Oxidative Stress in *Saccharomyces cerevisiae*

by

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COLLEGE OF AGRICULTURE, ENGINEERING AND SCIENCE

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I, Dr Patrick Govender as supervisor of the MSc study hereby consent to the submission of this MSc Thesis.

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SUMMARY

All aerobic organisms produces reactive oxygen species (ROS) via aerobic respiration such as superoxide anion radical ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($HO^{\bullet-}$) causing damage to cellular components such as DNA, protein and lipids. In humans, ROS plays a deleterious role as it causes cardiovascular disorders, neurodegenerative disorders, plays a role in diabetes and the aging process. Under normal physiological conditions, ROS is maintained at a steady state level in the cell via antioxidant defence mechanisms. Oxidative stress is caused through an accumulation of ROS within a cell.

Metabolism and aerobic respiration are the main contributors to oxidative stress in yeast. This finding has led to the development of the oxidative stress theory of aging which states that ROS that forms through normal metabolism causes damage to organelles and the accumulation of these ROS in the cell plays a pivotal role in aging and even organism death. Studies using functional genomics and computational modelling described a shift of the metabolic flux from glycolysis into the pentose phosphate pathway (PPP) under oxidative stress conditions. While most research has focused on the effects of oxidative stresses on cell viability, surprisingly very little work has been done on how changes in the supply of electrons affect the cell viability and the antioxidant capacity of the cell as well as the pathway the cell chooses to respond to stress. The data tentatively suggests that *S. cerevisiae* metabolism in the absence of G6PDH shifts to a salvage pathway that seems to more efficiently counteract the effects of otherwise deleterious effects of oxidative stress induced by H_2O_2.

Oxidative stress and the cells response via antioxidant defence mechanisms is important as this may shed light on various human associated diseases since *S. cerevisiae*, contains genes that are homologous to human genes. Current studies have used the stress spot assay as a visual representation to determine the effects of oxidative stress, however this assay does not provide great sensitivity and is a tedious procedure. Other quantitative assays that could be used as an alternative to the stress spot assay needed to be investigated. The data seemingly suggests that the commercially available ATP assay is a viable alternative for more concise or quantitative determination of the effect of an oxidative stress agent in *S. cerevisiae*.

This thesis is dedicated to
my daughter Kitana Inderpal, whom has motivated me to be the best I can be.

BIOGRAPHICAL SKETCH

Melissa Inderpal was born on the 4th of November 1987 and brought up in the small suburb of Shallcross. She matriculated in 2004 and achieved a distinction with merit and decided to further her studies in Science at the University of KwaZulu-Natal. She obtained her Bachelor of Science degree majoring in Genetics and Microbiology and her Bachelor of Science Honors degree in the field of Genetics. Melissa has a very intriguing mind and a curious nature hence her interest in research. Melissa enjoys a myriad of activities such as swimming, reading, dancing and listening to music.

ACKNOWLEDGEMENTS

I wish to express my sincere gratitude and appreciation to the following people and institutions:

- To **God**, for giving me the perseverance but most of all the strength to get through this degree and for enabling a positive attitude in all my endeavors as well as giving me a beautiful baby girl, **Kitana Inderpal** to keep me motivated and to see this degree through to completion.

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PREFACE

This dissertation is presented as a compilation of five chapters.

Chapter 1 **General Introduction and Project Aims**

Chapter 2 **Literature Review**

Chapter 3 **Research Results I**

The potential of cell viability assays as a rapid and more accurate measure of oxidative stress on *Saccharomyces cerevisiae*

Chapter 4 **Research Results II**

Oxidative stress in *Saccharomyces cerevisiae*

Chapter 5 **General Discussion and Conclusion**

CONTENTS

ABBREVIATIONS

ATM	Ataxia telangiectasia mutated
ATP	Adenosine triphosphate
CAT	Catalase
CR	Caloric restriction
G6PDH	Glucose-6-phosphate dehydrogenase
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GLR1	Glutathione reductase
GSH	Glutathione
GSSG	Glutathione disulfide
H_2O_2	Hydrogen peroxide
Hxp	Hexose transporters
NAD	Nicotinamide adenine dinucleotide
$NADP^+$	Nicotinamide adenine dinucleotide phosphate reduced form
NADPH	Nicotinamide adenine dinucleotide phosphate
$O_2^{\bullet-}$	Superoxide radical
$OH^{\bullet-}$	Hydroxyl radical
PPP	Pentose phosphate pathway
ROS	Reactive Oxygen Species
SOD	Superoxide dismutase
TCA	Tricarboxylic acid cycle
TPI	Triosephosphate isomerase
TRR	Thioredoxin reductase
Trx	Thioredoxin

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CHAPTER 1

Introduction and Project Aims

1.1 Introduction

Metabolism and aerobic respiration are contributors to oxidative stress in yeast. Molecular oxygen can be converted into ROS such as superoxides ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2) and hydroperoxides (ROOH) which can lead to DNA damage (mutations), lipid peroxidation and protein oxidation [1-3]. Although the cell has built-in antioxidant defence mechanisms to counteract the damaging effects of oxidants, in many instances these defences have been shown to be insufficient or impaired. This discovery has led to the development of the oxidative stress theory of aging which states that ROS that forms through normal metabolism causes damage to organelles and the accumulation of these ROS in the cell plays a pivotal role in aging and organism death [4]. In human-based research studies, oxidative stress has been shown to contribute to many pathologies such as cancer, cardiovascular disease, Down's syndrome, aging and age related diseases [3, 5, 6].

Studies using functional genomics and computational modelling inactivation of key enzymes in the glycolytic and related metabolic pathways is responsible for the rerouting of the carbohydrate flux from glycolysis into the pentose phosphate pathway (PPP) which increases the steady state NADPH level [7]. This is crucial as NADPH provide reducing power to the antioxidant defence network [8]. While most research has focused on the effects of oxidative stresses on cell viability, surprisingly very little work has been done on how changes in the supply of electrons affect the cell viability and the antioxidant capacity of the cell as well as the pathway the cell chooses to respond to stress.

The negative effects of oxidative stress warrants research in terms of developing or identifying quantitative cell viability assays for its analysis. To date, the spot assay is a widely used qualitative cellular viability method to determine the effects of oxidative stress [9, 10]. To quantitatively assess oxidative stress in yeast, a high throughput and reliable quantitative assay is much required as a research tool in this challenging and interesting research niche. In an attempt to source a suitable cellular viability assay, many factors need to be taken into consideration robustness, duration till completion, sensitivity and cost of the method.

1.2 Aims of this study

The main aim of the MSc study described within this dissertation is to evaluate the most suited cell viability assay to determine oxidative stress effects in *S. cerevisiae,* thereafter determining a relationship between glucose metabolism and oxidative stress response in *S. cerevisiae* as well as to ascertain a correlation between electron demand and antioxidant capacity of the cell with the best suited method identified. The dissertation is divided into five chapters, including this introductory **Chapter 1**.

In **Chapter 2**, a comprehensive literature review encompassing glucose transport and yeast metabolism focusing on its role in oxidative stress and antioxidant defence capacity of the cell. Furthermore it also incorporates a current view of cell viability assays used in oxidative stress determination studies.

In **Chapter 3**, evaluating two in vitro commercially available viability assays where compared to the commonly used qualitative spot assay to determine their ability to detect early cytotoxic events, establish the accuracy of these assays and to test the robustness of these methods using three genetically different yeast species under the effects of hydrogen peroxide (H_2O_2)

In **Chapter 4**, determining the relationship between glucose metabolism and oxidative stress response in *S. cerevisiae*, to ascertain a correlation between electron demand and antioxidant capacity of the cell.

Finally, **Chapter 5** reflects a general conclusion and ideas for future research.

1.3 References

1. Carmel-Harel O, Storz G: **Roles of the glutathione-and thioredoxin-dependent reduction systems in the Escherichia coli and Saccharomyces cerevisiae responses to oxidative stress**. *Annual Reviews in Microbiology* 2000, **54**(1):439-461.
2. Jones DP: **Radical-free biology of oxidative stress**. *American Journal of Physiology-Cell Physiology* 2008, **295**(4):849-868.
3. Thorpe GW, Fong CS, Alic N, Higgins VJ, Dawes IW: **Cells have distinct mechanisms to maintain protection against different reactive oxygen species: oxidative-stress-response genes**. *Proceedings of the National Academy of Sciences of the United States of America* 2004, **101**(17):6564-6569.

4. Harman D: **The biologic clock: the mitochondria?** *Journal of the American Geriatrics Society* 1972, **20**(4):145-147.
5. Costa V, Moradas-Ferreira P: **Oxidative stress and signal transduction in Saccharomyces cerevisiae: insights into ageing, apoptosis and diseases**. *Molecular aspects of medicine* 2001, **22**(4-5):217-246.
6. Busciglio J, Yankner BA: **Apoptosis and increased generation of reactive oxygen species in Down's syndrome neurons in vitro**. *Nature* 1995, **378**(6559):776-779.
7. Ralser M, Wamelink MM, Kowald A, Gerisch B, Heeren G, Struys EA, Klipp E, Jakobs C, Breitenbach M, Lehrach H: **Dynamic rerouting of the carbohydrate flux is key to counteracting oxidative stress**. *J Biol* 2007, **6**(10):1-18.
8. Tkach JM, Yimit A, Lee AY, Riffle M, Costanzo M, Jaschob D, Hendry JA, Ou J, Moffat J, Boone C: **Dissecting DNA damage response pathways by analysing protein localization and abundance changes during DNA replication stress**. *Nature cell biology* 2012, **14**(9):966-976.
9. Fomenko DE, Koc A, Agisheva N, Jacobsen M, Kaya A, Malinouski M, Rutherford JC, Siu K-L, Jin D-Y, Winge DR: **Thiol peroxidases mediate specific genome-wide regulation of gene expression in response to hydrogen peroxide**. *Proceedings of the National Academy of Sciences* 2011, **108**(7):2729-2734.
10. Paul S, Doering TL, Moye-Rowley WS: ***Cryptococcus neoformans* Yap1 is required for normal fluconazole and oxidative stress resistance**. *Fungal Genetics and Biology* 2015, **74**:1-9.

CHAPTER 2

Literature Review

2.1. Introduction

Yeast such as *Saccharomyces cerevisiae* are used extensively for the study of cell biology based on powerful genetic tools allowing for disruption, manipulation and analysis of genes. These tools can also be used in the improvement of analytical techniques encouraging our understanding of transcriptomic and proteomic studies [1-3]. A number of yeast proteins have been shown to be homologous to human proteins [4]. It's not surprising that these unicellular eukaryotic organisms have been used in an array of studies as a model for cytotoxicity and oxidative stress studies to analyse biochemical processes that may occur in humans.

Oxidative stress is defined as an imbalance of the redox homeostasis in the cell resulting in macromolecular damage and redox signalling disruption which is caused through the accumulation of reactive oxygen species (ROS) within the cell [5]. During aerobic respiration in yeast, molecular oxygen can be converted into ROS such as superoxides ($O_2^{\bullet-}$), hydroxyl radicals ($OH^{\bullet-}$), hydrogen peroxide (H_2O_2) and hydroperoxides (ROOH) which may lead to DNA damage (mutations), lipid peroxidation and protein oxidation [5-7]. Metabolism and aerobic respiration are the main contributors to oxidative stress in yeast. This discovery has led to the development of the oxidative stress theory of aging which states that ROS that forms through normal metabolism causes damage to organelles and the accumulation of these ROS in the cell contributes to aging and organism death [8]. In human research, oxidative stress has led to many diseases such as cancer, cardiovascular disease, neurodegenerative diseases and aging [7, 9, 10]. To counter the damaging effects of oxidants, cells have evolved antioxidant defence mechanisms.

There are two types of defence mechanisms that have been reported in yeast against oxidative stress and can be categorised as NADPH-independent enzymatic and NADPH-dependent non-enzymatic defence mechanisms [11]. The tripeptide glutathione (GSH), and the small protein thioredoxin (Trx) couple is considered NADPH-dependent defence mechanism [12] whereas the enzymatic NADPH-independent defence mechanisms

include enzymes such as superoxide dismutase (SOD), catalase (CAT) and peroxidases [7, 11, 13]. In recent years, there have been growing interest in the redirection of the glycolytic flux from glycolysis into the pentose phosphate pathway (PPP), also known as hexose monophosphate shunt, in response to oxidative stress [14]. Studies have shown that inactivation of key glycolytic enzymes redirects the metabolic flux into the pentose phosphate pathways to counteract oxidative stress resulting in the alteration of the homeostasis of cytoplasmic metabolites. Soluble sugars (carbohydrates) such as sucrose, fructose more importantly glucose, under oxidative stress, acts as nutrient and signalling molecules activating transduction pathways resulting in modified gene expression [15]. The metabolism of these sugars in yeast plays a central role in structure and functionality of the cell. Many studies using plant species have reported a direct link between carbohydrate metabolism and ROS production rates and regulation [15], however, very few studies have studied the regulation of carbohydrate metabolism and its effects on the antioxidant capacity of cell.

This literature review aims to provide an in-depth and up-to-date overview on yeast metabolism, oxidative stress and the defence mechanisms associated with the pentose phosphate pathway.

2.2. Yeast metabolism

Yeast metabolism refers to the anabolic and catabolic process of nutrients within a cell. Energy generation usually use $NADP^+$ and NAD^+ as co-factors like most other aerobic microorganisms, this occurs from the breakdown of organic compounds (sugars) usually in the form of ATP. The metabolic pathways are identical among various yeast species [16]. Nevertheless, the variation between species lies between the mechanisms for nutrient uptake and regulation of fermentation defining yeast as a highly diverse and complex metabolic species. Yeast species possess a broad range of carbon sources such as glucose, fructose and galactose. The metabolic system employed for the metabolism of hexose include glycolysis, tricarboxylic acid cycle (TCA) and the pentose phosphate pathway (PPP).

2.2.1. Glucose transport and energy metabolism

Glycolysis involves ten steps and is the main pathway for the metabolism of various sugars such as glucose (Figure 2.1). An essential step in metabolism of glucose is the transportation across the plasma membrane into the cell [17]. Glucose is transported by hexose transporter proteins by the energy independent symport system across the membrane [17]. There have been 20 hexose transport-related proteins identified (Hx1p-Hx17p, Gal2p, Rgt2p and Snf3p) and the type of hexose transport protein used depends on the concentration of sugar in the surrounding environment [17]. Once glucose has entered the cell it becomes phosphorylated by kinases to produce glucose-6-phosphate (G6P). G6P is then isomerized by phosphoglucose isomerase (PGI) to fructose 6-phosphate (F6P) which undergoes further enzymatic activity by phosphofructokinase (PFK) to produce fructose 1,6-biphosphate (FBP). Thereafter further enzymatic activity results in the formation of pyruvate. Pyruvate can then be further metabolised to form alcohol by anaerobic fermentation or CO_2 through aerobic respiration known as the Crabtree effect [16, 18].

Following glycolysis, mitochondrial respiration decarboxylates pyruvate to Acetyl-CoA and CO_2 [19]. Acetyl-CoA is then transported to the mitochondria where it is converted into various intermediates for biosynthetic processing and leads to the release of energy by the oxidation of the acetyl-group [19]. One molecule of Acetyl-CoA releases one molecule of ATP, however, the most amount of energy released occurs by the reduction of NADH and $FADH_2$ in the TCA cycle where one molecule of glucose is converted to 24 molecules of ATP [19]. NADPH is a key component of the pentose phosphate pathway (discussed below) and research has shown that regulation of the carbon flux is done here [16].

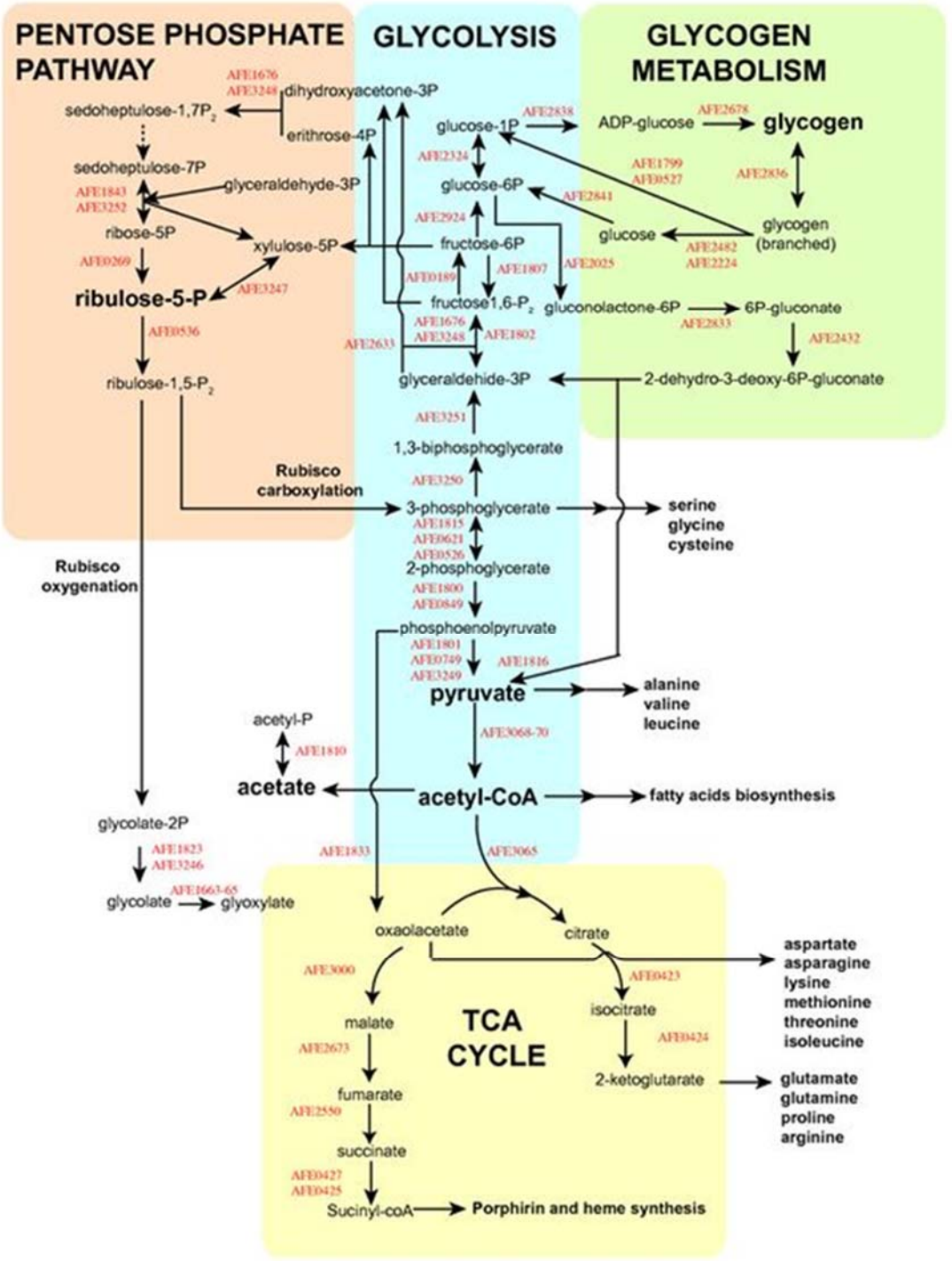

Figure 2.1. Simplified schematic diagram of glycolysis, pentose phosphate pathway (PPP), citric acid (TCA) cycle (adapted from [20]).

