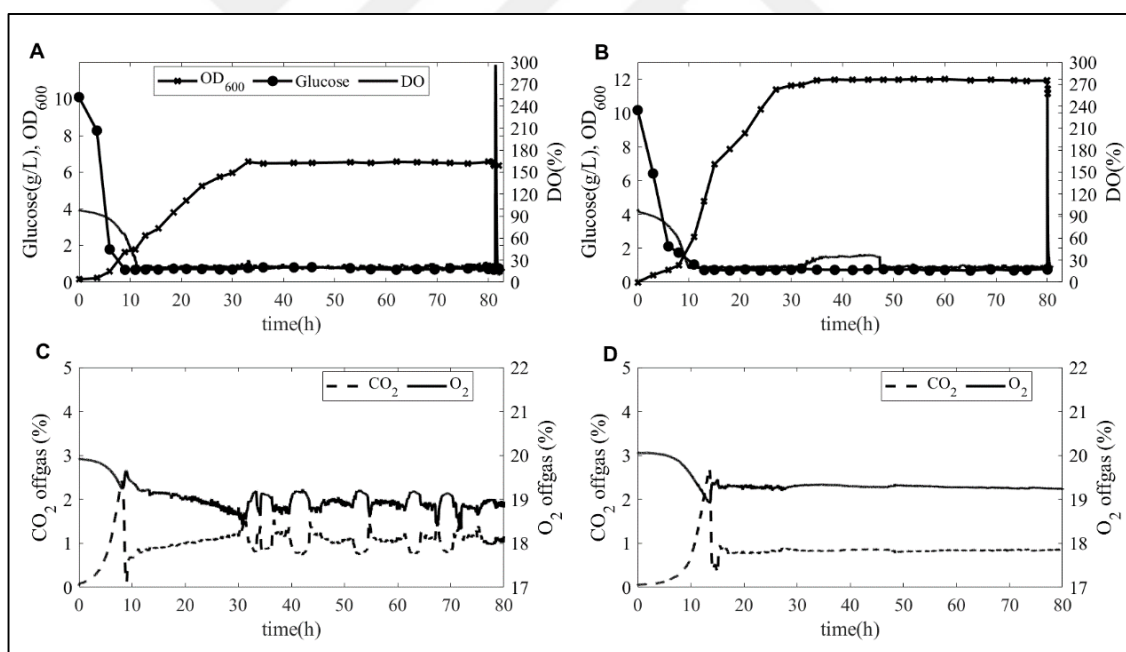


Figure 5.5. Physiological measurements of *S. cerevisiae* and *P. pastoris* during chemostat cultures for 5 mM hydrogen peroxide perturbation. Biomass accumulation, measured substrate concentration and dissolved oxygen level of (A) *S.cerevisiae* and (B) *P.pastoris*, and off-gas measurements of (C) *S.cerevisiae* and (D) *P.pastoris*

Table 5.3. Steady-state data in aerobic glucose-limited *S.cerevisiae* and *P.pastoris* chemostat cultures, $D=0.1 \text{ h}^{-1}$ The q-rates, are given in $\text{mmol DCW}^{-1} \text{ h}^{-1}$, the recoveries in percentages

	Measured (unbalanced) specific rates					
	$-q_s$	q_x	q_{CO_2}	$-q_{\text{O}_2}$	C-recovery	DoR-recovery
<i>S. cerevisiae</i>	1.044 ± 0.009	3.842 ± 0.005	2.428 ± 0.077	2.302 ± 0.198	100 ± 0.022	101 ± 0.041
<i>P. pastoris</i>	0.839 ± 0.008	3.694 ± 0.001	1.552 ± 0.007	1.606 ± 0.114	104 ± 0.010	109 ± 0.033

5.2.4. Chemostat Cultivation of *S.cerevisiae* and *P.pastoris*, and 40 mM of Hydrogen Peroxide Perturbation

To see the detrimental effect of H_2O_2 , glucose-limited chemostats were carried out, in duplicates, at a dilution rate of 0.1 h^{-1} for both yeasts and the system perturbed with 40 mM of H_2O_2 (Table 5.4). Upon oxidative stress to the culture at a steady-state, the dissolved oxygen level reached 300 percent within seconds and returned to its pre-stress value within 5 minutes (Figure 5.6A. and Figure 5.6B.). Furthermore, cell concentration was slightly decreased for both yeasts within 1 hour upon stress applied. In addition, an increase of 0.5-0.8°C in ambient temperature was observed when 40 mM of H_2O_2 stress was applied. Also, sudden pronounced foaming was observed after the addition of hydrogen peroxide.

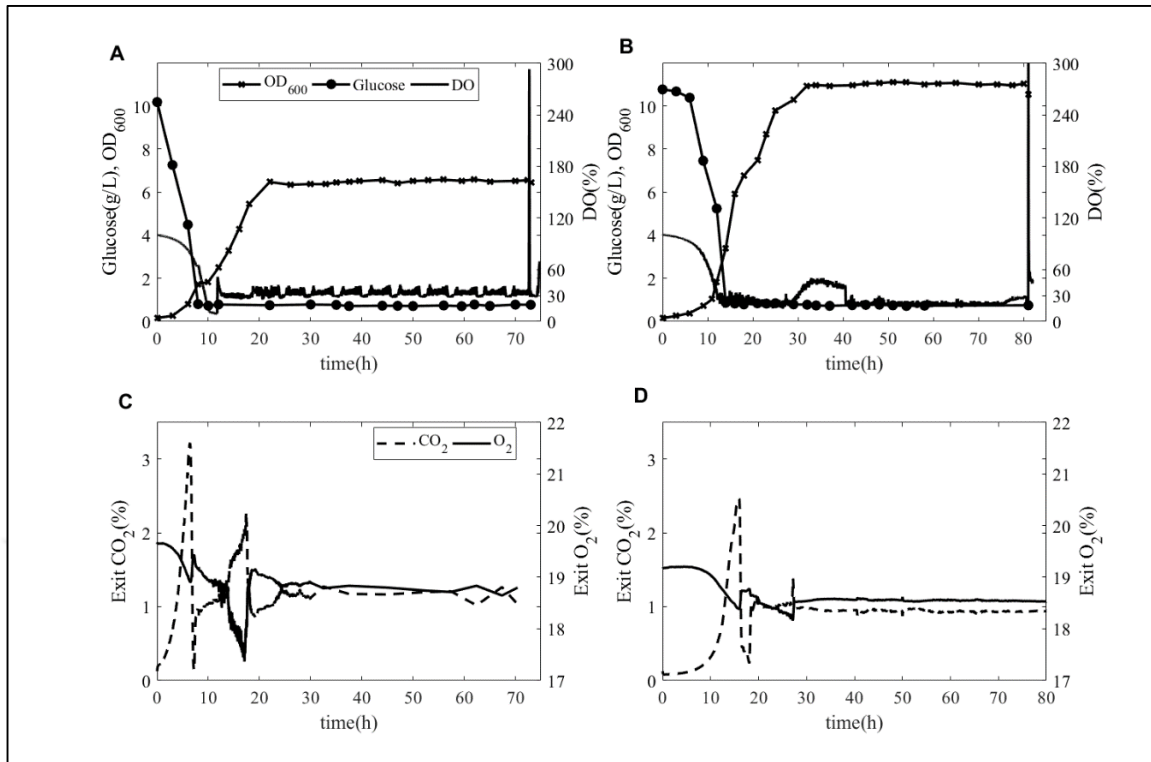


Figure 5.6. Physiological measurements of *S. cerevisiae* and *P. pastoris* during chemostat cultures for 40 mM hydrogen peroxide perturbation. Biomass accumulation, measured substrate concentration and dissolved oxygen level of (A) *S.cerevisiae* and (B) *P.pastoris*, and off-gas measurements of (C) *S.cerevisiae* and (D) *P.pastoris*

Table 5.4. Steady-state data in aerobic glucose-limited *S. cerevisiae* and *P. pastoris* chemostat cultures, $D=0.1 \text{ h}^{-1}$ The q-rates, are given in $\text{mmol DCW}^{-1} \text{ h}^{-1}$, the recoveries in percentages

	Measured (unbalanced) specific rates					
	$-q_s$	q_x	q_{CO_2}	$-q_{O_2}$	C-recovery	DoR-recovery
<i>S. cerevisiae</i>	1.047 ± 0.010	3.851 ± 0.001	2.471 ± 0.294	2.245 ± 0.059	101 ± 0.037	100 ± 0.001
<i>P. pastoris</i>	0.858 ± 0.006	3.731 ± 0.021	1.715 ± 0.081	1.271 ± 0.182	106 ± 0.019	101 ± 0.038

5.2.5. Chemostat Cultivation of *S. cerevisiae* and *P. pastoris*, and 20 mM of Dithiothreitol (DTT) Perturbation

For reductive stress, the chemostats were perturbed with 20 mM of dithiothreitol (DTT) (Table 5.5.). Upon reductive stress to the culture at steady-state, dissolved oxygen level increased from 20 to 60 percent within seconds and returned to its pre-stress value within 30 minutes. Furthermore, cell concentration was slightly decreased for both yeasts within 1 hour upon stress applied. This slight decrease can be caused by decreasing cell viability due to redox imbalance after DTT perturbation. On the other hand, in our study, the cells were growing far below the exponential growth regime due to the selected dilution rate, possibly rendering cells more tolerant to stress. The slight increase in dissolved oxygen level after adding DTT can be explained by the reaction of DTT with the air and trace metals in the medium, and it can produce H_2O_2 [154]. Therefore DO level increased back up to 60 percent.

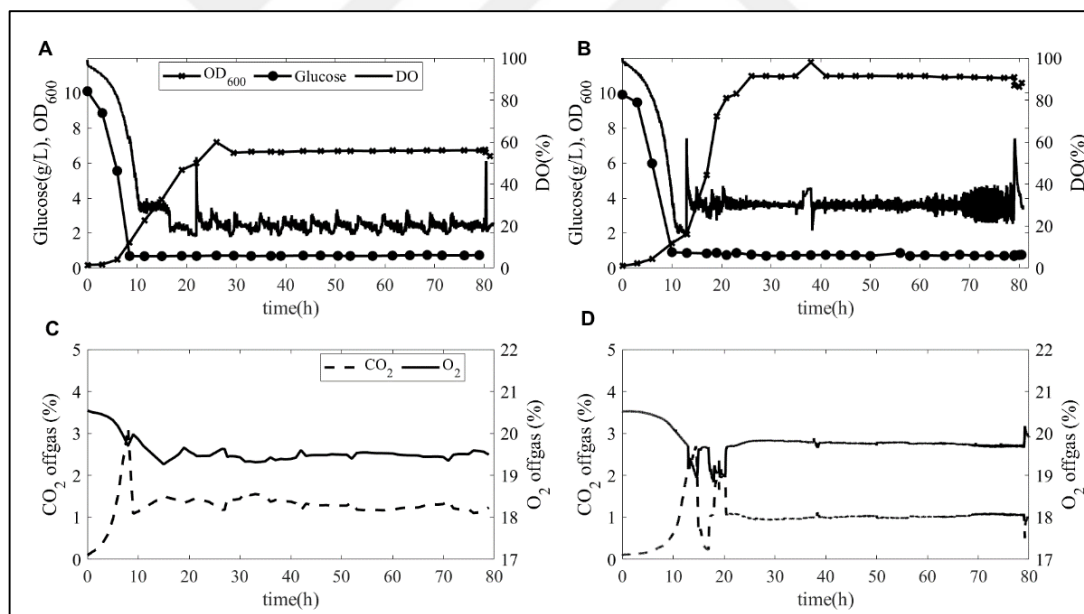


Figure 5.7. Physiological measurements of *S. cerevisiae* and *P. pastoris* during chemostat cultures for 20 mM DTT perturbation. Biomass accumulation, measured substrate concentration and dissolved oxygen level of (A) *S.cerevisiae* and (B) *P.pastoris*, and off-gas measurements of (C) *S.cerevisiae* and (D) *P.pastoris*

Table 5.5. Steady-state data in aerobic glucose-limited *S.cerevisiae* and *P.pastoris* chemostat cultures, $D=0.1 \text{ h}^{-1}$ The q -rates, are given in $\text{mmol DCW}^{-1} \text{ h}^{-1}$, the recoveries in percentages

	Measured (unbalanced) specific rates					
	$-q_s$	q_x	q_{CO_2}	$-q_{\text{O}_2}$	C-recovery	DoR-recovery
<i>S. cerevisiae</i>	1.028 ± 0.007	3.738 ± 0.002	2.841 ± 0.040	2.290 ± 0.179	107 ± 0.001	101 ± 0.022
<i>P. pastoris</i>	0.852 ± 0.012	3.688 ± 0.005	1.802 ± 0.016	1.444 ± 0.019	103 ± 0.013	104 ± 0.020

5.3. AMINO ACID RESPONSES TO OXIDATIVE AND REDUCTIVE STRESSES

Intracellular concentrations of alanine, arginine, asparagine, aspartic acid, cysteine, glutamine, glutamic acid, glycine, lysine, methionine, phenylalanine, proline, serine, tryptophan, tyrosine, valine, isoleucine+leucine were determined by HPLC. Unfortunately, the available HPLC methodology did not allow accurate quantification of isoleucine and leucine separately. These amino acids are evaluated as total concentrations.

A key question in such stimulus-response experiments is to decide when to collect the samples. Too early sampling results in unobservable responses, too late sampling results confounded responses. Two criteria are considered to decide on sampling time: (i) turnover time of intracellular metabolites and (ii) inspection of time-dependent transcriptomic response upon oxidative stress. Turn-over times of intracellular amino acids vary between 30-500 seconds at similar cultivation conditions [97,108]. To inspect the time-dependent transcriptomic response literature data GSE26169 has been used [155]. Around 300 genes has been found to be differentially expressed within 5 minutes, while around 1500 genes differentially expressed (fold change >2 , $p\text{-value} < 0.05$) within 20 minutes. Based on these two evidences metabolite sampling is decided to be the first 5 minutes, and sampling times after hydrogen peroxide and DTT perturbation were set to 1, 3, and 5 min. The response of each amino acid to oxidative/reductive stress is presented relative to its steady-state level. Two-tailed t-test statistical analyses allowed identifying those amino acid ratios significantly higher or lower than 1.

5.3.1. Amino Acid Responses of *S. cerevisiae*

The response of each amino acid against 5 mM of hydrogen peroxide-induced oxidative and 20 mM of DTT-induced reductive stress responses was evaluated for *S. cerevisiae*.

Pyruvate-derived amino acids indicated different trends in *S. cerevisiae* for oxidative and reductive stresses (Figure 5.8.). Alanine levels showed an opposite behavior after both stresses. After 20 mM of DTT, it has a decreasing trend up to 45 percent after 5 min. On the other hand, there was no significant change in alanine level after 5 mM hydrogen peroxide stress. Valine levels showed similar decreasing trends with high variances until after 3 min, and they followed opposite behavior after 5 min. Isoleucine+leucine levels had similar decreasing patterns after both stresses. The only delayed response was observed in DTT. In Christodoulou 2019, for minimal-medium growing *S. cerevisiae*, pyruvate level slightly decreased during 60 sec after 0.5 mM of H₂O₂ stress. Slightly decreased pyruvate level in the studies of Christodoulou 2019 can be a reason for slight changes in pyruvate-derived amino acids after 5 mM H₂O₂ stress for our study [18].

α -KG-derived amino acids represented different patterns after hydrogen peroxide induced-oxidative and DTT-induced reductive stresses (Figure 5.9.). Glutamate levels showed similar behavior until 3 min after both stresses. There was a breaking point after 5 min; its level turned back to nearly its initial levels after hydrogen peroxide stress; on the other hand, a nearly 27 percent decrease was observed after DTT stress. Arginine levels have an opposite trend for both stresses. There was no significant effect of DTT stress on arginine level while it increases after 3 min and (nearly) stabilizes after hydrogen peroxide stress. Proline levels showed a decreasing trend after 5 mM of hydrogen peroxide stress. However, it showed a significant increase (up to 80 percent) and a slight decrease (14 percent) after 1 min and 3 min of reductive stress. Also, an approximately 30 percent increase was observed after 5 min of reductive stress. There was no significant change in lysine levels after 20 mM of DTT stress and 5 mM of hydrogen peroxide stress, except after 3 min of hydrogen peroxide stress, having high variance. Proline and lysine had contradicting profiles for both stresses. Glutamine level showed opposite behavior after oxidative and reductive stresses until after 3 min. After oxidative stress, its level decreased after 1 min, and after 3 min, 33 percent increased following the initial decrease, and after 5 min, it reached up to its initial levels.

In contrast to this, after 20 mM of DTT, after 1 min, it increased, and then it started to decrease during 5 min. It was known that glutamate is the predominant metabolite in both yeasts[139], and also glutamate was a precursor amino acid for glutamine, proline, and arginine. It has been shown in various studies that proline is a protective metabolite in *S. cerevisiae* against many stresses such as freezing, ethanol, oxidation, and osmotic pressure. Also, recent studies have shown that proline accumulation increases stress tolerance against freezing, ethanol, and oxidation in *S. cerevisiae* [148–150].

