

## Article

# Distinct Short-Term Response of Intracellular Amino Acids in *Saccharomyces cerevisiae* and *Pichia pastoris* to Oxidative and Reductive Stress

Burcu Şirin Kaya  and Emrah Nikerel \* 

Department of Genetics and Bioengineering, Faculty of Engineering, Yeditepe University, Istanbul 34755, Turkey; burcusirinn@gmail.com

\* Correspondence: emrah.nikerel@yeditepe.edu.tr

**Abstract:** Despite being frequently encountered, the effect of oxidative or reductive stress on the intracellular metabolism and the response of the intracellular metabolome of yeasts is severely understudied. Non-conventional yeasts are attracting increasing attention due to their large substrate portfolio of non-canonical pathways as well as their production and secretion of proteins. To understand the effects of both stresses on yeast, the conventional model yeast *S. cerevisiae* and the non-conventional model yeast *P. pastoris* were perturbed with 5 mM of hydrogen peroxide for oxidative stress and 20 mM of dithiothreitol for reductive stress in well-defined chemostat cultures at a steady state, and fermentation profiles, intracellular amino acid levels, and intracellular glutathione levels were measured. Although stable profiles of extracellular metabolites were observed, significant changes were measured in intracellular amino acid levels within the first five minutes. Collectively, the amino acids ranged from 0.5 to 400  $\mu\text{mol/gDW}$ , with the most significant increase upon the induction of oxidative stress being seen in cysteine (up to 90%) for *S. cerevisiae* and in aspartate (up to 80%) for *P. pastoris*. Upon the induction of reductive stress, asparagine nearly halves in *S. cerevisiae*, while tryptophan decreases by 60% in *P. pastoris*. By inspecting the time traces of each amino acid, possible mechanisms of pathway kinetics are speculated. This work furthers our understanding of the response of metabolism to oxidative stress in two model yeasts.



**Citation:** Şirin Kaya, B.; Nikerel, E. Distinct Short-Term Response of Intracellular Amino Acids in *Saccharomyces cerevisiae* and *Pichia pastoris* to Oxidative and Reductive Stress. *Fermentation* **2024**, *10*, 166. <https://doi.org/10.3390/fermentation10030166>

Academic Editors: Agustín Aranda and Michael Breitenbach

Received: 29 December 2023

Revised: 1 February 2024

Accepted: 16 February 2024

Published: 15 March 2024



**Copyright:** © 2024 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (<https://creativecommons.org/licenses/by/4.0/>).

**Keywords:** intracellular amino acid; oxidative stress; *Pichia pastoris*; *Saccharomyces cerevisiae*; short-term stimulus–response

## 1. Introduction

An increasing level of oxygen in the atmosphere has given the potential to (micro)organisms, which originally evolved in a reducing environment, of efficient energy generation through respiration and also the capability to produce intermediates responsible for maintaining cellular homeostasis. Yet, excessive exposure to oxygen may cause damage to 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.

The response of yeast cells to oxidative stress is typically complex, tightly regulated, and involves several omic layers following a time hierarchy. Dedicated experiments exploiting this time hierarchy may ultimately allow for the isolation of a single-omic response to external stimuli. Such an approach has been adopted in stimulus–response experiments focusing on monitoring the system response upon the external perturbation of a well-defined metabolic state (e.g., steady state in a chemostat) within a couple of minutes [1]. The resulting transient data can be used to construct and parametrize a dynamic model for metabolic reaction networks [2–4]. A typical short-term stimulus–response experiment involves taking “snapshots of the metabolism” upon perturbation by rapidly quenching the metabolism to stop enzymatic activity, separating cells from the

fermentation medium, and extracting followed by quantifying intracellular metabolites. Focusing on the within-minute response of the cells and assuming that changes in enzyme concentration are negligible, the response can be attributed to the metabolic level and kinetic interactions [5,6].

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 a high potential to produce valuable biotechnological products including various recombinant proteins, several enzymes, and vaccines. 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*), a well-known methylotrophic yeast, is an essential host for recombinant protein production.

Despite the available interest, studies on the impact of environmental stresses on the physiology of *P. pastoris* are rather limited, especially on the effects of oxygen availability, which is one of the most common problems in industrial biotechnology. By significantly affecting cellular redox reactions, oxygen plays a key role in protein-folding reactions within cells as well as the growth and protein production of cells. Baumann et al. [7] 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, and they unraveled potentially distinct features of such an adaptation that have been described for *S. cerevisiae*.

Aerobic life induces oxidative stress, defined as high exposure to reactive oxygen species such as peroxides, alkyl hydroperoxides, and peroxide anions, 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 the beneficial and toxic effects on the cell. While cell proliferation and differentiation processes require low levels of ROS, even these low levels can damage cellular homeostasis and can drive cell aging and death. In the physiological state, cells maintain their ROS levels with dynamic equilibrium during ROS generation and elimination processes, antioxidant enzyme mechanisms (superoxide dismutase, catalase, and peroxidase reactions), glutaredoxin/thioredoxin systems, metabolic products (glutathione, which is used in almost all eukaryotes), and the upregulation of various transcription factors (Yap1p, Skn7p, Msn2/4p) [8].

Reductive stress is described as an increased level of reducing equivalents in the form of redox couples (GSH/GSSG, NAD(P)H/NAD(P)<sup>+</sup>). Although studies on understanding oxidative stress mechanisms and responses 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 O<sub>2</sub> 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 [9]. DTT can diffuse from membranes and prohibit 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 and Spellmann [10] showed that the transcriptional response of yeast cells to DTT-induced reductive stress was remarkably different from the general environmental stress response.

Redox couples, NAD(H), NADP(H), and GSH/GSSG are essential regulators of cell metabolism. Glutathione (GSH) is an antioxidant found in plants, animals, fungi, and some bacteria and archaea, which is capable of preventing damage to important cellular components caused by various sources including reactive oxygen species, free radicals, peroxides, lipid peroxides, and heavy metals. GSH, a tripeptide with a gamma peptide bond between the carboxyl group of the glutamate side chain and cysteine, acts as a linker

metabolite between amino acid metabolism and the redox state of cells [11]. Therefore, changes in the ratio of this redox couple may be key to understanding the reductive stress response. Besides its role in yeast metabolism, GSH is also significant for the bread and wine industries [12,13].

Recent studies on 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. Lin et al. [14] conducted a noteworthy study indicating that an oxidative stress response was induced with methanol in *P. pastoris*. Heterologous protein production increased with the new mutant cell being designed with the overexpression of genes related to an antioxidant mechanism against ROS [14]. Similar studies show that understanding the oxidative stress response mechanism is of high importance in terms of taking new steps to increase fermentation efficiency in various application fields from recombinant protein production to fine chemicals.

Microorganisms require an abundant supply of amino acids to maintain their driving force for proliferation. However, the role of amino acids in the cell is not limited to their primary role as the primary building block of proteins. Amino acids play diverse roles in the cell, including energy generation, nucleoside synthesis, homeostasis of cellular redox, as well as stabilizing cell osmolarity and pathogenesis. Amino acids and their derivatives, being the most abundant compounds among all detected metabolites, represent a pivotal focus in oxidative stress, and their associated amino acid metabolism can be easily modified [15].

