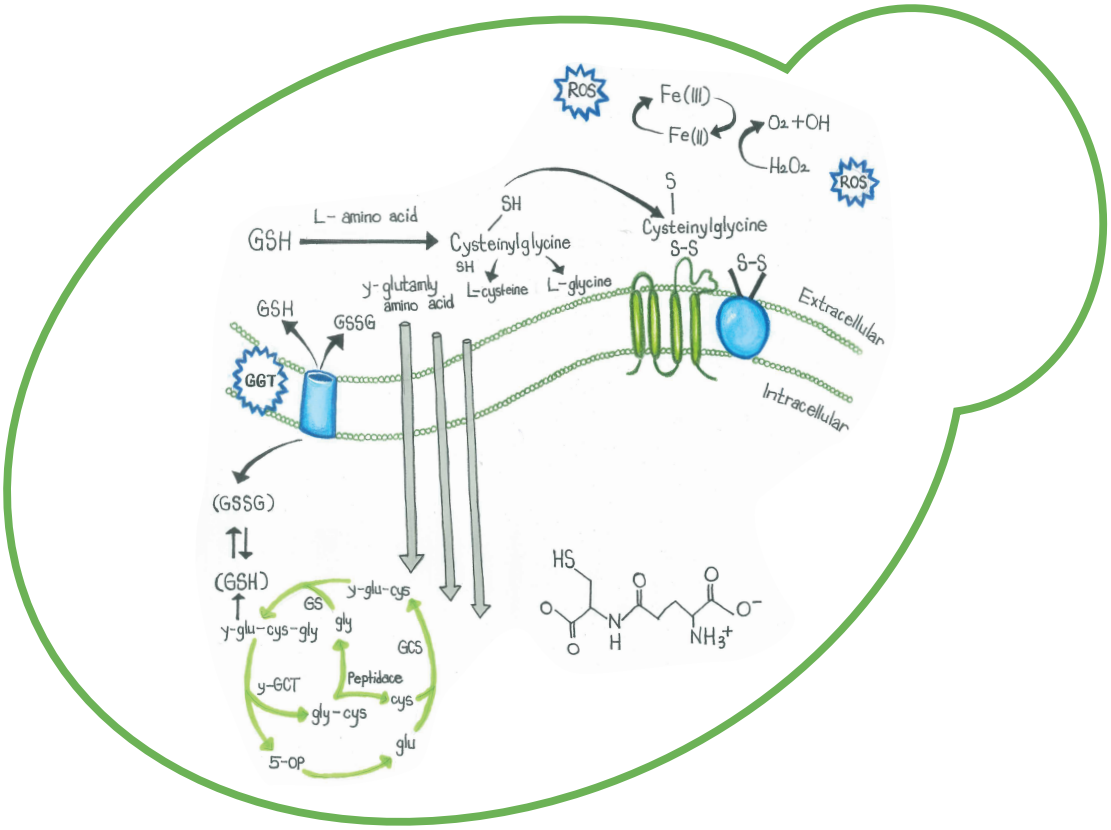
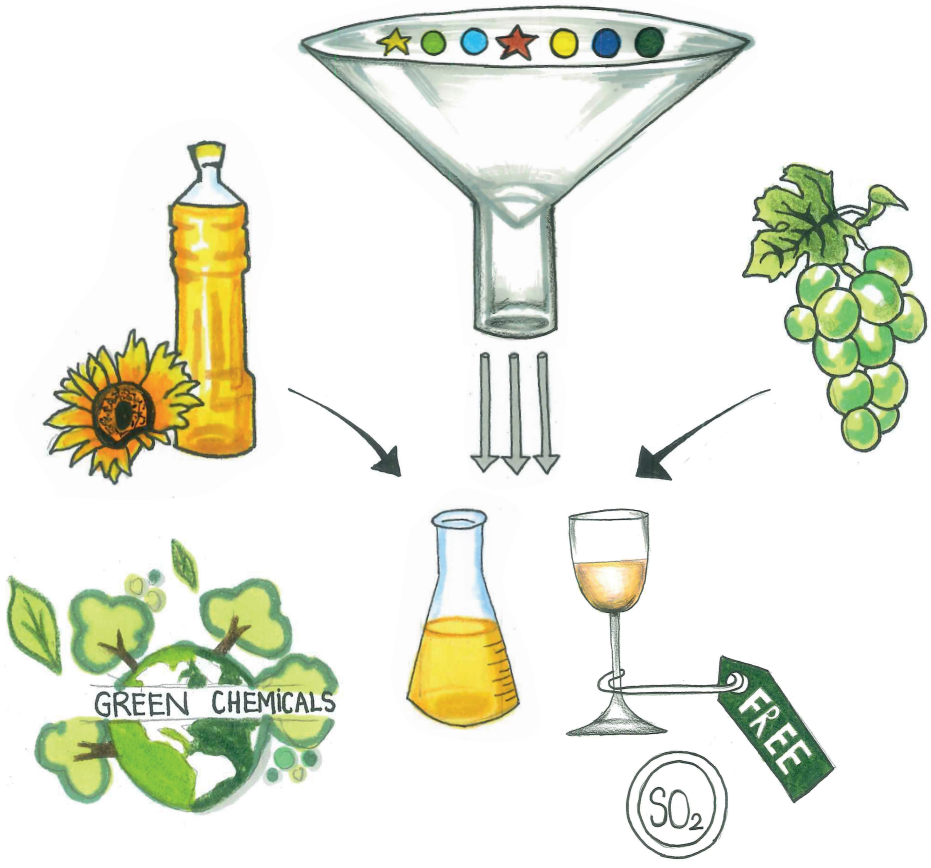
LIÈGE université
Gembloux
Agro-Bio Tech