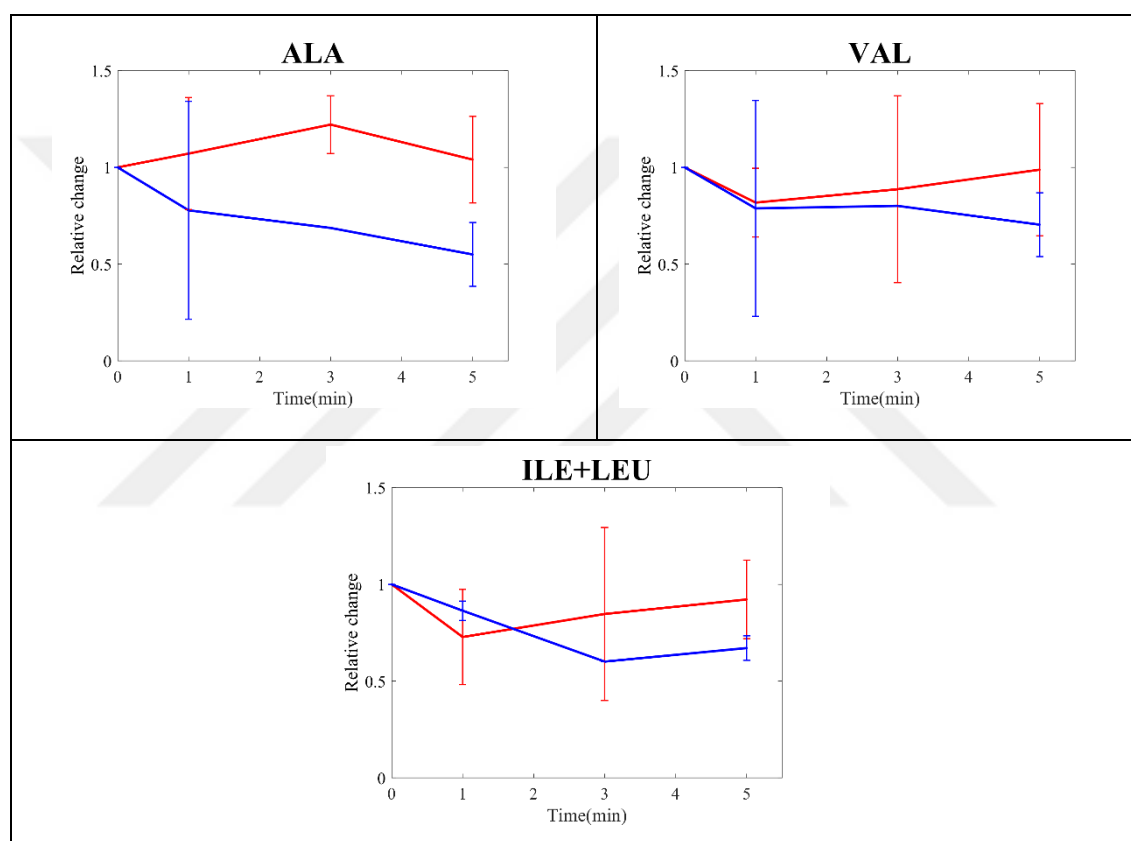


Figure 5.8. Pyruvate-derived amino acids in *S. cerevisiae* (Red lines indicate the 5 mM of H_2O_2 induced oxidative stress, blue lines indicate 20 mM of DTT induced reductive stress)

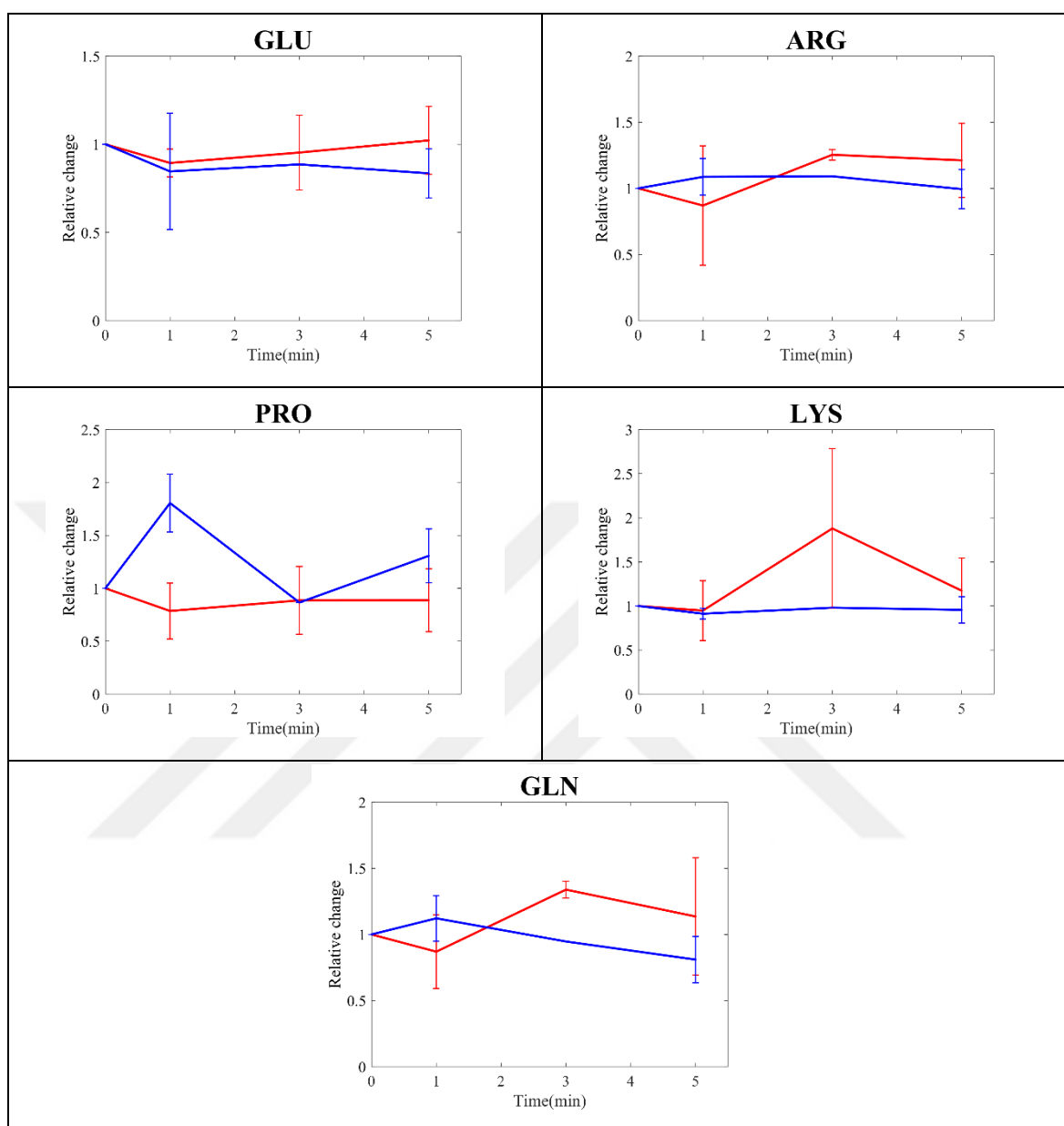


Figure 5.9. α -Ketoglutarate derived amino acids in *S. cerevisiae* (Red lines indicate the 5 mM of H₂O₂ induced oxidative stress, blue lines indicate 20 mM of DTT induced reductive stress)

3-PG-derived amino acids indicated different behaviors after both stresses (Figure 5.10.). Glycine level represented a slightly decreasing trend after DTT stress. On the other hand, there was a nearly unchanged level after hydrogen peroxide stress, except after 1 min, having high variance. Serine levels followed a decreasing pattern after DTT stress, while it showed an initial decrease after 1 min and nearly stabilized after 3 and 5 min of hydrogen peroxide

stress. Cysteine level showed decreasing trend after DTT stress, but there was a high variance in oxidative stress data.

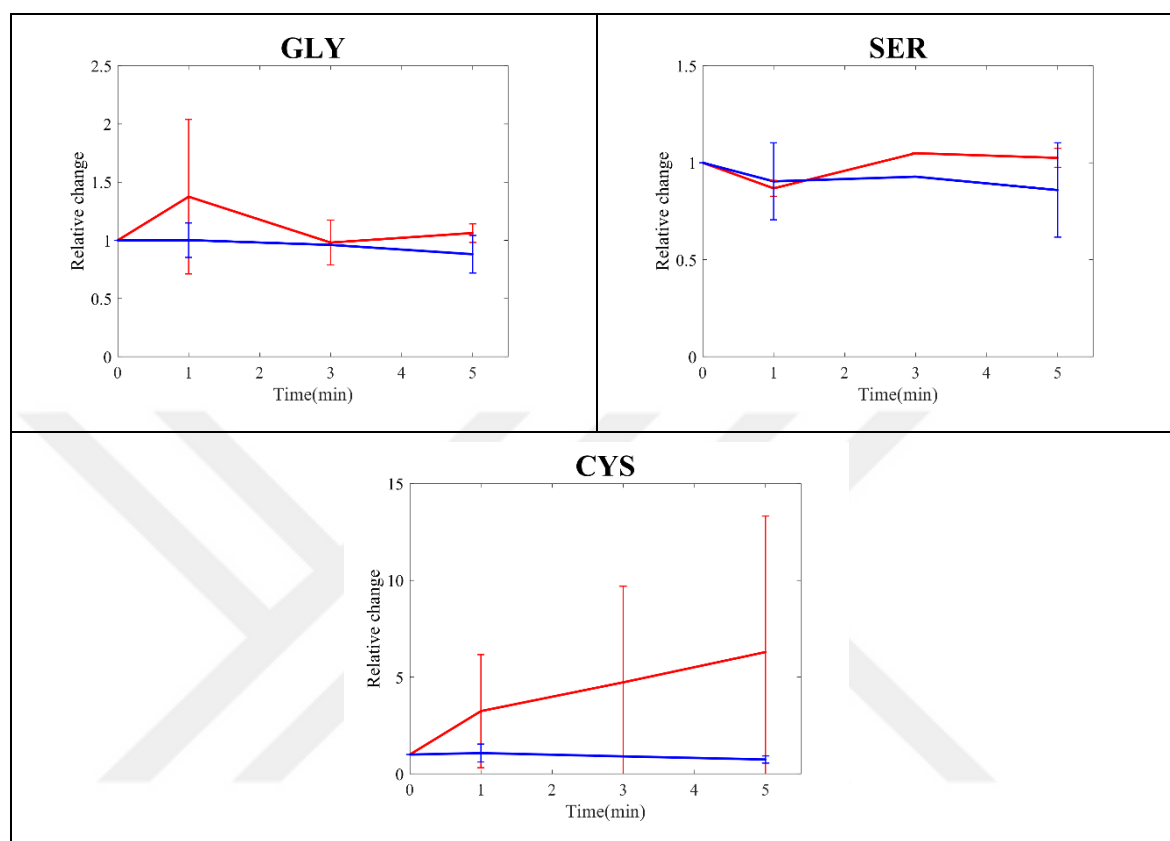


Figure 5.10. 3-PG-derived amino acids in *S. cerevisiae* (Red lines indicate the 5 mM of H_2O_2 induced oxidative stress, blue lines indicate 20 mM of DTT induced reductive stress)

OAA-derived amino acids also had different patterns after both stresses (Figure 5.11.). Aspartic acid, asparagine, and tyrosine showed similar decreasing trends after DTT stress. In contrast to this, methionine levels significantly increased after reductive stress. Interestingly, there was no significant change in aspartic acid and asparagine levels after hydrogen peroxide stress. Unfortunately, methionine data for oxidative stress had a high variance. Christodoulou et al., 2018 reported that when 1 mM H_2O_2 stress was applied to the exponentially growing *E.coli*, the intracellular aspartate level increased 1.6 fold within 60 seconds. Also, the glutamate level decreased by about 15 percent [19].

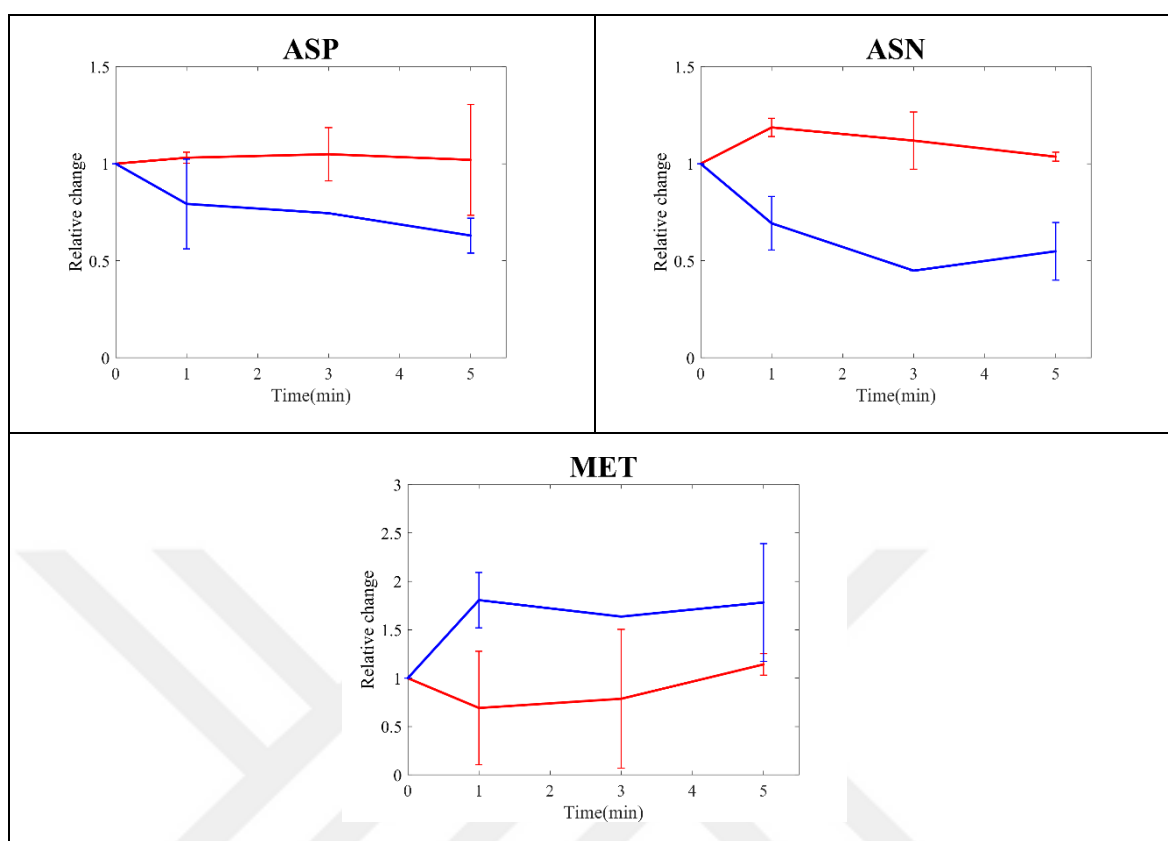


Figure 5.11. OAA- derived amino acids in *S. cerevisiae* (Red lines indicate the 5 mM of H_2O_2 induced oxidative stress, blue lines indicate 20 mM of DTT induced reductive stress)

There was no significant change in PEP-derived amino acids (Figure 5.12.). Tyrosine exhibits a significant decrease (up to 20 percent) after DTT stress, while it was nearly constant after hydrogen peroxide stress. Interestingly, there is no significant change in phenylalanine level after DTT stress. Phenylalanine level is constant after DTT stress, but it has a high variance in hydrogen peroxide data. Tryptophan levels have a high variance for both stresses' data. There are many studies showing that cells direct their metabolism to the pentose phosphate pathway (PPP) to produce NADPH in order to maintain their redox balance after oxidative stress [13,19]. Christodoulou et al., 2019 demonstrated that this mechanism is conserved among kingdoms (from bacteria to mammalian cells) under low oxidative stress. PPP acts as NADPH supplier for reductive steps of anabolic reactions and as intermediates of aromatic amino acids (Phe, Trp, Tyr) and nucleic acid biosynthesis [18].

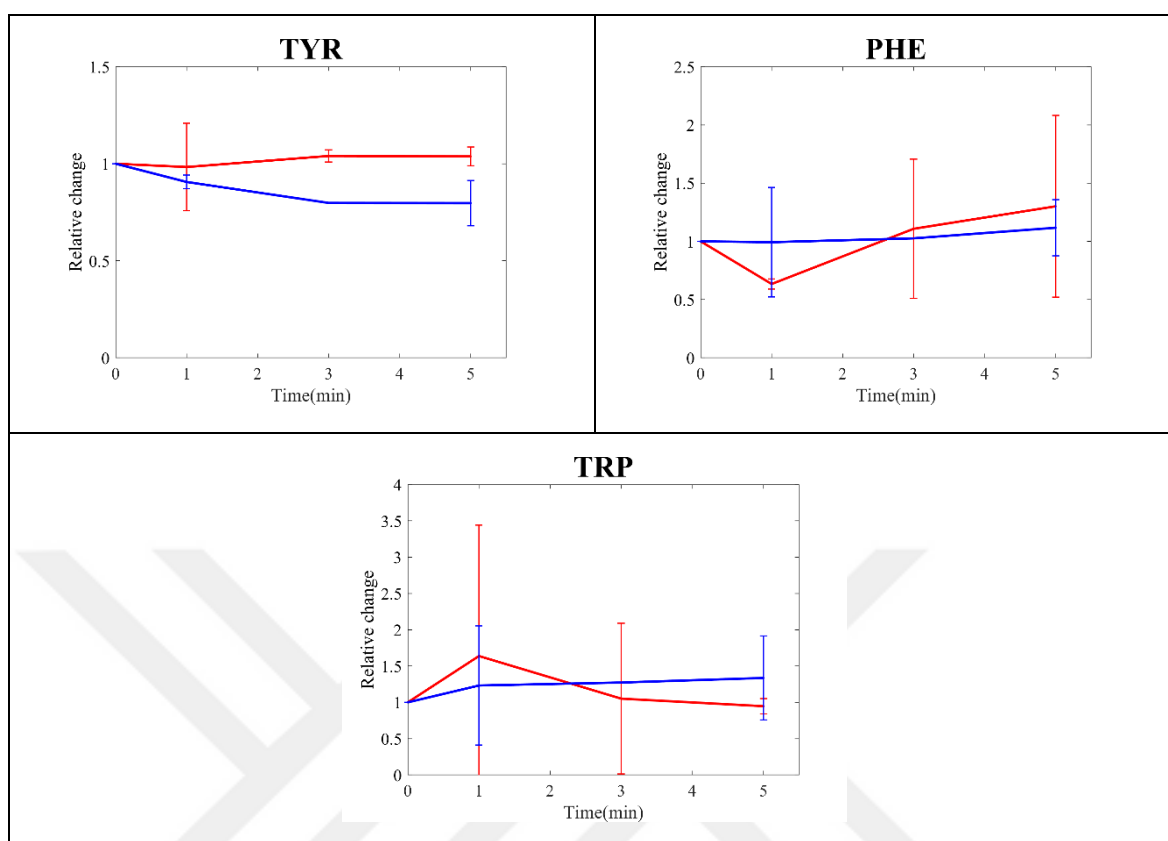


Figure 5.12. PEP-derived amino acids in *S. cerevisiae* (Red lines indicate the 5 mM of H_2O_2 induced oxidative stress, blue lines indicate 20 mM of DTT induced reductive stress)

5.3.2. Amino Acid Responses of *P. pastoris*

The response of each amino acid against 5 and 40 mM of hydrogen peroxide-induced oxidative and 20 mM of DTT-induced reductive stress responses were evaluated for *P. pastoris*.

Pyruvate-derived amino acids indicated similar trends on *P. pastoris* for 40 mM of hydrogen peroxide-induced (high) oxidative and 20 mM of DTT-induced reductive stresses. There was no significant change after 5 mM hydrogen peroxide-induced oxidative stress (Figure 5.13.). Valine and isoleucine+leucine levels showed a similar decrease after 40 mM hydrogen peroxide-induced oxidative stress and DTT stresses during 5 min after stresses. A significant decrease (about 50 percent) of valine and isoleucine+leucine levels were observed after 1 min of both stresses. Isoleucine+ leucine had a high variance in 5 mM hydrogen peroxide stress. Alanine and valine showed a nearly constant pattern (about a 7 percent decrease after

5 min) after 5 mM of hydrogen peroxide stress. Alanine levels showed a significant decrease in concentration up to 36 percent after 5 min of 40 mM of hydrogen peroxide. In contrast, a slight decrease was observed after 5 mM of hydrogen peroxide. Nevertheless, alanine had a higher variance on 20 mM DTT data.