2.2.2. Sugar metabolism

The two main environmental conditions influencing metabolic physiology in yeast is sugar composition within the media as well as oxygen availability [16]. The concentration and type of these sugars and oxygen availability has a great impact on whether the yeast cell will practice a fully respiratory or fermentative metabolism, occasionally both are mixed in respiratory-fermentative metabolism [16]. The external glucose levels control the switch between respiration and fermentation. Important reported effects occurring in *S. cerevisiae* were used to describe the energy-generating pathways involved in sugar metabolism in the presence or absence of oxygen described by diauxic shift, Pasteur effect, Crabtree effect, Warburg effect and caloric restriction [16].

2.2.2.1.Diauxic shift

S. cerevisiae naturally lives in glucose-rich environments, but if glucose levels decrease under aerobic conditions there is a switch from glucose metabolism to ethanol metabolism which is known as diauxic shift. It is also known to be correlated to changes in gene expression which is involved in cellular processes inducing carbon metabolism, protein synthesis or carbohydrate storage [21]. Responses of many genes have been described during the diauxic shift which may provide clues to the function of uncharacterized genes [21].

2.2.2.2. Pasteur effect

The Pasteur effect was first observed by Louis Pasteur and is a phenomenon whereby the presence of oxygen regulates glucose metabolism. Under anaerobic conditions, glycolysis is activated to produce less ATP, however, under aerobic conditions glucose metabolism decreases producing greater amounts of ATP, this is referred to as the Pasteur effect [22]. Glycolysis slows under aerobic conditions due to oxidized cytochrome inactivating 6-phosphofructokinase (6PFK) the third enzyme in the glycolytic pathway [23, 24]. The Pasteur effect can also be used to describe the effects in human ischaemic heart muscles. It was found that anaerobic production of ATP is used to meet the shortfall of the lower concentration of ATP produced by glycolysis [24].

2.2.2.3. Crabtree effect

The Crabtree effect is defined as the aerobic alcoholic fermentation in the presence of high glucose concentrations [23, 25, 26]. It has the opposite activity of the Pasteur effect and is said to give a competitive advantage to *S. cerevisiae* by allowing it to grow very fast during fermentation compared to other yeast species [23]. The Crabtree effect have also been described in tumour cells and is believed to be a possible silencing mechanism [27, 28].

2.2.2.4. Warburg effect

The Warburg effect was first described in 1924 by Otto Warburg who observed the metabolism of glucose by cancer cells were different compared to that of normal cells [29]. Warburg found that cancer cells ferment glucose under aerobic respiration in the support of mitochondrial oxidative phosphorylation resulting in cell proliferation [29]. It was found that an increase in cell proliferation causes higher lactate production suggesting that cancer cells depends on fermentation for the generation of ATP. The phenotypic change of the cell and an increase in the cell proliferation rate in the presence of oxygen is referred to as the Warburg effect.

In *S. cerevisiae,* sugar catabolism is activated by anaerobic respiration when cells are cultivated in excess sugar levels and a nitrogen source. Warburg effect takes place after the exhaustion of the nitrogen source [30]. In this state, *S. cerevisiae* is referred to as being in an inactive state as cells tend to respire between 25 to 100% in contrast to budding cells that respire 3 to 20% of the catabolised sugar suggesting a shift in metabolism therefore effecting energy regulation [31]. In its inactive state, *S. cerevisiae* shows a decrease in the formation of ATP upon an increase in the rate of glycolysis, there is also a loss of fermentation which converts respiration to the main catabolic pathway [31].

2.2.2.5. Caloric restriction

Calorie restriction (CR) or dietary restriction (DR) was reported to prolong the aging process in a wide range of organisms [32]. From yeast to humans, calorie restriction has

been described as reducing calories by altering the dietary intake of nutrients. CR in yeast is driven by carbon source limitation. Reducing the most preferred carbon source, glucose.

S. cerevisiae has gained popularity in studying the mechanism of CR-mediated lifespan. CR play a role in many pathways affecting many of the physiological processes in yeast longevity such as metabolic control and stress resistance. Of the many genes, silent information regulator *2* (*SIR2)* is the most studied [33]. *SIR2* encodes an NAD^+-dependent histone deacetylase that extracts acetyl groups from lysine residues which are found on the tails of histones H3 and H4 [34]. Deletion of the *SIR2* gene elevates the frequency of rDNA recombination resulting in over accumulation of extrachromosal rDNA which subsequently leads to premature aging while overexpressing *SIR2* sustains the replicative lifespan [35].

2.3. Free radicals and ROS

Free radicals are described as small, diffusible biological molecules with unpaired electrons [36]. These molecules are known as reactive oxygen species (ROS) and reactive nitrogen species (RNS) resulting from the oxidation/reduction (REDOX) reactions [37]. For the purpose of this study, we will focus our attention on ROS.

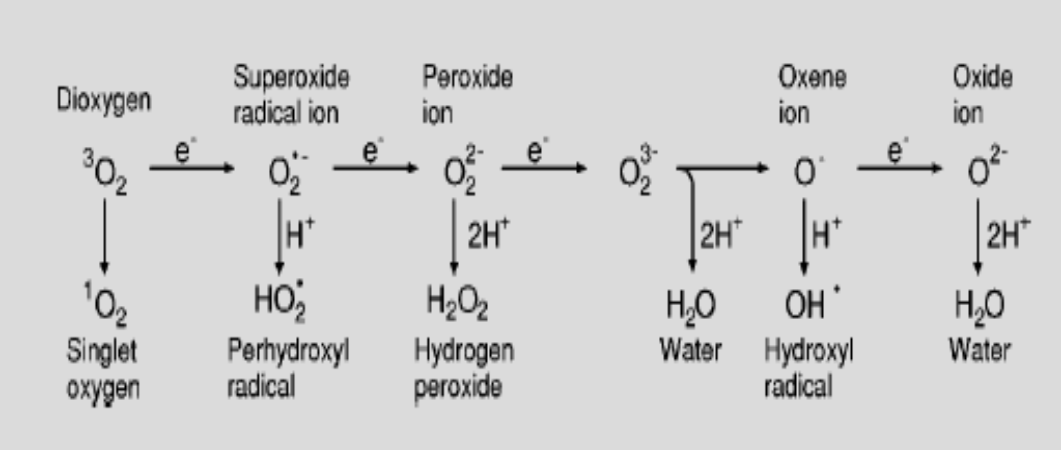

Figure 2.2. Generation of the different types of ROS by energy transfer [38].

ROS are characterised by a group of oxidants comprising of free radicals or molecular species with the ability to generate free radicals. ROS may occur as a result of metabolic processes or exposing cells to radiation, redox-cycling chemicals or heavy metals [39, 40]. All aerobically growing organisms are thus constantly exposed to ROS. The catabolism of molecular oxygen produces such as the superoxide anion ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2) and hydroxyl radicals ($HO^{\bullet -}$)[41]. The superoxide anion is dismutated either spontaneously or catalysed by superoxide dismutase (SOD) which produce H_2O_2. H_2O_2 is reduced to water via several peroxidases in the presence of transition metals.

2.3.1. Hydrogen peroxide

H_2O_2 is a commonly used oxidant to induce toxicity by redox cycling in cells and may contribute fundamentally to cellular redox regulation [42-44]. The redox signalling is regulated by post-translational modification whereby cysteine-containing regulatory proteins reversibly oxidises thiol groups of essential cysteine residues which results in alterations in protein catalytic activity [45]. In response to growth factors, hypoxia and other factors, at lowered intracellular levels, H_2O_2 has the ability to function as redox messengers in the signal transduction [46-48]. However, at higher concentrations studies have shown to be toxic to cells resulting in macromolecular damage to DNA, proteins and lipids. Possible mechanisms hypothesized for these results was the arrest of the yeast cell growth cycle possibly through chemical oxidation of cellular components leading to damage and eventually cell death, apoptosis and necrosis would follow suit [49-51].

2.4. Oxidative stress

Metabolism and aerobic respiration are contributors to oxidative stress in yeast. An accumulation of ROS may lead to DNA damage (mutations), lipid peroxidation and protein oxidation as seen in Figure 2.3 [5-7]. This discovery has led to the development of the oxidative stress theory of aging which states that ROS that forms through normal metabolism causes damage to organelles and the accumulation of these ROS in the cell plays a pivotal role in aging and even organism death [8].

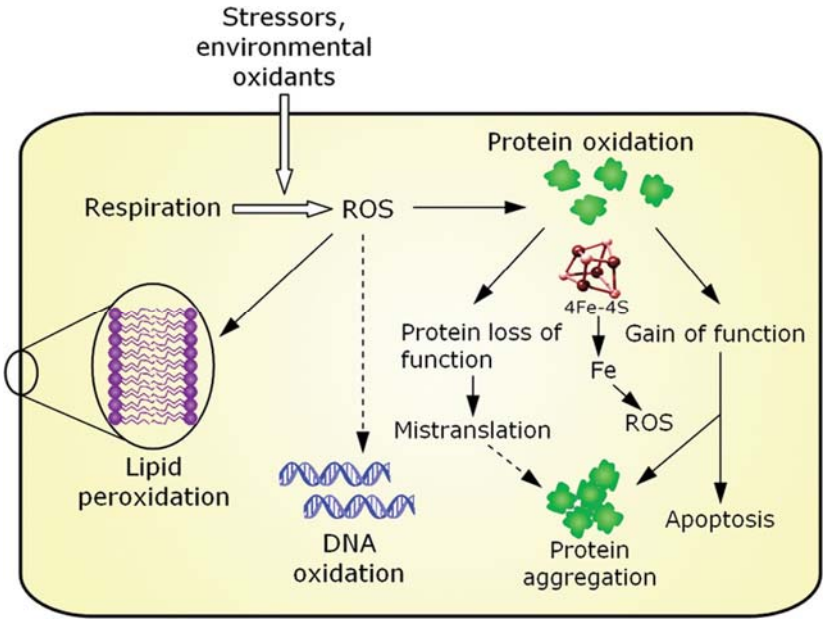

Figure 2.3. Diagram depicting the effects of oxidative stress on the cell resulting in protein oxidative, DNA oxidation and lipid peroxidation [52].

2.4.1. DNA damage

Reactions with ROS mainly $OH^{\bullet-}$, induces numerous alterations namely; cleavage of DNA, DNA-protein cross links and oxidation of purines. DNA mutations may arise due to erroneous base pairing occurring during replication leading to elevated mitochondrial permeability, released by cytochrome C, elevated intracellular Ca^{2+} followed by apoptosis [53]. Negatively charged DNA molecules bind to transition metal cations, Fe, and produce $OH^{\bullet-}$. $OH^{\bullet-}$ radical modifies DNA bases attacking the sugar backbone of DNA molecules causing intrachromosomal recombination. Studies have shown that oxidative DNA damage contributes to recombination of DNA in yeast cells [54]. Oxidative damage induced by mitochondrial genomic instability leading to respiratory dysfunction, plays a role in the ageing process [55].

2.4.2. Protein oxidation

Protein oxidation is defined as the covalent modification of a protein that induced by ROS or secondary by-products of oxidative stress. Protein oxidation adversely affect cellular homeostasis and reduces cell viability. The oxidation of proteins may result in the production of protein carbonyls. Protein carbonyl accumulation is detrimental to the cell

due to the cells inability to remove the formation of large protein aggregates through normal proteolytic pathways which may eventually lead to the disruption of cell homeostasis [56].

The $OH^{\bullet-}$ radical in the presence if O_2 stimulates protein aggregation leading to fragmentation damaging proteins in vivo. This reaction takes place on the alpha carbon and its side chains; leading to the formation of a carbon radicals ($C^{\bullet-}$) in the presence of O_2 yielding peroxyl radicals ($COO^{\bullet}$) and alkoxyl radicals ($CO^{\bullet}$). $COO^{\bullet}$ and $CO^{\bullet}$ is stabilised by the removal of hydrogen from another molecule becoming stabilised and promoting a chain reaction. At the amino level, hydroxylation alters amino acids by $OH^{\bullet-}$ which occurs on the following amino acids, phenylalanine, tyrosine and arginine resulting in the promoting in vivo, non-reversible radical conjunctions whereby two tyrosine residues combine to form bi-tyrosine. H_2O_2 stimulates the formation of disulphide bonds by the oxidation of sulphydryl (SH) groups of cysteine residues generating sulfenic acid which in turn oxidises another cysteine sulphydryl residue. Oxidative damage of proteins leads to cross-linking, an increased proteolytic susceptibility and therefore a decreased biological activity [57]. The accumulation of oxidized proteins have been shown to be related to various human diseases such as atherosclerosis, Alzheimer's disease and even ageing [58].

2.4.3. Lipid peroxidation

Lipid peroxidation occurs by $OH^{\bullet-}$ stimulated degradation of lipids by the oxidation of lipid hydroperoxides. Membrane-bound polyunsaturated fatty acids (PUFAs) are targets for lipid peroxidation such as arachidonic acid and linolenic acids and their by-products may lead to the formation of protein adducts as well as cause damage to cellular membranes. In humans, lipid peroxidation leads to diabetes and cardiovascular disease. Lipid hydroperoxides leads to the formation of further hydroperoxides and other reactive derivatives causing oxidative damage to cell biomolecules. In yeast, PUFAs are unable to be synthesized, however, upon cultivation in PUFA enriched media will readily incorporate into their membrane leading to lipid peroxidation upon exposure to oxidative stress [59].

2.5. Diseases associated with oxidative stress

Prolonged and/or sudden exposure to yeast cells to high levels of ROS, impairs their antioxidant defence resulting in apoptosis [60]. Apoptosis is a form of programmed cell death (PCD) maintaining disease control in multicellular organisms. Apoptosis changes cell morphology and structure resulting in the breakage of the cell into apoptotic bodies [61]. ROS damages cellular processes and contributes to a number of human diseases such as cancer, cardiovascular disease, stroke, neurodegenerative disorders and plays a role in the aging process (Figure 2.4) [58].

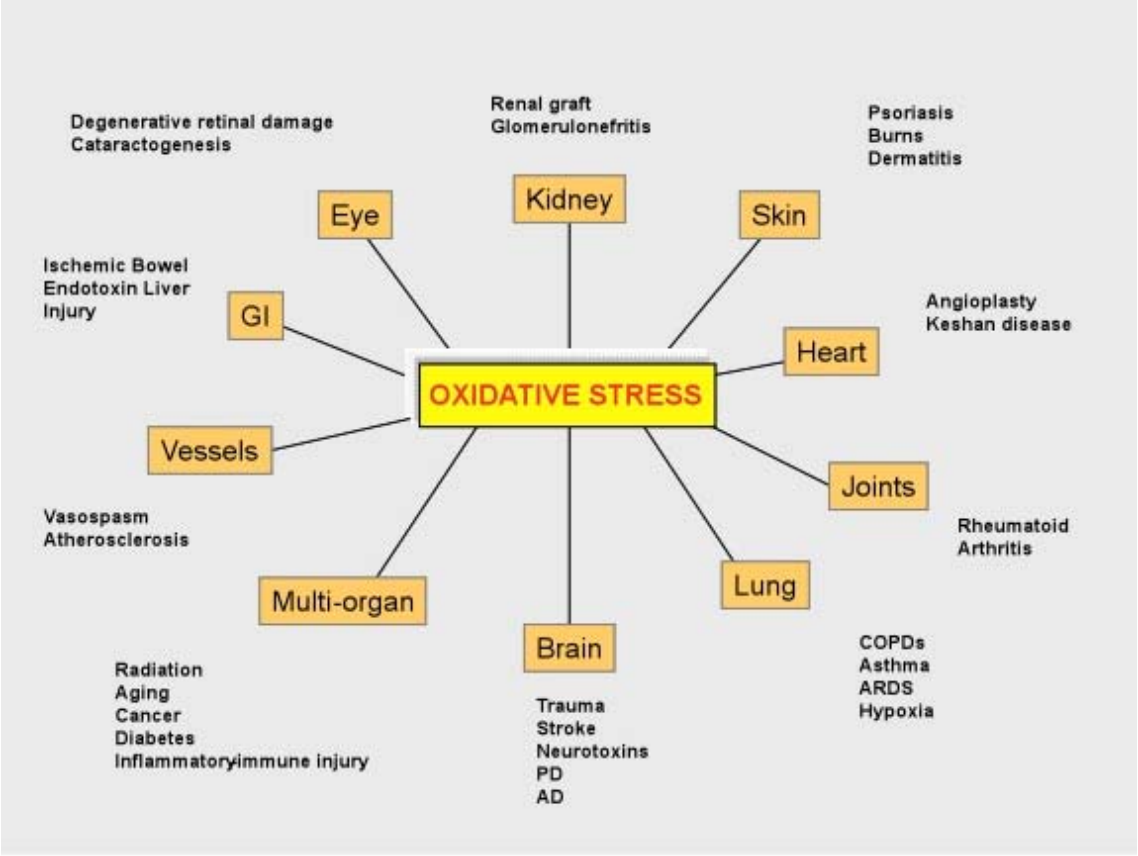

Figure 2.4. An illustration of the involvement of oxidative stress in human diseases characterized by chronic and over inflammation [62].

In humans, oxygen free radicals and many reactive oxygen species are produced through several physiological and biochemical processes [63]. Through aerobic metabolism oxygen related free radicals such as ($O_2^{\bullet -}$) and ROS such as H_2O_2 are generated in the body. Parallel to the production of ROS, antioxidants such as glutathione (GSH) and antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), glutathione

reductase (GLR1) and glutathione peroxidase (Gpx) facilitates the removal of free radicals. The inflation of free radicals in the body can lead to oxidative damage to lipids, proteins and DNA which ultimately precedes the development of many chronic diseases, cardiovascular diseases and age associated diseases namely, Multiple sclerosis (MS), Alzheimer's disease (AD) and Parkinson's disease (PD) [refer to Figure 2.4].