Gamma-glutamylcysteine synthesis in the yeast *Yarrowia lipolytica* and its application as antioxidant in food materials





Mümine Guruk

COMMUNAUTÉ FRANÇAISE DE BELGIQUE

UNIVERSITÉ DE LIÈGE – GEMBLOUX AGRO-BIO TECH

Gamma-glutamylcysteine synthesis in the yeast *Yarrowia lipolytica* and its application as an antioxidant in food materials

Mümine Guruk

Dissertation originale présentée en vue de l'obtention du grade de docteur en sciences agronomiques et ingénierie biologique

Promoteur : Patrick FICKERS
Promoteur : Huseyin ERTEN
Année académique: 2024 – 2025

Abstract

Mümine Guruk. (2025). Gamma-glutamylcysteine synthesis in the yeast *Yarrowia lipolytica* and its application as an antioxidant in food materails.
(PhD Dissertation in English). Gembloux, Belgium, Gembloux Agro-Bio Tech, University of Liège, 142p, 13 tables, 24 fig.

Abstract

Gamma-glutamyl cysteine (γGC) is the precursor of glutathione (GSH), the most abundant thiol compound in the cell, which plays an important role in many cellular mechanisms. Applications of GSH in medicine, food, cosmetics and biotechnology have long been known. Recent studies have also shown therapeutic potential of γGC. *Y. lipolytica* is an unconventional yeast and is of interest as a cell factory for pharmaceutical, food and feed applications.

In this thesis, we improve γGC production in *Y. lipolytica* primarily by deletion of the biosynthetic *GSH2*, which encodes the glutathione synthetase (GSS) enzyme, and by overexpression of the *GSH1* gene, which encodes the γ-glutamylcysteine ligase (GCL) enzyme, together with *MET4, GDH, CYSE* and *CYSF* genes, which are thought to have an effect on the glutamine and cysteine anabolism. Within 24 hours of cell culture, a γGC titre of 464 nmol mg-1 protein (93 mg gDCW-1) was attained using this approach.

Antioxidant and antimicrobial properties of γGC and GSH were tested. According to DPPH test, IC50 values were 0.29 mM and 0.36 mM for γGC and 0.19 mM and 0.22 mM for ABTS, respectively. According to agar diffusion test, both γGC and GSH were ineffective against *E. coli* growth, while the zone of inhibition for *Listeria monoycytogenes, Staphylococcus aureus* and *Bacillus cereus* was 15.20, 15.11, 12.95, 12.31 and 11.67, and 9.2 mm for γGC and GSH, respectively. The minimum inhibition concentration was determined as 10 mM, 5 mM, 10 mM, 5 mM, 5 mM for γGC and 5 mM, 5 mM, 10 mM, 10 mM, 10 mM for GSH in *E. coli, Listeria monoctogenes, Staphylococus aureus* and *Bacillus cereus*, respectively.

In the last part of the study, we chose two food products, sunflower oil and wine, which are prone to oxidative degradation, to see the antioxidant effect of GSH and γGC, in foods. The samples resistance to the generation of primary and secondary oxidation products was T>GCT>γGC>GSHT>GSH>C for up to 15 days under storage conditions. The antioxidant effect of two thiols, γ-glutamyl cysteine and glutathione, in sunflower oil under accelerated storage was studied. Oil samples were stored at 50°C for a period of 15 days. The samples resistance to the generation of primary and secondary oxidation products was T>GCT>γGC>GSHT>GSH>C for up to 15 days under storage conditions.