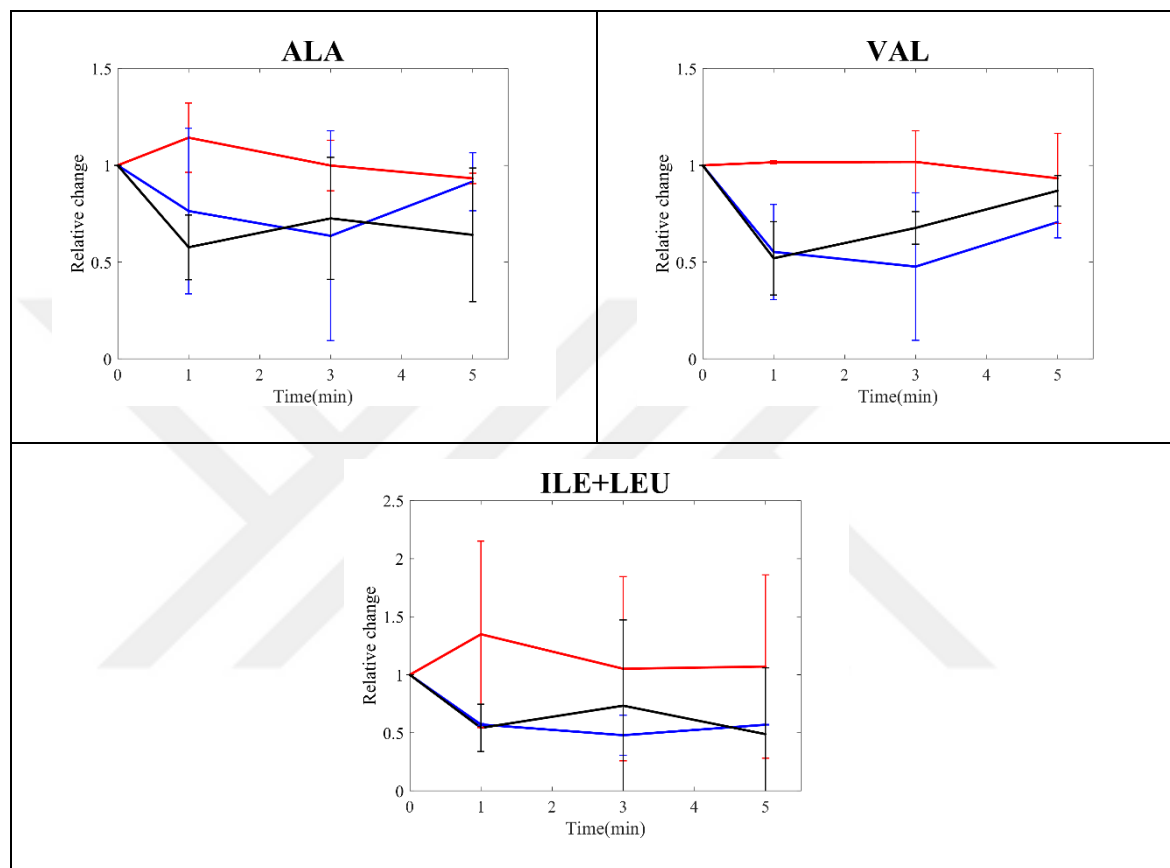


Figure 5.13. Pyruvate-derived amino acids in *P. pastoris* (Red lines indicate the 5 mM of H₂O₂ induced oxidative stress, black lines indicate the 40 mM of H₂O₂ induced oxidative stress, blue lines indicate 20 mM of DTT induced reductive stress)

α -KG derived amino acids represented different patterns after hydrogen peroxide induced-oxidative and DTT-induced reductive stresses (Figure 5.14.). Glutamate levels had a high variance for both stresses. Arginine level was nearly constant after 5 mM of hydrogen peroxide-induced stress, while a decreasing trend was observed after 40 mM of hydrogen peroxide-induced oxidative and DTT stresses. Glutamine level decreased after 40 mM of hydrogen peroxide-induced oxidative and DTT stresses. In contrast, after 5 mM hydrogen peroxide-induced oxidative stress, glutamine levels did not significantly change. Its level increased after 1 min and continued after 3 min up to 16 percent increase, and after 5 min it

reached up to nearly its initial levels. Proline levels had a high variance for DTT-stress and hydrogen peroxide-induced stress data. After 5 mM of hydrogen peroxide stress, the lysine level showed an increasing trend after 1 and 3 min, and it turned back to its initial levels after 5 min.

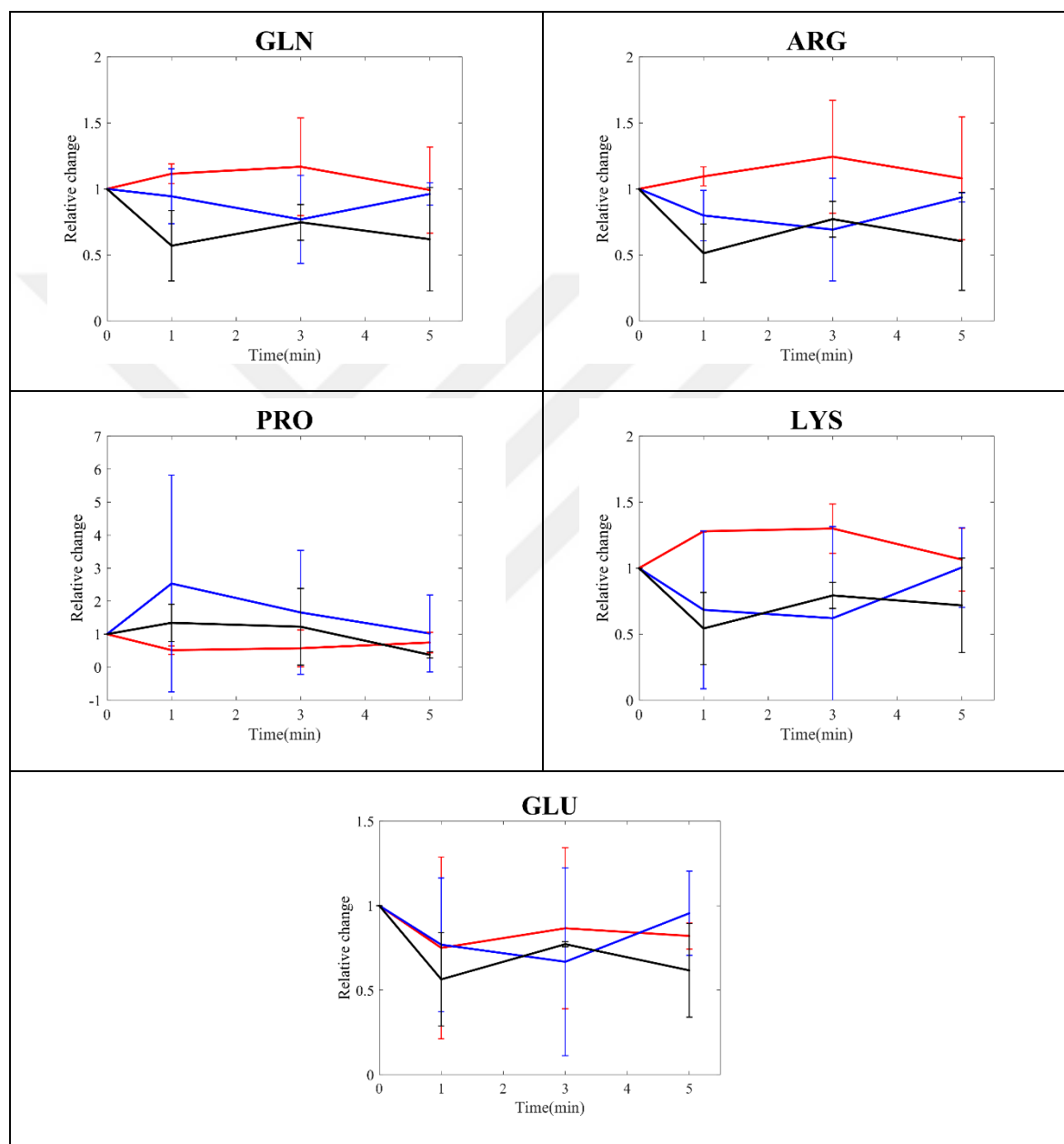


Figure 5.14. α -ketoglutarate derived amino acids in *P. Pastoris* (Red lines indicate the 5 mM of H₂O₂ induced oxidative stress, black lines indicate the 40 mM of H₂O₂ induced oxidative stress, blue lines indicate 20 mM of DTT induced reductive stress)

3-PG-derived amino acids indicated different behaviors after both stresses (Figure 5.15.). Glycine levels represented an unchanged trend after both stresses, but serine levels had a high variance for DTT stress data. Cysteine levels indicated opposite behavior after both concentrations of oxidative stress. While cysteine level showed an initial increasing trend after 1 min of 5 mM of hydrogen peroxide stress, it reached up to initial levels after 3 and 5 min. There was a 36 percent decrease after 1 min of 40 mM of hydrogen peroxide stress, and around a 15 percent decrease was observed after 3 and 5 min. Unfortunately, there was a high variance of DTT stress data.

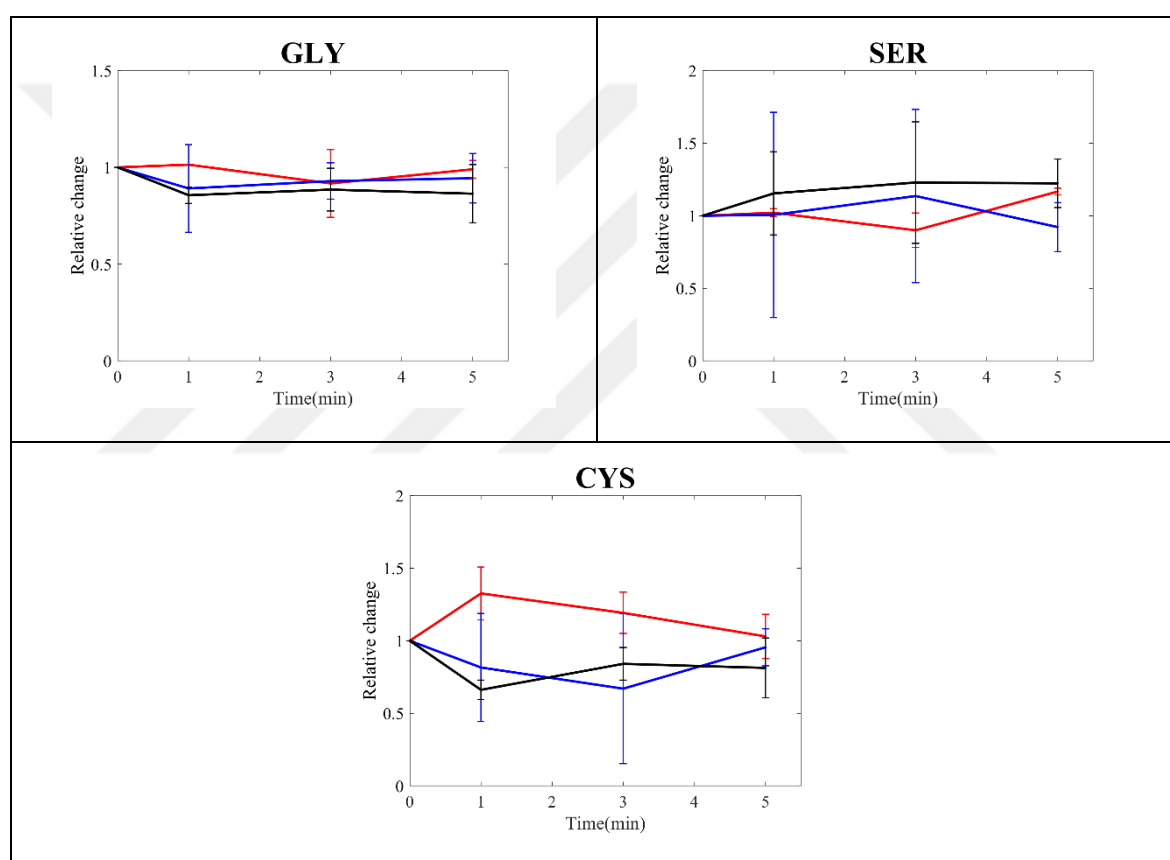


Figure 5.15. 3-PG-derived amino acids in *P. pastoris* (Red lines indicate the 5 mM of H₂O₂ induced oxidative stress, black lines indicate the 40 mM of H₂O₂ induced oxidative stress, blue lines indicate 20 mM of DTT induced reductive stress)

OAA-derived amino acids also had different patterns after both stresses (Figure 5.16.). While methionine levels had a similar trend after both concentrations of oxidative stress, there was an opposite behavior against reductive stress. Its level increased up to 33 percent after 1 min and stabilized around its initial level after 3 and 5 min after hydrogen peroxide stresses. In

contrast to this, it showed decreasing trend up to 3 min, and its level increased up to 20 percent after reductive stress. There was no significant change in asparagine levels after stress. A significant increasing trend (up to 40-70 percent) was observed for aspartic acid levels after 5 mM of hydrogen peroxide stress and an approximately 25 percent increase after 40 mM of hydrogen peroxide stress. Aspartic acid levels had opposite patterns with high variances of DTT stress.

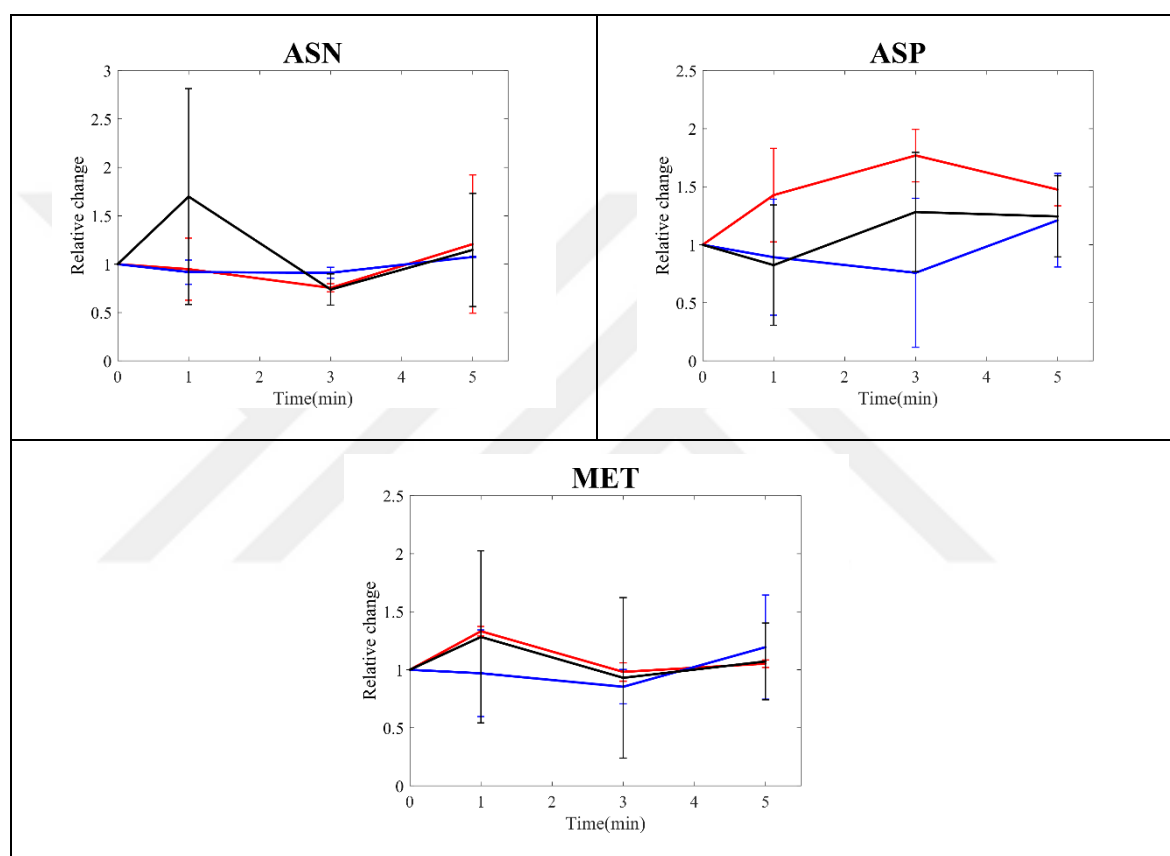


Figure 5.16. OAA- derived amino acids in *P. Pastoris* (Red lines indicate the 5 mM of H₂O₂ induced oxidative stress, black lines indicate the 40 mM of H₂O₂ induced oxidative stress, blue lines indicate 20 mM of DTT induced reductive stress)

PEP-derived amino acids also had different patterns for both stresses (Figure 5.17.). Tyrosine level indicated a significant decrease (up to 30 percent) after 3 min of DTT stress, and it returned to the initial level after 5 min. While tyrosine levels had opposite behavior after both concentrations of oxidative stress, after 5 min, they showed a similar trend. Phenylalanine showed decreasing trend after both stresses, except after 1 min of 5 mM of hydrogen peroxide-induced oxidative stress. Tryptophan levels had similar decreasing

behavior for both stresses except after 1 min of 40 mM of hydrogen peroxide-induced oxidative stress. There was a high variance for 40 mM of hydrogen peroxide-induced oxidative stress data.

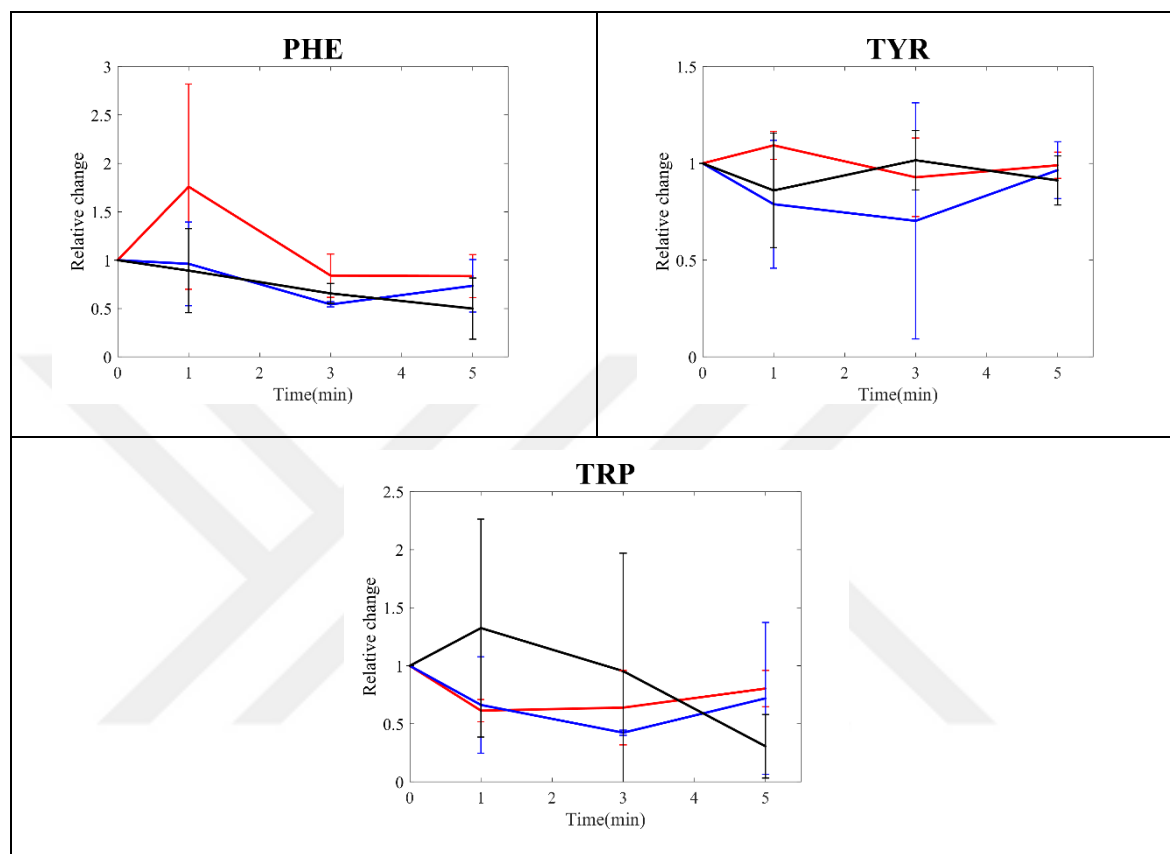


Figure 5.17. PEP-derived amino acids in *P. Pastoris* (Red lines indicate the 5 mM of H_2O_2 induced oxidative stress, black lines indicate the 40 mM of H_2O_2 induced oxidative stress, blue lines indicate 20 mM of DTT induced reductive stress)

5.4. OXIDATIVE STRESS RESPONSE ON 150 L OF EXPONENTIALLY-GROWING YEASTS

S. cerevisiae and *P. pastoris* cells were cultivated in a 150 L bioreactor to see the oxidative stress effects on the scale-up. The fermentation was run in a fed-batch mode instead of a chemostat due to practical reasons at a larger scale. Yeasts were aerobically grown in a bioreactor with a 100 L initial working volume. Fed-batch cultivations were carried out, in duplicates, at a constant feeding rate, and the system was perturbed with 5 mM of H_2O_2 at the mid-exponential phase.