2.6. Antioxidant defence mechanisms

The unicellular eukaryote *Saccharomyces cerevisiae* (baker's yeast) is the ideal model to study oxidative defence mechanisms as it contains the same defence mechanisms as higher eukaryotic organisms (humans) and can be manipulated genetically and biochemically [11]. A number of NADPH-dependent and NADPH-independent defence mechanisms exist in the cell to counteract ROS both enzymatically and non-enzymatically [11]. Non-enzymatic NADPH-dependent defence mechanisms include the tripeptide glutathione (GSH) and the small protein thioredoxin (Trx) whereas the enzymatic NADPH-independent defence mechanisms include enzymes (Figure 2) such as superoxide dismutase (SOD), catalase and peroxidase [7, 11, 13].

2.6.1. Enzymatic defence mechanisms

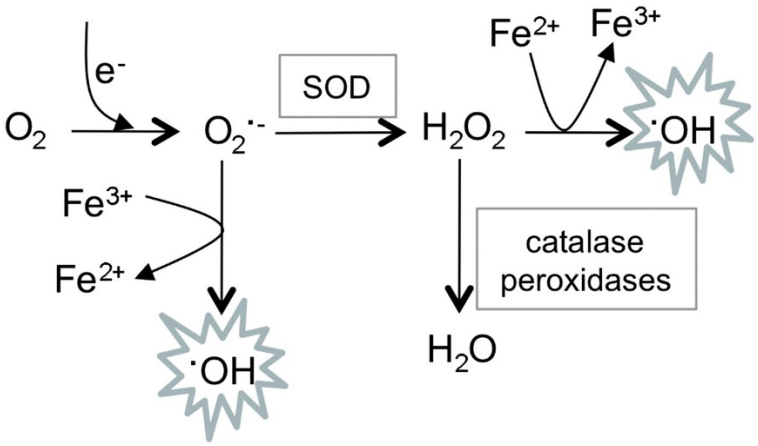

Figure 2.5. Illustration depicting the breakdown of ROS by the enzymatic defence mechanisms superoxide dismutase (SOD), catalase (CAT) and peroxidase to prevent ROS generation.

2.6.1.1.Superoxide dismutase (SOD)

SOD is able to enzymatically convert $O_2^{\bullet-}$ into H_2O_2 and O_2 which has a transition metal in its centre comprising of either iron, copper or manganese and therefore does not require the use of NADPH to initiate its activity. *C*opper and zinc SOD's are found in *S. cerevisiae*, which is found in the mitochondrial inter-membrane as well as other cellular compartments [53]. Other SOD's found are the Mn-SOD found in the mitochondrial matrix considered the primary $O_2^{\bullet-}$ scavenging enzyme for the $O_2^{\bullet-}$ protection during respiration. Cells that lack both *SOD1* and *SOD2* genes are still viable but have more profound defects when compared to single mutants. Studies have therefore concluded that the *sod1* single mutant is essential for the protection against $O_2^{\bullet-}$ toxicity leading to poor growth rate, methionine and lysine auxotrophy and even spontaneous mutagenesis [64].

2.6.1.2.Catalase (CAT)

The enzyme catalase oxidises H_2O_2 to O_2 and H_2O. There are two catalase enzymes, catalase A and catalase T encoding for *CTA1* and *CTT1* respectively found in *S. cerevisiae*. Catalase A principal function is to remove H_2O_2 generated by fatty acid β-oxidation in the peroxisome [65]. Catalase T's function still remains unclear, however it has been hypothesized that the *CTT1* gene is regulated under oxidative stress and starvation conditions. *Cta*1p and *Ctt1*p mutant strains were shown to be sensitive to H_2O_2. Single and double mutants are inefficient to escalate an adaptive response to H_2O_2. Essentially both catalases are vital for tolerance against H_2O_2 [53].

2.6.1.3.Peroxidase

Peroxidases reduce inorganic and organic peroxides into alcohols that use cysteine thiols. Three classes of peroxidases are known to date viz glutathione peroxidase (Gpx) and thioredoxin peroxidase (Tpx) and peroxiredoxin (Prx). These peroxidases donate electrons to thiols, however, but can occasionally use peroxidases as donors [66]. Tpx is known to induce the reduction of H_2O_2 and alkyl hydroperoxides to H_2O or their

corresponding alcohols. Prx are different from that of Trx and Gpx in that there is one conserved domain with cysteine active-sites. These cysteine active sites can be classified into one or two cysteine residues dependent on the presence of the active-sites cysteine [53]. *S. cerevisiae* cells possess four 2-cysteine residues and one 1-cysteine residue differing in subcellular localisation and peroxide specificities [53].

There are five different Tpx found in *S. cerevisiae* found in the cytosol in different cellular compartments; Tsa1, Tsa2 and Aph1. The mitochondria contains the nuclear Prx1 and Dot5 [66]. Tsa1 was the first of the thiol specific antioxidant to be identified with cys47 and cys170 as the active-site cysteines functioning as a peroxidase. The 2-Cys Prx moiety catabolises of H_2O_2 by Cys47 to sulfenic acid. The oxidized form of Cys47 can then react with the Cys170 of alternate molecule forming a disulphide anti-parallel homodimer which is then reduced via the thioredoxin system [53].

Gpx utilises GSH catabolising H_2O_2 to water and organic peroxide alcohols. There are two forms of Gpx described, the classical Gpx and the phospholipid hydroperoxide glutathione peroxidases, PHGpx [53]. In *S. cerevisiae*, Gpx1 and Gpx2 can be induced by caloric restriction and Gpx3 is involved in the regulation of intracellular hydroperoxide levels that transduce redox signals. There have been only three Gpxs described in *S. cerevisiae*, with the location of Gpx1 and Gpx3 to be unknown and Gpx2 to be located in the cytosol [66].

2.6.2. Non-enzymatic defence mechanisms

Non-enzymatic defence mechanisms relies on the reduction of NADPH through two distinct electron flow systems such as the glutathione and the thioredoxin pathways as seen in Figure 2.6. These two pathways are essential for the maintenance of a reduced thiol redox balance in the cytoplasm also acting as electron donors for enzymes with a reducing step in their catalytic cycle [53].

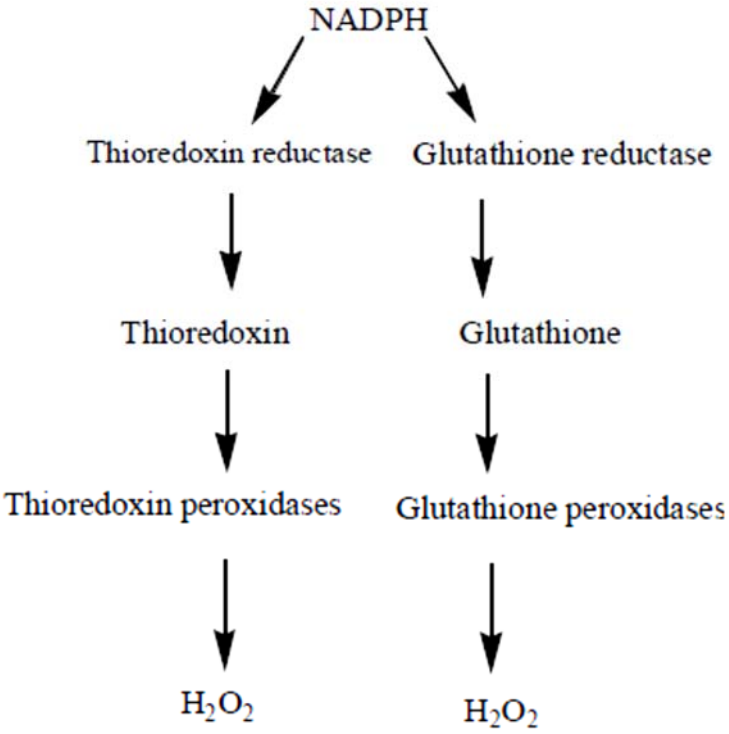

Figure 2.6. Schematic representation of glutathione (GSH) and thioredoxin pathways depicting the flow of electrons from NADPH to oxidised H_2O_2.

2.6.2.1.Glutathione system

GSH, is synthesised via γ-glutamylcysteine synthetase (GCS) and glutathione synthase (GSS). GCS catalyses the condensation of cysteine onto the gamma carbon of glutamate with GSS forming GSH via the addition of glycine. Cysteine residues plays a role in the redox balance within the cell as GSH has a very low redox potential of -240 mV allowing donation of its electrons to other cysteine residues. [41]. GSH dimerises the formation of the disulphide GSSG which is then reduced back to GSH by the NADPH-dependent glutathione reductase (GLR1) maintaining the cytoplasmic reduced ratio of GSH:GSSG at 30:1 to 100:1. GSH has the characteristics of the redox buffer due to the high GSH:GSSG ratio and the low redox potential therefore allowing it to play a role in reducing the cytoplasmic thiol redox balance and in absorbing oxidising equivalents [53].

2.6.2.2.Thioredoxin system

The two active cysteines of Trx is found within a conserved active motif of Trp-Cys-Gly-Pro-Cys. These cysteine residues become oxidised by a disulphide bonded substrate to an intramolecular disulphide via a thiol-disulphide [53]. The NADPH-dependent thioredoxin reductase (TRR) reduces Trx to an active dithiol form having higher reducing redox potential of -270mV allowing it to reduce the disulphide bonds of other proteins. TRR along with GSH aids in maintaining a reduced cytoplasmic thiol redox balance [53].

2.6.3. The pentose phosphate pathway and oxidative stress defence

The key product in the oxidative pentose phosphate pathway is NADPH which is used for biosynthetic processes and also maintains redox homeostasis to protect the cell against ROS [67]. NADPH-dependent oxidative stress defence mechanisms in this section. It has been shown that most mammalian cells can adapt to oxidative stress by alteration of gene expression including genes (transcriptional and translational) encoding for antioxidants and metabolic enzymes [68]. It was also found post-translationally, kinase ataxia telangiectasia mutated (ATM) (which is a stress-responsive regulator) stimulates the pentose phosphate pathway allowing for detoxification of the cell as well as to continuously oxidize molecules and synthesize nucleotides under stress [69]. The activation of ATM promoted formation Hsp27-G6PDH complex [69, 70]. The increase activity of this complex increased the metabolic input of the pentose phosphate pathway resulting in increased NADPH as well as increased nucleotide production [70]. It was found that one of the key enzymes responsible for the oxidant defence of the cell is glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and triose-phosphate isomerise (TPI) (Figure 1) [14, 69]. These enzymes are responsible for the shift of metabolic flux from glycolysis to the pentose phosphate pathway which in turn is responsible for the oxidant defence of the cell . Redox homeostasis is a balance of the NADH/NAP^+ and NADPH/$NADP^+$ couples. Also, the inactivation of G6PDH reroutes the carbohydrate flux to maintain NADPH/$NADP^+$ equilibrium to prevent oxidative stress from damaging the cell depicted by Figure 2.7 [14].

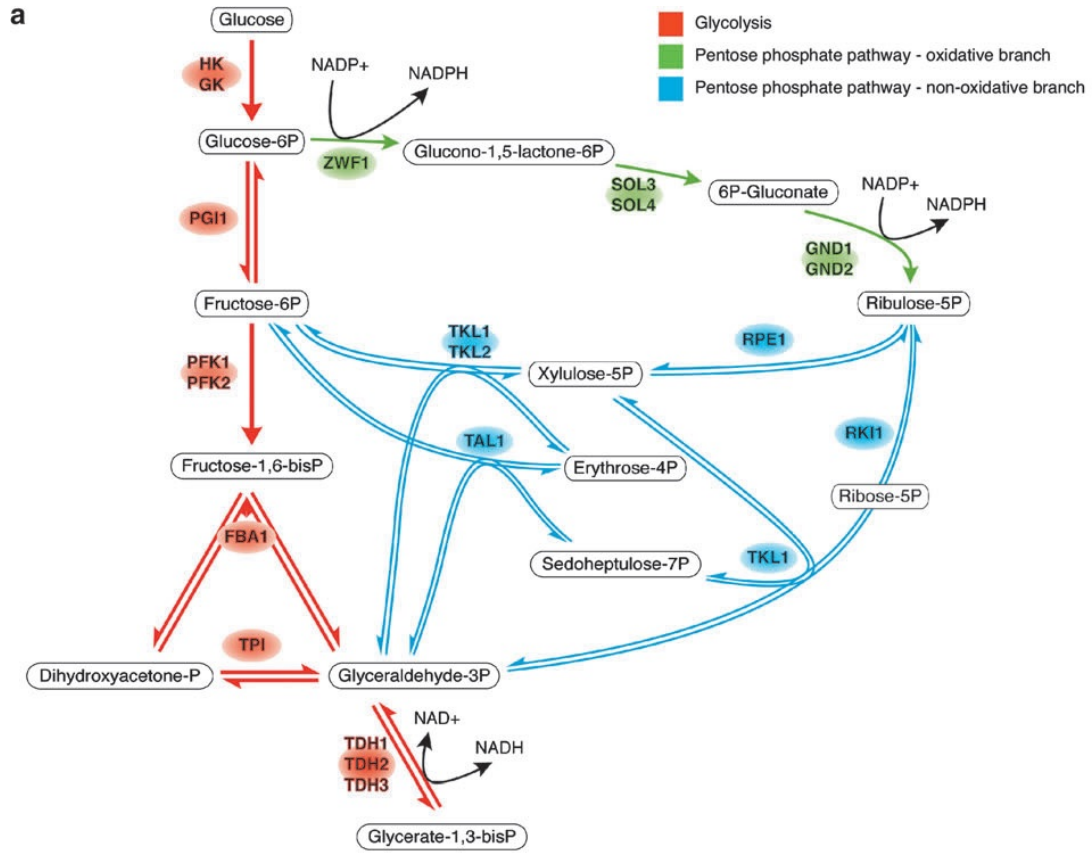

Figure 2.7. Schematic of a subset of biochemical reactions of the glycolytic pathway (red) and the associated pentose phosphate pathway (blue). One directional arrows represent direct, one-step biochemical reactions, and two-directional arrows represents reversible reactions [52].

2.7. Cell Viability Assays

The determination of cellular viability is imperative for the analysis and understanding of cytotoxicity events under various toxic conditions in this study, oxidative stress. There are myriad of assays available allowing detection of oxidative stress in mammalian cells, however, unspecific to yeast species. Oxidative stress can be evaluated by cell viability and vitality. Cell viability is defined as the percentage of live cells in a whole population whereas cell vitality describes the internal (metabolic) function of the cell [71]. The determination of cell viability is still one of the most commonly used methods to analyse chemical, physical and environmental factors [71]. Currently the preferred method is the qualitative stress spot assay which is known to be tedious, time consuming and may sometimes result in inaccuracies.

Other cell viability assays available are based on various cellular functions such as enzyme activity, cell membrane permeability, cell adherence, ATP production, co-enzyme production, and nucleotide uptake activity that applies to mammalian, fungi and certain yeast species. For the purpose of this literature review, we will focus on two commercially available quantitative cell viability assays viz. the CellTiter-Blue® Cell Viability Assay (Alamar Blue assay) and the RealTime-Glo™ MT Cell Viability Assay (ATP assay).

2.7.1. Spot assay

The spot assay is commonly used as a measure of oxidative stress in *S. cerevisiae*. This assay is advantageous in that it is inexpensive, does not require the use of expensive equipment. However, the greatest disadvantages are it is not suitable for large sample sizes, the time taken to complete an experiment and obtain results is very extensive also inaccuracies result from varying spot sizes and insensitivity of the method [71, 72].

2.7.2. Alamar Blue assay

The Alamar Blue assay has been used in many studies to measure cellular viability in fungi, the method is robust, has a short duration till completion (approximately 2 hours), sensitive and can be used for big sample sizes therefore making this assay a suitable candidate for *S. cerevisiae* related research. The ATP assay was selected mainly on the basis that a recent research study reported its suitability in measuring cellular viability in *S. cerevisiae* as a function of thermotolerance [73].

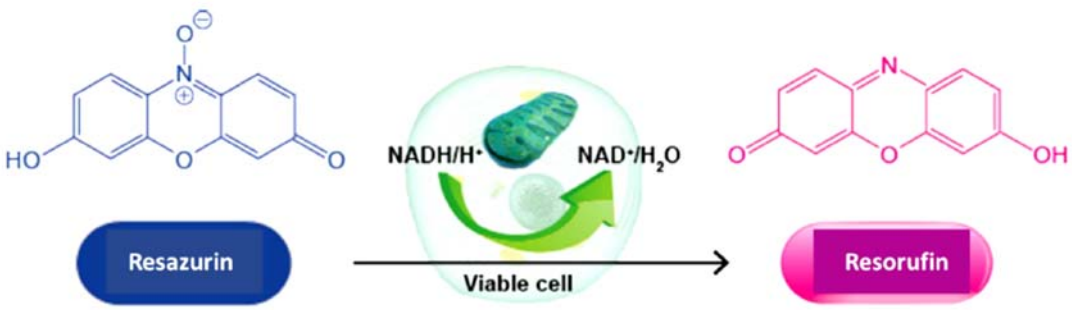

Figure 2.8. Illustration depicting the reduction of the blue coloured resazurin to the pink coloured resorufin (adapted from [74]).

Alamar Blue monitors the reducing environment of a living cell using the water soluble, non-toxic REDOX indicator resazurin (IUPAC name: 7-hydroxy-10-oxidophenoxazin-10-ium-3-one) allowing for continuous monitoring of cells in culture. Resazurin (+380 mV at pH 7.0, 25 °C) is a non-fluorescent blue dye that is reduced by NADPH (E_o = 320 mV), FADH (E_o = 220 mV), FMNH (E_o = 210 mV), NADH (E_o = 320 mV), as well as cytochromes (E_o = 290 mV to +80 mV) to the pink highly fluorescent resorufin (Figure 2.8) [75]. It has also been documented that Alamar Blue can be reduced by other enzymes in addition to mitochondrial reductases such as diaphorases (dihydrolipoamine dehydrogenase), dihydrolipoamine dehydrogenase, NAD(P)H:quinone oxidoreductase and flavin reductase [76-78]. This reduction from resazurin to resorufin makes detection of cell viability flexible since measurements can be quantitative as colorimetric and fluorometric readings or qualitative where the colour intensity of resorufin would indicate viable cells.

2.7.3. ATP assay

The ATP assay is based on the detection of ATP as an indicator of cellular function. Adenosine triphosphate, ATP, is an important molecule in all living organisms and is indicative of metabolically active cells. It is generated by living cells through respiration and is subsequently utilised to carry out biological processes that are necessary for growth, survival and replication [79]. Intracellular ATP plays a role in storing and supplying energy to metabolism and enzymatic reactions whereas extracellular ATP is essential for signalling functions suggesting that ATP is a multipurpose molecule and supplier of energy in both eukaryotic and prokaryotic organisms [79]. Cell injury or oxygen/substrate depletion produce non-viable cells. Non-viable cells lose the ability to synthesize ATP as well as endogenous ATPases rapidly deplete the existing ATP [80, 81]. Therefore, ATP detection can be considered a reasonable measurement to study metabolically active systems or living cells.