Departing from the question of how the non-conventional yeast model *P. pastoris* responds to oxidative stress in comparison to the conventional yeast model *S. cerevisiae*, this study focuses on the quantitative investigation of intracellular amino acids and glutathione levels in *P. pastoris* and compares them to those of the conventional yeast *S. cerevisiae*. Adopting the systems biology approach, the data generated, including fermentation profiles as well as measured targeted metabolome data and transcriptome data from the literature, are collectively taken into account in response to oxidative and reductive stress for conventional and non-conventional yeasts.

## 2. Materials and Methods

### 2.1. Organisms and Media

*Saccharomyces cerevisiae* CEN.PK113-7D (MAT $\alpha$ , MAL2-8c SUC2, obtained from the EUROSCARF collection) and *Pichia pastoris* ATCC 28485 (obtained from ATCC) were kept in 30 percent glycerol at  $-80\text{ }^{\circ}\text{C}$  prior to use in this study. Yeast cells were grown to the stationary phase in 250 mL Erlenmeyer flasks with 50 mL of a double mineral medium at 180 rpm and  $30\text{ }^{\circ}\text{C}$  [16]. 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  $1000\times$  vitamin solution, and 20 mL of  $100\times$  trace elements (Table S1). The medium was sterilized at  $121\text{ }^{\circ}\text{C}$  15 min prior to use, and the glucose solution was separately sterilized at  $121\text{ }^{\circ}\text{C}$  for 3 min. Both  $1000\times$  vitamin solution and  $100\times$  trace element solution were filter-sterilized.

### 2.2. Chemostat Cultivation and Application of Oxidative Stress

Both yeasts were aerobically cultivated in a double mineral medium at a dilution rate of  $0.1\text{ h}^{-1}$  in 4 L bioreactors (Minifors 2, Infors HT, Bottmingen, Switzerland) with a working volume of 1.5 L. The medium is supplemented with  $200\text{ }\mu\text{L L}^{-1}$  Antifoam Y-30 emulsion (Sigma Aldrich, St. Louis, MO, USA). Chemostat cultivation was preceded by a batch phase performed on a medium identical to the feed medium. At the end of the batch phase (evidenced by a sharp decrease in the carbon dioxide level in the off-gas and a sharp increase in the oxygen profile of the off-gas), medium feeding was started. The culture temperature was kept at  $30\text{ }^{\circ}\text{C}$ , and the pH was controlled at 5.5 using 3 M orthophosphoric acid or 3 M sodium hydroxide. The cultures were sparged with sterile air with a  $0.75\text{ Lh}^{-1}$  flow rate and stirred initially at 500 rpm. The dissolved oxygen (DO) level was maintained

above 20% through the cascaded control of the agitation speed. The fractions of carbon dioxide and oxygen in the off-gas were followed with the BlueInOneFerm gas analyzer (BlueSens, GmbH, Herten, Germany). The amounts of added acid, base, and antifoam were monitored and recorded during the entire course of fermentation.

Chemostat cultures were assumed to be at a steady state when the culture biomass production, carbon dioxide, and oxygen compositions of the off-gas readings did not change more than 5 percent after 5 volume changes. Furthermore, glucose limitation was confirmed by measuring the residual glucose concentration under  $0.9 \text{ g L}^{-1}$  at a steady state. To apply oxidative or reductive stress to the culture, corresponding agents 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. Just before the application of hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) perturbation, 10 mL of culture broth was sampled, and all the data from that sample were used as a reference state. The oxidative stress was mimicked by pulsing 5 mM of  $\text{H}_2\text{O}_2$ , and the reductive stress was mimicked by pulsing 20 mM Dithiothreitol (DTT) to the reactor. To observe the response of the yeasts, broth samples were rapidly withdrawn and quenched 1, 3, and 5 min after exposure to  $\text{H}_2\text{O}_2$  and DTT, and metabolites were analyzed after extraction.

### 2.3. Rapid Sampling, Quenching, and Extraction of Intracellular Metabolites

10 mL of broth samples were rapidly withdrawn and quenched into 35 mL pure methanol at  $<-40 \text{ }^\circ\text{C}$ , quickly vortexed, and stored back at  $-40 \text{ }^\circ\text{C}$ . To remove the extracellular medium, samples were centrifuged at  $5000 \times g$  for 5 min and at  $-12 \text{ }^\circ\text{C}$  (1580R, Gyrozen, Daejeon, Republic of Korea), and supernatants were stored at  $-80 \text{ }^\circ\text{C}$  for the determination of extracellular metabolites [2]. Intracellular metabolites were extracted using the boiling ethanol extraction method, following the earlier work carried out by Lange et al. [17], particularly for the measurement of the intracellular amino acid levels. A total of 35 mL of 75% ethanol was heated in a water bath at  $95 \text{ }^\circ\text{C}$  for 5 min, the boiling ethanol was quickly poured onto the cell pellet, and the tubes were vortexed as fast as possible and placed on the water bath for 3 min. The samples were then cooled and centrifuged at  $7000 \times g$  for 10 min at  $4 \text{ }^\circ\text{C}$  (1580R, Gyrozen, Daejeon, Republic of Korea), and the extracts were stored at  $-80 \text{ }^\circ\text{C}$  [18]. Finally, the extracts were concentrated through a rotary evaporator (Heidolph, Schwabach, Germany), and then the samples were dried using a freeze-dryer (Labconco, Kansas City, Missouri, USA). Before each analysis, the extracts were resuspended on 500–1000  $\mu\text{L}$  double-distilled water [2]. Broth samples collected prior to  $\text{H}_2\text{O}_2$  perturbation were analyzed similarly and used as reference state measurements.

### 2.4. Analytical Methods

The biomass concentration was followed through the optical density measured using UV spectrometry (Thermo Scientific Genesys 10S UV-vis, Waltham, MA, USA) at 600 nm as well as the measurement of the dry weights of the cells. To measure the dry weight of the cells, 40 mL of the sample was taken in pre-weighted tubes and centrifuged at 10,000 rpm, followed by washing the pellets with distilled water or PBS solution. Then, the washed pellets were dried at  $105 \text{ }^\circ\text{C}$  for at least 24 h. Residual sugar was measured with the dinitrosalicylic acid (DNS) method [19]. Acetate, ethanol, and glycerol were analyzed by HPLC (LC20AD, Shimadzu, Kyoto, Japan) equipped with an RI detector and IC-Pak ion exclusion column ( $7 \mu\text{m}$ ,  $7.8 \times 300 \text{ mm}$ , Waters, Milford, MA, USA) maintained at  $50 \text{ }^\circ\text{C}$  using 5 mM sulfuric acid as mobile phase as  $0.6 \text{ mL min}^{-1}$ .