White wine were maturated for two months following the separate addition of SO_2, γGC, and GSH at the concentration of 30 mg/L. As a result, the protective properties of γGC and GSH additives on

phenolic compounds and decelerating the browning degree in white wine were eluciated. At the end of the maturation period, γGC (218,43 GAE/L), GSH (215,22 GAE/L) treatments had the highest amount of phenolic compounds, followed by SO_2 (205,57 GAE/L) and control (192,23 GAE/L). Furthermore, γGC (OD_{420} 0.032) added wine displayed almost the same level of browning with the SO_2 (OD_{420} 0,031) added wine.

Keywords: γ-glutamylcysteine (γGC), GSH1, GSH2, antioxidant, oil oxidation, white wine

Résumé

Mümine Guruk. (2025). Gamma-glutamylcysteine synthesis in the yeast *Yarrowia lipolytica* and its application as an antioxidant in food materials

(Thèse de doctorat en anglais). Gembloux, Belgique, Gembloux Agro-Bio Tech, Université de Liège, p142, tables13, fig24.

Résumé

Le gamma-glutamylcystéine (γGC) est le précurseur du glutathione (GSH), le composé thiol le plus abondant dans la cellule, qui joue un rôle important dans de nombreux mécanismes cellulaires. Les applications du GSH en médecine, en alimentation, en cosmétique et en biotechnologie sont connues depuis longtemps. Des études récentes ont également montré le potentiel thérapeutique du la γGC. *Yarrowia* est une levure non conventionnelle qui présente un intérêt en tant qu'usine cellulaire pour les applications pharmaceutiques, alimentaires et animales.

Dans cette thèse, nous avons amélioré la production de γGC dans *Y. lipolytica* principalement par la déletion du gene *GSH2* biosynthétique, qui code l'enzyme glutathion synthétase (GSS), et la surexpression du gène *GSH1*, qui code l'enzyme γ-glutamylcystéine ligase (GCL), et des gènes *MET4, GDH, CYSE et CYSF,* qui sont censés avoir un effet sur la voie métabolique du GSH. Après 24 heures de culture, un titre de γGC de 464 nmol mg-1 de protéine (93 mg gDCW-1) a été atteint en utilisant cette approche.

Les propriétés antioxydantes et antimicrobiennes du γGC et du GSH ont été testées. Selon le test DPPH, les valeurs IC50 sont respectivement de 0,29 mM et 0,36 mM pour le γGC et de 0,19 mM et 0,22 mM pour l'ABTS. Selon le test de diffusion en gélose, le γGC et le GSH sont tous deux inefficaces contre la croissance d'*E. coli*, tandis que la zone d'inhibition pour *Listeria monoycytogenes, Staphylococcus aureus* et *Bacillus cereus* était de 15,20; 15,11; 12,95; 12,31 et 11,67, et 9,2 mm pour le γGC et le GSH, respectivement. La concentration minimale d'inhibition a été déterminée à 10 mM, 5 mM, 10 mM, 5 mM, 5 mM pour γGC et 5 mM, 5 mM, 10 mM, 10 mM, 10 mM pour GSH dans *E. coli, L. monoctogenes, S. aureus* et *B. cereus*, respectivement.

Dans la dernière partie de l'étude, nous avons choisi deux produits alimentaires, l'huile tournesol et le vin, qui sont sujets à la dégradation oxydative, pour observer l'effet antioxydant du glutathion et de la γ-glutamylcystéine (γGC) dans les aliments. La résistance des échantillons à la génération de produits d'oxydation primaires et secondaires était T>GCT>γGC>GSHT>GSH>C jusqu'à 15 jours dans des conditions de stockage. L'effet antioxydant de deux thiols, la γGC et le GSH, dans l'huile de tournesol sous stockage accéléré a été étudié. Français Les échantillons d'huile ont été stockés à 50°C pendant une période de 15 jours. La résistance des échantillons à la génération de produits d'oxydation primaires et secondaires était T>GCT>γGC>GSHT>GSH>C pendant 15 jours maximum dans des conditions de stockage.

Le vin blanc a été vieilli pendant deux mois après l'ajout séparé de SO_2, de γGC et de GSH à une concentration de 30 mg/L. En conséquence, les propriétés protectrices des additifs γGC et GSH sur les composés phénoliques et le ralentissement du degré de brunissement du vin blanc ont été obtenus. À la fin de la période de maturation, les traitements γGC (218,43 GAE/L), GSH (215,22 GAE/L) présentaient la plus grande quantité de composés phénoliques, suivis du SO_2 (205,57 GAE/L) et du témoin (192,23 GAE/L). De plus, le γGC ajouté au vin (OD420 0,032) a affiché presque le même niveau de brunissement que lorsque du SO_2 est utiliser (OD420 0,031).