The principle behind this assay involves an oxidative reaction whereby the enzyme luciferase in combination with cellular ATP, a metallic cation as well as molecular oxygen catalyzes the conversion of firefly luciferin, to an excited oxyluciferin species (Figure 2.9) [82-84].

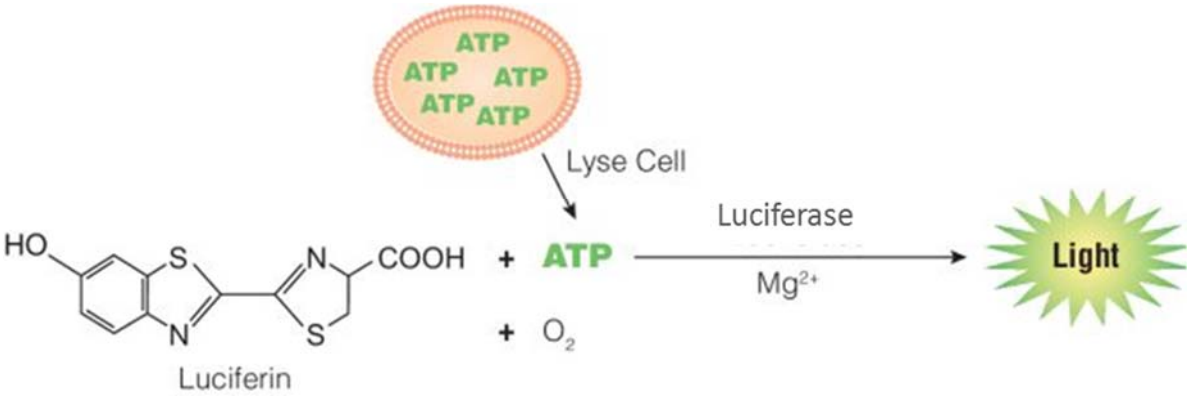

Figure 2.9. Illustration depicting the reaction of luciferin converted to light by the enzyme luciferase (adapter from [85]).

This reaction releases pyrophosphate and ATP is converted to AMP emitting light (at a wavelength of 470-700 nm) which is directly proportional to the amount of intracellularly located ATP. The ATP assay is advantageous in that it is sensitive as the limit of detection is 5 cells per 100 µL, simple to perform, rapid and can be used for a large number of samples [86]. An important precaution to users of this assay is that the ATP-dependent luciferase reaction rate is susceptible to fluctuations in temperature which may result in misleading data. It is therefore important to equilibrate to room temperature to minimize thermal gradients created by plate well position.

2.8. Conclusion

Up until now, a multitude of scientific research and review papers covering oxidative stress in *S. cerevisiae* have been reported. According to the emerging research into the metabolic flux shift from glycolysis to the PPP under oxidative stress conditions, this is key in antioxidant defence of the cell. However considering the increasing number of studies, the regulation of these events is yet to be fully understood. As such research continuously strives towards a more comprehensive understanding of antioxidant defence capacity of the cell. To date, there have been many viability assays developed determining oxidative stress. However not many of these assays are specific to yeast. Strides should be made in the direction of yeast specific viability assays.

CHAPTER 3

Research Results I

The potential of cell viability assays as a rapid and more accurate measure of oxidative stress on *S. cerevisiae*

The potential of cell viability assays as a rapid and more accurate measure of oxidative stress on *S. cerevisiae*

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3.1. Abstract

To date oxidative stress in *S. cerevisiae* has been the focal point of a multitude of research studies primarily driven by the need to understand the fundamental biochemistry of oxidative stress. Interest has also been fuelled by industrial technologies where *S. cerevisiae* is used as cellular factories for the production of commercially attractive commodities. In this scenario oxidative stress during early fermentation can negatively impact on productivity and it is imperative to reduce or effectively manage such deleterious events. Given the fact that *S cerevisiae* is widely accepted as a simple and easy to work with eukaryotic model organism it is used to study aspects of many oxidative stress disorders afflicting humans. Currently the spot assay is the favoured method to evaluate the effect of oxidative stress reagents in yeast. However, its limitations include its requirement for expertise, tedious nature and generation of qualitative data that does not facilitate differentiation of subtle differences. To provide a more robust method, this study uses a multi yeast strain approach that evaluates two commercially available cytotoxic assays against the spot assay viz, RealTime-Glo™ MT Cell Viability Assay (ATP assay) and the CellTiter-Blue® Cell Viability Assay (Alamar Blue assay). Hydrogen peroxide (H_2O_2) is selected as the oxidant since its mechanism of action is known and it has been studied extensively. Both these assays measure metabolic activity as an indicator of cell vitality. The data strongly suggests that the ATP assay displays a similar cell survival trend to that of the spot assay whereas the data obtained from the Alamar Blue assay was inconsistent. Moreover, the ATP assay generated data demonstrated good repeatability and could quantitatively differentiate cell viability for different oxidative stress regimes in the three different *S cerevisiae* genetic backgrounds used in this study. The data seemingly suggests that the ATP assay is a viable alternative for more concise or quantitative determination of the effect of an oxidative stress agent in *S cerevisiae*.

3.2. Introduction

The concept of oxidative stress was first introduced in 1985 and has since been extensively investigated in human and yeast models to gain insight into biochemical pathways leading to various human disorders as well as to reduce the deleterious effects altering flavour, ethanol production and overall quality of beer in fermentation studies [1-4]. Oxidative stress is caused by an accumulation of ROS within the cell leading to DNA damage, lipid peroxidation and protein oxidation [5, 6]. Oxidative stress can be evaluated by cell viability and vitality. Cell viability is defined as the percentage of live cells in a whole population whereas cell vitality describes the internal (metabolic) function of the cell [7]. The determination of cell viability is still one of the most commonly used methods to analyse chemical, physical and environmental factors [7].

Currently, the preferred method to measure oxidative stress in yeast is the spot assay which was presented 5 decades ago [8]. Since then, the spot assay has been manipulated and many variations have been described [9-12]. The assay is inexpensive to perform, however, the greatest disadvantages are the long duration (three days) of the assay but more importantly it only provides an estimation of the degree of growth inhibition and is not suitable for studies with large sample sizes. This qualitative assay fails to differentiate and provide the estimation of viable yeast cells or cells unable to reproduce yet it is still a widely used method in cyto- and genotoxicity studies [7, 13]. Therefore, other established cell viability methods need to be evaluated to provide a more precise or quantitative measurement of cell viability. Factors that need to be taken into consideration are cost, duration, sensitivity and ease of detection.

Currently, many cell viability assays are based on various cellular functions such as enzyme activity, cell membrane permeability, cell adherence, ATP production, co-enzyme production, and nucleotide uptake activity that applies to mammalian, fungi and certain yeast species. In this study two assays were strategically selected to measure cellular viability, the CellTiter-Blue® Cell Viability Assay (Alamar Blue assay) and the RealTime-Glo™ MT Cell Viability Assay (ATP assay). The Alamar Blue assay has been used in many studies to measure cellular viability in fungi, the method is robust, has a

short duration till completion (approximately 2 hours), sensitive and can be used for big sample sizes therefore making this assay a suitable candidate for *S. cerevisiae* related research. The ATP assay was selected mainly on the basis that a recent research study reported its suitability in measuring cellular viability in *S. cerevisiae* as a function of thermotolerance [14].

$NADH + H^+ + \text{acceptor (resazurin)} \rightarrow NAD^+ + \text{reduced acceptor (resorufin)}$

Alamar Blue monitors the reducing environment of a living cell using the water soluble, non-toxic REDOX indicator resazurin (IUPAC name: 7-hydroxy-10-oxidophenoxazin-10-ium-3-one) allowing for continuous monitoring of cells in culture. Resazurin (+380 mV at pH 7.0, 25 °C) is a non-fluorescent blue dye that is reduced by NADPH (E_o = 320 mV), FADH (E_o = 220 mV), FMNH (E_o = 210 mV), NADH (E_o = 320 mV), as well as cytochromes (E_o = 290 mV to +80 mV) to the pink highly fluorescent resorufin (see reaction above) [15]. It has also been documented that Alamar Blue can be reduced by other enzymes in addition to mitochondrial reductases such as diaphorases (dihydrolipoamine dehydrogenase), dihydrolipoamine dehydrogenase, NAD(P)H:quinone oxidoreductase and flavin reductase [16-18]. This reduction from resazurin to resorufin makes detection of cell viability flexible since measurements can be quantitative as colorimetric and fluorometric readings or qualitative where the colour intensity of resorufin would indicate viable cells.

The ATP assay is based on the detection of ATP as an indicator of cellular function. Adenosine triphosphate, ATP, is an important molecule in all living organisms and is indicative of metabolically active cells. It is generated by living cells through respiration and is subsequently utilised to carry out biological processes that are necessary for growth, survival and replication [19]. Intracellular ATP plays a role in storing and supplying energy to metabolism and enzymatic reactions whereas extracellular ATP is essential for signalling functions suggesting that ATP is a multipurpose molecule and

supplier of energy in both eukaryotic and prokaryotic organisms [19]. Cell injury or oxygen/substrate depletion produce non-viable cells. Non-viable cells lose the ability to synthesize ATP as well as endogenous ATPases rapidly deplete the existing ATP [20, 21]. Therefore, ATP detection can be considered a reasonable measurement to study metabolically active systems or living cells.

The ATP luciferin–luciferase system from the firefly *Photinus pyralis* was first described in 1963 [20]. The principle behind this assay involves an oxidative reaction whereby the enzyme luciferase in combination with cellular ATP, a metallic cation as well as molecular oxygen catalyzes the conversion of firefly luciferin, to an excited oxyluciferin species (as shown in the equation below) [22-24].

$$\text{Luciferin} + O_2 + \text{ATP} + Mg^{2+} \xrightarrow{\text{Luciferase}} \text{Oxyluciferin} + \text{AMP} + \text{PPi} + CO_2 + \text{light}$$

This reaction releases pyrophosphate and ATP is converted to AMP emitting light (at a wavelength of 470-700 nm) which is directly proportional to the amount of intracellularly located ATP. The ATP assay is advantageous in that it is sensitive as the limit of detection is 5 cells per 100 µL, simple to perform, rapid and can be used for a large number of samples [25]. An important precaution to users of this assay is that the ATP-dependent luciferase reaction rate is susceptible to fluctuations in temperature which may result in misleading data. It is therefore important to equilibrate to room temperature to minimize thermal gradients created by plate well position.

As mentioned, the qualitative spot assay is widely used in yeast viability research. Other assays need to be investigated and optimized for viability research in yeast to provide a quantitative measurement. In the present study two in vitro viability assays where compared to the commonly used qualitative spot assay to determine their ability to detect early cytotoxic events, establish the accuracy of these assays and to test the robustness of these methods using three genetically different yeast species induced by the oxidant H_2O_2. We report in this study on the suitability of the commercially available ATP assay as a distinctively more quantitative robust, time efficient (approximately 15 mins), sensitive and high throughput assay to monitor oxidative stress in *S. cerevisiae*.

3.3. Materials and Methods

Chemicals, solvents and media used in this study were of molecular biology grade.

3.3.1. Strains. The *S. cerevisiae* laboratory strains employed in this study are listed in Table 3.1. All strains are derived from *S288c* genetic background.

Table 3.1 Strains employed in this study

Strain	Genotype	Reference
BY4742	*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*	EUROSCARF
Σ1278b	*MATα ura3Δ leu2Δ his3*	EUROSCARF
BJ2168	*MATa leu2 trp1 ura3-52 prb1-1122 pep4-3 prc1-407 gal2*	ATCC

3.3.2. Media and cultivation conditions. Yeast strains were routinely cultivated at 30°C in synthetic chemically defined complete (SCD) medium, containing 0.67% (w/v) yeast nitrogen base without amino acids (Difco™) and 2% (w/v) glucose, supplemented with the appropriate auxotrophic requirements. Agar (Difco™) was used for all solid media in the cultivation process. Single yeast colonies were obtained from three-day old SCD solid media plate cultures and were used to inoculate the starter culture in 20 mL SCD medium contained in 100 mL Erlenmeyer flasks which were incubated with shaking (150 rpm) for 18 hours at 30°C. Thereafter, the starter culture was used to inoculate fresh experimental cultures 50 mL SCD medium contained in 250 mL Erlenmeyer flasks at an optical density (OD_{600}) of 0.1. Experimental cultures were grown with shaking at 30°C until an OD_{600} of 1.0 was attained reflecting the exponential phase at which cells are metabolically active. Survival was normalised to control samples with control samples defined as being 100% viable. All experiments were performed in triplicate or otherwise stated.

3.3.3. Spot assay. The stress spot assay was adapted from Formenko, D.E. and colleagues [11]. Experimental cultures in their exponential phase of growth were treated with sub-lethal concentrations (1, 5 and 10 mM) of H_2O_2 for 1 hour to avoid complete apoptosis of yeast cells and to specifically induce oxidative stress in *S. cerevisiae*. Cell viability was then determined by performing a 10 fold serial dilution of cultures exposed

to varying concentrations of H_2O_2 into sterile distilled water (dH_2O). Serially diluted cell suspensions (10^{-2}, 10^{-3}, 10^{-4}) were homogenised by vortexing and 5 µL of each dilution were spotted onto preheated SCD agar together with the undiluted sample (negative control). To prevent smearing the spots were allowed to air dry for a few minutes. Thereafter plates were parafilmed and incubated in an inverted position at 30°C for two days. Experiment was performed in duplicate.

3.3.4. ATP assay. This assay is a homogenous method for determining viable, metabolically active cells based on the quantification of ATP and was performed using the RealTime-Glo MT Cell Viability Assay (Promega Corporation, Madison, USA) according to the manufacturers' instructions. Experimental cultures in their exponential phase of growth were treated with sub-lethal concentrations of H_2O_2 (1, 5 and 10 mM) and incubated with shaking at 30 °C for 1 hour. In each test well equal volumes of RealTime-Glo MT Cell Viability Assay reagent was added to the culture medium to a final volume of 200 µL. Experiments were performed in black opaque 96-well plates (Greiner Bio-One, Frickensausen, Germany) to prevent background and luminescence cross-interference from adjacent wells. In addition, controls to rule out autoluminescence and luminescence quenching were included in the experimental design. Plates were then wrapped in foil and incubated with shaking (150 rpm) for 10 mins at 30 °C. Bioluminescence as a measure of viability was determined in a Synergy™ HT Multi-Mode Microplate Reader (Biotek Instruments, USA). Data is represented as percentage of viability.

3.3.5. Alamar Blue Assay. The CellTiter-Blue® Cell Viability Assay (Promega Corporation, Madison, USA) was used to determine metabolically active cells by the reduction of resazurin to resorufin. Analysis were performed in clear 96-well plates (Greiner Bio-One, Frickensausen, Germany). Following exposure to H_2O_2 (1, 5 and 10 mM), 20 µL of the Alamar Blue reagent was added to each test well containing 100 µL of experimental culture. Plates were then wrapped in foil and incubated with shaking (150 rpm) for 2 hours at 30°C. Fluorescence at 560 nm (excitation) and 590 nm (emission) was measured using a Synergy™ HT Multi-Mode Microplate Reader (Biotek Instruments, USA). Data is represented as percentage viability.

3.3.6. Statistical Analysis. Statistical analysis was performed using Graphpad Instat version 3.05 32 bit for Windows 95/NT (GraphPad Software, San Diego California). Data sets were analysed by one-way ANOVA and the Tukey's post hoc test. A *p*-value of <0.05 was considered to be statistically significant.

3.4. Results

The spot assay has been used extensively in measuring cellular viability and growth inhibition of yeast cells. There are many variations of this assay, some measuring the diameter of the spot and others numerating the single colonies at a specific dilution. However previous studies using both these attempted numeration procedures were unable to produce consistently accurate quantitative assessments. As such, the spot assay is used as a qualitative method to determine oxidative stress in this study.

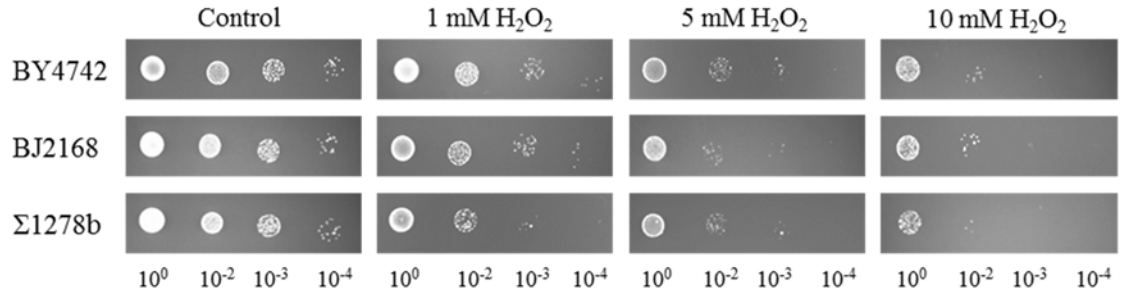

Figure 3.1. Spot assay determination of cellular viability under H_2O_2 for three genotypically different yeast strains (BY4742, BJ2168 and Σ1278b). H_2O_2 sensitivity was determined by visually evaluating cell growth after 2 days.

According to the data (Figure 3.1), there is a definite negative correlation between higher H_2O_2 concentration and lower survival rates across all yeast strains. Most growth was observed in the presence of least oxidant (1 mM H_2O_2) and the least growth was observed in the presence of most oxidant (10 mM H_2O_2). The data seems to suggest that there is no marked difference in the growth pattern of all strains after exposure to H_2O_2.