The initial fermentation profile of *S.cerevisiae* is shown in Figure 5.18A. Upon oxidative stress to the culture at the mid-exponential phase, the dissolved oxygen level reached 300 percent within seconds and returned to its pre-stress value within 5 minutes. Before hydrogen peroxide (H_2O_2) perturbation, 10 mL of culture broth was rapidly sampled as a reference state. Then, broth samples were rapidly withdrawn and quenched 1, 3, and 5 min after exposure to H_2O_2 , and metabolites were analyzed after extraction.

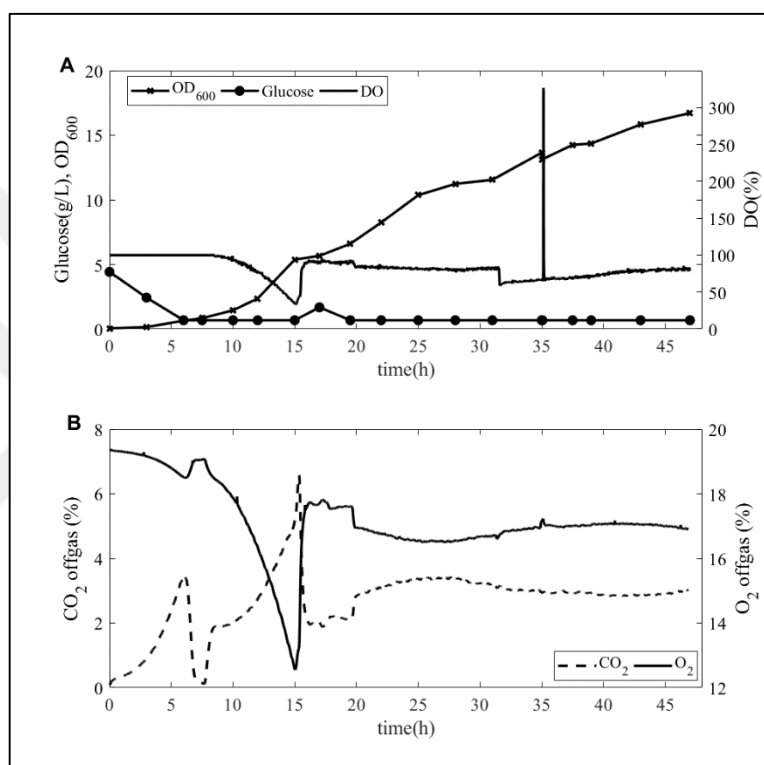


Figure 5.18. Physiological measurements of *S.cerevisiae* during fed-batch cultures (A) Biomass accumulation, measured substrate concentration, and dissolved oxygen level, and (B) off-gas measurements

Similarly, the initial fermentation profile of *P. pastoris* is shown in Figure 5.19A. Cell concentration reached OD₆₀₀ 27, and the mid-exponential phase was determined as OD₆₀₀ 20 around 24 hours.

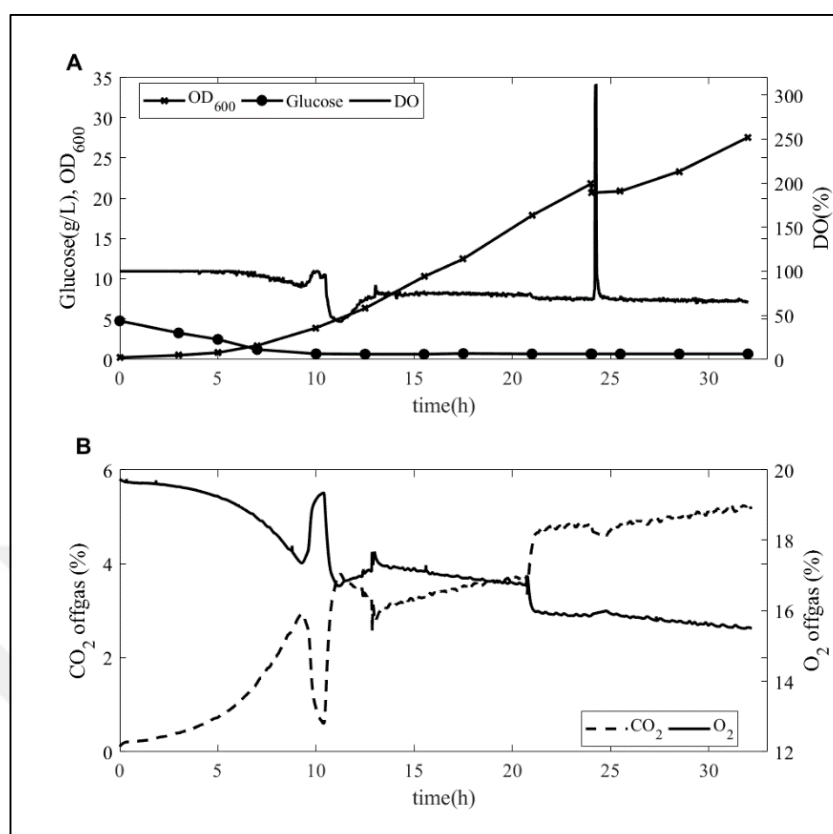


Figure 5.19. Physiological measurements of *P. pastoris* during fed-batch cultures (A) Biomass accumulation, measured substrate concentration and dissolved oxygen level, and (B) off-gas measurements

5.4.1. Amino Acid Responses of *S. cerevisiae* and *P. pastoris* in Pilot Scale

The response of each amino acid against 5 mM of hydrogen peroxide-induced oxidative stress responses were evaluated for *S. cerevisiae* and *P. pastoris*.

5.4.1.1. Comparison of *S. cerevisiae* and *P. pastoris* amino acid levels

The intracellular amino acid levels were compared as a reference state of 150L scale, for both yeasts (Figure 5.20.). The graph was presented in log-scale as the data spans several orders of magnitude. We observed that there is increasing discrepancy among organisms at low concentrations. The amino acid levels were lower than *S. cerevisiae* by a factor of 4 for *P. pastoris* in 150L scale.

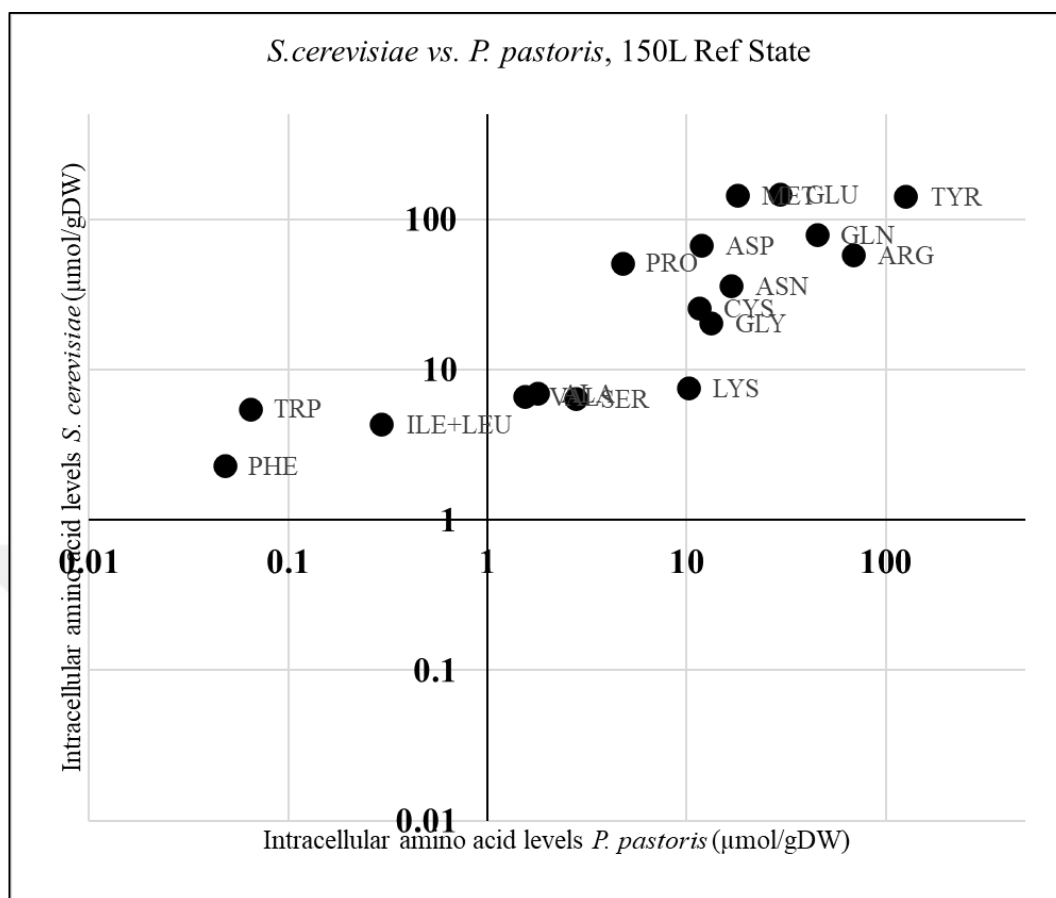


Figure 5.20. The intracellular amino acid levels for *S. cerevisiae* and *P. pastoris* at 150 L scale

5.4.1.2. Amino acid responses of *P. pastoris*

Pyruvate-derived amino acids indicated similar trends on *P. pastoris* for 5 mM hydrogen peroxide-induced oxidative stress. Alanine, valine, and isoleucine+leucine levels sharply decreased (up to 90 percent) after 1 min. While alanine and isoleucine+leucine levels decreased to 30 percent, valine levels reached their initial values after 3 min (Figure 5.21.).

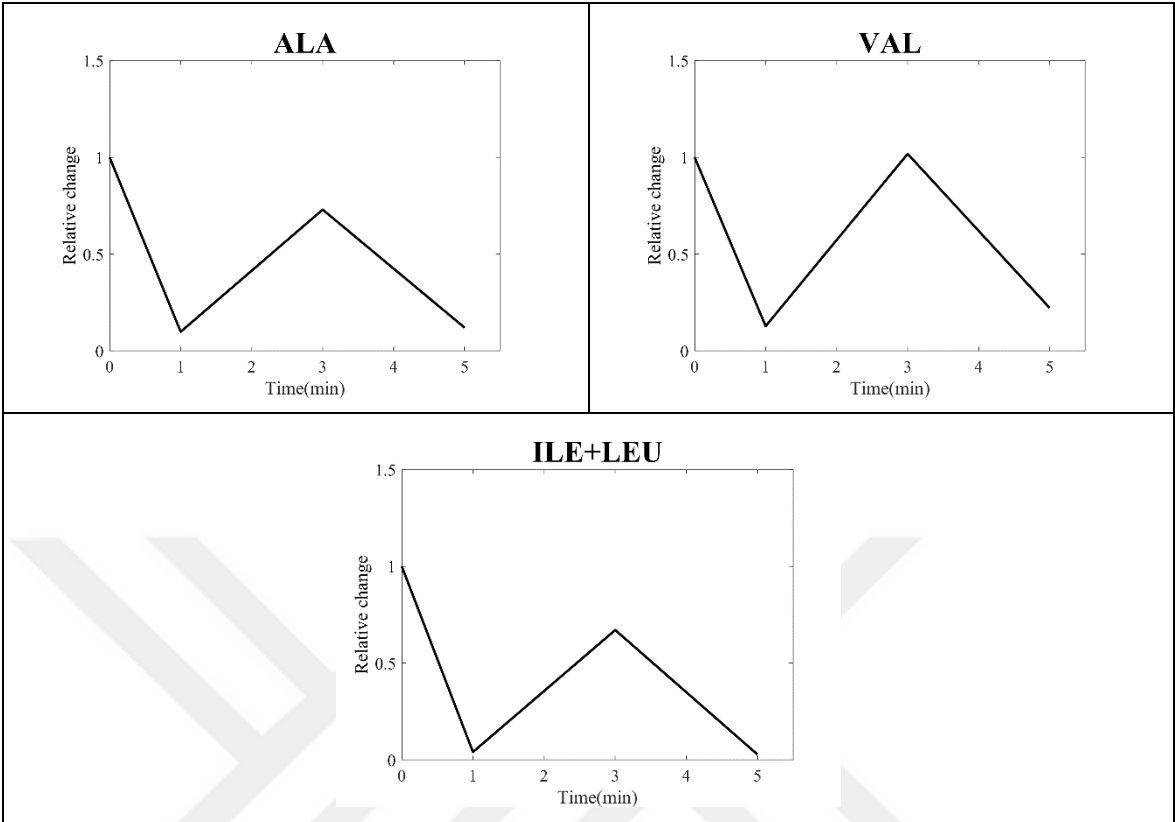
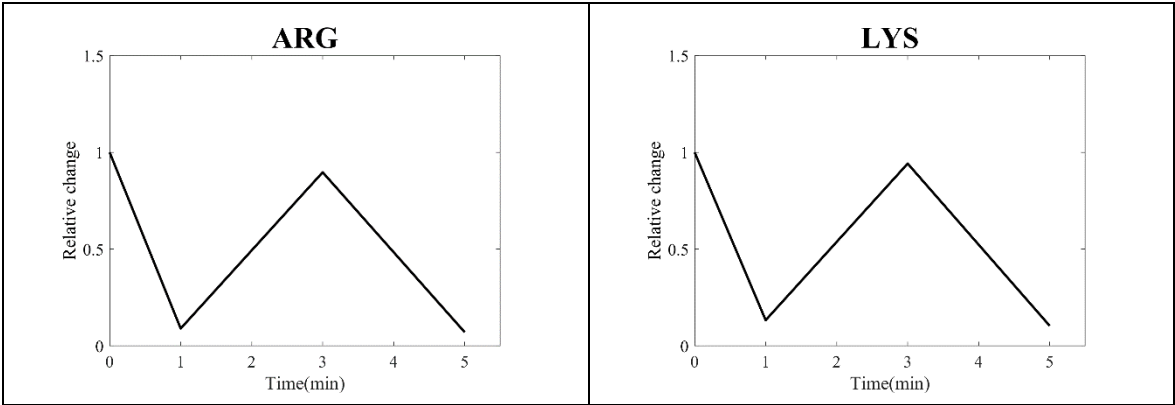


Figure 5.21. Pyruvate-derived amino acids in *P.pastoris* after 5 mM of H₂O₂ induced oxidative stress in 150 L

α -KG-derived amino acids represented similar patterns after hydrogen peroxide induced-oxidative stress (Figure 5.22.).



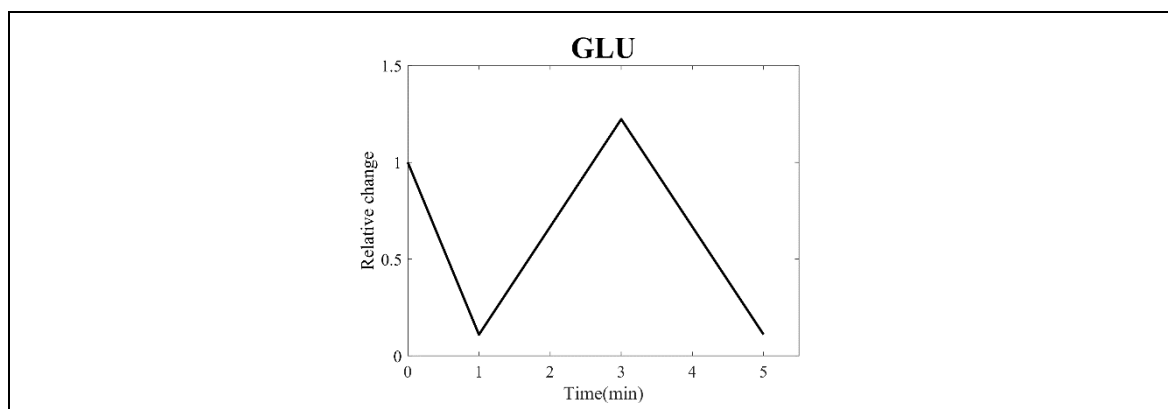


Figure 5.22. α -ketoglutarate derived amino acids in *P. pastoris* 5 mM of H_2O_2 induced oxidative stress in 150 L

3-PG-derived amino acids indicated different behaviors after 5 mM of hydrogen peroxide induced oxidative stress (Figure 5.23.). Glycine levels showed an unchanged trend after stress, while serine and cysteine levels showed similar trends.