Identification and quantification of intracellular amino acids were carried out by first derivatizing using diethyl ethoxymethylenemalonate (DEEMM) and then quantification using HPLC (LC20AD, Shimadzu, Kyoto, Japan) equipped with a UV detector (280 nm) at  $25 \text{ }^\circ\text{C}$  using an XBridge C18 column ( $5 \mu\text{m}$   $4.6 \times 150 \text{ mm}$ , Waters) eluted with two mobile phases as follows: 25 mM sodium acetate buffer (pH 5.8) with 0.02% sodium azide and 80:20 acetonitrile/methanol (4:1 (v/v) at  $0.8 \text{ mL min}^{-1}$  [20]. Commercial amino acids (L-tryptophan (T0254), L-phenylalanine (P5482), L-alanine (A7627), L-valine (V0500),

L-isoleucine (I7403), L-leucine (L8912), glycine (G7403), L-cysteine (C7352), L-serine (S4311), L-glutamic acid monosodium salt hydrate (G1626), L-proline (P0380), DL-aspartic acid potassium salt (A2025), L-arginine (A8094), L-methionine (M9625), L-asparagine (A0884), L-glutamine (G8540), and L-lysine (L9037) from Sigma Aldrich, USA) were used as the external standards for HPLC (Table S2). The available HPLC methodology did not allow for the accurate quantification of isoleucine and leucine separately. These amino acids were evaluated as total concentrations.

For glutathione, a UV detector at 210 nm with the same column was used, this time eluted with 25 mM sodium phosphate monobasic (pH 3.5) at 0.3 mL min<sup>-1</sup> [21]. Commercial reduced glutathione (G6013) and oxidized glutathione (G4501-Sigma Aldrich, USA) were used as external standards for glutathione measurements.

### 2.5. Elemental Balances, Statistical Evaluations

Biomass-specific rates of growth ( $\mu$ ), glucose consumption ( $-q_S$ ), oxygen consumption ( $-q_{O_2}$ ), and carbon dioxide production ( $q_{CO_2}$ ) were calculated/estimated using the steady-state mass balances. The cumulative carbon balance accounts for the carbon from the substrate used and the accumulation of the carbon in biomass, carbon dioxide, and by-products if any. In the same manner, the cumulative electron balance accounts for the electrons from glucose, transferred to biomass and oxygen, with each being weighed by the corresponding degree of reduction. The biomass formula for *S. cerevisiae* used was that as described in [17], and for *P. pastoris* the one used was that in [22]. The calculated average values of the specific rates were reconciled under the constraint of satisfying the elemental conservation relations [23].

Statistical significance in the change in intracellular metabolite levels was assessed using a paired Student's *t*-test (prior and posterior to the oxidative stress) under the null hypothesis that there is no change. The critical level of significance is taken to be 0.05.

## 3. Results and Discussion

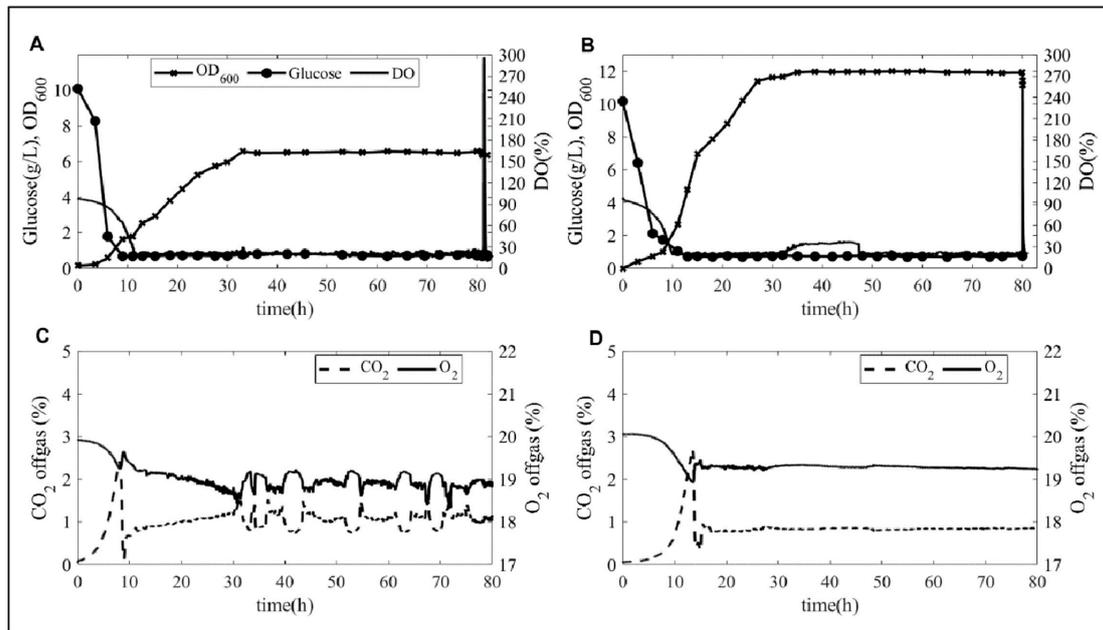
This work aims to further our understanding of the response of two model yeasts (conventional and non-conventional) to oxidative and reductive stress using short-term stimulus–response experiments. With this aim, glucose-limited chemostats well characterized in their fermentation profiles (both the initial batch phase as well as its steady state) were carried out at a dilution rate of 0.1 h<sup>-1</sup> for both yeasts. For the oxidative stress response, H<sub>2</sub>O<sub>2</sub> perturbation experiments were conducted, and DTT perturbation experiments were conducted for the reductive stress response. Prior to the perturbation experiments, the patch/plate assays were used to determine the toxic levels of DTT on the yeast cells. In line with our results, Trotter et al. [24] reported that the wild-type *S. cerevisiae* was extremely resistant, growing on plates containing DTT concentrations as high as 16 mM. Therefore, 20 mM of DTT was selected in subsequent perturbation experiments.

### 3.1. Fermentation Profiles

Glucose-limited chemostats were carried out at a dilution rate of 0.1 h<sup>-1</sup> for both yeasts. The time profiles from the start of the batch until the steady state is reached are presented in Figure 1. Both yeasts have fairly stable glucose, biomass, and DO profiles. At the end of the batch phase (around the 10th hour), the CO<sub>2</sub> level reaches around 4%, the feeding starts, and the CO<sub>2</sub> level quickly decreases after feeding. Upon stress application, the DO levels instantaneously increase up to 300 % for H<sub>2</sub>O<sub>2</sub> perturbation (Figure 1I) and up to 60% for DTT perturbation (Figure 1II), with the latter being attributed to the interaction of DTT with air and trace metals in the medium producing H<sub>2</sub>O<sub>2</sub> [25]. As for the microbial physiology, due to the respiro-fermentative characteristics of *S. cerevisiae*, the yield of biomass on glucose is lower than the yield of biomass on glucose of *P. pastoris* in the batch fermentations. While *P. pastoris* was reported to be Crabtree-negative in earlier publications [19], low levels of ethanol were measured in our batch experiments in the presence of excess glucose in line with the recent literature [20,21] although the levels are far