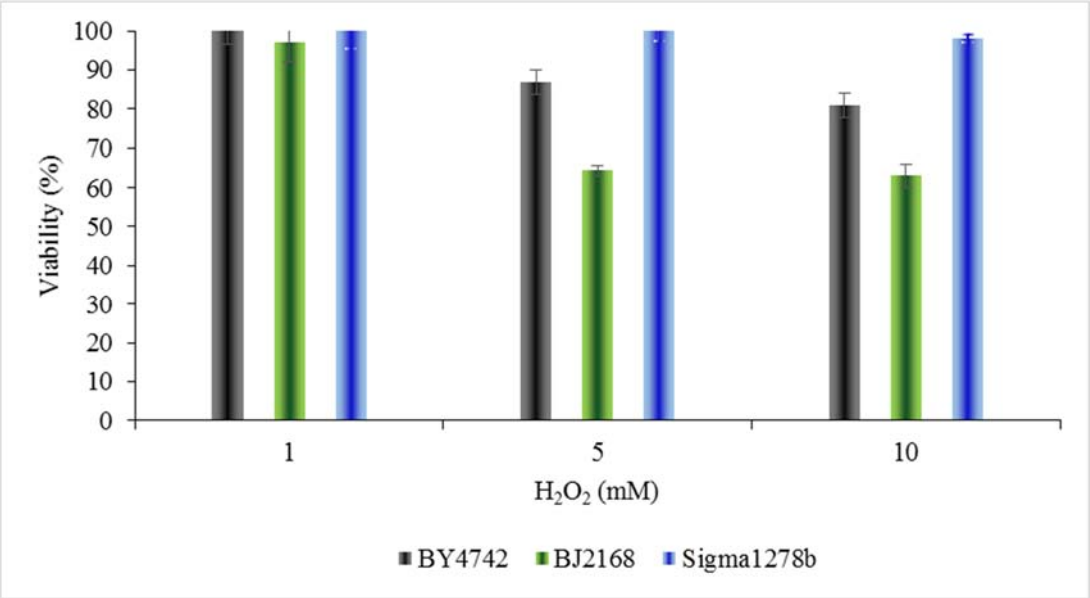

Figure 3.2. Cell viability of three genotypically distinct yeast strains (BJ2168, BY4742 and Σ1278b) after exposure to H_2O_2 (1, 5 and 10 mM) as determined by the Alamar Blue assay. The data reflected is the mean (±SD) of five determinants.

According to the data obtained from the Alamar Blue assay (Figure 3.2), BY4742 and BJ2168 showed a negative correlation between increased H_2O_2 concentration and decreased cell viability. Surprisingly, there were no significant differences (p>0.05) observed in the survival trends for Σ1278b after exposure to increasing concentrations to H_2O_2. In addition and importantly the cell viability rates across all strains after exposure to all concentrations of H_2O_2 appears to be higher when evaluated against the spot assay. For BJ2168, there was no significant difference (p>0.05) between the 5 mM and 10 mM treatment regimes. For BY4742, all data was statistically significantly different. In comparison to the spot assay, it appears that the Alamar Blue assay data is fairly inconsistent.

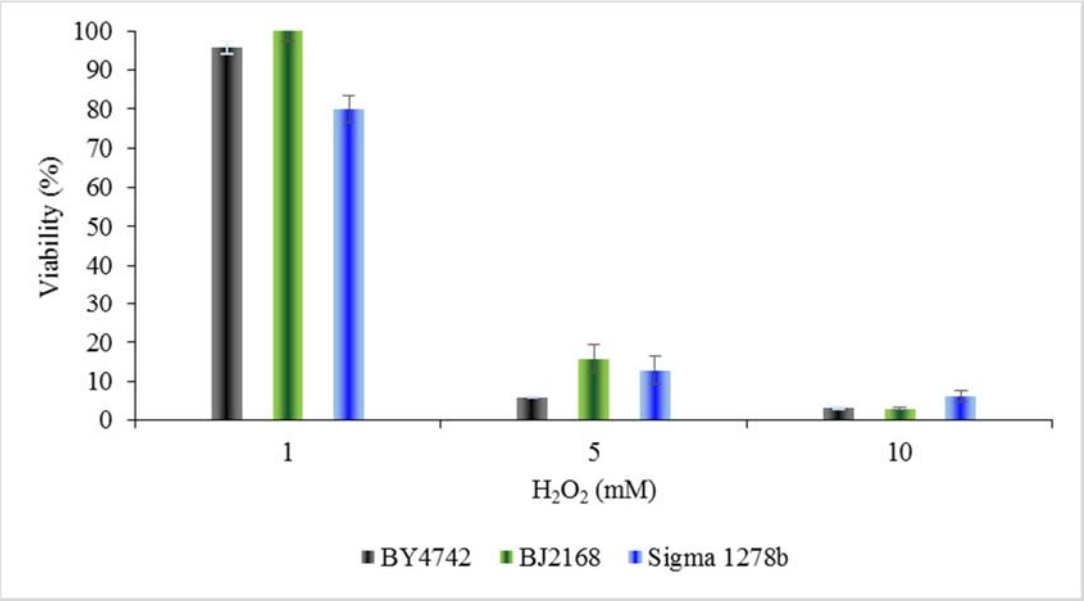

Figure 3.3. Cell viability of three genotypically distinct yeast strains (BJ2168, BY4742 and Σ1278b) after exposure to H_2O_2 (1, 5 and 10 mM) as determined by the ATP assay. The data reflected is the mean (±SD) of five determinants.

A strong negative correlation exists between cell viability and H_2O_2 as determined by the ATP assay (Figure 3.3). Data suggests an increase in H_2O_2 concentration results in a decrease in cellular growth. There was a noticeable difference between all H_2O_2 concentrations, data was significantly different (p<0.05). The ATP assay shows similarities to the trend displayed by the spot assay, increased oxidative stress causes a decrease in yeast growth with an increase in H_2O_2 concentration.

3.5. Discussion

The well-established spot assay has been extensively used in determining the effects of stressors on yeast cell fitness. This method is used as a qualitative visual method in numerous studies usually for screening purposes to select a range of concentrations of the test compound/s as well as to test the sensitivity of yeast cells to a stress [26]. Even though the spot assay shows a correlation between reduced cellular growth with increased H_2O_2 concentration, the method is very time consuming, is performed over a period of 3 days and is very tedious [27].

It should be noted that determination of viability with the spot method is a subjective matter since small variations in the experimental conditions such as change in media, growth temperature, duration of exposure to a test compound or the use of solid or liquid media may reduce or enhance sensitivity [11, 26, 27]. Therefore, while there is a positive correlation between sensitivity, growth rate and increased concentration of the test compound, the results may vary and sometimes be inconsistent. Important advantages of this method is that it is not yeast species-specific and there is no need for expensive equipment to obtain data. As mentioned previously, there are many disadvantages associated with the spot assay, time constraints, tedious, inaccuracies due to varying spot sizes and most importantly from the data it is clearly evident the spot assay although showing a survival trend for oxidative stress in the presence of H_2O_2 concentrations increasing from 1 to 10 mM, it does not quantitate the population of viable cells [7].

In contrast to the spot assay, the Alamar Blue assay is a rapid, non-toxic means to measure cell viability in mammalian, bacterial, fungi and few yeast species [28-31]. However, results observed in this study with three genotypically distinct yeast strains (BJ2168, BY4742 and Σ1278b) generally showed a higher cell viability rates when compared to the spot assay. Moreover, no significant differences were observed when strain Σ1278b was exposed to higher concentrations of H_2O_2. This is clearly of concern as it seems contradictory to the results obtained with the well-established spot assay. The results obtained from Alamar Blue assay is not well aligned to the visuals of the spot assay. The inconsistent data seems to suggest that an undiluted population of cells after exposure to H_2O_2 with Alamar Blue reagent produces relatively high fluorescence which precludes their exact quantification. This stems from the observation that cell viability ranges from 70% to 100% without the minimum of 0% being attained under experimental conditions in this study. This scenario most probably coincides with maximum fluorescence detection limit of the multimodal plate reader apparatus employed in this study. In this regard and which should be the subject of further study, is to determine the cell viability rates of serially diluted yeast cell populations after exposure to H_2O_2. As such it seems that the Alamar Blue assay specifications require further optimization to determine its suitability in determining cell viability in response to oxidative stress reagents.

The data of this study demonstrates that the ATP assay shows reliability, sensitivity, and time efficiency [14, 25]. Interestingly cell viability data using the ATP assay seems closely aligned to that of the survival trends obtained with the spot assay. This seems to suggest that the design and specifications of the ATP assay are suitable for *S. cerevisiae*. More concisely the manufacturer prescribed method of cell lysis which has been reported to be effective with mammalian, fungal and bacterial cells seems to be satisfactory for lysis of *S. cerevisiae* used in this study. Cell viability ranges from 0% to 100% under the experimental conditions used in this study. The latter seemingly indicates that the maximum and minimum sensitivities of the commercially available ATP assay is well suited to quantitatively determining the effects of oxidative stress on *S. cerevisiae* without manipulation or modification of the experimental procedure. The ATP assay seems an attractive and robust alternative to the spot assay in determining cell viability from undiluted yeast cell suspensions after exposure to H_2O_2.

3.6. Conclusion

Following evaluation of two commercially available cell viability assays against the conventionally accepted spot assay, we confirm that the ATP assay can be used to quantitatively evaluate oxidative stress induced by the oxidant H_2O_2. The ATP assay is the preferred method because it provides quantitative, sensitive, reproducible results as compared to the qualitative spot and Alamar Blue assays. Further studies with the Alamar Blue assay need to be conducted to determine its suitability in the event that serially diluted yeast cell populations after exposure to H_2O_2 is employed in the assay procedure.

3.7. Acknowledgements

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3.8. References

1. Engskog MK, Ersson L, Haglöf J, Arvidsson T, Pettersson C, Brittebo E: **β-N-Methylamino-l-alanine (BMAA) perturbs alanine, aspartate and glutamate metabolism pathways in human neuroblastoma cells as determined by metabolic profiling**. *Amino Acids* 2017:1-15.
2. Kunikata H, Ida T, Sato K, Aizawa N, Sawa T, Tawarayama H, Murayama N, Fujii S, Akaike T, Nakazawa T: **Metabolomic profiling of reactive persulfides and polysulfides in the aqueous and vitreous humors**. *Scientific Reports* 2017, **7**:41984.
3. Zhao F, Du Y, Bai P, Liu J, Lu W, Yuan Y: **Enhancing Saccharomyces cerevisiae reactive oxygen species and ethanol stress tolerance for high-level production of protopanoxadiol**. *Bioresource Technology* 2017, **227**:308-316.
4. Shi X, Zou Y, Chen Y, Zheng C, Ying H: **Overexpression of a Water-Forming NADH Oxidase Improves the Metabolism and Stress Tolerance of Saccharomyces cerevisiae in Aerobic Fermentation**. *Frontiers in Microbiology* 2016, **7**.
5. Mikhed Y, Daiber A, Steven S: **Mitochondrial oxidative stress, mitochondrial DNA damage and their role in age-related vascular dysfunction**. *International journal of molecular sciences* 2015, **16**(7):15918-15953.
6. Yuzefovych LV, Musiyenko SI, Wilson GL, Rachek LI: **Mitochondrial DNA damage and dysfunction, and oxidative stress are associated with endoplasmic reticulum stress, protein degradation and apoptosis in high fat diet-induced insulin resistance mice**. *PloS one* 2013, **8**(1):e54059.
7. Kwolek-Mirek M, Zadrag-Tecza R: **Comparison of methods used for assessing the viability and vitality of yeast cells**. *FEMS yeast research* 2014, **14**(7):1068-1079.
8. Nakano M, Kishimoto Y, Saito K: **Spot method in agar for the quantitative assay of anti□ Salmonella antibody**. *Japanese journal of microbiology* 1968, **12**(3):364-366.
9. Hays SL, Firmenich AA, Berg P: **Complex formation in yeast double-strand break repair: participation of Rad51, Rad52, Rad55, and Rad57 proteins**. *Proceedings of the National Academy of Sciences* 1995, **92**(15):6925-6929.
10. Wu A, Wemmie JA, Edgington NP, Goebl M, Guevara JL, Moye-Rowley WS: **Yeast bZip proteins mediate pleiotropic drug and metal resistance**. *Journal of Biological Chemistry* 1993, **268**(25):18850-18858.
11. Fomenko DE, Koc A, Agisheva N, Jacobsen M, Kaya A, Malinouski M, Rutherford JC, Siu K-L, Jin D-Y, Winge DR: **Thiol peroxidases mediate specific genome-wide regulation of gene expression in response to hydrogen peroxide**. *Proceedings of the National Academy of Sciences* 2011, **108**(7):2729-2734.
12. Mukhopadhyay K, Kohli A, Prasad R: **Drug susceptibilities of yeast cells are affected by membrane lipid composition**. *Antimicrobial Agents and Chemotherapy* 2002, **46**(12):3695-3705.
13. Drotschmann K, Clark AB, Tran HT, Resnick MA, Gordenin DA, Kunkel TA: **Mutator phenotypes of yeast strains heterozygous for mutations in the MSH2 gene**. *Proceedings of the National Academy of Sciences* 1999, **96**(6):2970-2975.

14. Paciello L, Falco FC, Landi C, Parascandola P: **Strengths and weaknesses in the determination of Saccharomyces cerevisiae cell viability by ATP-based bioluminescence assay**. *Enzyme and microbial technology* 2013, **52**(3):157-162.
15. Kreft S, Kreft M: **Quantification of dichromatism: a characteristic of color in transparent materials**. *JOSA A* 2009, **26**(7):1576-1581.
16. Matsumoto K, Yamada Y, Takahashi M, Todoroki T, Mizoguchi K, Misaki H, Yuki H: **Fluorometric determination of carnitine in serum with immobilized carnitine dehydrogenase and diaphorase**. *Clinical chemistry* 1990, **36**(12):2072-2076.
17. Belinsky M, Jaiswal AK: **NAD(P)H:quinone oxidoreductase1 (DT-diaphorase) expression in normal and tumor tissues**. *Cancer and Metastasis Reviews* 1993, **12**(2):103-117.
18. Chikuba K, Yubisui T, Shirabe K, Takeshita M: **Cloning and nucleotide sequence of a cDNA of the human erythrocyte NADPH-flavin reductase**. *Biochemical and biophysical research communications* 1994, **198**(3):1170-1176.
19. Mempin R, Tran H, Chen C, Gong H, Ho KK, Lu S: **Release of extracellular ATP by bacteria during growth**. *BMC microbiology* 2013, **13**(1):1.
20. McElroy W: **Crystalline firefly luciferase: LH_2+ ATP⇄ LH_2 · AMP+ PP LH_2 · AMP+ O_2→ L-AMP+ light+ H_2O**. *Methods in enzymology* 1963, **6**:445-448.
21. Crouch S, Kozlowski R, Slater K, Fletcher J: **The use of ATP bioluminescence as a measure of cell proliferation and cytotoxicity**. *Journal of immunological methods* 1993, **160**(1):81-88.
22. McElroy WD: **The energy source for bioluminescence in an isolated system**. *Proceedings of the National Academy of Sciences* 1947, **33**(11):342-345.
23. McElroy WD: **Properties of the reaction utilizing adenosinetriphosphate for bioluminescence**. *Journal of biological chemistry* 1951, **191**(2):547-557.
24. Deluca M: **Firefly luciferase**. *Adv Enzymol Relat Areas Mol Biol* 1976, **44**:37-68.
25. Riss TL, Moravec RA, Niles AL, Benink HA, Worzella TJ, Minor L: **Assay Guidance Manual [Internet]**. In. Bethesda (MD): Eli Lilly & Company and the National Center for Advancing Translational Sciences; 2013 May 1 [updated 2016 July 1].
26. Spencer J, Phister TG, Smart KA, Greetham D: **Tolerance of pentose utilising yeast to hydrogen peroxide-induced oxidative stress**. *BMC research notes* 2014, **7**(1):1.
27. Memarian N, Jessulat M, Alirezaie J, Mir-Rashed N, Xu J, Zareie M, Smith M, Golshani A: **Colony size measurement of the yeast gene deletion strains for functional genomics**. *BMC bioinformatics* 2007, **8**(1):117.
28. O'Brien J, Wilson I, Orton T, Pognan F: **Investigation of the Alamar Blue (resazurin) fluorescent dye for the assessment of mammalian cell cytotoxicity**. *European Journal of Biochemistry* 2000, **267**(17):5421-5426.
29. Barua P, You MP, Bayliss K, Lanoiselet V, Barbetti MJ: **A rapid and miniaturized system using Alamar blue to assess fungal spore viability: implications for biosecurity**. *European Journal of Plant Pathology* 2016:1-12.
30. Twigg R: **Oxidation-reduction aspects of resazurin**. *Nature* 1945, **155**(3935):401-402.
31. Tiballi RN, He X, Zarins LT, Revankar SG, Kauffman CA: **Use of a colorimetric system for yeast susceptibility testing**. *Journal of clinical microbiology* 1995, **33**(4):915-917.

CHAPTER 4

Research Results II

Oxidative stress in *Saccharomyces cerevisiae*

Oxidative stress in *Saccharomyces cerevisiae*

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4.1. Abstract

S. cerevisiae can be used as a model organism to study the effects of various diseases such as neurodegenerative diseases, cancer and aging caused by oxidative stress. All aerobic organisms require molecular oxygen to respire efficiently or to process energy. When molecular oxygen is reduced there is a production of reactive oxygen species (ROS). Understanding the biochemical metabolic pathways involved in antioxidant defence is limited and further research will shed light on various human-related diseases. As such, the current study evaluates the effects of oxidative stress on the haploid laboratory yeast strain *S. cerevisiae* BY4742 and its four deletion mutant strains (BY4742, *Δglr1*, *Δtdh3*, *Δtel1* and *Δzwf1*) when cultivated aerobically with varying concentrations of glucose (0.5, 2 and 5%) and hydrogen peroxide [H_2O_2 (1, 5 and 10 mM)]. The oxidative stress agent H_2O_2 was strategically selected as its mechanism of action is well understood and it has been researched extensively in *S. cerevisiae*. With the exception of *Δzwf1* deletion strain, an increase in glucose concentration seemingly displayed no impact on cell survival rates of the other strains upon exposure to H_2O_2. Interestingly, the *Δzwf1* strain which is deficient in the enzyme glucose-6-phosphate dehydrogenase (G6PDH), a rate limiting enzyme of the pentose phosphate pathway exhibited higher survival rates across all glucose concentrations than the parental or other deletion strains. The *Δzwf1* strain performed best on 0.5% glucose/H_2O_2 containing media seemingly demonstrating caloric restriction characteristics. *Δglr1, Δtdh3* and *Δtel1* displayed reduced survival rates upon exposure to H_2O_2 suggesting that these mutants play a role in oxidative stress defence. The data tentatively suggests that *S. cerevisiae* metabolism with a G6PDH deficiency shifts to another salvage pathway that more efficiently counteracts the effects of otherwise deleterious oxidative stress induced by H_2O_2.

4.2. Introduction

All aerobic organisms use molecular oxygen as a terminal electron acceptor to produce energy by respiration [1, 2]. However, reducing molecular oxygen to water produces reactive oxygen species (ROS) such as superoxide anion radicals ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2) and the hydroxyl radicals ($OH^{\bullet-}$) which may cause damage to cellular processes and components (DNA, proteins and lipids) leading to mutations and even death [3, 4]. ROS derived from the environment are a result of many factors such as radiation and pollution. In addition it can be generated via cellular metabolism as by-products during aerobic respiration or REDOX-catalysed reactions [4]. An accumulation of ROS results in a cellular status of oxidative stress.