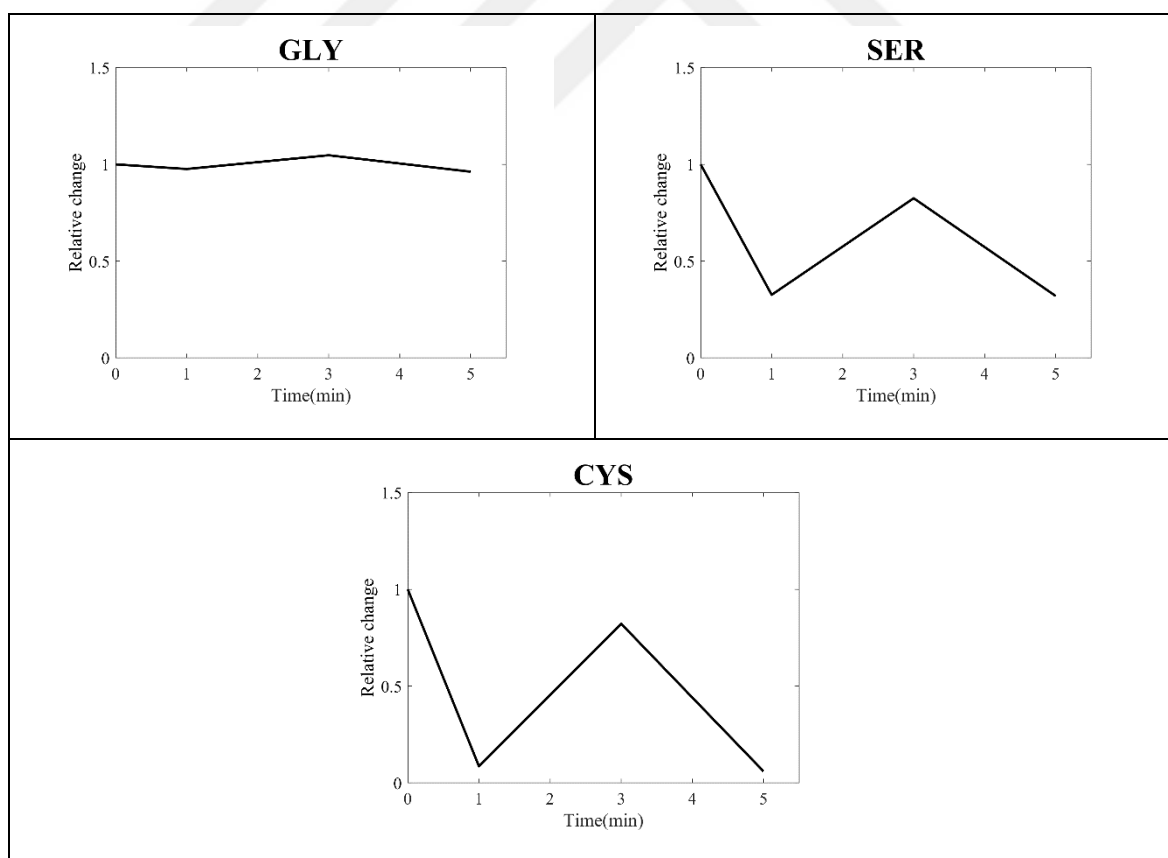


Figure 5.23. 3-PG-derived amino acids in *P. pastoris* 5 mM of H_2O_2 induced oxidative stress in 150 L

OAA-derived amino acids also had different patterns after both stresses (Figure 5.24.). While asparagine levels showed a decreasing trend during 5 min after oxidative stress, aspartic acid and methionine levels sharply decreased after 1 min and showed recovery behavior after 3 min and followed by a decrease in 5 min.

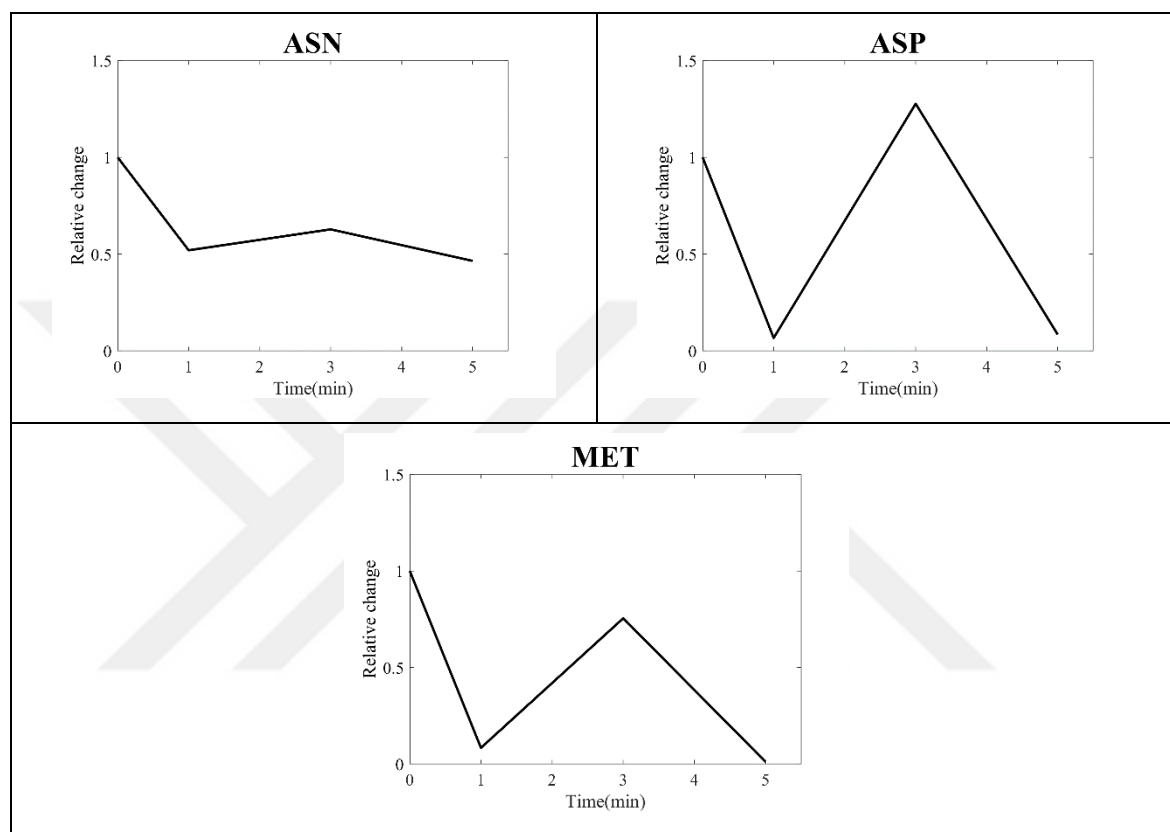


Figure 5.24. OAA- derived amino acids in *P. pastoris* 5 mM of H₂O₂ induced oxidative stress in 150 L

PEP-derived amino acid tyrosine showed decreasing pattern after stress in *P. pastoris* (Figure 5.25.).

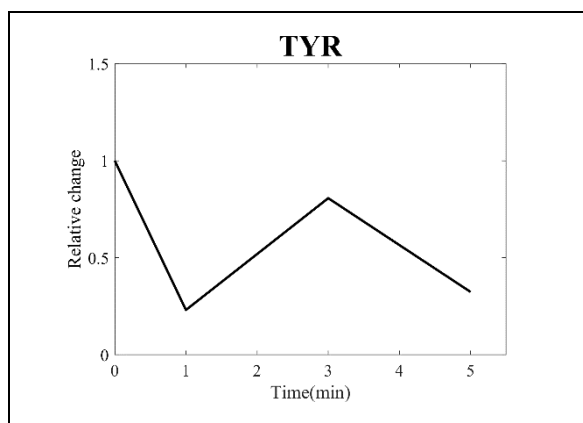
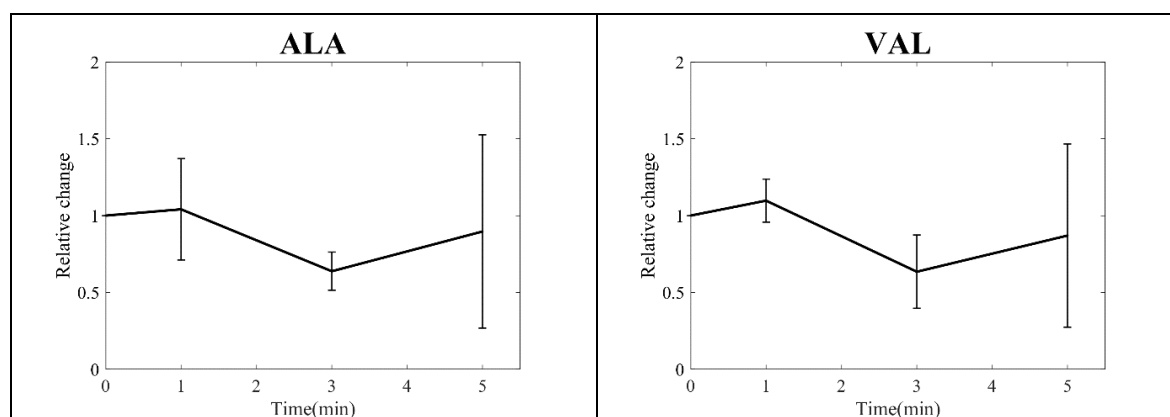


Figure 5.25. PEP-derived amino acids in *P. pastoris* 5 mM of H_2O_2 induced oxidative stress in 150 L

5.4.1.3. Amino acid responses in *S. cerevisiae*

The response of each amino acid against 5 mM of hydrogen peroxide-induced oxidative stress was evaluated for *S. cerevisiae* on a 150L scale.

Pyruvate-derived amino acids have similar patterns after 5 mM of H_2O_2 -induced oxidative stress at a 150L scale. Although there was high variance after 5 min results, there was a 25 percent decrease after 3 min and recovery of these amino acids after 5 min (Figure 5.26.).



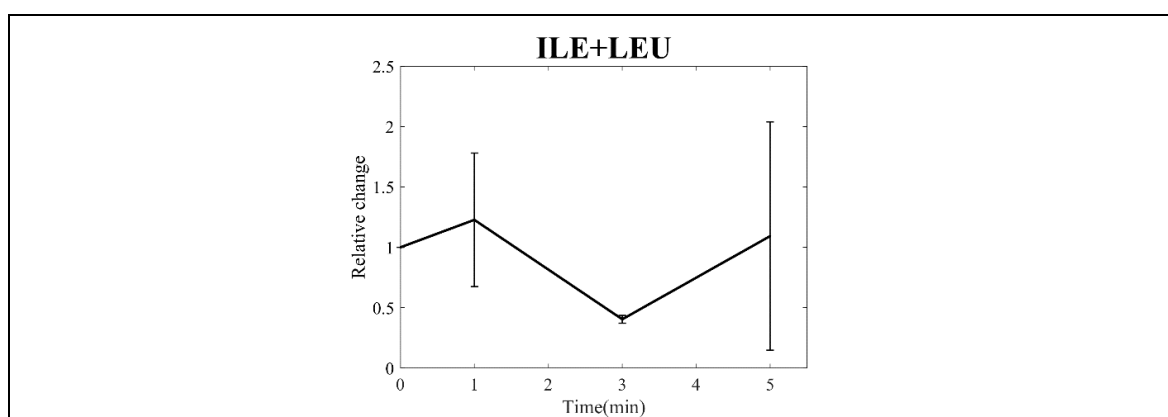
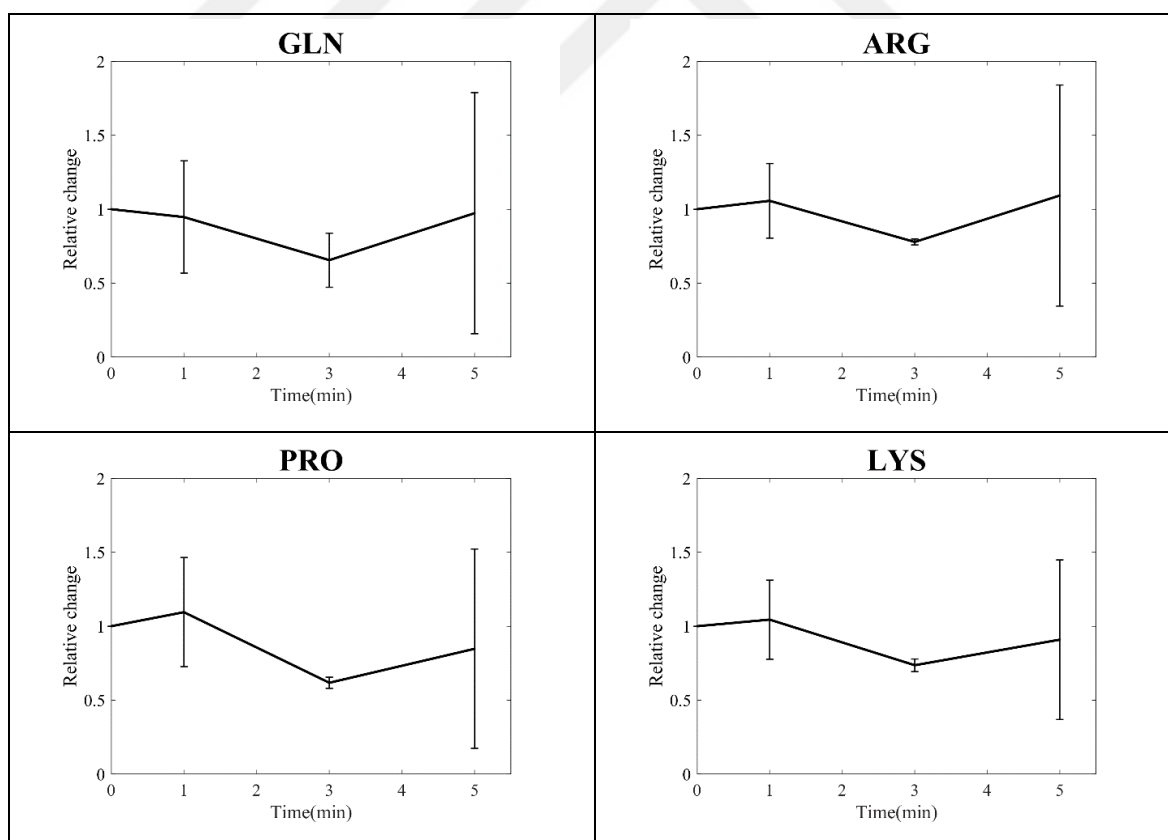


Figure 5.26. Pyruvate-derived amino acids in *S. cerevisiae* after 5 mM of H_2O_2 induced oxidative stress in 150 L

α -KG derived amino acids represented similar patterns after hydrogen peroxide induced-oxidative stress (Figure 5.27.). They showed decreasing trend after 3 min, and recovery of these amino acids were started after 5 min.



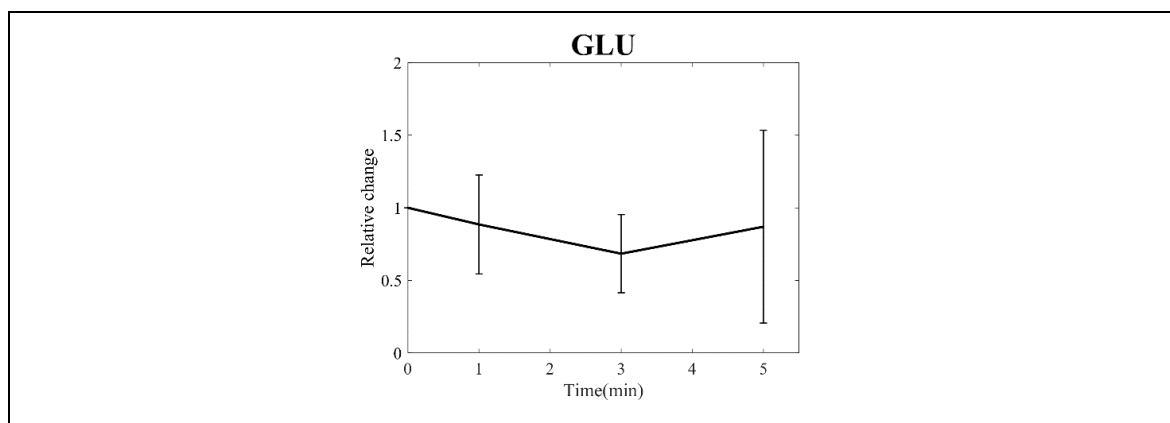


Figure 5.27. α -ketoglutarate derived amino acids in *S. cerevisiae* on 150 L scale

3-PG-derived amino acids indicated different behaviors after 5 mM of hydrogen peroxide-induced oxidative stress (Figure 5.28.). While serine and cysteine levels showed similar decreasing trends after 3 min and recovery of these amino acids was shown after 5 min, glycine levels showed an unchanged trend after stress.

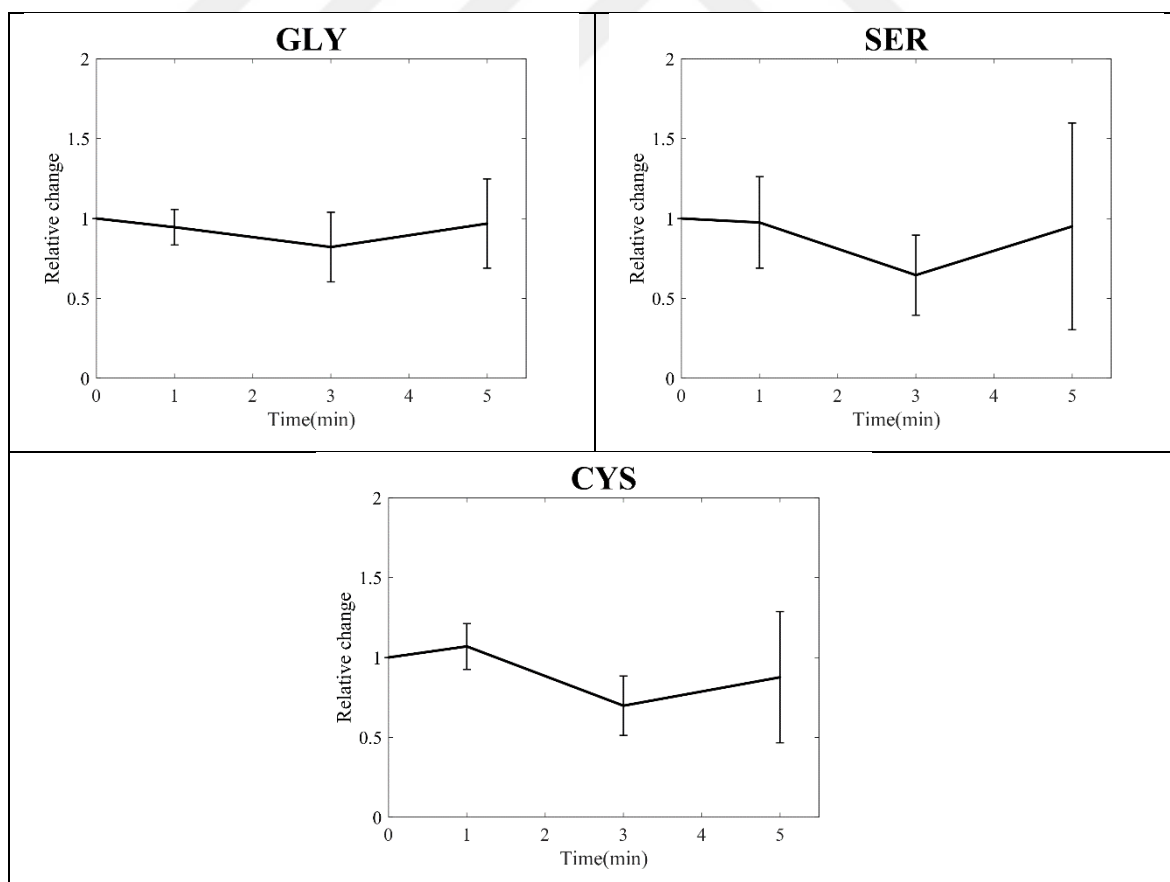


Figure 5.28. 3-PG-derived amino acids in *S. cerevisiae*, on 150L scale

OAA-derived amino acids also had similar decreasing patterns after 3 min and recovery of amino acids after 5 min (Figure 5.29.).