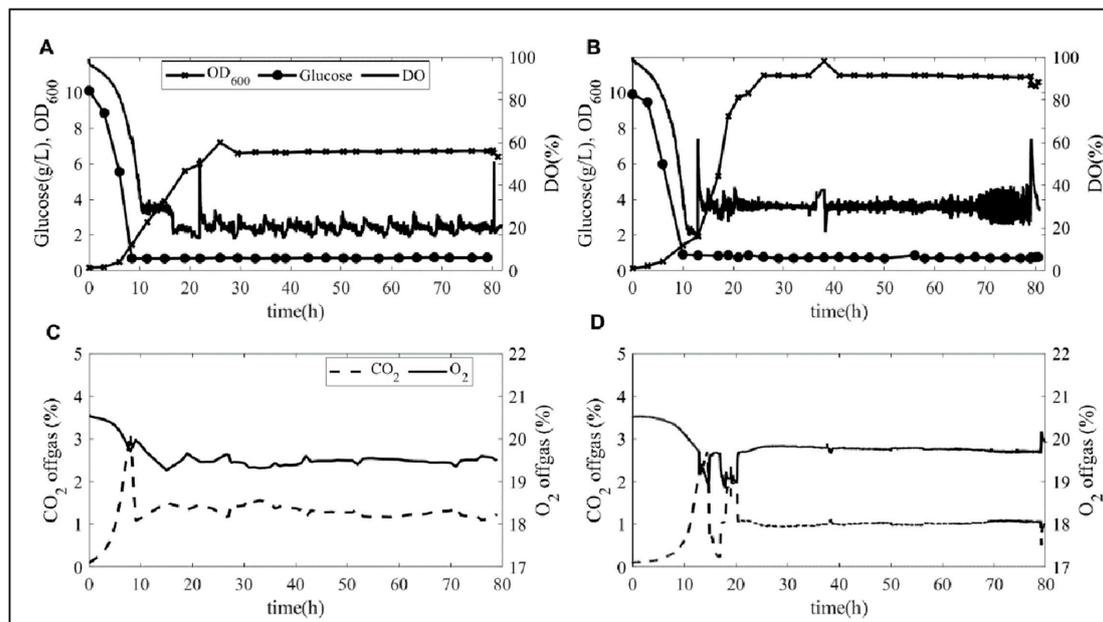
less than what was measured for *S. cerevisiae* (3–4 g/L vs. 0.5–1.5 g/L ethanol for *S. cerevisiae* and *P. pastoris*, respectively). Additionally, *S. cerevisiae* produces 0.3–0.5 g/L glycerol and acetate during the batch culture, while there is no detectable by-product formation carried out by *P. pastoris*. The biomass-specific uptake and secretion rates, as well as carbon and the degrees of reduction recoveries at a steady state, were presented in Table 1.

**Response to oxidative stress (5mM H<sub>2</sub>O<sub>2</sub>)**



(I)

**Response to reductive stress (20 mM DTT)**



(II)

**Figure 1.** (I). Glucose, biomass, and dissolved oxygen levels (DO(%)) observed for oxidative stresses (top panel (I)) and reductive (bottom panel (II)) stresses. (A) *S. cerevisiae*, (B) *P. pastoris*, off-gas measurements of (C) *S. cerevisiae*, and (D) *P. pastoris* during chemostat cultures are in each panel. The feeding started right around the CO<sub>2</sub> peak, which was around the 9th hour for *S. cerevisiae* and around the 12th hour for *P. pastoris*.

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

	Measured (Unbalanced) Specific Rates					
	$-q_s$	$q_x$	$q_{CO_2}$	$-q_{O_2}$	C-Recovery	DoR-Recovery
<i>S. cerevisiae</i>	$1.046 \pm 0.001$	$3.847 \pm 0.004$	$2.450 \pm 0.022$	$2.274 \pm 0.029$	$100 \pm 0.00$	$100 \pm 0.01$
<i>P. pastoris</i>	$0.848 \pm 0.010$	$3.713 \pm 0.019$	$1.633 \pm 0.081$	$1.438 \pm 0.167$	$104 \pm 0.01$	$104 \pm 0.04$

All the experimental data contain the average values of the biomass-specific rates and the % for recovery values of six chemostats prior to perturbation.

### 3.2. Amino Acid Profiling of *S. cerevisiae* and *P. pastoris* after Oxidative and Reductive Stress

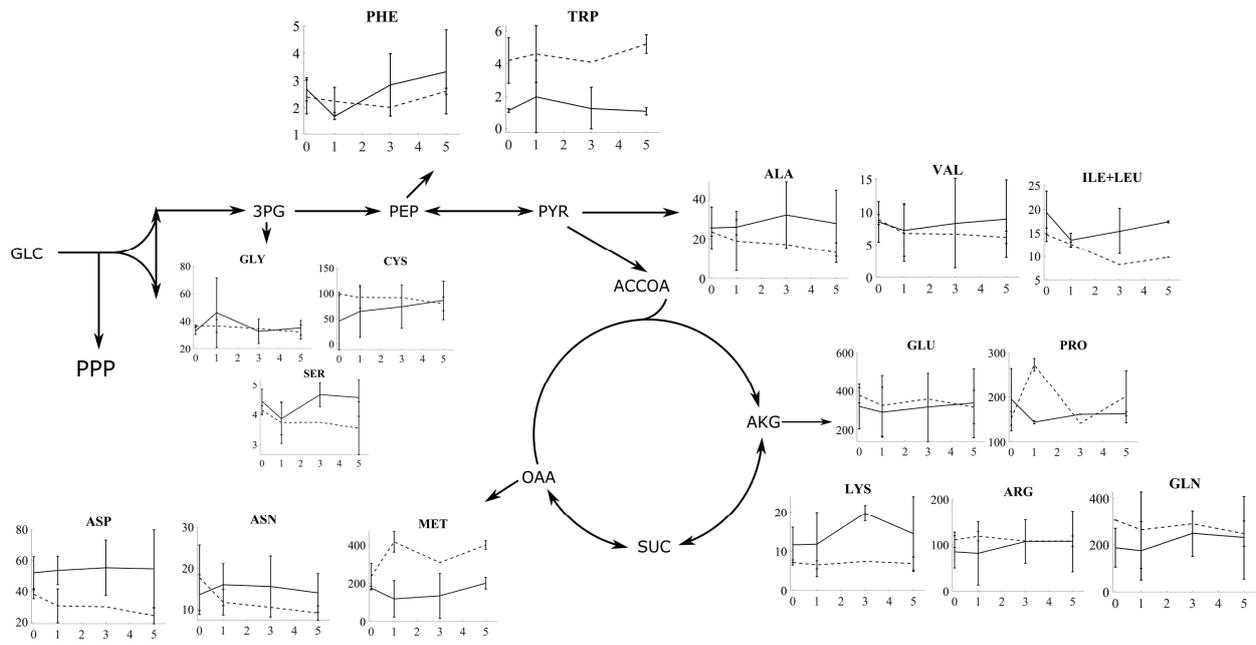
Quantitative measurement of intracellular metabolite levels and their time profiles suffer from several challenges, e.g., a high level of (accumulated) noise as these typically result from a series of elaborate procedures (quenching, extraction, quantification), the high background at the steady state resulting in minimal relative changes upon perturbation, and the choice of the time-window to observe the dynamics.

The sampling time is very important to decide since the long-term response is typically confounded with secondary effects, while the shorter term response is not enough for observing enough dynamics. Here, the intracellular concentrations of amino acids prior to and after  $\text{H}_2\text{O}_2$  and DDT perturbation were measured. To decide the time of the sampling points, the turnover times of the intracellular amino acids are considered, and these vary between 30 and 500 s under similar cultivation conditions [4]. Based on this, samples were collected until 300 s following perturbation. The effect of the 5 mM  $\text{H}_2\text{O}_2$ -induced oxidative stress as well as the 20 mM DTT-induced reductive stress on intracellular amino acid levels is presented in  $\mu\text{mol/gDW}$  in Figure 2A,B for *S. cerevisiae* and *P. pastoris*, respectively, as well as in Supplementary Tables S3 and S4.