Mots-cles: γ-glutamylcysteine (γGC), GSH1, GSH2, antioxydant, oxydation de l'huile, vin blanc

Acknowledgements

I would like to express my sincere thanks to my supervisors Prof. Dr. Huseyin ERTEN and Prof. Dr. Patrick FICKERS for their guidance, support, constant encouragement and for always being there. Also I am thankful to them for this great journey that allowed me to grow both professionally and personally. I would also like to thank the respectable committee members of my PhD Thesis, Prof. Dr. Marc ONGENA, Prof. Dr. Filiz OZCELIK, Prof. Dr. Serkan SELLI and Prof.Dr. Yeşim SOYER KUCUKSENEL for their guidance, valuable insight and constructive suggestions which help me to improve my PhD thesis.

I would like to acknowledge to Çukurova University Scientific Research Project Unit for supporting my PhD studies (Project Number: FDK-2022-14872). Also I am thankful to Erasmus+ program for their financial support for one year. I would like to thank to Çukurova University International Office and Semra SADIK KRUPKA for her help in the Erasmus program in the international office and Seher OZER UTUK and Pauline ANTOINE for all her help with the joint degree process.

I am also so thankful to colleagues Dr. Imen Ben TAHAR, Technician Olivia DENIES, Technician Sebastian STEEL, MSc Cristina BUSTOS and Dr. Bilal AGIRMAN, Assoc. Dr. Merve DARICI, Res. Assist. Abdullah ÖZONUR, Dr. Akram Ben GHORBAL, and other people from the department for their help, support and friendly working atmosphere.

My special thanks come to my late father, Mustafa GURUK, who was very enthusiastic about my academic success. My nephews, Azize GUNDEM, Misal DADAK and other family members who accompanied me the entire time on this challenging journey. My last thanks to my friends Arif BOZDEVECI, Demet EKTIREN, Cemre AGIRMAN, Ali DOGAN, and Mete KARABOYUN. They have always given strength and courage to go forward in this work. Thanks them with all of my heart for their understanding, patience, consolatory words and always believing in me.

Mümine Guruk.

List of publications

Article 1 (Partly mentioned in Chapter 2):

Diem Do*, **Mümine Guruk***, Małgorzata Kus-Liśkiewicz, Christian Damblon, Anthony Arguelles-Arias, Huseyin Erten, Patrick Fickers. Biosynthesis of the antioxidant γ-glutamyl-cysteine with engineered *Yarrowia lipolytica.* Biotechnology Journal 2023, 19,1-9

*The first two authors contributed equally to this work.

ARTICLE 2 (Partly mentioned in Chapter 4):

Guruk, M., Fickers, P., Selli, S., & ERTEN, H. (2023). Investigation of the antioxidant effect of two thiols,γ-glutamyl cysteine and glutathione, in sunflower oil under accelerated storage. Carpathian Journal of Food Science and Technology 2024, 16(1), 89-101

ARTICLE 3 (Partly mentioned in Chapter 5):

Guruk, M., Fickers, P., Agirman, B., Darıcı, M., Erten, H. (2025). Evaluating the potential of gamma-glutamylcysteine and glutathione as substitutes for SO_2 in white wine**.** Food Science & Nutrition Journal 2025.

List of communications during scientific events

Oral communication:

Potential of Gama Glutamyl Cysteine in Biotechnology

7th International Congress on Applied Biological Sciences, Çukurova University, 01-02/11/2022

Oral communication:

Synthesis of thiol containing metabolites in *Yarrowia lipolytica*

47th Annual Conference on Yeasts, 26/05/2023

Oral communication:

Investigation of the antioxidant and antimicrobial potantial of two thiols, γ-glutamyl cysteine and glutathione

International Food Innovation and Sustainability Congress, 16-18/05/2024

Poster:

Importance of *Yarrowia lipolytica* for industrial applications

The 35th Interntional Specialized Symposium on Yeasts, 21-25/10/2019

Table of contents

List of figures

List of tables

List of abbreviations

°C : Centigrade Degree
µg : Microgram
µL : Microliter
µm : Micrometre
γGC : gamma glutamyl cysteine
ABS : Absorbance
ABTS : 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid
ANOVA : Analysis of Variance
ARS : Autonomously replicaring sequences
ATTC :American type culture collection
BHA : Butylated hydroxyanisole
BHT : Butylated hydroxytoluene
bp : Base Pair
BSA : Bovine serum albumin
CDW : Cell dry weight
CEN : Centomere
CFU : Colony Forming Unit
cm : Centimetre
CYS : Cysteine
d : Day
DNA : Deoxyribonucleic Acid
DPPH : 2,2-diphenyl-1-picrylhydrazyl
EYK : Erythrulose kinase
FAO : Food and Agriculture Organization
FDA : Food and Drug Administration
FFA : Free fatty acid
FID : Flame ionization detector
g : Gram
GC : gamma glutamyl cysteine
GCL : glutamyl cysteine ligase
GC-MS : Gas Chromatography-Mass Spectrometry
GDH : Glutamate deyhydrogenase
GPX :Glutahione peroxidase
GRAS : Generally Recognized as Safe
GS : glutathione synthetase