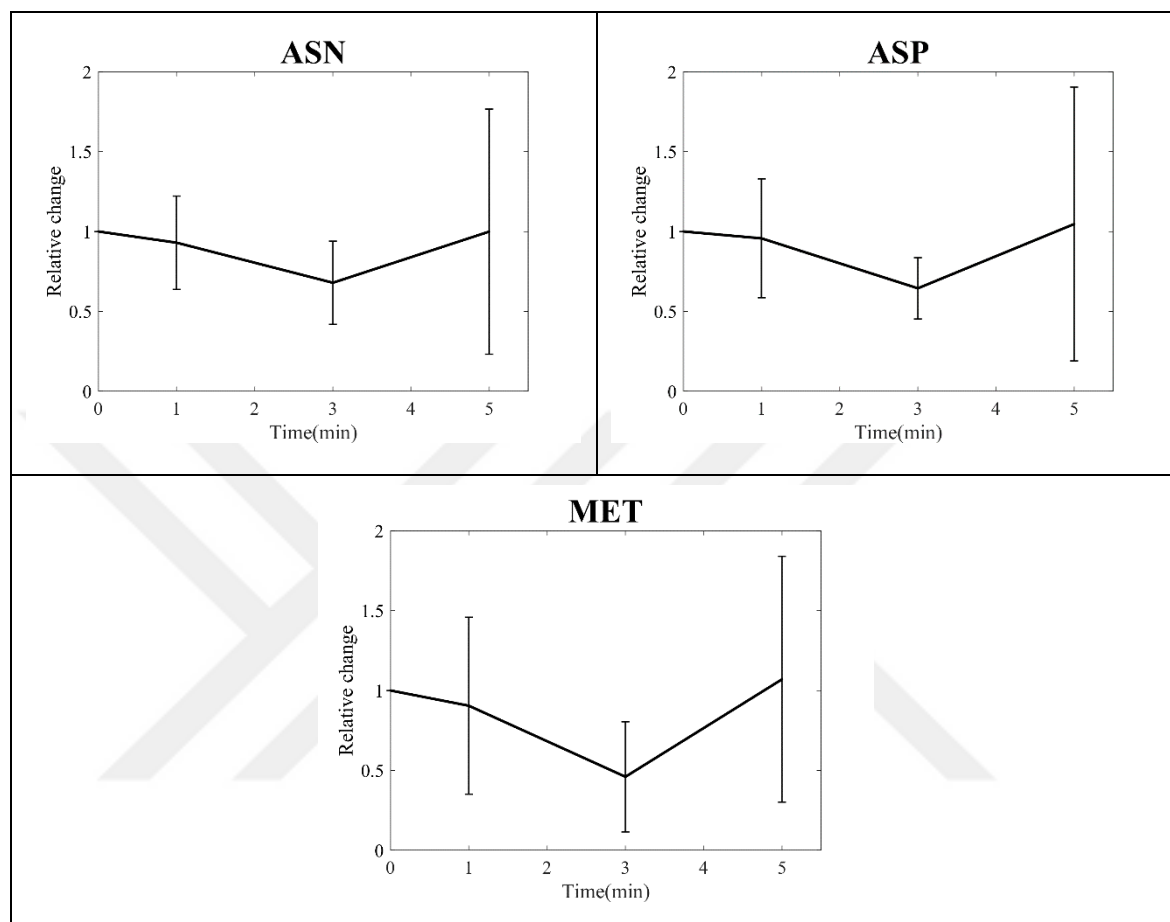


Figure 5.29. OAA- derived amino acids in *S. cerevisiae* on 150L scale

PEP-derived amino acids showed different behavior after 5 min. Tyrosine and tryptophan levels showed decreasing trend after 3 min and turned back its initial levels after 5 min. On the other hand, phenylalanine levels continued to show decreasing trend also after 5 min (Figure 5.30.).

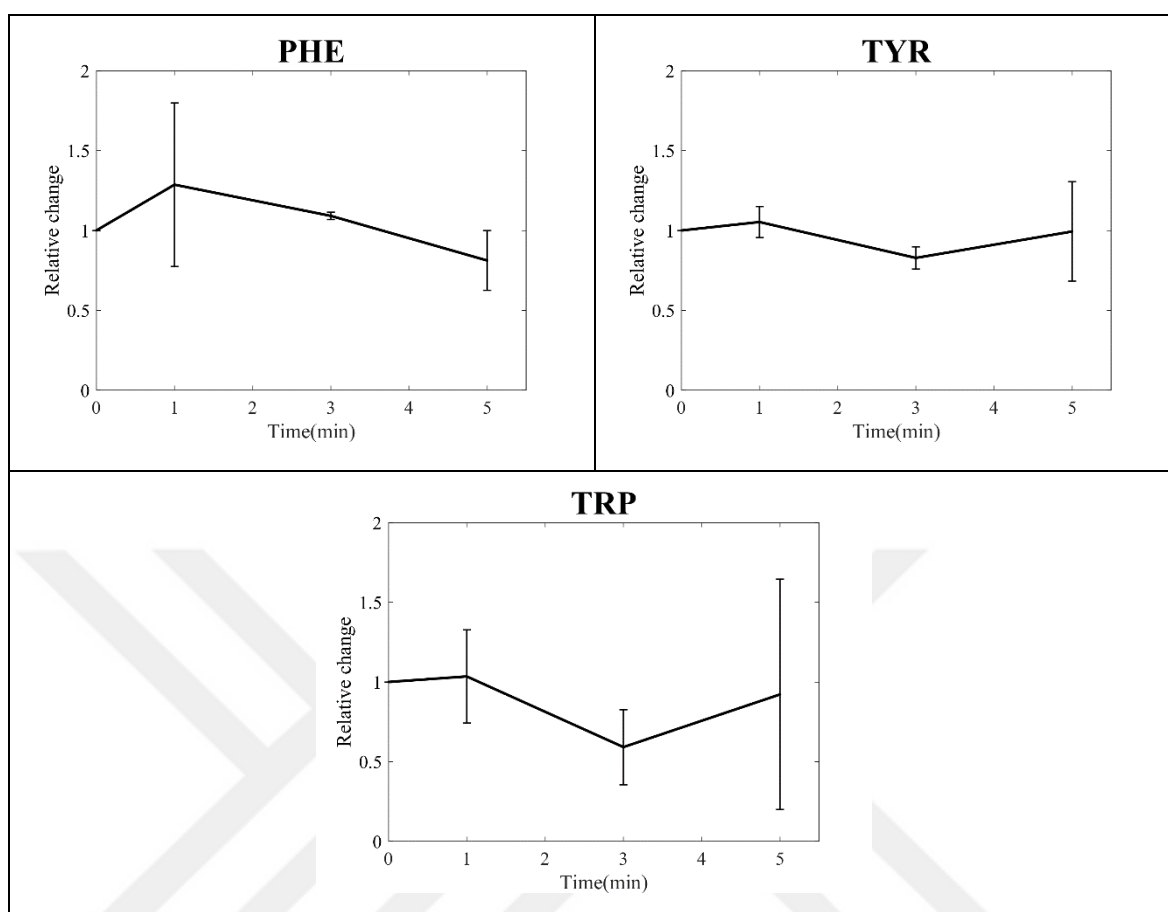


Figure 5.30. PEP-derived amino acids in *S. cerevisiae* on 150 L scale

5.4.1.4. Comparison of amino acid responses in two scales

To compare the amino acid responses of both yeasts in two scales, the graphs were presented in log scales as the data spans several orders of magnitude (Figure 5.31. and

Figure 5.32.). At low concentrations, there is increasing discrepancy at scales and among organisms. Overall we can see that the amino acid levels are lower on 150L scale by a factor of 3-5 for both yeasts.

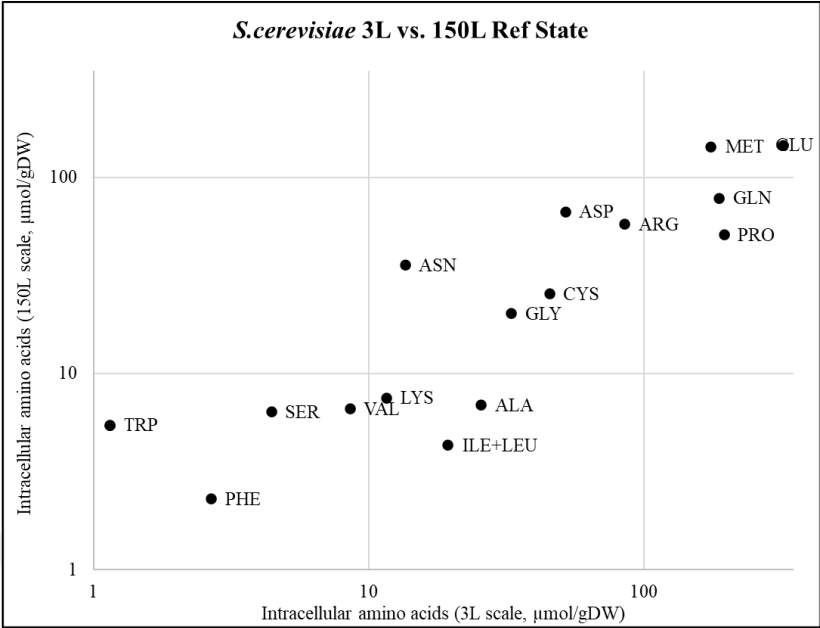


Figure 5.31. Comparison of amino acids in *S. cerevisiae* in 3L and 150L scales

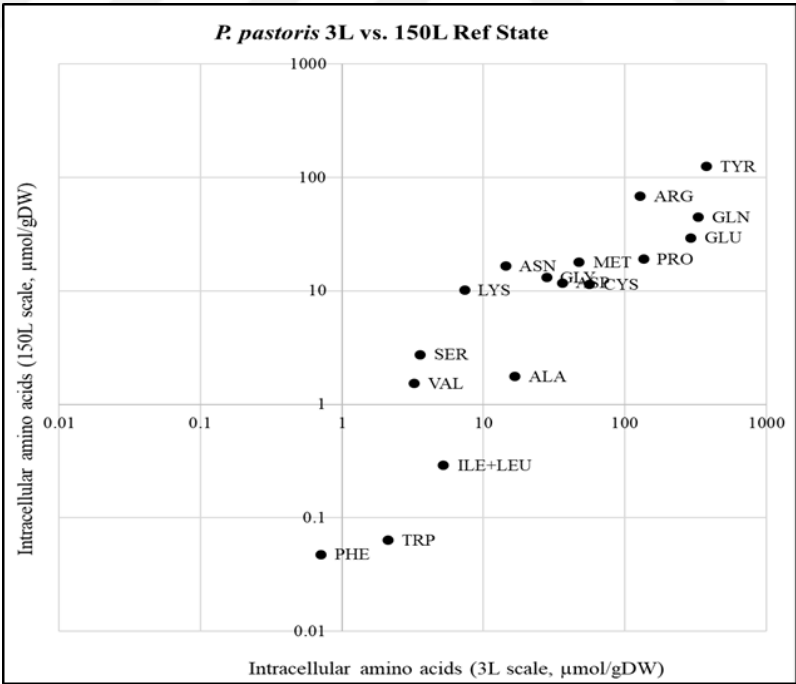


Figure 5.32. Comparison of amino acids in *P. pastoris* in 3L and 150L scales

5.5. QUANTIFICATION OF TOTAL GLUTATHIONE

The total glutathione levels were evaluated as percent of biomass as shown in Figure 5.33. There was no clear trend for glutathione levels after each stress conditions. We showed that glutathione levels increased with 5 mM of hydrogen peroxide-induced oxidative stress at chemostat (5OxScer) in *S.cerevisiae* and decreased with 20 mM of DTT-induced reductive stress (20RedScer). We observed that total glutathione levels in biomass were lower in *P.pastoris* than *S.cerevisiae* in 3L scale chemostat experiments. There was an opposing behaviour for total glutathione levels during 5 min for *P.pastoris* in 3L and 150L scales of oxidative stresses, but each scale, recovery processes were observed after 5 min.

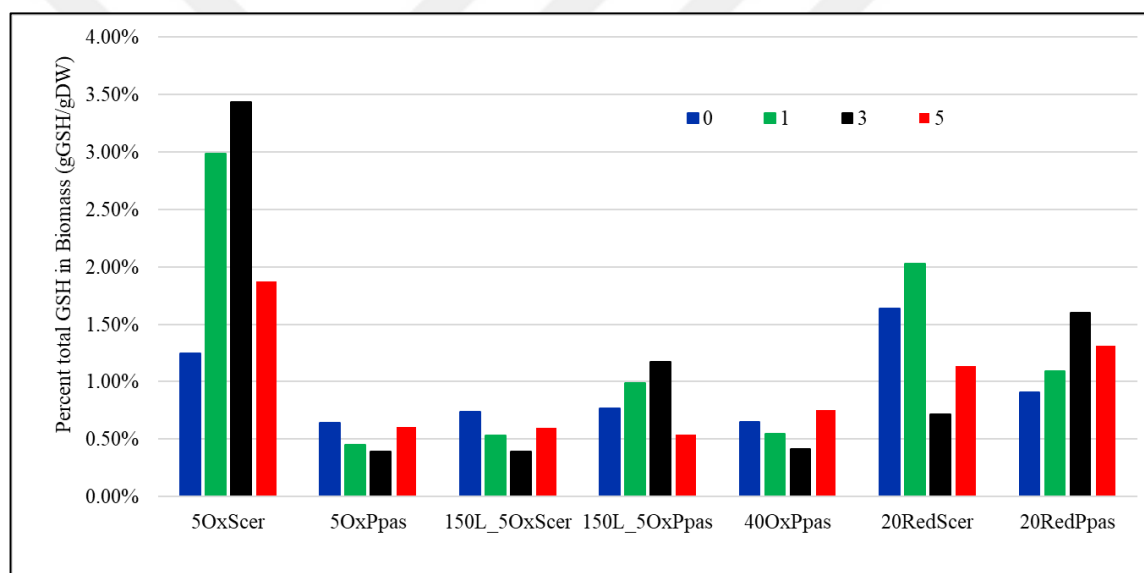


Figure 5.33. Total glutathione per biomass. The blue bars indicate that the glutathione amount in the reference state, green bars indicate the after 1 min of stress, black bars indicate that the after 3 min of stress, and red bars indicate that after 5 min of stress

5.6. FLUX BALANCE ANALYSIS

5.6.1. Simulations for *S. cerevisiae* and *P. pastoris*

Five different scenarios was simulated for both yeasts using genome-scale metabolic models of Yeast 8.5.0 and *P.pastoris* iMT1026v3 for *S.cerevisiae* and *P.pastoris*, respectively. The

objectives and constraints of scenarios are shown in Table 5.6. Estimated consumption rates of glucose and oxygen and production rate of carbon dioxide are shown in Figure 5.34. For the first scenario, maximization of growth was selected as an objective function and optimized to obtain metabolic flux distributions in FBA by constraining the specific consumption rate of glucose as $-10 \text{ mmol DCW}^{-1} \text{ h}^{-1}$. The growth rate was estimated as 0.867 h^{-1} while non-growth associated maintenance was 0, carbon dioxide production rate as $22.64 \text{ mmol DCW}^{-1} \text{ h}^{-1}$, oxygen consumption rate as $-21.19 \text{ mmol DCW}^{-1} \text{ h}^{-1}$ for *S. cerevisiae* (Figure 5.34). For *P. pastoris*, the growth rate was estimated as 1.060 h^{-1} while non-growth associated maintenance was 0, carbon dioxide production rate as $22.19 \text{ mmol DCW}^{-1} \text{ h}^{-1}$, oxygen consumption rate as $-20.74 \text{ mmol DCW}^{-1} \text{ h}^{-1}$ (Figure 5.35.).

Table 5.6. Scenarios simulated with FBA

Scenario	Objective	Organism	Constraints				
			-qS	μ	m _{ATP}	q _{CO2}	-q _{O2}
Unconstrained growth	Growth maximization	<i>S. cerevisiae</i>	10	-	-	-	-
		<i>P. pastoris</i>					
m _{ATP} constrained growth	Growth maximization	<i>S. cerevisiae</i>	10	-	0.7	-	-
		<i>P. pastoris</i>	10	-	0.55	-	-
Maximum m _{ATP} chemostat	m _{ATP} maximization	<i>S. cerevisiae</i>	1.2 -0.89	0.1	-	2.21-2.70	3.19-1.70
		<i>P. pastoris</i>	0.96-0.72	0.09-1	-	1.47-1.8	1.8-1.08
Unconstrained qGSH	GSH exchange maximization	<i>S. cerevisiae</i>	10	-	-	-	-
		<i>P. pastoris</i>					
Constrained qGSH	GSH exchange maximization	<i>S. cerevisiae</i>	1.2 -0.89	0.1	0.6	2.21-2.70	3.19-1.70
		<i>P. pastoris</i>	0.96-0.72	0.09	0.18	1.47-1.8	1.8-1.08

For the second scenario, maximization of growth reaction was selected as an objective function and optimized to obtain metabolic flux distributions in FBA by constraining the specific consumption rate of glucose as $-10 \text{ mmol DCW}^{-1} \text{ h}^{-1}$ and non-growth associated maintenance as $0.7 \text{ mmol ATP DCW}^{-1} \text{ h}^{-1}$ for *S. cerevisiae*. The growth rate was estimated as 0.864 h^{-1} , while the carbon dioxide production rate was $22.77 \text{ mmol DCW}^{-1} \text{ h}^{-1}$, oxygen

consumption rate was $-21.32 \text{ mmol DCW}^{-1} \text{ h}^{-1}$ (Figure 5.34.). Biomass yield on glucose was calculated as 0.481 for the first and 0.48 for the second scenarios. For *P. pastoris*, the growth rate was maximized as an objective function and optimized to obtain metabolic flux distributions in FBA by constraining the specific consumption rate of glucose as $-10 \text{ mmol DCW}^{-1} \text{ h}^{-1}$ and non-growth associated maintenance as $0.55 \text{ mmol ATP DCW}^{-1} \text{ h}^{-1}$. The growth rate was estimated as 1.058 h^{-1} , while the carbon dioxide production rate was $22.26 \text{ mmol DCW}^{-1} \text{ h}^{-1}$ and the oxygen consumption rate was $-20.81 \text{ mmol DCW}^{-1} \text{ h}^{-1}$ (Figure 5.35.). Biomass yield on glucose was calculated as 0.589 for the first and 0.588 for the second scenarios.