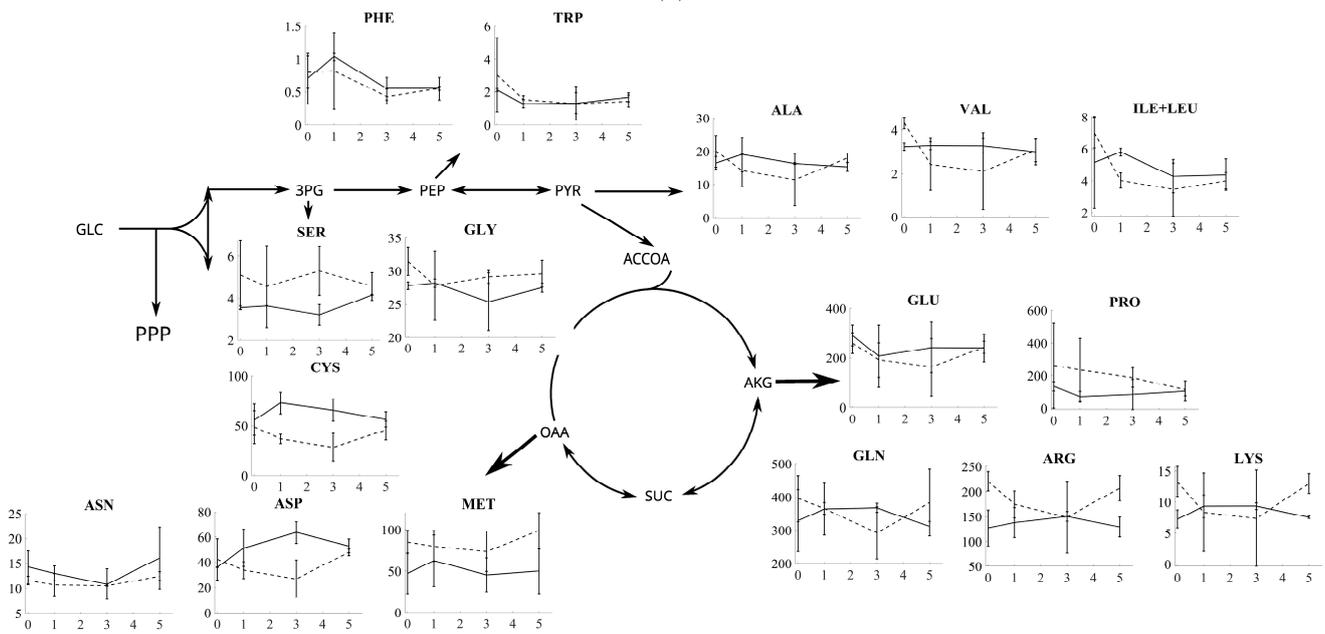
The amino acid levels at a steady state, prior to stress application, are compared to assess batch-to-batch variation and the overall quality of the data. For *S. cerevisiae*, the amino acid levels are well-stable and are measured from several independent chemostats. An exception to this is cysteine (Cys), as well as glycine (Gly) to a lesser extent, with both of them having a high level of batch-to-batch biological variation at a steady state. For *P. pastoris*, however, a handful of amino acids, i.e., cysteine (Cys), glutamate (Glu), asparagine (Asn), methionine (Met), and lysine (Lys), exhibit high batch-to-batch variation.

Overall, the levels of valine (Val), serine (Ser), and proline (Pro) followed similar decreasing profiles for the first 3 mins and increasing profiles between 3 and 5 min after oxidative stress for both yeasts. Aspartate (Asp), glutamine (Gln), methionine (Met), phenylalanine (Phe), tryptophan (Trp), and proline changed significantly after the first minute upon 5 mM  $\text{H}_2\text{O}_2$ -induced oxidative stress for *P. pastoris*. Aspartate, glutamate, phenylalanine (Phe), tryptophan, and proline levels were significantly changed after 3 and 5 min for *P. pastoris*. Asparagine, arginine (Arg), and glutamine levels were significantly changed after 1 and 3 min for *S. cerevisiae*.

As part of the response to 20 mM-DTT-induced reductive stress, tryptophane, phenylalanine, and lysine followed an increasing trend in course of 5 min as observed for *S. cerevisiae*. Conversely, arginine, glutamine, cysteine, alanine, aspartate, and glycine had a decreasing trend in the course of 5 min. Alanine, arginine, methionine, lysine, valine, glutamate, and serine showed a similar behavior. Also, proline, asparagine, and isoleucine+leucine (ile+leu) followed decreasing trends. Cysteine and proline are the only amino acids that follow a contradictory profile in the course of 5 min as observed for both stresses in *S. cerevisiae*.



(A)



(B)

**Figure 2.** (A) The amino acid response to oxidative and reductive stress for *S. cerevisiae*. The amino acids are grouped according to the metabolite in the central carbon metabolism from which they are produced. In each plot, solid lines represent the response to oxidative stress, while dashed lines represent the response to reductive stress, with the *y*-axis in each plot being the intracellular concentration in  $\mu\text{mol/gDW}$  and the *x*-axis being the time in minutes. (B) The amino acid response to oxidative and reductive stress for *P. pastoris* (bottom panel). The amino acids are grouped according to the metabolite in the central carbon metabolism from which these are produced. In each plot, solid lines represent the response to oxidative stress, while dashed lines represent the response to reductive stress, with the *y*-axis in each plot being the intracellular concentration in  $\mu\text{mol/gDW}$  and the *x*-axis being the time in minutes.

For *P. pastoris*, alanine, arginine, glutamine, tryptophan, and isoleucine+leucine followed similar trends that were more or less constant; aspartate, cysteine, glutamate, lysine,

methionine, and valine displayed similar decreasing trends after 20 mM DTT stress. Proline followed a decreasing trend in the course of 5 min after reductive stress. Asparagine also followed more stable profiles after 20 mM-DTT stress. Serine is the only amino acid that follows a profile of decreasing, increasing, and then decreasing again in response to reductive stress. Arginine, lysine, glutamine, aspartate, cysteine, and serine have opposite profiles against oxidative and reductive stress for *P. pastoris*.

Earlier work on the oxidative stress response of yeasts typically focuses on the transcriptomic response to either hydrogen peroxide [26] or cumene hydroperoxide [27], while (targeted) metabolome approaches are scarcely available [28].

Sha et al. [27] focused on the early temporal transcriptional response of *Saccharomyces cerevisiae* to oxidative stress induced by a cumene peroxide. Interesting for that study, a considerable number of transcripts responded to the stress, thereby suggesting a highly dynamic profile for the metabolome. They also focused on glutathione, and they additionally noted that the cells respond distinctively to the two peroxides applied. Additionally, the authors noted that the genes downregulated only by cumene hydroperoxide are mainly related to the mitochondrial function. This is in qualitative agreement with our results in the sense that the levels of more than half of the amino acids from the TCA cycle (Figure 2A) decrease in the first 5 min. As for *Pichia pastoris*, proline, glutamate, asparagine, and tryptophan decrease in the first minute upon stress application. Inspecting differentially expressed genes in the work of Sha et al. [27], we see that amino acid-related genes are typically linked to the transport processes (permeases, transporters, etc.) and protein kinases. In a similar work by Zhao et al. [26], where the transcriptomic response to oxidative stress is measured, aminotransferases, glutamate decarboxylases, and glutamate dehydrogenases are upregulated, while several lyases are downregulated. All in all, several signaling pathways related to amino acid biosynthesis have been differentially changed, thereby contributing to the time hierarchy of the corresponding amino acids.