GSH : Glutathione

GSH1 : glutamyl csyteine synthetase

GSH2 : glutathione synthetase

GSSG : Glutathione disulphide

h : Hour

H_2O : Water

H_2O_2 : hydrogen peroxide

HCl : Hydrochloric Acid

HPLC : High Performance Liquid Chromatography

L : Litre

LAB : Lactic acid bacteria

LTR : Long terminal repeats

log : Logarithmic

M : Molar

mg : Milligram

min : Minute

mL : Millilitre

mM : Milimolar

mm : Millimetre

Mb : Mega base

MHB : Muller hinton broth

NaCl : Sodium Chloride

ng : Nano Gram

OD : Optical Density

PBS : Phosphate buffered saline

PCR : Polymerase chain reaction

PDA : Potato Dextrose Agar

ppm : Parts Per Million

P-Av : P- anisidine value

PV : Peroxide value

ROS : Reactive Oxygen Species

RPM : Rotation Per Minute

s : Second

S : *Saccharomyces*

SPME : Solid Phase Micro Extraction

SO_2 : Sulphur dioxide

TTC : 2,3,5 triphenyltetrazolium chloride

TY : Total yeast

UAS : Upstream activation sequence

UPLC : Ultra performance liquid chromatography

UV : Ultraviolet

Q-TOF : quadrupole time of light

v/v : Volume/Volume

VOCs : Volatile Organic Compounds

w/v : Weight/Volume

Y : *Yarrowia*

YNB : Yeast Nitrogen Base

YNBD : Yeast Nitrogen Base Dextrose

YPD : Yeast Extract Peptone Dextrose

Preface

Glutathione (GSH) is the most abundant low molecular weight thiol in cells and plays an important role in the maintenance and regulation of the thiol-redox state of the cell. Therefore, it can be argued that homeostasis of GSH at optimal concentrations and reduced/oxidation ratios in cell compartments is fundamental for a healthy cellular redox. Cytosolic GSH de novo synthesis occurs in all mammalian cells by two sequential ATP-dependent enzyme-catalysed reactions. In the first, glutamate cysteine ligase (GCL) forms the unusual γ-peptide bond between L-glutamic acid and L-cysteine to produce γ-glutamylcysteine (γGC). In the latter, glutathione synthetase (GS) then adds glycine to γGC to form GSH. Cellular GSH homeostasis is controlled by non-allosteric feedback inhibition exerted by GSH on GCL activity.

γGC can be synthesised chemically and biotechnologically. However, chemical synthesis on a large scale is a complex process. Moreover, the process has low yields (less than 10 per cent) and requires the use of toxic solvents, leading to environmental and safety concerns. Therefore, microbial production research and applications are in high demand. With the development of recombinant protein technology and increased research in this field, γGC has also become of medical or biotechnological interest. Besides its therapeutic effect, it can react with saccharides in foods to produce a roast meat flavour.

Increasing the production efficiency of γ-glutamyl cysteine, which has the potential for medical and biotechnological use, by microbial means and knowing its potential for use as an additive will accelerate production studies.

The main aim of this thesis was to evaluate *Yarrowia lipolytica* as a producer of high-value γ-glutamylcysteine and to evaluated its potential as an antioxidant and antimicrobial biocompound by using some food materials. The **first chapter** *Yarrowia lipolytica* and γ-glutamylcysteine were briefly summarised. A short review carried out on the usability of γ-glutamylcysteine in the food industry in third section. In the **second chapter** designed genes considered to be important in the metabolic pathway of GSH in yeast, *GSH1, GSH2, MET4, GDH, CYSTE, CYSTF,* were studied. They were designed to increase γ-glutamylcysteine yield through overexpression and deletion of genes responsible for GSH synthesis in the production of GSH in the glutathione metabolic pathway. In the **third chapter** we evaluated the potential of γ-glutamylcysteine as an antioxidant and antimicrobial biocompound. The **fourth chapter** was focused on how γ-glutamylcysteine, which has promise in antioxidant testing, works against commercial antioxidants when added to sunflower oil, which is the most commonly used oil in industry and is oxidation-prone. In the **fifth chapter** we examined the potential of γ-glutamylcysteine as a sulfur (SO_2) substitute by incorporation into wine during maturation and determined its effect on wine aroma.