The concept of oxidative stress was introduced three decades ago and is an active field of research [5]. Oxidative stress is defined as the imbalance of oxidants to pro-oxidants resulting in a disruption to the redox homeostasis of the cell [6]. The role of ROS in many human disorders such as cancer, diabetes, neurodegenerative diseases, cardiovascular diseases and also the aging process has been well studied [7, 8]. Similarly the role of anti-oxidant defence mechanisms from enzymes to small molecules, as well as transcriptomic and proteomic responses have also been extensively described in yeast [9]. Studies using functional genomics and computational modelling showed one of the key enzymes responsible for the antioxidant defence machinery of the cell was glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Under oxidative stress conditions, inactivation of this enzyme is responsible for the rerouting of the carbohydrate flux from glycolysis into the pentose phosphate pathway (PPP) which increases the steady state NADPH level. This is crucial as NADPH provide reducing power to the antioxidant defence network [10].

The glucose-6-phosphate dehydrogenase (G6PDH) is the rate limiting enzyme catalysing the first step of the PPP generating NADPH from $NADP^+$ [11]. NADPH in turn maintains the level of glutathione (GSH) present and thereby maintaining the redox homeostasis of the cell as well as offering protection against oxidative damage [2]. Since G6PDH is a rate limiting enzyme it is regarded important for biosynthesis of the sugar moiety for nucleic acid production [12].

It has also been suggested that NADPH dependent-glutathione reductase (GLR) and NADPH dependent-thioredoxin reductase (TRR) are responsible for protection of the yeast cell against oxidative stress through maintaining the yeast intracellular homeostasis [13]. GLR is a flavoenzyme responsible for the maintenance of the intracellular homeostasis by catalysing the reduction of GSSG to GSH using the reducing power of NADPH [5]. NADPH is also used as a co-factor in the breakdown of thioredoxin by thioredoxin reductase and hence we can conclude that both the glutathione and thioredoxin systems are linked due to NADPH used as a source for reducing equivalents [14]. It has also been shown that the thioredoxin system is responsible for controlling the balance of GSH/GSSG once the glutaredoxin system becomes unavailable [15].

The *TEL1* gene that encodes for protein kinase which is primarily involved in telomere length regulation, contributes to cell cycle checkpoint control in response to DNA damage and regulating P-body formation induced by replication stress [10, 16]. It is homologous to the human ataxia-telangiectasia mutated (*ATM*) gene which is responsible for ataxia telangiectasia (AT) [17]. It has been proposed that mutants in *ATM* and *TEL1* result in shortened telomeres and display some aspects of premature ageing, hence, further research needs to be done to understand the relationships between ageing, damage response pathways, telomeres and oxidative stress [18].

While most research has focused on the effects of oxidative stresses on cell viability, surprisingly very little work has been done on how changes in the supply of electrons affect the cell viability and the antioxidant capacity of the cell as well as the pathway the cell chooses to respond to stress. Therefore the aim of this study is to determine the relationship between glucose metabolism and oxidative stress response in *S. cerevisiae*, to determine a correlation between electron demand and antioxidant capacity of the cell.

4.3. Materials and Methods

Chemicals, solvents and media used in this study were of molecular biology grade.

4.3.1. Strains. The yeast strains employed in this study are listed in Table 3.1. All deletion strains were derived from wild type BY4742. Deletion strains were grown with G418 (200 µg/mL) for verification.

Table 4.1 Strains employed in this study

Strain	Genotype	Reference
BY4742	*MATα; his3Δ1; leu2Δ0; lys2Δ0; ura3Δ0*	EUROSCARF
Δglr1	*BY4742; MATα; ura3Δ0; leu2Δ0; his3Δ1; lys2Δ0; YPL091w::KanMX4*	EUROSCARF
Δtdh3	*BY4742; MATα; ura3Δ0; leu2Δ0; his3Δ1; lys2Δ0; YGR192c::KanMX4*	EUROSCARF
Δtel1	*BY4742; MATα; ura3Δ0; leu2Δ0; his3Δ1; lys2Δ0; YBL088c::KanMX4*	EUROSCARF
Δzwf1	*BY4742; MATα; ura3Δ0; leu2Δ0; his3Δ1; lys2Δ0; YNL241c::KanMX4*	EUROSCARF

4.3.2. Media and cultivation conditions. Yeast strains were routinely cultivated at 30°C in synthetic chemically defined complete (SCD) medium, containing 0.67% (w/v) yeast nitrogen base without amino acids (Difco™) and 2% (w/v) glucose, supplemented with the appropriate auxotrophic requirements and agar (Difco™) was used for all solid media in the cultivation process. Single yeast colonies were obtained from three-day old SCD solid media plate cultures and were used to inoculate the starter culture in 20 mL SCD medium contained in 100 mL Erlenmeyer flasks which were incubated with shaking (150 rpm) for 18 hours at 30°C. Thereafter, the starter culture was used to inoculate fresh experimental cultures 50 mL SCD medium contained in 250 mL Erlenmeyer flasks at an optical density (OD_{600}) of 0.1. Experimental cultures were grown with shaking at 30°C until an OD_{600} of 1.0 was attained reflecting the exponential phase at which cells are metabolically active. Survival was normalised to control samples with control samples defined as being 100% viable. All experiments were performed in triplicate or otherwise stated.

4.3.3. Isolation of yeast chromosomal DNA

Yeast chromosomal DNA was isolated by the method described by Ausubel and co-workers [19]. The yeast strains were grown at 30°C with shaking at 150 rpm in a mechanical shaker for 16 hours in 10 mL yeast extract peptone dextrose (YEPD) medium. The yeast cells were thereafter collected via centrifugation at 3000 rpm at 4 °C for 2 minutes. The YEPD supernatant was discarded and cells were resuspended in 500 µL of sterile distilled water. The cell suspension was transferred to a sterile 1.5 mL microcentrifuge tube and centrifuged at 12000 rpm for 30 seconds at 4°C.

The supernatant was discarded and the pellet was disrupted by vortexing briefly in the residual liquid. The cells were resuspended in of 200 µL of breaking buffer [2% (v/v) Triton X-100, 1% (v/v) SDS, 100 mM NaCl, 10 mM Tris-HCl pH 8.0, 1 mM EDTA pH 8.0], approximately 0.3 g of glass beads (Sigma-Aldrich, USA) and 200 µL phenol/chloroform/isoamyl alcohol (PCI) [25:24:1 (v/v/v)] reagent. Thereafter the yeast cells were vortexed at high speed for 3 minutes. This was followed by the addition of 200 µl of TE (tris-ethylene diamine tetra-acetic acid) buffer (10 mM Tris-HCl and 1 mM EDTA pH8.0) and the tubes were briefly vortexed. The microcentrifuge tubes were centrifuged at 13000 rpm for 10 minutes at 4°C. Supernatant was transferred to a sterile 1.5 mL microcentrifuge tube and treated with 1 mL of 100% ethanol and mixed by inversion.

The samples were stored at for 20 minutes to facilitate DNA precipitation. The microcentrifuge tubes were then centrifuged at 13000 rpm for 5 minutes at 4°C and supernatant was discarded. The pellet was resuspended in 400 µl TE buffer. RNA contaminants were removed by the addition of 3 µL Ribonuclease A (10 mg/mL) (Sigma Aldrich Incorporated, USA) and incubating the solution at 37°C for 5 minutes. Thereafter 10 µL of 4 M ammonium acetate together with 1 mL of 100% ethanol was added to the microcentrifuge tube and mixed by inversion. The samples were stored at -80°C for 20 minutes. The microcentrifuge tubes were centrifuged at 13 000 rpm at 4°C for 3 minutes. The DNA pellet remained in the tube after discarding the supernatant and resuspended in 100 µL of TE buffer.

4.3.4. Deletion yeast strain verification

All mutant yeast strains were verified using a PCR based strategy. The Takara Ex Taq™ DNA polymerase (Takara Bio Inc., Otsu, Japan) system was used according to the manufacturer's guidelines. All primers for the respective mutant strain is listed in Table 4.2. The integration of the Kanamycin (KanMX) cassette into the correct locus was confirmed by PCR using homologous primer sets which contained forward and reverse primers that is complementary to sequences from outside the region of integration (Primers A and D) as well as within the open reading frame (Primers B and C). Primers B and C were used together with primers A and D respectively to amplify PCR products indicating an unsuccessful gene deletion. However, when primers A and D amplified PCR products together with primes kan B and kan C respectively, this is indicative of a successful gene deletion. PCR products were run on a 1% agarose gel and stained with ethidium bromide (EtBr).

Table 4.2. Primers used in this study

Primer Name	**Primer Sequence (5´ → 3´)**
Δglr1 confirmation A	TTTATACGTACATAATTGGCAAGCA
Δglr1 confirmation B	CTTTTCTGAACTTCAACATTACCGT
Δglr1 confirmation C	GCCATGTACTATGCTATGTTGAGTG
Δglr1 confirmation D	TTTGAAGGCTTAAAGTTAGAAAGCA
Δtdh3 confirmation A	CATCAGTTCATAGGTCCATTCTCTT
Δtdh3 confirmation B	ATCTTCTTACCATCGACAATGATGT
Δtdh3 confirmation C	GTTTTCAAGGAATTAGACACTGCTC
Δtdh3 confirmation D	AATATCCCCAAAATTATTAAGAGCG
Δtel1 confirmation A	CACATGATATTATGAGCGTGATAGG
Δtel1 confirmation B	ATTTTGTGTGTTCAGATGCAAGTAA
Δtel1 confirmation C	TGAAATAAAACCGCAGTTAAGAAAC
Δtel1 confirmation D	ATCTACGTCGATTTCTTTCATTTTG
Δzwf1 confirmation A	ATTATTAATGTGGGATTTTTGGCTC
Δzwf1 confirmation B	CTTGAAGAACTGTTCGACCTTAGAG
Δzwf1 confirmation C	CGCTGTGTACCTAAAGTTTAATGCT
Δzwf1 confirmation D	TCAATGATAAGTACAAGTCCAATCG

4.3.5. Spot assay. The stress spot assay was adapted from the method describe by Formenko, D.E. and colleagues [9]. Experimental cultures in their exponential phase of growth were treated with sub-lethal concentrations (1, 5 and 10 mM) of H_2O_2 for 1 hour to avoid complete apoptosis of yeast cells and to specifically induce oxidative stress in *S. cerevisiae*. Cell viability was then determined by performing a 10 fold serial dilution of cultures exposed to varying concentrations of H_2O_2 into sterile distilled water (dH_2O). Serially diluted cell suspensions (10^{-2}, 10^{-3}, 10^{-4}) were homogenised by vortexing and 5 µL of each dilution were spotted onto preheated SCD agar together with the undiluted sample (negative control). To prevent smearing the spots were allowed to air dry for a few minutes. Thereafter plates were parafilmed and incubated in an inverted position at 30°C for two days. Experiment was performed in duplicate.

4.3.6. ATP assay. This assay is a homogenous method for determining viable, metabolically active cells based on the quantification of ATP and was performed using the RealTime-Glo MT Cell Viability Assay (Promega Corporation, Madison, USA) according to the manufacturers' instructions. Experimental cultures in their exponential phase of growth were treated with sub-lethal concentrations of H_2O_2 (1, 5 and 10 mM) and incubated with shaking at 30 °C for 1 hour. In each test well equal volumes of RealTime-Glo MT Cell Viability Assay reagent was added to the culture medium to a final volume of 200 µL. Experiments were performed in black opaque 96-well plates (Greiner Bio-One, Frickensausen, Germany) to prevent background and luminescence cross-interference from adjacent wells. In addition, controls to rule out autoluminescence and luminescence quenching were included in the experimental design. Plates were then wrapped in foil and incubated with shaking (150 rpm) for 10 mins at 30 °C. Bioluminescence as a measure of viability was determined in a Synergy™ HT Multi-Mode Microplate Reader (Biotek Instruments, USA). Data is represented as percentage of viability.

4.3.7. Statistical Analysis. Statistical analysis was performed using Graphpad Instat version 3.05 32 bit for Windows 95/NT (GraphPad Software, San Diego California). Data sets were analysed by one-way ANOVA and the Tukey's post hoc test. A p-value of <0.05 was considered to be statistically significant.

4.4. Results

4.4.1. PCR verification of mutant yeast strains

The deletion by integration of the KanMX gene in the *GLR1*, *TDH3*, *TEL1* AND *ZWF1* ORF in the respective mutant BY4742ΔGLR1, BY4742ΔTDH3, BY4742ΔTEL1 and BY4742ΔZWF1 strains was confirmed via a PCR strategy according to the method as described [27]. The correct integration of the KanMX gene into the GLR1, TDH3, TEL1 AND ZWF1 loci was verified by the appearance of relevant amplicons (Figure 4.1) using gene-specific primer sets Conf A with KanB and KanC with Conf D (Table 4.2). In addition the absence of a PCR product using gene-specific primers sets with one from inside the region of integration of the deletion cassette viz; Conf A with Conf B and Conf C with Conf D indicate the successful deletion of native ORF (Figure 4.1).

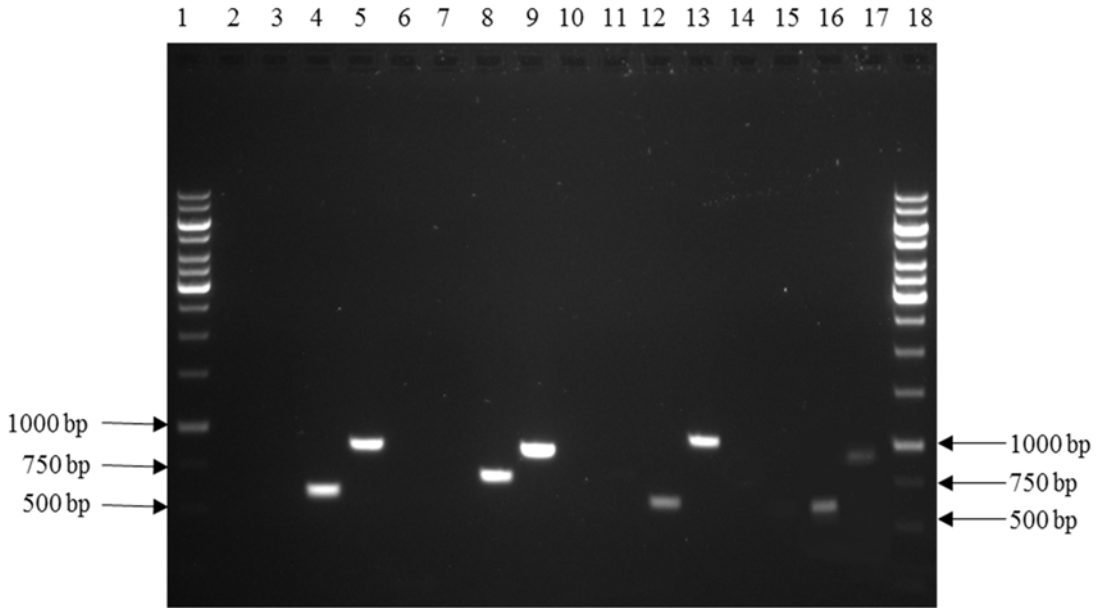

Figure 4.1. PCR verification of mutant yeast strains BY4742*Δtel1*, BY4742*Δtdh3,* BY4742*Δzwf1* and BY4742*Δglr1* on an EtBr stained 1% agarose gel. Lanes 1 and 18 contains 1 kb DNA molecular weight marker (Thermo Fisher Scientific, USA). Lanes 2, 3, 6, 7, 10, 11, 14 and 15 shows no amplicon of the ORF fragments whereas lanes 4, 5, 8, 9, 12, 13, 16, 17 contain PCR amplification of the KanMX gene verifying yeast strains BY4742*Δtel1* (591 and 891 bp), BY4742*Δtdh3* (696 and 882 bp), BY4742*Δzwf1* (573 and 988 bp) and BY4742*Δglr1* (623 and 977 bp) respectively.

4.4.2. Oxidative stress response

In this study, the quantitative ATP bioluminescence and qualitative spot assays (not all data shown) were used to evaluate oxidative stress response in BY4742 and its four deletion mutant strains after exposure to varying concentrations of H_2O_2 and cultivation on varying glucose concentrations.

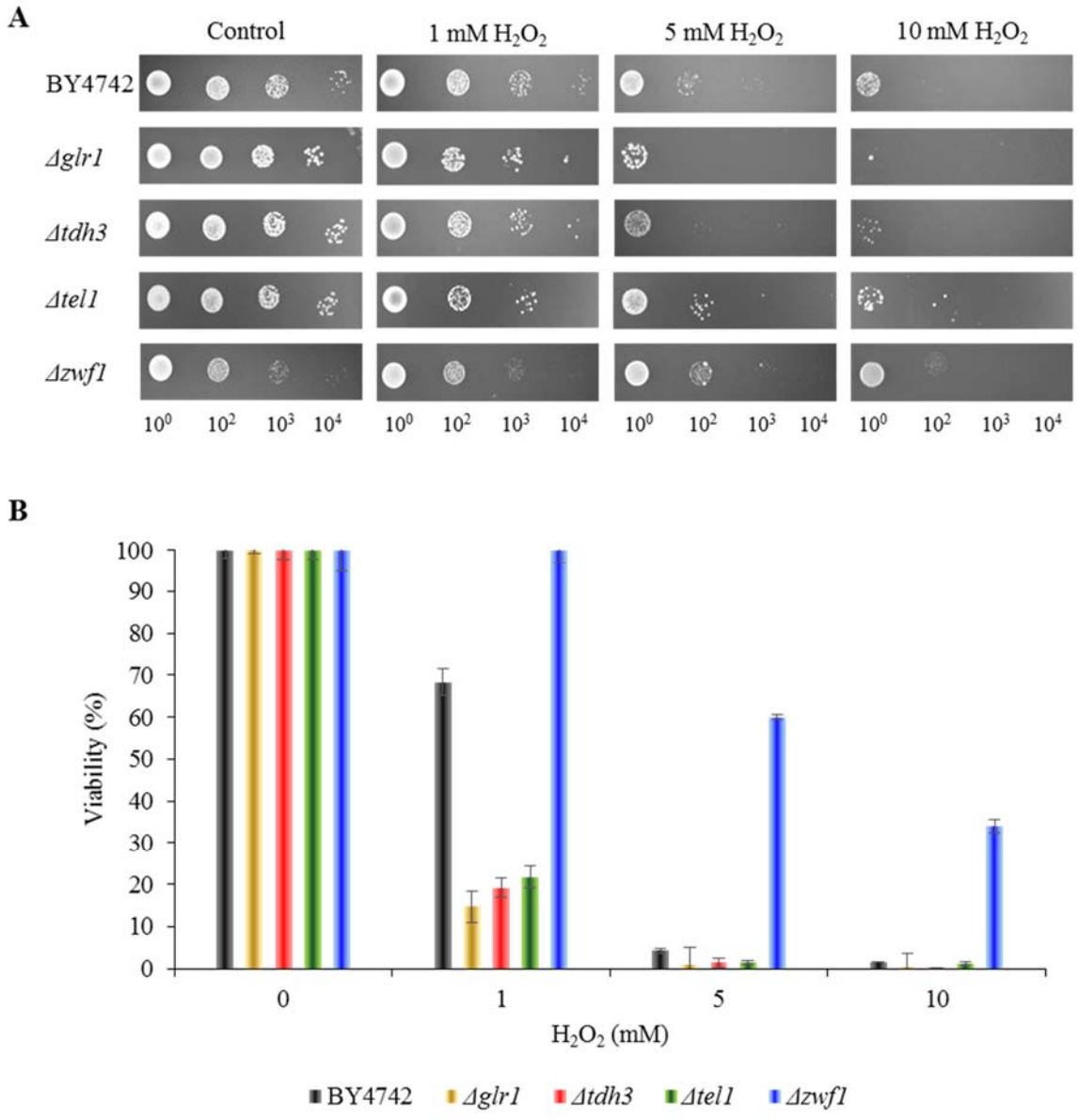

Figure 4.2. Cell viability of five yeast strains (BY4742, *Δglr1*, *Δtdh3*, *Δtel1* and *Δzwf1*) after exposure to H_2O_2 (1, 5 and 10 mM) grown on 0.5% glucose as determined by the (A) ATP assay and (B) spot assay. The data reflected by (A) was performed in duplicate and (B) is the mean (±SD) of five replicates.