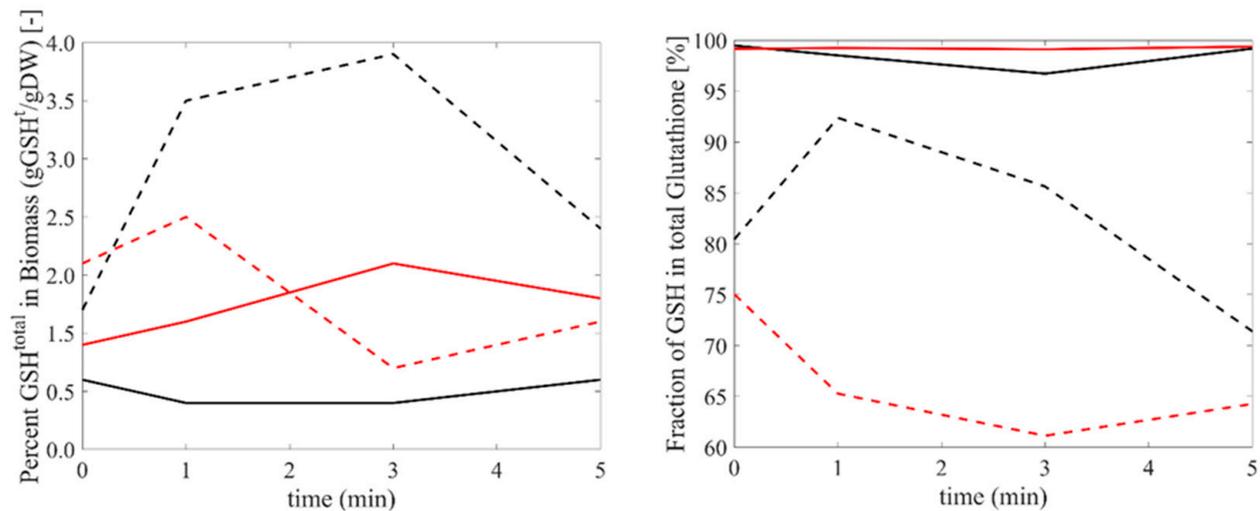
The work of Christodolou et al. [28] compares the stress response of *E. coli*, *H. sapiens*, and *S. cerevisiae* vis-à-vis their oxidative response to search for a potentially conserved mechanism related to the rapid response using metabolomic data and an ensemble kinetic modeling approach. Interestingly, common to all three species, the normally inhibited oxidative pentose phosphate pathway (PPP) gets increased pathway flux for detoxification upon stress application, and the authors name this as “reserve flux”. This concept of a reserve flux is in line with our above-discussed observations as well as the transcriptomic response measured by Sha et al. [27] of how having a pentose phosphate pathway as a reserve allows the levels of PPP-related amino acids to increase upon stress application. This is indeed what we observe for tryptophan, which does increase for *S. cerevisiae*.

Several precursors to amino acids have been quantified by Christodolou et al. [28]. Our work and their work can be seen as complementary since a number of precursor metabolites to amino acids have already been reported by them. Two of these are striking as follows: pyruvate and  $\alpha$ -ketoglutarate ( $\alpha$ -KG). For pyruvate, the levels decrease in the first two minutes before restoring back afterwards. In our work, the amino acids from pyruvate (PYR), namely valine and ile+leu, also decrease initially before restoring back after the first minute. This correlated profile points (closely) to the mass action kinetics for the corresponding biochemical reactions. Similarly, for  $\alpha$ -KG, its level is measured as decreasing immediately after stress, as reported by Christodolou et al. [28], and the amino acids of glutamate and proline also follow a similar trend, thereby indicating clearly linear kinetics for these synthesis reactions. Lastly, 3-phosphoglycerate (3-PG) only correlates with serine among 3-PG-derived amino acids.

### 3.3. Glutathione Response to Oxidative and Reductive Stress

The intracellular levels of glutathione (GSH, GSSG, and total) are additionally measured upon oxidative and reductive perturbation for both yeasts (Figure 3). Although the reduced form of glutathione (GSH) is dominant in both yeasts, the reducing power (the ratio of GSH/GSSG) is far higher in *Pichia pastoris* (with the ratio being around 5–20 for

*S. cerevisiae* and above 100 for *P. pastoris*), which might (partially) explain why *P. pastoris* is more resilient to oxidative stress. Interestingly, both the levels of total glutathione and the distribution among the reduced and oxidized forms change significantly in *S. cerevisiae*, with these changes being limited in *P. pastoris*. Furthermore, total glutathione response for reductive stress is almost a mirror image of the response to oxidative stress in both yeasts. The total amount of GSH first increases and then decreases for oxidative stress, but is otherwise for reductive stress, thereby indicating a distinct response of the two yeasts.



**Figure 3.** The change in the biomass of total glutathione (**left panel**) as well as the fraction of the reduced form (GSH) (**right panel**) as a function of reductive (red) and oxidative (black) stresses for both yeasts (*S. cerevisiae*, dashed lines; *P. pastoris*, solid lines).

Lastly, when taken together, the time profiles of glutathione and the precursor amino acid profiles allows us to speculate on the potential bottlenecks in glutathione production in both yeasts. We speculate that the bottlenecks are different among the two as follows: both cysteine and glycine may be the bottleneck for *S. cerevisiae*, while a supply of glycine seems to be the bottleneck for *P. pastoris*, with these observations being based on the time profiles of these amino acids and GSH. This bottleneck is deduced by noting that the glutathione time profile is correlated with the time profiles of cysteine and glycine for *S. cerevisiae*. For *P. pastoris* glutathione, the time profile correlates with the glycine and glutamate time profiles. These potential targets for improved GSH production can easily be pursued, e.g., by co-feeding with corresponding amino acid(s) along with the oxidative stress as an example of a push strategy. There are various studies on supplementation, especially that of the precursor amino acids of glutathione in yeast culture media [29–31]. Wen et al. [32] showed that cysteine is the major limiting compound for glutathione biosynthesis. Also, a study by Wang et al. [29] and Liang et al. [33] supported how with the addition of cysteine into the culture media, the intracellular GSH concentration significantly increased. In a similar attempt, Christodolou et al. [28] measured GSH levels. Reduced GSH decreases in the first 40 s, increases until the second minute, and decreases afterwards. GSH is measured in our experiments as increasing until the first minute and then stably decreasing afterwards. The profiles are in semi-quantitative agreement after two minutes, but a seeming difference in the first two minutes points to a highly dynamic response upon stress application. It should be noted that the metabolic response to oxidative stress is not limited to glutathione but that other enzymatic mechanisms may also play a role (catalase, SODs, etc.). It is worth discussing that, as also mentioned by Zhao et al. [26], other non-enzymatic, metabolite antioxidants such as ascorbate, tocopherol, and uric acid may play roles in scavenging reactive oxygen species, which is omitted in this work as GSH is known to be dominant in both yeasts.