Chapter 1

Introduction

1. Glutathione homeostasis-maintaining dipeptide, γ-glutamyl cysteine: history, role in the cell, biosynthesis, limitation and industrial production

1.1 History

Gamma-glutamylcysteine (γGC, GC, gGC), found in the cytoplasm of almost all cells, and it is synthesized from L-glutamic acid and L-cysteine, catalyzed by the enzyme glutamate cysteine ligase (γ-glutamylcysteine synthase, GCL, EC 6.3.2.2,). γGC production plays a key role in glutathione (GSH) synthesis.

In 1983, Mary E. Anderson and Alton Meister first demonstrated that γ-glutamylcysteine could enhance the cellular GSH levels in a mouse model. Their research revealed that γGC synthesized in their lab was effectively taken up by cells, circumventing the rate-limiting step of the GCL enzyme, leading to increased GSH production [1,2]. Control experiments using constitutive amino acids, such as L-glutamic acid and L-cysteine, were ineffective in achieving similar results. Despite the promising findings, subsequent studies on γGC were limited due to its unavailability in commercial markets. However, with its recent commercialization, research into γGC's efficacy has resumed, highlighting its potential in various health applications.

1.2 Occurance and role in cells

γGC is detected intracellularly at 5–10 μM [3] In the cells, γGC reacts rapidly with glycine to yield GSH; hence the intracellular concentrations are generally low. GSH biosynthesis ends with the second and last reaction step catalysed by glutathione synthetase (GSS) [4].

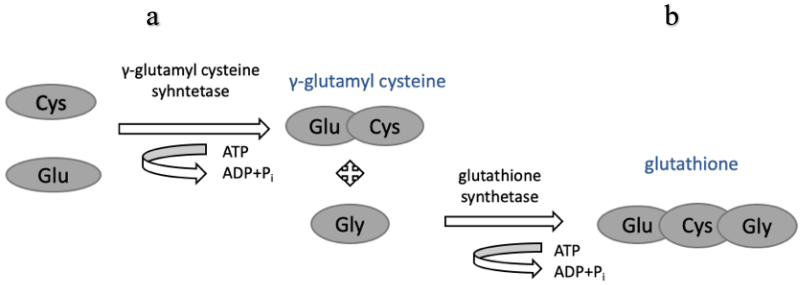


Figure 1. Glutathione synthesis pathway in the cell (a: γ-glutamylcysteine synthesis by γ-glutamylcysteine synthetase; b: glutathione synthesis by glutathione synthetase)

The possibility of the GSH precursor γGC being an important antioxidant in the cell has been emphasised. This dipeptide protects the γ-glutamyl bond, ensuring GSH stability and resistance to degradation [5]. Recently, γGC has been reported to be capable of functioning as a GPx1 cofactor in mitochondrial H_2O_2 detoxification, similar to GSH [6]. Furthermore, in a mouse model of

neurodegeneration, it has been shown that sufficient mitochondrial γGC production can prevent neuronal death (**Figure 2**). The antioxidant effect of γGC is believed to be responsible for survival in both cultured cells and GSS deficient individuals [7,8].

γGC is fundamental to cellular biochemistry and physiology, acting as a precursor to GSH, contributing to cellular redox balance [9,10]. In GSH-deficient cells, γGC may assume the biological role of GSH [11,12]. In a related study, it was reported that mice lacking the GCL gene died before birth and could not pass the embryonic stage. Considering that human cellular γGC production decreases with aging and the course of many chronic diseases, it has been suggested that γGC supplements may offer health advantages [13]. When GSH is acutely reduced to sub-optimal levels, such as during periods of intense exercise, trauma or intoxication, such γGC supplementation may also be beneficial.