There is a distinct relationship observed between yeast cell viability and H_2O_2 toxicity. Figure 4.2 A shows an increase in the H_2O_2 concentration decreases the yeast survival rate as determined by the spot assay. The ATP assay shows similarities to the trend displayed by the spot assay, increased oxidative stress causes a decrease in yeast cell viability upon increase of H_2O_2 concentration.

In Figure 4.2 B for BY4742, *Δglr1, Δtdh3* and *Δtel1*, there was no significant difference (p>0.05) between the 5 mM and 10 mM treatment regimes. For *Δzwf1*, all data was statistically significantly different. Interestingly, *Δzwf1* exhibited higher growth when grown on 0.5% glucose added to the media as compared to the other yeast strains across all concentrations of H_2O_2. Also, *Δzwf1* exhibited a higher growth rate on 0.5% glucose containing media as compared to the other glucose concentrations (2% and 5%).

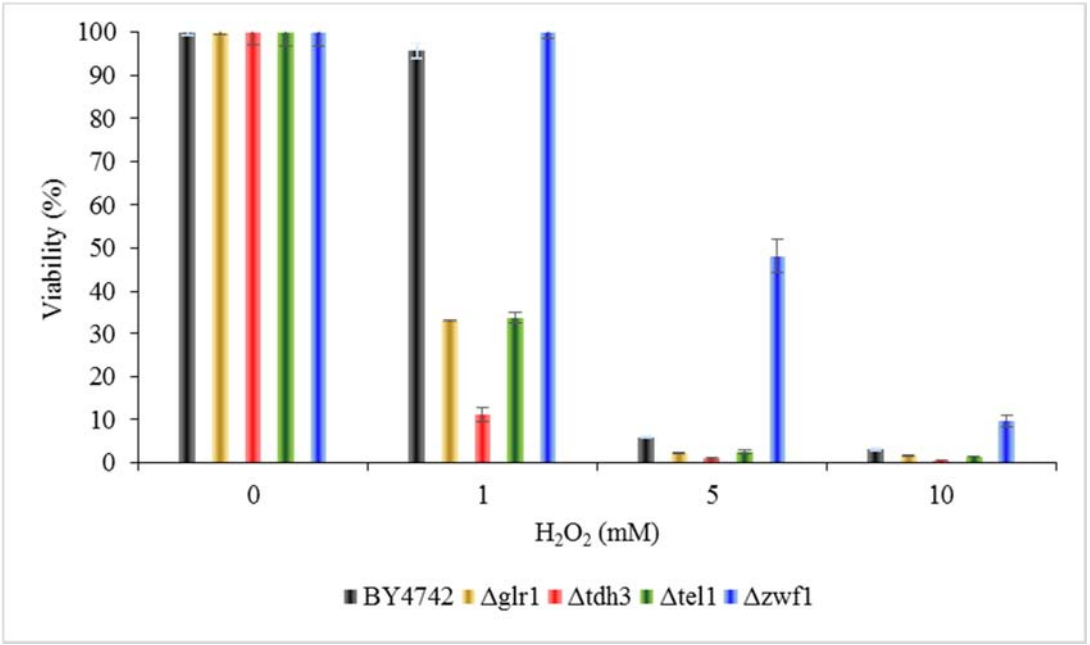

Figure 4.3. Cell viability of five yeast strains (BY4742, *Δglr1*, *Δtdh3*, *Δtel1* and *Δzwf1*) after exposure to H_2O_2 (1, 5 and 10 mM) grown on 2% glucose as determined by the ATP assay. The data reflected is the mean (±SD) of five replicates.

Data suggests an increase in H_2O_2 concentration results in a decrease in cellular growth (Figure 4.3). For BY4742 and *Δzwf1*, all data was statistically significantly different. On 2% glucose media, *Δzwf1* exhibited higher growth compared to the other yeast strains across all concentrations of H_2O_2. For *Δglr1, Δtdh3* and *Δtel1*, there was no significant difference (p>0.05) between the 5 mM and 10 mM treatment regimes.

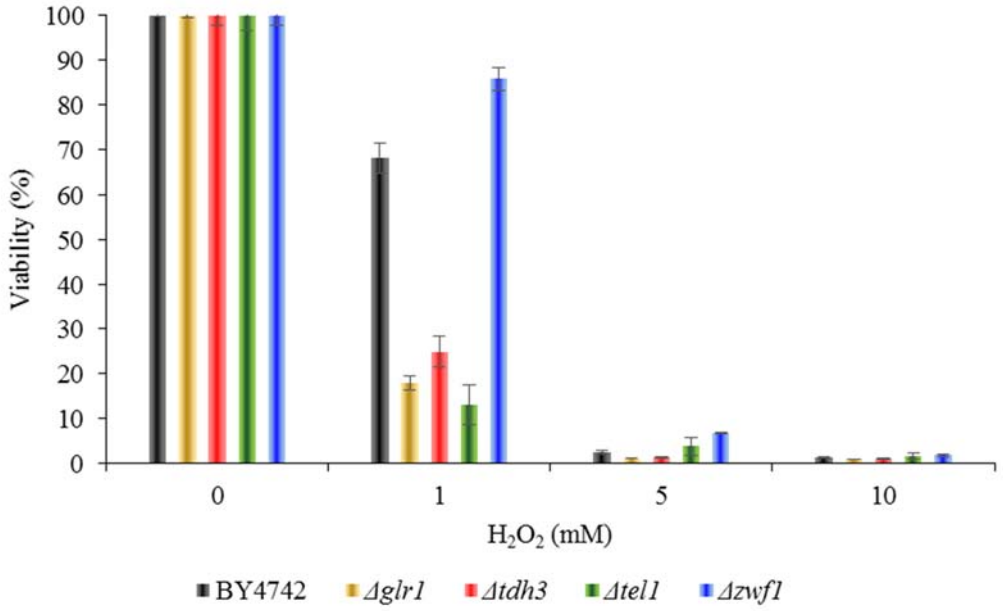

Figure 4.4. Cell viability of five yeast strains (BY4742, *Δglr1*, *Δtdh3*, *Δtel1* and *Δzwf1*) after exposure to H_2O_2 (1, 5 and 10 mM) grown on 5% glucose as determined by the ATP assay. The data reflected is the mean (±SD) of five replicates.

The data suggests a clear relationship between decreased cell viability and increased concentration of H_2O_2 as determined by the ATP assay (Figure 4.4). For *Δzwf1* and *Δtel1*, all data was statistically significantly different. On 5% glucose media, *Δtdh3* exhibited higher growth compared to the other yeast strains across all concentrations of H_2O_2. For BY4742, *Δglr1* and *Δtdh3*, there was no significant difference (p>0.05) between the 5 mM and 10 mM treatment regimes.

Additionally across all glucose concentrations the following was observed (Figure 4.2 to Figure 4.4). *Δtdh3* exhibited a higher growth rate when exposed to 1 mM H_2O_2 and cultivated on 5% glucose media as compared to the other glucose concentrations (0.5% and 2%). Also, *Δglr1* and *Δtel1* exhibited higher growth rates when grown on 2% glucose in the media as compared to the other glucose concentrations (0.5% and 5%).

4.5. Discussion

In this study, the effects of energy regulation and oxidative stress on *S. cerevisiae* was investigated. In recent years it has been established in yeast that a key protective mechanism against ROS is the switch of the carbohydrate flux from glycolysis to the pentose phosphate pathway. However, the mode of action is still yet to be fully elucidated although various research validation research studies have been attempted to date [20-23].

The *tel1* protein is a protein kinase recognised to be involved in telomere length regulation as well as play a role in DNA damage repair, recombination, and regulation of the cell cycle [24, 25]. From the data, a deletion of *TEL1* gene exhibited drastically reduced cellular growth upon exposure to increasing concentrations of H_2O_2 across all glucose concentrations. The data indicates that *Δtel1* mutant strain plays a role in oxidative stress defence. Considering that the conditions of this study is aligned to exponential growth phase of yeast that demands a high synthetic turnover of DNA for cell division, a non-functional *TEL1* gene would limit the yeast in this regard and contribute negatively to its oxidative stress defence.

Surprisingly very few studies have been conducted on the supply of electrons via the anabolism of glucose and its effect on antioxidant capacity under oxidative stress conditions which is partially investigated in this study. The transport of glucose into the cell is a rate-limiting step for sugar metabolism and therefore controls glycolytic flux which plays a major role in metabolism and growth as well as triggers stress defence mechanisms by manipulating the expression level of proteins [26, 27]. As mentioned before, when cells experience increased oxidative stress there is a shift of the flux from the glycolytic pathway to the pentose phosphate pathway in order for the cells to repair any damage caused [23, 28]. Our assumption for this study was an increase in glucose concentration will have a positive effect on the antioxidant protection of the cell by providing more reducing equivalents to the pentose phosphate pathway under oxidative stress.

GLR1 and *TDH3* are both intermediates of the PPP. *GLR1* is known to play a central role in intracellular redox homeostasis by catalysing the reduction of GSSG to GSH using the reducing power of NADPH which is known to play an important role in oxidative stress defence [5]. *TDH3* encodes for GAPDH responsible protein S-thiolation which has been proposed that protein S-thiolation protects proteins by preventing the irreversible oxidation of cysteine residues [29]. Deficiency of these genes *GLR1* and *TDH3* genes possess similar cellular viability trends. The trends shows a decrease in cellular survival rates when there is an increase in H_2O_2 concentration. In a study conducted by Ralser and colleagues, it was proposed that GAPDH inactivation may contribute to the redirection of the carbohydrate flux from glycolysis to the PPP in yeast species increasing PPP metabolites and NADPH resulting in an increased cellular survival rate [23, 30-32]. However, the data shows that mutant yeast strain *Δtdh3* does not conform to the above mentioned findings. Although *Δtdh3* showed increased growth on 5% glucose, the survival rates were negligible across all glucose concentrations. Many studies confirm the findings in this study whereby *Δglr1* results in decreased survival rates upon exposure to H_2O_2 due to the cells inability to reduce oxidized glutathione (GSH) to its reduced form to maintain REDOX homeostasis [33-35]. Upon comparison of *Δglr1* and *Δtdh3* to the wild type BY4742, the data seemingly suggests that *GLR1* and *TDH3* genes may have some importance against oxidative stress since deletion of these genes clearly affects the yeast ability to cope.

The results are not supportive of the findings by Barros and colleagues as well as Russell and colleagues whom indicated high glucose levels is associated with toxicity and pathogenesis through increased production of ROS in yeasts and in mammalian cells [36, 37].

An interesting finding of this study is the increased survival rate of mutant strain *Δzwf1*. At the lowest glucose (0.5%) concentration, yeast strain exhibited the highest survival rates across all concentrations of H_2O_2 compared to other yeast strains. The data suggests that *Δzwf1* which encodes for the enzyme glucose-6-phosphate dehydrogenase (G6PD) exhibits caloric restriction (CR) characteristics whereby there is a strong association between lifespan extension and resistance to oxidative stress when grown on low glucose

concentrations [38]. This reduced calorie intake, has been studied for many years and is known to extend life span in organisms ranging from yeast to mice [39]. The data is consistent with findings in recent studies on *Saccharomyces cerevisiae*, which shows that low glucose and calorie restriction strongly decreased mitochondrial ROS production and increased chronological life span.

G6PD is a rate limiting enzyme leading into the PPP known to maintain cellular redox homeostasis under oxidative stress by rapidly producing NADPH [12]. A deficiency of this enzyme would result in the cell being sensitive to oxidative stress. The mutant strain *Δzwf1* seemingly shifts away from the accepted views of other research studies which indicate that a deficiency of G6PDH results in increased sensitivity to oxidative stress, and their data generated using the spot assay grown on rich YEPD medium. When nutrient limited conditions are used, there will is a bigger demand on intracellular metabolism to produce building blocks whereas if a nutrient rich medium is used, most metabolites and other essential nutrients can be assimilated from the nutritional environment. Due to differences in experimental design, most specifically growth conditions, concentrations of stress agent and type of stress agent the data is difficult to compare. Although the data produced by this study is seemingly interesting further study into this phenomenon employing different media conditions would be beneficial in this regard.

Taking a closer look at the viability rates of the yeast strains upon H_2O_2 exposure, it should be noted that determination of viability is a subjective matter since small variations in the experimental conditions such as change in media, growth temperature, exposure of test compound at a specific growth phase in the yeast cycle, duration of exposure to a test compound or the use of solid or liquid media may reduce or enhance sensitivity [9, 40, 41]. Therefore, while there is a positive correlation between sensitivity, growth rate and increased concentration of the test compound, the results may vary and sometimes be inconsistent when comparing results with other studies' findings. It should be noted that this study was performed with exponential growing cells. When yeast cell growth is closer to stationary phase, there is an accumulation of ROS within in the cell producing erroneous results. Further studies should be performed to determine oxidative stress effects on stationary phase cell population.

It's immensely interesting that the knockout of the *ZWF1* gene would seemingly shut down the PPP and thereby limit the yeast cells ability to deal with oxidative stress which is not actually the case with the results observed in this study. The absence of the *ZWF1* gene resulted in the cell becoming more resistant to H_2O_2 concentrations, showing survival rates exceeding that of the parental strain The results seemingly suggests that the metabolic flux shifts to another metabolic pathway to compensate for the imbalance of $NADPH/NADP^+$.

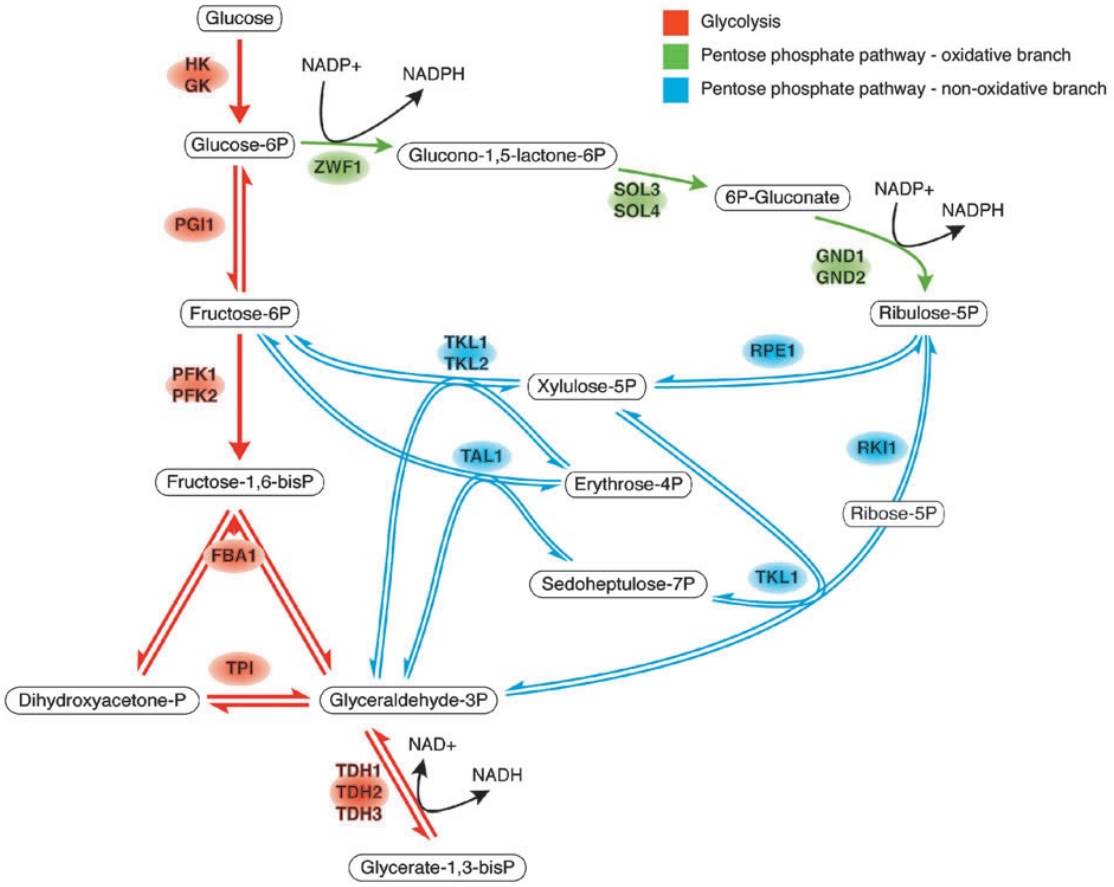

Figure 4.5. Schematic representation illustrating glycolysis together with the oxidative and nonoxidative branches of the PPP (Adapted from [21]).