However, within the short time frame, we assumed that the levels of these enzymes would not substantially change. Further investigation might also include the activities of these enzymes at a steady state, even if they are constant within the time frame. Further extensions of this work may be on the untargeted quantification of entire metabolomics as well as on multi-omic approaches.

#### 4. Conclusions

To further our understanding of the mechanism to cope with oxidative and reductive stress in yeast cells, this study provides a new dataset consisting of fermentation data and intracellular amino acid and intracellular glutathione levels for two different yeasts (*S. cerevisiae* and *P. pastoris*) at 5 mM H<sub>2</sub>O<sub>2</sub>-induced oxidative stress and 20 mM DTT-induced reductive stress. The amino acids range from 0.5 to 400 µmol/gDW. Upon oxidative stress, cysteine significantly increased (up to 90%) in *S. cerevisiae* and aspartate significantly increased (up to 80%) in *P. pastoris*. *S. cerevisiae* loses approximately half of its asparagine under reductive stress, while *P. pastoris* loses 60% of its tryptophan. Overall, it is extremely hard, if ever possible, to isolate the effect of both stresses in a single-omic layer or a single pathway, and when also considering the metabolic reaction networks, it affects the NAD/NADH balance and thereby numerous redox reactions. The data presented in this work point to the kinetics of amino acid production pathways as well as to potential bottlenecks in glutathione production pathways. Departing from the results of earlier studies discussed by presenting additional metabolome data under comparable conditions, the results presented here are still far from depicting the entire multi-omic landscape. Rather, this work constitutes a stepping stone for further quantitative study on metabolomics with a systems biology approach.

**Supplementary Materials:** The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/fermentation10030166/s1>. A supplementary spreadsheet file is provided as a supplementary file. Table S1: Ingredients of Vitamin solution and Trace elements for Verduyn Medium; Table S2: The amino acid standards used as an external calibration for HPLC; Table S3: The effect of 5 mM H<sub>2</sub>O<sub>2</sub>-induced oxidative stress as well as 20 mM DTT-induced reductive stress on intracellular amino acid levels is presented in µmol/gDW for *S. cerevisiae*; Table S4: The effect of 5 mM H<sub>2</sub>O<sub>2</sub>-induced oxidative stress as well as 20 mM DTT-induced reductive stress on intracellular amino acid levels is presented in µmol/gDW *P. pastoris*.

**Author Contributions:** Conceptualization, B.Ş.K. and E.N.; methodology, B.Ş.K.; formal analysis, B.Ş.K. and E.N.; investigation, B.Ş.K.; writing—review and editing, B.Ş.K. and E.N.; supervision, E.N.; project administration, B.Ş.K.; funding acquisition, E.N. All authors have read and agreed to the published version of the manuscript.

**Funding:** This research was funded by Ministry of Agriculture and Forestry, grant number TAGEM-18/AR-GE/26.

**Institutional Review Board Statement:** Not applicable.

**Informed Consent Statement:** Not applicable.

**Data Availability Statement:** All data used in this study are shared in the main article.

**Acknowledgments:** The financial support for Burcu Şirin through the TÜBİTAK 2211C Program (1649B03) is gratefully acknowledged.

**Conflicts of Interest:** The authors declare no conflicts of interest.

#### References

1. Mashego, M.R.; van Gulik, W.M.; Vinke, J.L.; Visser, D.; Heijnen, J.J. In Vivo Kinetics with Rapid Perturbation Experiments in *Saccharomyces Cerevisiae* Using a Second-Generation BioScope. *Metab. Eng.* **2006**, *8*, 370–383. [CrossRef] [PubMed]
2. Canelas, A.B.; Ten Pierick, A.; Ras, C.; Seifar, R.M.; Van Dam, J.C.; Van Gulik, W.M.; Heijnen, J.J. Quantitative Evaluation of Intracellular Metabolite Extraction Techniques for Yeast Metabolomics. *Anal. Chem.* **2009**, *81*, 7379–7389. [CrossRef] [PubMed]
3. Nikerel, I.E.; Canelas, A.B.; Jol, S.J.; Verheijen, P.J.T.; Heijnen, J.J. Construction of Kinetic Models for Metabolic Reaction Networks: Lessons Learned in Analysing Short-Term Stimulus Response Data. *Math. Comput. Model. Dyn. Syst.* **2011**, *17*, 243–260. [CrossRef]