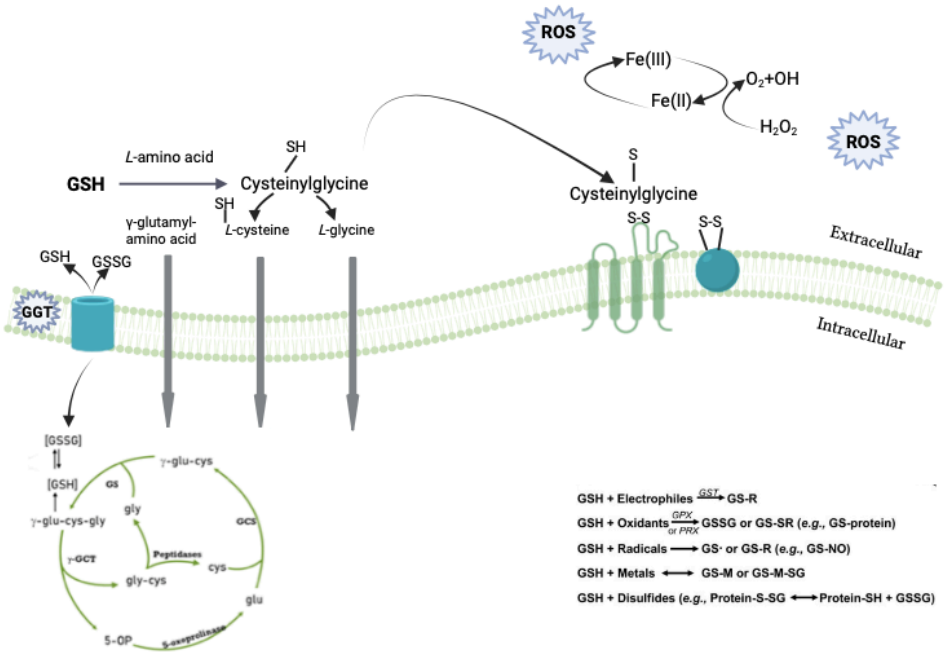


Figure 2. Homeostasis of reduced glutathione (GSH) and oxidized/disulfide (GSSG) glutathione in cells. ROS: oxidative stress (Reactive Oxygen Species), GGT:gamma glutamyl transferase, GS: Glutathione Synthetase, GCS: gamma glutamyl synthetase (adapted from Gaucher et al., 2018)

1.3 Bioavailability and biosynthesis

The homeostasis of GSH is maintained through de novo synthesis, recycling of glutathione oxidized (GSSG) and catabolism of GSH [9]. GSH is synthesised in two consecutive steps requiring ATP, the first of which is the formation of γ-glutamylcysteine from cysteine and glutamate by GCL. Glycine is then bound to γGC through the action of GSS to yield GSH. The GCL enzyme is regulated by non-allosteric feedback inhibition exerted by GSH, which competes with glutamate for binding at the enzyme active site [14,15]. GSS shows higher activity than GCL and there is no inhibitory regulation [2]. Consequently, the intracellular γGC level is low because it is immediately converted to GSH by GSS [3]. The rate of GSH synthesis is limited by both GCL activity and the availability of cysteine, whose

intracellular concentration is significantly lower than that of glutamate and glycine [5,16]. In the recycling pathway, GSSG is reduced by glutathione reductase in the presence of the cofactor NADPH, while γ-glutamyltranspeptidase (γGT) catalyses the hydrolysis of GSH with the release of free cysteine [5]. In normal physiological state, the GSH pool is mainly in reduced form [17].

1.4 Importance

The onset of ageing in mammals is associated with a significant decrease in both GCL activity and cysteine availability [18]. This low de novo GSH synthesis has been associated with the development of age-related diseases such as Alzheimer's disease [19] or Parkinson's disease [20]. Since cysteine availability is a rate-limiting factor for GSH synthesis, various cysteine prodrugs such as N-acetylcysteine (NAC) or NAC ethyl ester have been used in some clinical applications [18]. However, the aforementioned low level of GCL activity still limits a new synthesis. Similarly, external supply of GSH is not effective in human treatment. In fact, GSH has a half-life of 1.6 min in plasma [21] and passive transport of GSH from plasma into cells is thermodynamically unfavourable [1]. γGC has been proposed as an alternative drug to increase the intracellular GSH level [22,23]. Recent studies have shown that its oral administration increased GSH level in lymphocyte cell of healthy human [24], while it improved specific memory, neuroapoptosis and oxidative stress in mice with Alzheimer's disease [25]. γGC has also demonstrated the ability to reduce oxidative stress damage and neuroinflammation triggered by soluble amyloid β-oligomers in human astrocytes [26] and also in human endothelial cells [27]; it may also alleviate insulin resistance and hepatic steatosis [28]. In addition, it has been shown that γGC can substitute GSH as a cofactor of glutathione peroxidase 1 (GPX1), an enzyme commonly found in many human tissues that protects cells from oxidative stress [6]. All of these emphasise that γGC is promising in preventing or partially ameliorating chronic diseases or age-related disorders.

1.5 Limitation in industrial production

It is possible to synthesise γGC chemically and biologically. However, chemical synthesis on a large scale consists of complex steps such as protecting, coupling, unprotecting and forming the unconventional γ-glutamyl peptide bond to the reactive sulfhydryl group of cysteine. In addition, the technique utilises highly hazardous chemicals while yielding less than 10%, which raises safety and environmental questions. Over the years there have been numerous attempts to produce biological γGC by fermentation, none of which have been successfully commercialised. Although metabolic engineering and bioreactor processes have improved, some disadvantages of biotechnological production such as low volumetric yields, longer fermentation times, complex purification processes as well as high equipment costs limit commercial production [29-32].