A possible mechanism and other yet to be elucidated mechanisms to describe the increased survival rate under oxidative stress is the redirection of the metabolic flux from the glycolytic pathway into the PPP through fructose-6-phosphate (F6P) and glyceraldehyde-3-phosphate (G3P) as seen in Figure 4.5 above. Following inactivation

of the rate limiting enzyme G6PDH the metabolic flux is unable to enter the oxidative irreversible part of the PPP which produces cellular defence intermediates such as Trx and GSH. The metabolic flux reroutes and now enters the PPP through F6P and G3P via the nonoxidative reversible part. There is possibly an increase in the production of ribose-5 phosphate (R5P) which is responsible for nucleotide synthesis as well as increase the production of other intermediates to aid in the production of lipids and amino acids downstream of the PPP. In this regard, further research should be conducted on the above mentioned metabolites (G6P, F6P and G3P) which may shed light on the link between glycolysis and the pentose phosphate pathway and in so doing will confirm oxidative stress defence mechanisms either producing increased production of macromolecules or reducing equivalents such as GSH and/or Trx. Additionally, metabolic computational modelling studies should be initiated to shed light on possible metabolic salvage mechanisms in response in a *ZWF1* deletion.

4.6. Conclusion

Following the evaluation of various laboratory yeast species grown on different concentrations of glucose and exposed to varying concentrations of oxidative stress we have tentatively deduced that increased glucose levels is inversely related to the yeast ability to cope with oxidative stress. Interestingly upon evaluation of the genes involved in oxidative stress, *Δzwf1* mutant exhibited increased cell survival rates across all concentrations of H_2O_2. However, higher survival rates were observed when *Δzwf1* was grown on 0.5% glucose in the media seemingly suggesting caloric restriction played a role in the increased growth. Further studies need to be conducted on G6PDH in combination with TPI and GAPDH to determine the effects of oxidative stress on the metabolic flux of the cell and to better understand the flux through the pentose phosphate pathway. Glucose enters yeast cells through glucose-sensing transporters (Hxtp) and is then phosphorylated by hexokinases to generate G6PD [42, 43]. Further research on mutation of the glucose-sensing transporters may be conducted to further understand the relationship between glucose and increased survival rate of the yeast cell.

4.7. Acknowledgments

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4.8. References

1. Tran LT, Miki T, Kamakura M, Izawa S, Tsujimoto Y, Miyabe S, Inoue Y, Kimura A: **Oxidative stress response in yeast: induction of glucose-6-phosphate dehydrogenase by lipid hydroperoxide in Hansenula mrakii**. *Journal of fermentation and bioengineering* 1995, **80**(6):606-609.
2. Izawa S, Inoue Y, Kimura A: **Oxidative stress response in yeast: effect of glutathione on adaptation to hydrogen peroxide stress in Saccharomyces cerevisiae**. *FEBS letters* 1995, **368**(1):73-76.
3. Bostek C: **Oxygen toxicity: an introduction**. *AANA journal* 1989, **57**(3):231-237.
4. Apel K, Hirt H: **Reactive oxygen species: metabolism, oxidative stress, and signal transduction**. *Annu Rev Plant Biol* 2004, **55**:373-399.
5. Sies H: **Oxidative stress: introductory remarks**. London: Academic Press, Inc.; 1985.
6. Sies H: **Oxidative stress: a concept in redox biology and medicine**. *Redox biology* 2015, **4**:180-183.
7. Kayama Y, Raaz U, Jagger A, Adam M, Schellinger IN, Sakamoto M, Suzuki H, Toyama K, Spin JM, Tsao PS: **Diabetic cardiovascular disease induced by oxidative stress**. *International journal of molecular sciences* 2015, **16**(10):25234-25263.
8. Harman D: **Aging: a theory based on free radical and radiation chemistry**: University of California Radiation Laboratory; 1955.
9. Fomenko DE, Koc A, Agisheva N, Jacobsen M, Kaya A, Malinouski M, Rutherford JC, Siu K-L, Jin D-Y, Winge DR: **Thiol peroxidases mediate specific genome-wide regulation of gene expression in response to hydrogen peroxide**. *Proceedings of the National Academy of Sciences* 2011, **108**(7):2729-2734.
10. Tkach JM, Yimit A, Lee AY, Riffle M, Costanzo M, Jaschob D, Hendry JA, Ou J, Moffat J, Boone C: **Dissecting DNA damage response pathways by analysing protein localization and abundance changes during DNA replication stress**. *Nature cell biology* 2012, **14**(9):966-976.
11. Nogae I, Johnston M: **Isolation and characterization of the ZWF1 gene of *Saccharomyces cerevisiae*, encoding glucose-6-phosphate dehydrogenase**. *Gene* 1990, **96**(2):161-169.
12. Pandolfi PP, Sonati F, Rivi R, Mason P, Grosveld F, Luzzatto L: **Targeted disruption of the housekeeping gene encoding glucose 6-phosphate dehydrogenase (G6PD): G6PD is dispensable for pentose synthesis but essential for defense against oxidative stress**. *The EMBO journal* 1995, **14**(21):5209.
13. Grant CM, Collinson LP, Roe J-H, Dawes IW: **Yeast glutathione reductase is required for protection against oxidative stress and is a target gene for yAP-1 transcriptional regulation**. *Molecular microbiology* 1996, **21**(1):171-179.

14. Greetham D, Grant CM: **Antioxidant activity of the yeast mitochondrial one-Cys peroxiredoxin is dependent on thioredoxin reductase and glutathione in vivo**. *Molecular and cellular biology* 2009, **29**(11):3229-3240.
15. Jun KO, Song C-H, Kim Y-B, An J, Oh JH, Choi SK: **Activation of translation via reduction by thioredoxin-thioredoxin reductase in Saccharomyces cerevisiae**. *FEBS letters* 2009, **583**(17):2804.
16. Ritchie KB, Petes TD: **The Mre11p/Rad50p/Xrs2p complex and the Tel1p function in a single pathway for telomere maintenance in yeast**. *Genetics* 2000, **155**(1):475-479.
17. Greenwell PW, Kronmal SL, Porter SE, Gassenhuber J, Obermaier B, Petes TD: ***TEL1*, a gene involved in controlling telomere length in *S. cerevisiae*, is homologous to the human ataxia telangiectasia gene**. *Cell* 1995, **82**(5):823-829.
18. Zhou B-BS, Elledge SJ: **The DNA damage response: putting checkpoints in perspective**. *Nature* 2000, **408**(6811):433-439.
19. Ausubel F, Brent R, Kingston R, Moore D, Seidman J, Smith J, Struhl K: **Short protocols in molecular biology**. In. New York: John Wiley & Sons; 1995.
20. Cosentino C, Grieco D, Costanzo V: **ATM activates the pentose phosphate pathway promoting anti□oxidant defence and DNA repair**. *The EMBO journal* 2011, **30**(3):546-555.
21. Krüger A, Grüning N-M, Wamelink MM, Kerick M, Kirpy A, Parkhomchuk D, Bluemlein K, Schweiger M-R, Soldatov A, Lehrach H: **The pentose phosphate pathway is a metabolic redox sensor and regulates transcription during the antioxidant response**. *Antioxidants & redox signaling* 2011, **15**(2):311-324.
22. Cosentino C, Grieco D, Costanzo V: **ATM activates the pentose phosphate pathway promoting anti-oxidant defence and DNA repair**. *The EMBO journal* 2010, **30**(3):546-555.
23. Ralser M, Wamelink MM, Kowald A, Gerisch B, Heeren G, Struys EA, Klipp E, Jakobs C, Breitenbach M, Lehrach H: **Dynamic rerouting of the carbohydrate flux is key to counteracting oxidative stress**. *J Biol* 2007, **6**(10):1-18.
24. Keith CT, Schreiber SL: **PIK-related kinases: DNA repair, recombination, and cell cycle checkpoints**. *Science* 1995, **270**(5233):50.
25. Jackson SP: **The recognition of DNA damage**. *Current opinion in genetics & development* 1996, **6**(1):19-25.
26. Boles E, Hollenberg CP: **The molecular genetics of hexose transport in yeasts**. *FEMS microbiology reviews* 1997, **21**(1):85-111.
27. Ozcan S, Dover J, Rosenwald AG, Wölfl S, Johnston M: **Two glucose transporters in Saccharomyces cerevisiae are glucose sensors that generate a signal for induction of gene expression**. *Proceedings of the National Academy of Sciences* 1996, **93**(22):12428-12432.
28. Belinha I, Amorim MA, Rodrigues P, de Freitas V, Moradas-Ferreira P, Mateus N, Costa V: **Quercetin increases oxidative stress resistance and longevity in Saccharomyces cerevisiae**. *Journal of agricultural and food chemistry* 2007, **55**(6):2446-2451.
29. Grant CM, Quinn KA, Dawes IW: **Differential protein S-thiolation of glyceraldehyde-3-phosphate dehydrogenase isoenzymes influences sensitivity to oxidative stress**. *Molecular and cellular biology* 1999, **19**(4):2650-2656.

30. Chuang D-M, Hough C, Senatorov VV: **Glyceraldehyde-3-phosphate dehydrogenase, apoptosis, and neurodegenerative diseases**. *Annu Rev Pharmacol Toxicol* 2005, **45**:269-290.
31. Shenton D, Grant CM: **Protein S-thiolation targets glycolysis and protein synthesis in response to oxidative stress in the yeast Saccharomyces cerevisiae**. *Biochemical Journal* 2003, **374**(2):513-519.
32. Le Goffe C, Vallette G, Charrier L, Candelon T, Chantal B-H, Bouhours J-F, Laboisse CL: **Metabolic control of resistance of human epithelial cells to H_2O_2 and NO stresses**. *Biochemical Journal* 2002, **364**(2):349-359.
33. Grant CM, MacIver FH, Dawes IW: **Glutathione is an essential metabolite required for resistance to oxidative stress in the yeast Saccharomyces cerevisiae**. *Current genetics* 1996, **29**(6):511-515.
34. Penninckx MJ: **An overview on glutathione in *Saccharomyces* versus non-conventional yeasts**. *FEMS yeast research* 2002, **2**(3):295-305.
35. Grant CM: **Role of the glutathione/glutaredoxin and thioredoxin systems in yeast growth and response to stress conditions**. *Molecular microbiology* 2001, **39**(3):533-541.
36. Barros MH, Bandy B, Tahara EB, Kowaltowski AJ: **Higher respiratory activity decreases mitochondrial reactive oxygen release and increases life span in Saccharomyces cerevisiae**. *Journal of Biological Chemistry* 2004, **279**(48):49883-49888.
37. Russell JW, Golovoy D, Vincent AM, Mahendru P, Olzmann JA, Mentzer A, Feldman EL: **High glucose-induced oxidative stress and mitochondrial dysfunction in neurons**. *The FASEB Journal* 2002, **16**(13):1738-1748.
38. Raffaghello L, Lee C, Safdie FM, Wei M, Madia F, Bianchi G, Longo VD: **Starvation-dependent differential stress resistance protects normal but not cancer cells against high-dose chemotherapy**. *Proceedings of the National Academy of Sciences* 2008, **105**(24):8215-8220.
39. Weindruch R, Walford RL: **Retardation of aging and disease by dietary restriction**: CC Thomas; 1988.
40. Spencer J, Phister TG, Smart KA, Greetham D: **Tolerance of pentose utilising yeast to hydrogen peroxide-induced oxidative stress**. *BMC research notes* 2014, **7**(1):1.
41. Memarian N, Jessulat M, Alirezaie J, Mir-Rashed N, Xu J, Zareie M, Smith M, Golshani A: **Colony size measurement of the yeast gene deletion strains for functional genomics**. *BMC bioinformatics* 2007, **8**(1):117.
42. Özcan S, Johnston M: **Function and regulation of yeast hexose transporters**. *Microbiology and Molecular Biology Reviews* 1999, **63**(3):554-569.
43. Walsh RB, Kawasaki G, Fraenkel D: **Cloning of genes that complement yeast hexokinase and glucokinase mutants**. *Journal of bacteriology* 1983, **154**(2):1002-1004.

CHAPTER 5

General Discussion and Conclusion

5.1. General Discussion and Conclusions

The first aspect of this study reports on the evaluation of two commercial viability assays viz, RealTime-Glo™ MT Cell Viability Assay (ATP assay) and the CellTiter-Blue® Cell Viability Assay (Alamar Blue assay) in terms of their suitability to quantitatively determine oxidative stress in genotypically different yeast species. Currently the spot assay is the favoured method to evaluate the effect of oxidative stress reagents in yeast. However, its limitations include its requirement for expertise, tedious nature and generation of qualitative data that does not facilitate differentiation of subtle differences. This data generated by this method is visually assessed and is used as a qualitative method in numerous studies usually for screening purposes to select a range of concentrations of the test compound/s as well as to test the sensitivity of yeast cells to a stress [1].

The Alamar Blue assay is a rapid, non-toxic means to measure cell viability in mammalian, bacterial, fungi and few yeast species [2, 3]. However, results observed in this study with three genotypically distinct yeast strains (BJ2168, BY4742 and Σ1278b) generally showed a higher cell viability rates and which were not well-aligned that of the spot assay. The inconsistency seems to suggest that an undiluted population of cells after exposure to H_2O_2 with Alamar Blue reagent produces relatively high fluorescence which precludes their exact quantification. This scenario most probably coincides with maximum fluorescence detection limit of the multimodal plate reader apparatus employed. In this regard and which should be the subject of further study, is to determine the cell viability rates of serially diluted yeast cell populations after exposure to H_2O_2. As such it seems that the Alamar Blue assay specifications require further optimization to determine its suitability in determining cell viability in response to oxidative stress reagents.

Interestingly cell viability data using the ATP assay seems closely aligned to that of the survival trends obtained with the spot assay. This seems to suggest that the design and specifications of the ATP assay are suitable for *S. cerevisiae*. The ATP assay seems an attractive and robust alternative to the spot assay in determining cell viability from

undiluted yeast cell suspensions after exposure to H_2O_2 and is therefore the preferred method because it provides quantitative, sensitive, reproducible results as compared to the qualitative spot and Alamar Blue assays

The second aspect of this study was to evaluate the effects of oxidative stress on the haploid laboratory yeast strain *S. cerevisiae* BY4742 and its four deletion mutant strains (BY4742, *Δglr1*, *Δtdh3*, *Δtel1* and *Δzwf1*) when cultivated aerobically in chemically defined growth medium containing increasing concentrations of glucose (0.5, 2 and 5%) and hydrogen peroxide [H_2O_2 (1, 5 and 10 mM)] using the spot and ATP assays.. In recent years it has been established a key protective mechanism against ROS in yeast is the switch of the carbohydrate flux from glycolysis to the pentose phosphate pathway, however, the mode of action is still yet to be established [4-6]. Surprisingly very few studies have been conducted on the supply of electrons by the breakdown of glucose and its effect on the antioxidant capacity of the cell under oxidative stress which will be our focus in this study.

Based on previous research reports, the assumption for this study was that an increase in glucose concentration will indeed have a positive effect on the antioxidant protection of the cell by providing more reducing equivalents to the pentose phosphate pathway under oxidative stress. The results obtained this theory, showing an increase in glucose concentration seemingly displayed no impact on yeast survival rates across yeast strains upon exposure to H_2O_2 except in the case of one mutant strain, *Δzwf1*. Deficiency of genes *TDH3* encoding glyceraldehyde-3-phospate and *GLR1* encoding glutathione reductase genes possess similar cellular viability trends. A study conducted by Ralser and colleagues (2007) proposed that GAPDH inactivation may contribute to the redirection of the carbohydrate flux from glycolysis to the PPP acting as a switch in yeast increasing cellular survival rate [5]. Many studies confirmed that *GLR1* gene deletion resulted in reduced cellular growth due to the cells inability to catalyse the reaction of oxidized glutathione to its reduced form to maintain cellular REDOX homeostasis and oxidative stress defence [7, 8].

It's immensely interesting that the knockout of the *ZWF1* gene would seemingly shut down the PPP and thereby limit the yeast cells ability to deal with oxidative stress which is not actually the case with the results observed in this study. The absence of the *ZWF1* gene resulted in the cell becoming more resistant to H_2O_2 concentrations, showing survival rates exceeding that of the parental strain. The results seemingly suggest that the metabolic flux is redirected to another metabolic pathway to compensate for the imbalance of NADPH/$NADP^+$. A possible mechanism is the redirection of the metabolic flux from the glycolytic pathway into the reversible nonoxidative part of the PPP via the metabolites fructose-6-phosphate (F6P) and glyceraldehyde-3-phosphate (G3P). The increased flux through this pathway stimulates the production of nucleic acids and nucleotides by ribose-5-phosphate (R5P) as well as other intermediates downstream for increased amino acid and lipid production to counteract oxidative stress damaging effects. Further experimentation and computational modelling should be done to shed light on these possible metabolites and their role in oxidative stress defence.

5.2. References

1. Spencer J, Phister TG, Smart KA, Greetham D: **Tolerance of pentose utilising yeast to hydrogen peroxide-induced oxidative stress**. *BMC research notes* 2014, **7**(1):1.
2. O'Brien J, Wilson I, Orton T, Pognan F: **Investigation of the Alamar Blue (resazurin) fluorescent dye for the assessment of mammalian cell cytotoxicity**. *European Journal of Biochemistry* 2000, **267**(17):5421-5426.
3. Barua P, You MP, Bayliss K, Lanoiselet V, Barbetti MJ: **A rapid and miniaturized system using Alamar blue to assess fungal spore viability: implications for biosecurity**. *European Journal of Plant Pathology* 2016:1-12.
4. Cosentino C, Grieco D, Costanzo V: **ATM activates the pentose phosphate pathway promoting anti□oxidant defence and DNA repair**. *The EMBO journal* 2011, **30**(3):546-555.
5. Ralser M, Wamelink MM, Kowald A, Gerisch B, Heeren G, Struys EA, Klipp E, Jakobs C, Breitenbach M, Lehrach H: **Dynamic rerouting of the carbohydrate flux is key to counteracting oxidative stress**. *J Biol* 2007, **6**(10):1-18.
6. Krüger A, Grüning N-M, Wamelink MM, Kerick M, Kirpy A, Parkhomchuk D, Bluemlein K, Schweiger M-R, Soldatov A, Lehrach H: **The pentose phosphate pathway is a metabolic redox sensor and regulates transcription during the antioxidant response**. *Antioxidants & redox signaling* 2011, **15**(2):311-324.
7. Grant CM: **Role of the glutathione/glutaredoxin and thioredoxin systems in yeast growth and response to stress conditions**. *Molecular microbiology* 2001, **39**(3):533-541.
8. Grant CM, MacIver FH, Dawes IW: **Glutathione is an essential metabolite required for resistance to oxidative stress in the yeast Saccharomyces cerevisiae**. *Current genetics* 1996, **29**(6):511-515.