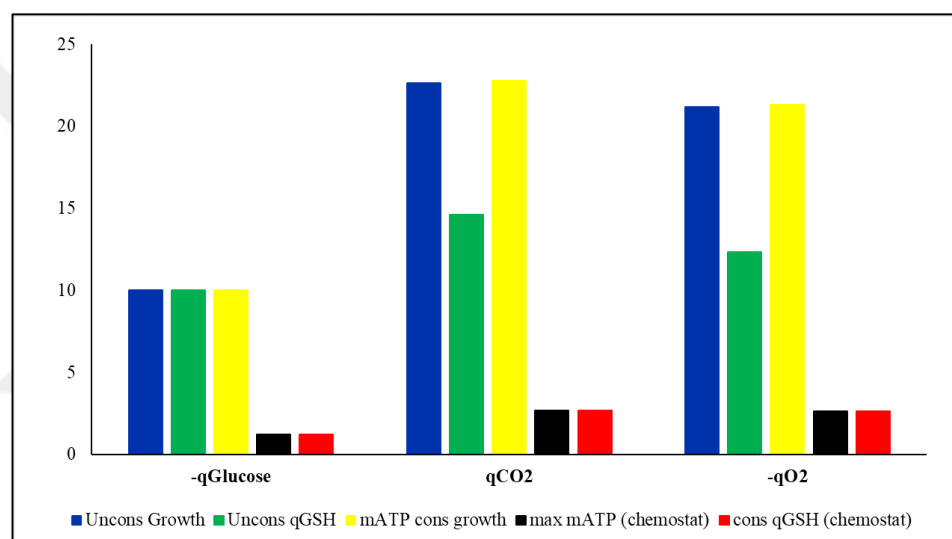


Figure 5.34. Estimated specific rates for consumption of glucose and oxygen and carbon dioxide production from FBA analysis of five scenarios for *S. cerevisiae*

For the third scenario, maximization of non-growth associated maintenance reaction was selected as an objective function and optimized to obtain metabolic flux distributions in FBA by constraining the specific consumption rates of glucose, oxygen, and carbon dioxide production rates. The simulation was performed by using the rates from the steady-state of fermentations. Non-growth associated maintenance was estimated as $0.684 \text{ mmol ATP DCW}^{-1} \text{ h}^{-1}$, while carbon dioxide production rate was $2.695 \text{ mmol DCW}^{-1} \text{ h}^{-1}$, oxygen consumption rate was $-2.625 \text{ mmol DCW}^{-1} \text{ h}^{-1}$ (Figure 5.34.). Biomass yield on glucose was calculated as 0.463. For *P. pastoris*, non-growth associated maintenance was estimated as $0.181 \text{ mmol ATP DCW}^{-1} \text{ h}^{-1}$, the theoretical value was reported as $0.55 \text{ mmol ATP DCW}^{-1}$

h^{-1} , while carbon dioxide production rate was $1.777 \text{ mmol DCW}^{-1} \text{ h}^{-1}$, oxygen consumption rate as $-1.798 \text{ mmol DCW}^{-1} \text{ h}^{-1}$ (Figure 5.35.).

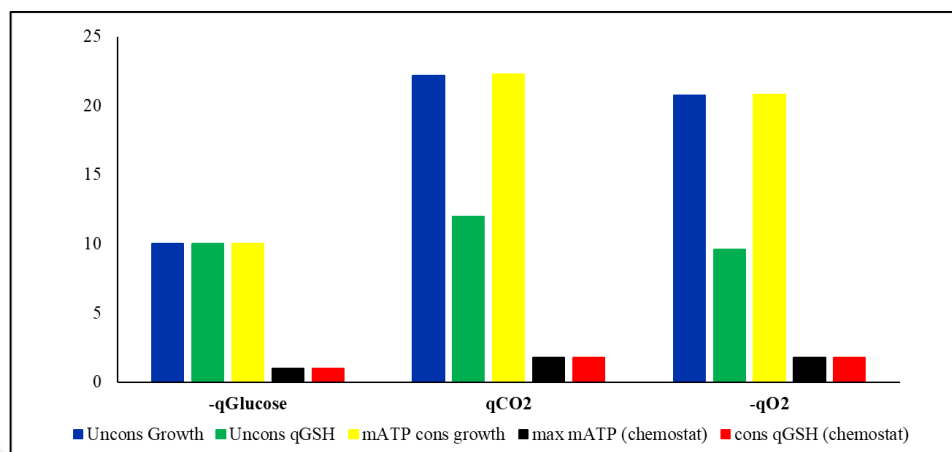


Figure 5.35. Estimated specific rates for consumption of glucose and oxygen and carbon dioxide production from FBA analysis of five scenarios for *P. pastoris*

For the fourth scenario, maximization of glutathione exchange reaction was selected as an objective function and optimized to obtain metabolic flux distributions in FBA by constraining the specific consumption rate of glucose as $-10 \text{ mmol DCW}^{-1} \text{ h}^{-1}$. The glutathione production rate was estimated as $4.54 \text{ mmol DCW}^{-1} \text{ h}^{-1}$ while there is no flux for growth and non-growth associated maintenance reactions under these conditions. Cell routes to metabolism for glutathione production and respiration and therefore, carbon dioxide production rate and oxygen consumption rate were estimated as $14.62 \text{ mmol DCW}^{-1} \text{ h}^{-1}$ and $-12.35 \text{ mmol DCW}^{-1} \text{ h}^{-1}$, respectively (Figure 5.34.). For *P. pastoris*, the glutathione production rate was estimated as $4.8 \text{ mmol DCW}^{-1} \text{ h}^{-1}$ while there is no flux for growth and non-growth associated maintenance reactions. Cell routes to metabolism for glutathione production and respiration and therefore, carbon dioxide production rate and oxygen consumption rate were estimated as $12.0 \text{ mmol DCW}^{-1} \text{ h}^{-1}$ and $-9.60 \text{ mmol DCW}^{-1} \text{ h}^{-1}$, respectively (Figure 5.35.).

For the fifth scenario, the glutathione exchange reaction was selected as an objective function and optimized to obtain metabolic flux distributions in FBA by constraining the specific consumption rates of glucose, oxygen, and production rates of carbon dioxide and non-growth associated maintenance. The simulation was performed by using the rates from the steady-state of fermentations. The glutathione production rate was estimated as $18 \text{ } \mu\text{mol}$

$\text{DCW}^{-1} \text{ h}^{-1}$, while carbon dioxide production and oxygen consumption rates were estimated as 2.695 and $-2.615 \text{ mmol DCW}^{-1} \text{ h}^{-1}$ (Figure 5.34.). For *P. pastoris*, the glutathione production rate was estimated as $0.145 \mu\text{mol DCW}^{-1} \text{ h}^{-1}$, while carbon dioxide production and oxygen consumption rates were estimated as 1.777 and $-1.798 \text{ mmol DCW}^{-1} \text{ h}^{-1}$ (Figure 5.35.).

To investigate the link between central carbon metabolism and glutathione production succinate dehydrogenase, glyceraldehyde-3-phosphate dehydrogenase, glutamate dehydrogenase, glycine hydroxymethyltransferase, phosphoserine transaminase, pyruvate kinase, glucose-6-phosphate dehydrogenase, phosphogluconate dehydrogenase, and isocitrate dehydrogenase reactions have been monitored under simulated scenarios for *S. cerevisiae* (Figure 5.36.) and *P. pastoris* (Figure 5.37.).

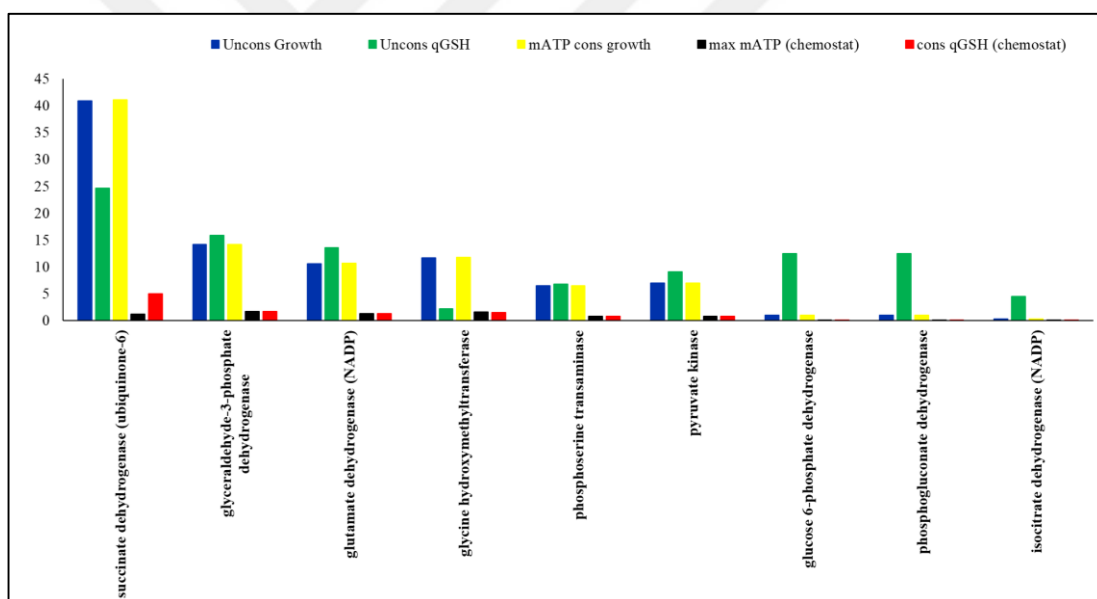


Figure 5.36. Estimated fluxes from FBA analysis for five different scenario for *S. cerevisiae*

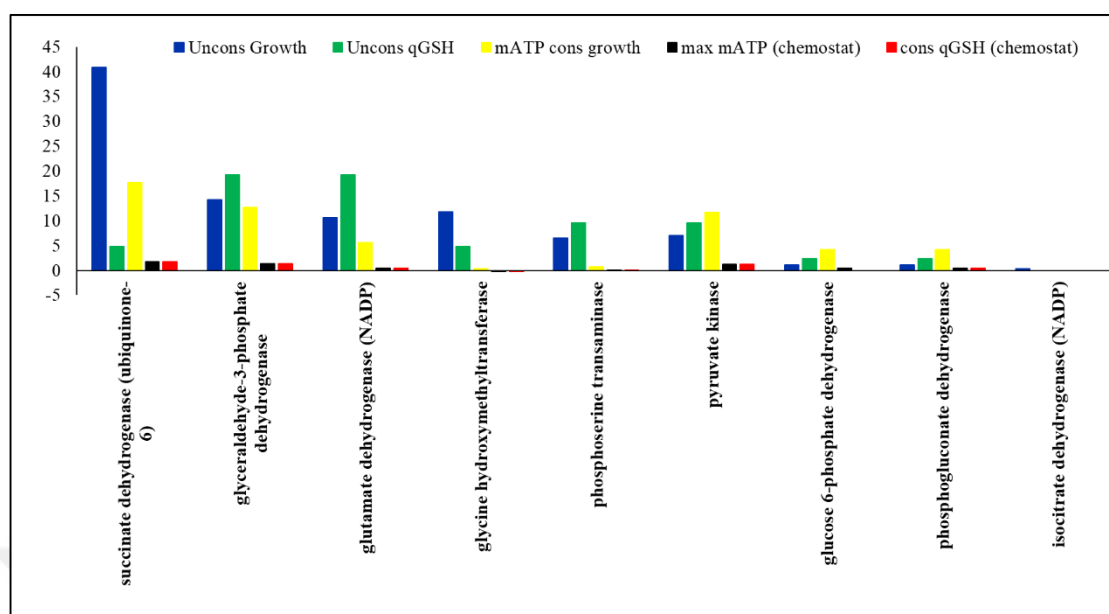


Figure 5.37. Estimated fluxes from FBA analysis for five different scenarios for *P. pastoris*

The unconstrained glutathione maximization model predicted that there would be an increased flux to glucose-6-phosphate dehydrogenase, phosphogluconate dehydrogenase, isocitrate dehydrogenase, glutamate dehydrogenase, and glyceraldehyde-3-phosphate dehydrogenase reactions. Glucose-6-phosphate dehydrogenase and phosphogluconate dehydrogenase are key enzymes for oxidative stress upon oxidative stress [13,18]. Sudden oxidative stress leads to detoxifying ROS with glutathione-based mechanisms and concomitant oxidation of NADPH. The decreased NADPH requires the rapid replenishing of NADPH pools. Glucose 6-phosphate (G6P) dehydrogenase and phosphogluconate (6PG) dehydrogenase in the oxidative branch of the pentose phosphate (PP) pathway catalysis are the main replenishing reactions in bacteria, *S. cerevisiae*, and most mammalian cells. Under oxidative stress, all cells increase the rate of NADP^+ to NADPH reduction, primarily by redirecting the glycolytic flux to the PP pathway [13,18,19].

The model-based investigation of response to oxidative stress via adding hydrogen peroxide transport reaction ($\text{H}_2\text{O}_2 \rightarrow \text{s}_{-0837}$), which is based on the diffusion of hydrogen peroxide inside the cell, to the model. Different scenarios were simulated to understand the effects of the addition of hydrogen peroxide on growth via setting the growth reaction as an objective function. In the flux distribution of both yeast models, the main differences are monitored. While the oxidative stress level was increased by the hydrogen peroxide transport reaction

added to the model, the flux to cytosolic catalase increased for *S.cerevisiae*, while no change was observed in the maximized growth rate. On the other hand, the growth rate of *P. pastoris* decreases with increasing hydrogen peroxide concentration and the flux for thioredoxin reductase reaction increases (Figure 5.38.).

FBA is also used to predict the phenotypic effects arising from the gene deletion. To understand the effects of deletion of cytosolic catalase gene for *S. cerevisiae*, the reaction of catalase was set as 0; the growth rate decreased from 0.8667 to 0.116 h⁻¹, and flux of thioredoxin reductase increased. When the reaction of thioredoxin reductase was set at 0, there was no growth in *P. pastoris*. Conventional yeast *S. cerevisiae* and non-conventional yeast *P. pastoris* have different coping mechanisms with hydrogen peroxide-induced oxidative stress. Studies show that *S. cerevisiae* has both cytosolic and peroxisomal/mitochondrial catalases in the literature. In contrast, when grown in glucose-containing media, *P. pastoris* has a relatively equal catalase distributed between peroxisomes and mitochondria [156].

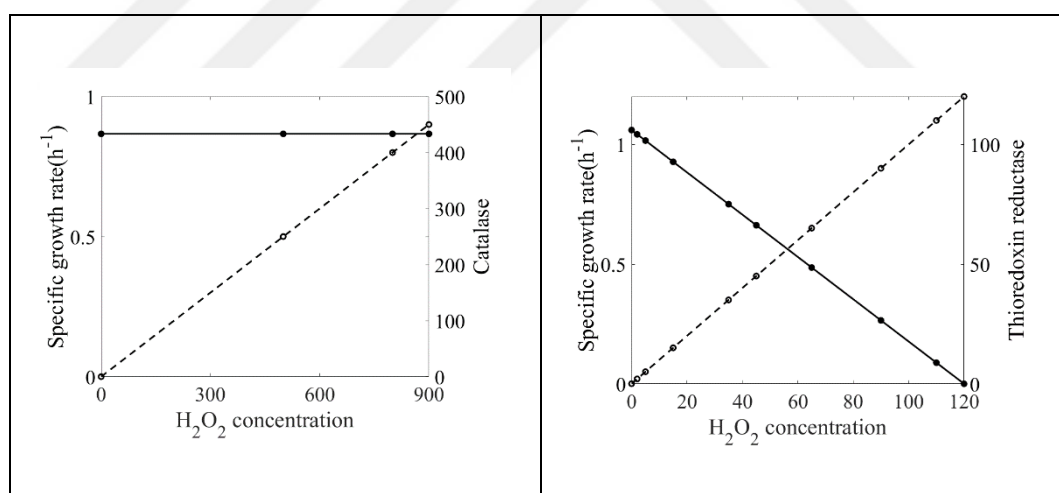


Figure 5.38. The effects of hydrogen peroxide-induced oxidative stress on the growth (left axis) and selected stress coping mechanism of *S. cerevisiae* (left panel) and *P. pastoris* (right panel)

The third scenario was simulated to compare GSH-overproducer and the wild type *S. cerevisiae*. The flux of reactions involved in the pentose phosphate pathway (e.g., glucose-6-phosphate dehydrogenase, phosphogluconate dehydrogenase, and transketolase) increased up to 4 fold, the reactions responsible for sulfate metabolism (sulfate adenylyltransferase,

sulfite reductase, sulfate exchange) increased around 15-20 fold for GSH-overproducer strain. This can be explained by glutathione production requiring supply cofactors such as NADPH for redox equilibrium. Also, it was known that all cells increase the rate of NADP⁺ to NADPH reduction, mostly by rerouting glycolytic flux into the pentose phosphate pathway when exposed to oxidative stress [13,18]. Sulfate metabolism is also important for glutathione metabolism to synthesize glutathione precursors [157]. The flux for cystathionine beta-synthase, O-succinylhomoserine lyase (L-cysteine), tyrosine transaminase, glycine-cleavage complex (lipoamide) increases in the GSH-overproduction scenario. The same scenario was simulated for *P.pastoris*. For the GSH-overproducer strain, the flux of reactions involved in sulfate metabolism (sulfate adenylyltransferase, sulfite reductase, sulfate exchange) increased around 25-fold for the strain which overproduced GSH. Glucose-6-phosphate dehydrogenase flux decreases, and phosphogluconate dehydrogenase and transketolase activities increase up to 2.5 and 1.5 fold in stress and overproducer scenarios. The flux of hexokinase decrease up to 70 percent, and amino acid-related pathways (alpha-ketoglutarate/malate transporter, glycine hydroxymethyltransferase, glutamate dehydrogenase, phosphoserine transaminase, and phosphoserine phosphatase) increase in GSH-overproduction scenario, in contrast to this they decrease under hydrogen peroxide-induced oxidative stress scenario.

5.7. GENERATING GSH PRODUCERS USING CLASSICAL STRAIN OF *YARROWIA LIPOLYTICA*

5.7.1. UV Mutagenesis of *Y. lipolytica*

The toxicity level of hydrogen peroxide on the *Y.lipolytica* was determined using patch/plate assays, and 10 mM is the lethal concentration of H₂O₂. Therefore, the cultures were spread on YPD agar containing 10, 15, and 20 mM of H₂O₂ after UV irradiation to select potential oxidative stress-resistant mutants. The colonies growing on the 10 and 15 mM of H₂O₂-containing agars were selected as resistant to oxidative stress. The cultures of these strains were used to make stock cultures and kept at -80°C. While after UV irradiation for 30 minutes did not see any grown colonies on YPD agar containing 10 mM H₂O₂, a few colonies were observed in those exposed for 40 minutes (Figure 5.39.). In addition, lots of colonies

(up to 86) were observed on YPD agar containing 15 mM of H_2O_2 after both exposure times (Figure 5.40.).