4. Nikerel, I.E.; Verheijen, P.J.T.; van Gulik, W.M.; Heijnen, J.J. Model-Based Design of Superior Cell Factory: An Illustrative Example of *Penicillium Chrysogenum*. In *Systems Metabolic Engineering*; Springer: Dordrecht, The Netherlands, 2012; pp. 221–270.
5. Nikerel, I.E. Managing Complexity of Cellular Systems: Theoretical Tools for Dynamic Modeling of Metabolic Reaction Networks. Ph.D. Thesis, Delft University of Technology, Delft, The Netherlands, 2009; 231p.
6. Kresnowati, M.T.A.P.; Suarez-Mendez, C.M.; van Winden, W.A.; van Gulik, W.M.; Heijnen, J.J. Quantitative Physiological Study of the Fast Dynamics in the Intracellular pH of *Saccharomyces Cerevisiae* in Response to Glucose and Ethanol Pulses. *Metab. Eng.* **2008**, *10*, 39–54. [[CrossRef](#)] [[PubMed](#)]
7. Baumann, K.; Carnicer, M.; Dragosits, M.; Graf, A.B.; Stadlmann, J.; Jouhten, P.; Maaheimo, H.; Gasser, B.; Albiol, J.; Mattanovich, D.; et al. A Multi-Level Study of Recombinant *Pichia Pastoris* in Different Oxygen Conditions. *BMC Syst. Biol.* **2010**, *4*, 141. [[CrossRef](#)]
8. Diezmann, S. Oxidative Stress Response and Adaptation to H<sub>2</sub>O<sub>2</sub> in the Model Eukaryote *Saccharomyces Cerevisiae* and Its Human Pathogenic Relatives *Candida Albicans* and *Candida Glabrata*. *Fungal Biol. Rev.* **2014**, *28*, 126–136. [[CrossRef](#)]
9. Korge, P.; Calmettes, G.; Weiss, J.N. Increased Reactive Oxygen Species Production during Reductive Stress: The Roles of Mitochondrial Glutathione and Thioredoxin Reductases. *Biochim. Biophys. Acta Bioenerg.* **2015**, *1847*, 514–525. [[CrossRef](#)]
10. Gasch, A.; Spellman, P. Genomic Expression Programs in the Response of Yeast Cells to Environmental Changes. *Mol. Biol. Cell* **2000**, *11*, 4241–4257. [[CrossRef](#)]
11. Kurylenko, O.O.; Dmytruk, K.V.; Sibirny, A. *Glutathione Metabolism in Yeasts and Construction of the Advanced Producers of This Tripeptide*; Springer: Cham, Switzerland, 2019; ISBN 9783030211103.
12. Chen, Y.; Yang, X.; Zhang, S.; Wang, X.; Guo, C.; Guo, X.; Xiao, D. Development of *Saccharomyces Cerevisiae* Producing Higher Levels of Sulfur Dioxide and Glutathione to Improve Beer Flavor Stability. *Appl. Biochem. Biotechnol.* **2012**, *166*, 402–413. [[CrossRef](#)]
13. Schmachl, M.; Lorenz, E.; Senz, M. Microbial Production of Glutathione. *World J. Microbiol. Biotechnol.* **2017**, *33*, 106. [[CrossRef](#)] [[PubMed](#)]
14. Lin, N.X.; He, R.Z.; Xu, Y.; Yu, X.W. Oxidative Stress Tolerance Contributes to Heterologous Protein Production in *Pichia Pastoris*. *Biotechnol. Biofuels* **2021**, *14*, 160. [[CrossRef](#)] [[PubMed](#)]
15. Pan, Y.; Cheng, J.H.; Sun, D.W. Metabolomic Analyses on Microbial Primary and Secondary Oxidative Stress Responses. *Compr. Rev. Food Sci. Food Saf.* **2021**, *20*, 5675–5697. [[CrossRef](#)] [[PubMed](#)]
16. Verduyn, C.; Postma, E.; Scheffers, W.A.; Van Dijken, J.P. Effect of Benzoic Acid on Metabolic Fluxes in Yeasts: A Continuous-culture Study on the Regulation of Respiration and Alcoholic Fermentation. *Yeast* **1992**, *8*, 501–517. [[CrossRef](#)] [[PubMed](#)]
17. Lange, H.C.; Heijnen, J.J. Statistical Reconciliation of the Elemental and Molecular Biomass Composition of *Saccharomyces Cerevisiae*. *Biotechnol. Bioeng.* **2001**, *75*, 334–344. [[CrossRef](#)] [[PubMed](#)]
18. Lange, H.C.; Eman, M.; Van Zuijlen, G.; Visser, D.; Van Dam, J.C.; Frank, J.; De Teixeira Mattos, M.J.; Heijnen, J.J. Improved Rapid Sampling for in Vivo Kinetics of Intracellular Metabolites in *Saccharomyces Cerevisiae*. *Biotechnol. Bioeng.* **2001**, *75*, 406–415. [[CrossRef](#)] [[PubMed](#)]
19. Miller, G.L. Use of Dinitrosalicylic Acid Reagent for Determination of Reducing Sugar. *Anal. Chem.* **1959**, *31*, 426–428. [[CrossRef](#)]
20. Pudlik, A.M.; Lolkema, J.S. Rerouting Citrate Metabolism in *Lactococcus Lactis* to Citrate-Driven Transamination. *Appl. Environ. Microbiol.* **2012**, *78*, 6665–6673. [[CrossRef](#)]
21. Rollini, M.; Manzoni, M. Influence of Different Fermentation Parameters on Glutathione Volumetric Productivity by *Saccharomyces Cerevisiae*. *Process Biochem.* **2006**, *41*, 1501–1505. [[CrossRef](#)]
22. Carnicer, M.; Ten Pierick, A.; Van Dam, J.; Heijnen, J.J.; Albiol, J.; Van Gulik, W.; Ferrer, P. Quantitative Metabolomics Analysis of Amino Acid Metabolism in Recombinant *Pichia Pastoris* under Different Oxygen Availability Conditions. *Microb. Cell Fact.* **2012**, *11*, 83. [[CrossRef](#)]
23. van der Hijden, R.T.J.M.; Heijnen, J.J.; Hellinga, C.; Romein, B.; Luyben, K.C.A.M. Linear Constrain Relations in Biochemical Reaction Systems: I. Classification of the Calculability and the Balanceability of Conversion Rates. *Biotechnol. Bioeng.* **1994**, *43*, 3–10. [[CrossRef](#)]
24. Trotter, E.W.; Grant, C.M. Thioredoxins Are Required for Protection against a Reductive Stress in the Yeast *Saccharomyces Cerevisiae*. *Mol. Microbiol.* **2002**, *46*, 869–878. [[CrossRef](#)] [[PubMed](#)]
25. Netto, L.E.S.; Stadtman, E.R. The Iron-Catalyzed Oxidation of Dithiothreitol Is a Biphasic Process: Hydrogen Peroxide Is Involved in the Initiation of a Free Radical Chain of Reactions. *Arch. Biochem. Biophys.* **1996**, *333*, 233–242. [[CrossRef](#)] [[PubMed](#)]
26. Zhao, H.; Chen, J.; Liu, J.; Han, B. Transcriptome Analysis Reveals the Oxidative Stress Response in *Saccharomyces Cerevisiae*. *RSC Adv.* **2015**, *5*, 22923–22934. [[CrossRef](#)]
27. Sha, W.; Martins, A.M.; Laubenbacher, R.; Mendes, P.; Shulaev, V. The Genome-Wide Early Temporal Response of *Saccharomyces Cerevisiae* to Oxidative Stress Induced by Cumene Hydroperoxide. *PLoS ONE* **2013**, *8*, e74939. [[CrossRef](#)]
28. Christodoulou, D.; Kuehne, A.; Estermann, A.; Fuhrer, T.; Lang, P.; Sauer, U. Reserve Flux Capacity in the Pentose Phosphate Pathway by NADPH Binding Is Conserved across Kingdoms. *iScience* **2019**, *19*, 1133–1144. [[CrossRef](#)]
29. Wang, B.; Liang, G.; Zhou, Q.; Xie, J.; Mo, Y. Combined Amino Acids Modulation with H<sub>2</sub>O<sub>2</sub> Stress for Glutathione Overproduction in *Candida Utilis*. *Afr. J. Biotechnol.* **2010**, *9*, 5399–5406.
30. Wei, G.Y.; Wang, D.H.; Chen, J. Overproduction of Glutathione by L-Cysteine Addition and a Temperature-Shift Strategy. *Biotechnol. Bioprocess. Eng.* **2008**, *13*, 347–353. [[CrossRef](#)]

31. Wang, Z.; Tan, T.; Song, J. Effect of Amino Acids Addition and Feedback Control Strategies on the High-Cell-Density Cultivation of *Saccharomyces Cerevisiae* for Glutathione Production. *Process Biochem.* **2007**, *42*, 108–111. [[CrossRef](#)]
32. Wen, S.; Zhang, T.; Tan, T. Maximizing Production of Glutathione by Amino Acid Modulation and High-Cell-Density Fed-Batch Culture of *Saccharomyces Cerevisiae*. *Process Biochem.* **2006**, *41*, 2424–2428. [[CrossRef](#)]
33. Liang, G.B.; Du, G.C.; Chen, J. A Novel Strategy of Enhanced Glutathione Production in High Cell Density Cultivation of *Candida Utilis*-Cysteine Addition Combined with Dissolved Oxygen Controlling. *Enzym. Microb. Technol.* **2008**, *42*, 284–289. [[CrossRef](#)]

**Disclaimer/Publisher’s Note:** The statements, opinions and data contained in all publications are solely those of the individual author(s) and contributor(s) and not of MDPI and/or the editor(s). MDPI and/or the editor(s) disclaim responsibility for any injury to people or property resulting from any ideas, methods, instructions or products referred to in the content.