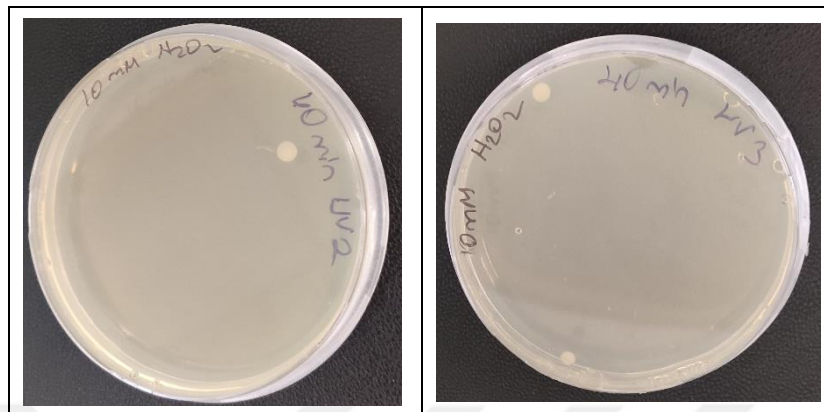


Figure 5.39. Colonies growing on YPD agar plate containing 10 mM of H_2O_2 after UV irradiation for 40 min

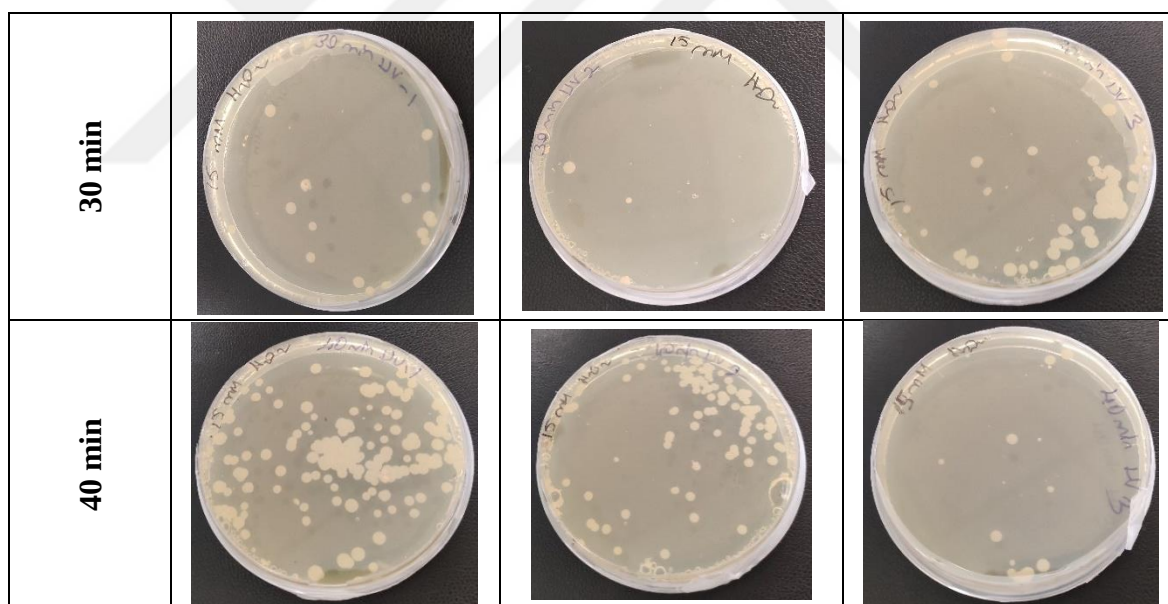


Figure 5.40. Colonies growing on 15 mM of H_2O_2 containing YPD agar after UV irradiation for 30 and 40 min

5.7.2. Screening of Strains that Are Overproducing GSH

First, reference strain and colonies grew after UV mutation, and hydrogen peroxide stress was screened via fermentation in a 2XVerduyn medium until 29 h. Then different concentrations (0, 15, 50, and 100 mM) of H_2O_2 were pulsed, and the cells were harvested

at 32 h of fermentation. Biomass and glucose concentrations were followed, cells were harvested, and total glutathione levels were determined with a quantification kit for oxidized and reduced glutathione. For this study, wild-type *Y. lipolytica* was used as a control group (reference strain) to determine GSH over-producing strain (Figure 5.43.).

UV-mutated strain 15-40-1-22 was more resistant to hydrogen peroxide-induced oxidative stress than the reference strain. After 3 hours of 100 mM of H_2O_2 pulse, only a transparent medium remained in the reference strain shown in Figure 5.41.

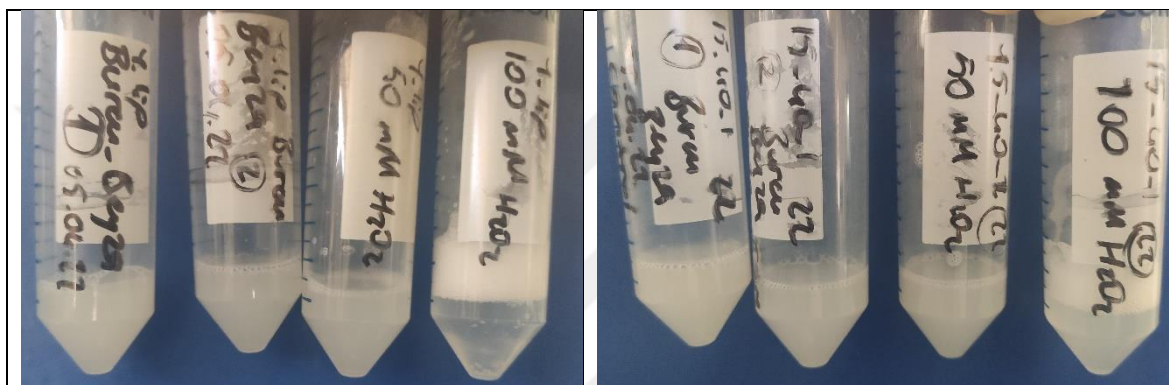


Figure 5.41. Different concentrations of hydrogen peroxide (oxidative) stresses for reference (left panel) and UV-mutated 15-40-1-22 strains (right panel)

While OD600 decreases with increasing H_2O_2 concentration in reference strain, UV mutated strain is more resistant to increasing H_2O_2 concentration. A significant decrease in OD600 was observed only after 100 mM of hydrogen peroxide pulse for UV mutated strain (Figure 5.42.).

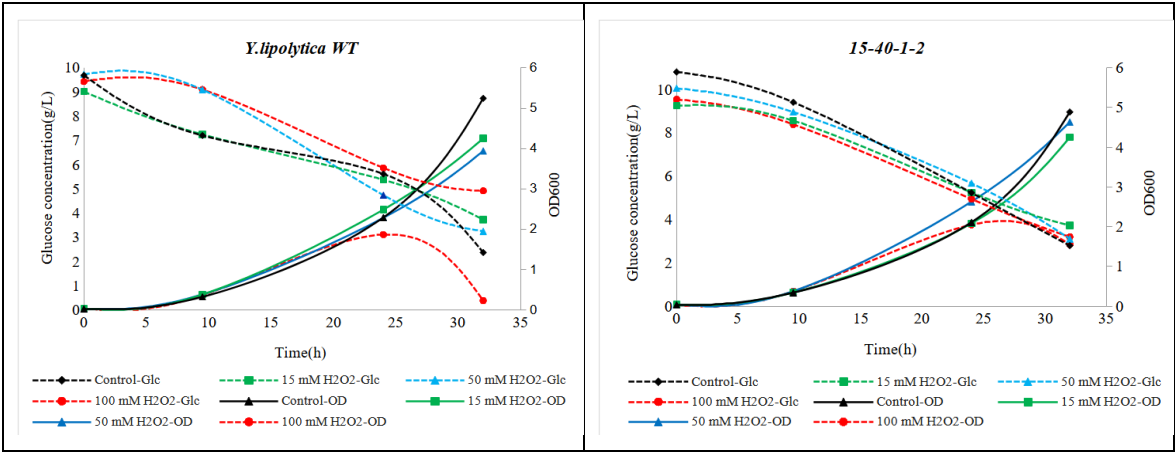
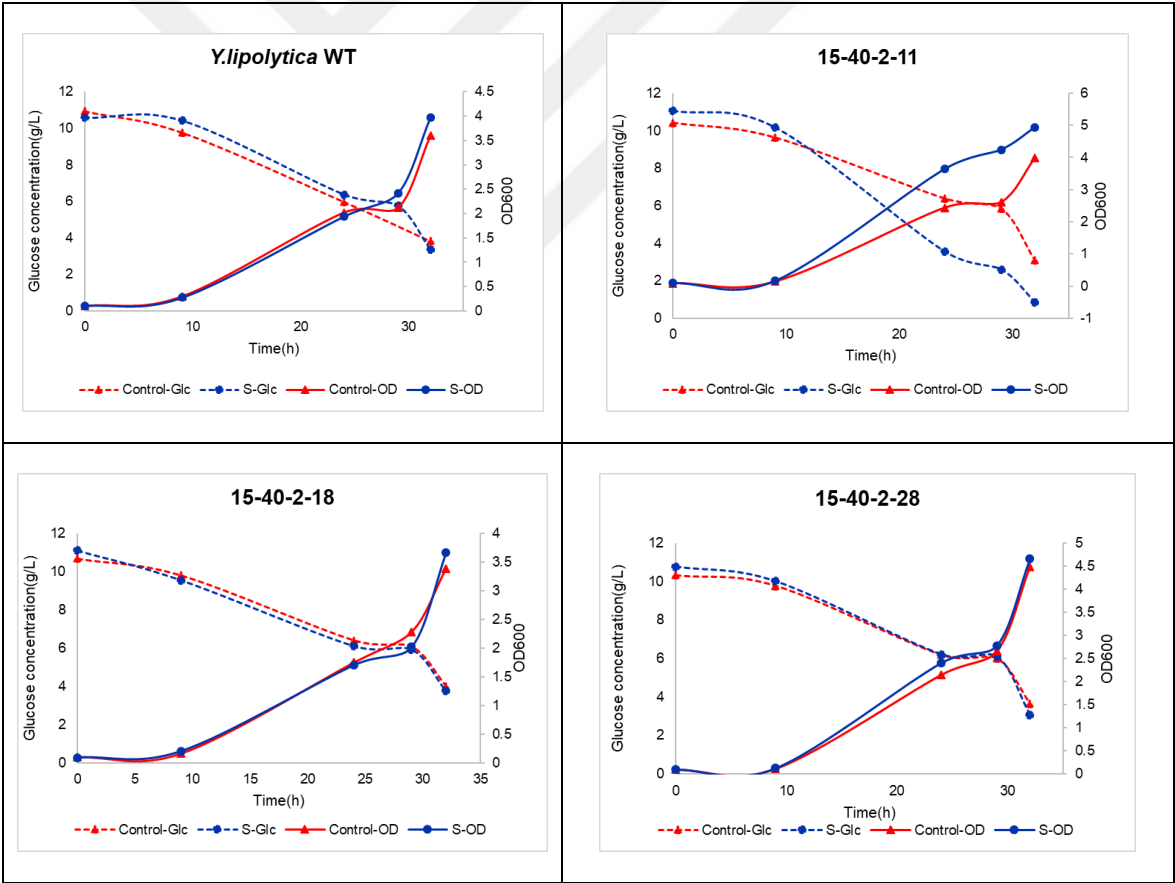


Figure 5.42. Biomass and glucose concentrations of the reference strain of *Y. lipolytica* and UV mutated strain-15-40-1-22



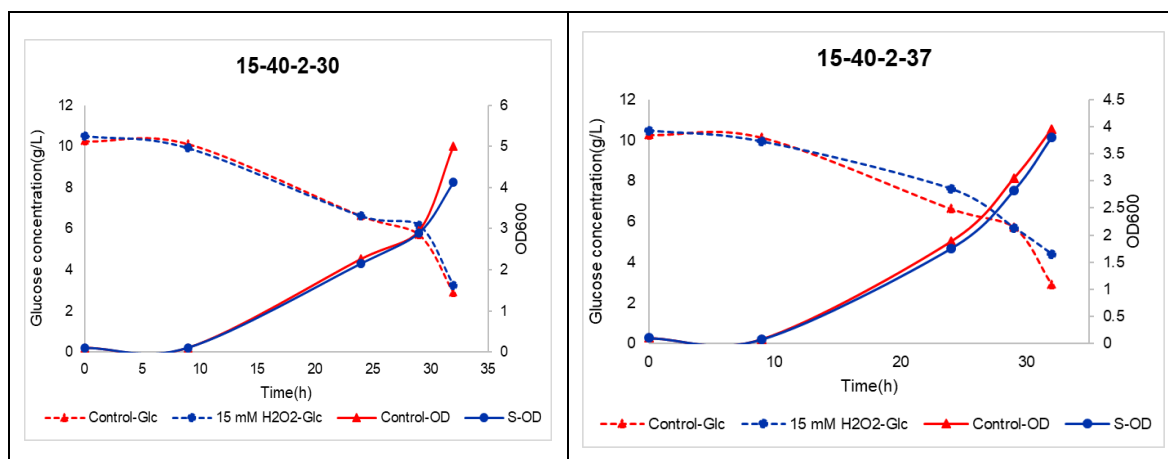


Figure 5.43. Fermentation profiles of *Y. lipolytica* WT and various UV-mutated strains

Wild type (reference strain) and UV-mutated strains were compared according to the percentage of total glutathione amount (g/mole) per biomass concentration in Figure 5.44. It was determined that the strains of 15-40-1 25 were the highest producers, and 15-30-3 2, 15-40-2 19 produced 50 percent more glutathione than the reference strain. 15-40-2-28 and 15-40-2-37 strains produced low glutathione than the reference strain.

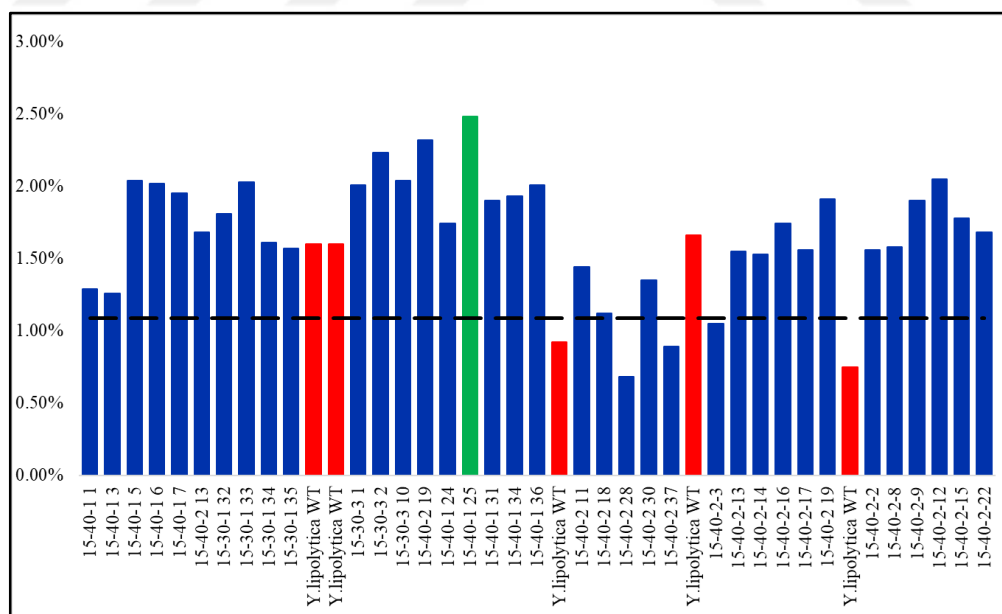


Figure 5.44. Total glutathione per biomass. The black dashed line indicates that the average of GSH amount in *Y. lipolytica* WT cells, red columns indicate the *Y. lipolytica* WT cells and green column indicates that the highest producers

6. CONCLUSION

Non-conventional yeasts are gaining popularity in fermentation industry. Despite growing interest, research on the response of NCY metabolism to various environmental stimuli is still in its infancy. Using systems biology approach, this thesis aimed to understand the oxidative stress response and to ultimately decipher stress-induced interactions among central carbon, energy metabolism, and amino acid metabolism. Therefore, it focuses on the comparison of conventional to non-conventional yeast metabolism in fed-batch and chemostat-grown cultures. The experimental data was expanded to include simulations of genome-scale models to understand the intracellular flux distribution.

This thesis provides a novel dataset consisting of fermentation data intracellular amino acid and glutathione levels for two different yeasts (*S. cerevisiae* and *P. pastoris*) at two different stress conditions (oxidative vs. reductive stresses) and two different scales (3L vs. 150L).

The non-conventional yeasts *P. pastoris* and *Y. lipolytica* (10 mM of H_2O_2 is the toxic concentration) were found to be more resistant to oxidative stress than the conventional yeast, *S. cerevisiae* (5 mM of H_2O_2 is the toxic concentration) via plate assays. In addition, 40 mM of DTT was the lethal concentration for *S. cerevisiae* and *P. pastoris*.

There is a discrepancy between organisms at low concentrations of amino acids. Furthermore, the amino acid levels were less on the 150L scale by a factor of 3-5 for both yeasts. Oxidative stress induces a different short-term response among amino acid levels for both yeasts cultivated in a chemostat. There is a delayed amino acid response for *S. cerevisiae* with respect to *P. pastoris*. The glutathione levels increased with hydrogen peroxide-induced oxidative stress (5 mM) at chemostat in *S. cerevisiae* and decreased with (20 mM) DTT-induced reductive stress. Comparing batch to batch (around 50 percent relative standard deviation), sample to sample (less than 20 percent relative standard deviation) and injection to injection (less than 10 percent relative standard deviation) variances, it can be concluded that targeted quantitative metabolomics approach was successfully applied in this thesis.

The response to oxidative stress was investigated using FBA. *S. cerevisiae* and non-conventional yeast *P. pastoris* have different coping mechanisms against hydrogen peroxide-induced oxidative stress. The flux distribution of cytosolic catalase increased for

S. cerevisiae, and the thioredoxin reductase increased for *P. pastoris* in hydrogen peroxide-induced oxidative stress scenarios simulated by FBA.

Oxidative stress-tolerant strains of *Y. lipolytica* and glutathione over-producer strains of *Y. lipolytica* were obtained via classical UV-mutagenesis and screening under H₂O₂ stress. The strains which produce high GSH have up to a 50 percent increase in GSH content compared to the wild-type strain.

This study demonstrated a potential to understand that the oxidative stress response differs in intracellular amino acid and glutathione levels by supporting it with flux distributions for conventional and non-conventional yeasts. In addition, transcriptome responses of yeasts can be evaluated to complete the systems biology approach. Future studies can focus on sequencing of strains that produce higher GSH and follow by detailed phenotype characterization. Responses to oxidative stress between wild-type and mutant strain of *Y. lipolytica* can be evaluated from the perspective of this thesis using stimulus-response technology. In addition, the generated strains which produce higher GSH can be alternative producer to conventional yeast in the industry. Moreover, to increase GSH production, high biomass concentrations can be targeted with high cell density fermentation, and glutathione production can be increased by feeding precursor amino acids (cysteine, glutamic acid, and glycine).

7. PUBLICATIONS

7.1. JOURNAL ARTICLES

Tanıl E, Şirin B, Nikerel E. Meta-analysis of transcriptome data on oxidative stress response in *Saccharomyces cerevisiae* cells underlines regulation of carbon, redox and glutathione metabolism. *Biotech Studies*.2020;29(1):8-17.

Özcan E, Seven M, Şirin B, Çakır,T, Nikerel E, Teusink B, & Toksoy Öner E. Dynamic co-culture metabolic models reveal the fermentation dynamics, metabolic capacities and interplays of cheese starter cultures. *Biotechnology and Bioengineering*. 2021;118(1): 223-237.

7.2. POSTER PRESENTATIONS

06/2019 Sirin B, Nikerel E. Quantitative metabolome analysis of glutathione pathway in non-conventional yeasts. Presented at 7th Conference of Physiology of Yeasts Filamentous Fungi (PYFF) 24-27 June 2019, Milan, ITALY

7.3. CONFERENCE PROCEEDINGS

10/2019 Sirin B, Nikerel E. World of Small Molecules in Non-conventional Yeasts. Proceeding of The 35th International Specialized Symposium on Yeasts (ISSY 35, Antalya (Turkey), October 21-25), p.46

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