1.6 Biological process of γGC in yeast

The *GSH1* gene in *Yarrowia lipolytica*, *ylGSH1*, encodes a protein that consists of a fragment of the 1884 bp, 678 amino acid residues. It bears 46% similarity in sequence of the corresponding gene in

Saccharomyces cerevisiae [33]. The *GSH1* gene is also upregulated in response to oxidative and nitrosative stress conditions, such as exposure to H_2O_2 and various chemicals or electrophiles, as part of the transcriptional activation process linked to the upstream heavy metal response. The transcription factor Yap1p, considered a central modulator in response to oxidative stress, is essential for *GSH1* gene induction [34-37]. In addition to the components of the oxidative stress response, regulators of sulfur assimilation pathways (specifically bZIP protein Met4p, an activator of most SUL1-3 and other sulfate utilization genes) are necessary for normal expression from this promoter. Met4p, Met31/32p and Cbf1p proteins have been noted by Bachhawat et al., 2009 [38] to be involved in the regulation of *GSH1*, Cbf1 is more of a repressor, and deletion of CBF1 causes derepression in the *GSH1* gene. As detailed in section 1.3, in all living organisms the *GSH1* gene is repressed due to a feedback process in the GSH metabolic pathway. In yeast cells, the functioning of the *GSH1* gene is inhibited by the increase of GSH. Therefore, this gene has been studied for a long time [39]. It has been reported that the *S. cerevisiae* YHT178 yeast strain, in which the γ-glutamylcysteine synthetase gene promoter has been replaced by a strong transcriptional promoter ΔP8, produces large amounts of γ-glutamylcysteine synthetase in its cell [40]. It was reported that strain YHT178, with increased expression of GCL, could accumulate a maximum of 1.69% of γGC in its cells in synthetic minimal medium. However, the growth rate of yeast in such environment has not been reported. Although the growth rate in YPD medium, which is more nutritious than synthetic minimal medium, has been reported, it cannot be said that the required growth rate has been reached at the industrial level even in YPD medium [32].

In this metabolic pathway, *GSH2* is the gene encodes a protein that called glutathione synthetase which has critical role in the biosynthesis GSH. Transcriptional regulation of the *GSH2* gene in response to oxidative stress is highly similar to that of the *GSH1* gene and is modulated by Yap1p. *GSH2* encodes the GSH with a length of 1473 bp and 491 amino acids. When the amount of glutathione increases in the cell, this creates a repressive effect on the Gsh1 protein. Thus, the synthesis of γGC stops. It has been reported that the knockdown of the *GSH2* gene increases the production of γGC in *S. cerevisia* [7]. Moreover, the reported γGC content of the YL1 strain in which the GSS gene is disrupted is as low as 0.533% and is unacceptable for practical use at the industrial level. Additionally, since the phenotype of the YL1 strain corresponds to the phenotype of a strain with partially reduced glutathione synthetase, it was noted that GSS was not completely eliminated from it [7]. However, the YL1 strain differs substantially from the GSS attenuated strain of the present invention in that it shows significantly different proliferation abilities during the logarithmic growth phase in a glutathione-containing medium and in a GSH-free medium.

In another report, Ootake and colleagues reported that GSH was not detected in the glutathione synthetase-deficient *S. cerevisiae* YL1 strain [40]. Inoue et al. reported that there was a disruption in the GS gene on a chromosome, and the glutathione content of the strain with the disrupted gene was measured, but GSH was not detected [41]. Therefore, it is thought that knockdown of the *GSH2* gene in *Y. lipolytica* will increase the production of γGC.

The dipeptide γGC plays a key role in the synthesis of cysteine and subsequently GSH. Cysteine, released from the sulphide reduced in sulfur metabolism through the cysteine synthetase enzyme, provides regulation in glutathione metabolism. The cysteine synthetase enzyme, consisting of three consecutive genes (YALI0D25168g, YALI0E08536g, YALI0F14047g), is involved in the last step of cysteine synthesis.

α-ketoglutarate, which plays an important role in the citric acid cycle, forms an crucial link between the carbon and nitrogen metabolism. The conversion of α-ketoglutarate to glutamate is a reversible reaction and is carried out by glutamate dehydrogenases (GDH). *GDH1, GDH2* and *GDH3* genes responsible for the Gdh enzyme have been extensively studied in *S. cerevisiae*. The studies were also carried out on two genes responsible for glutamate synthesis (*GDH1*, YALI0F17820g; *GDH2*, YALI0E09603g) in *Y. lipolytica* [42]. While α-ketoglutarate production is increased by deleting the *GDH1* gene and overexpression the *GDH2* gene, the opposite situation also occurs. γGC is synthesized by attaching the α-carboxyl group of glutamate to the amino group of cysteine. Therefore, γGC synthesis is closely related to the intracellular concentration of glutamate.

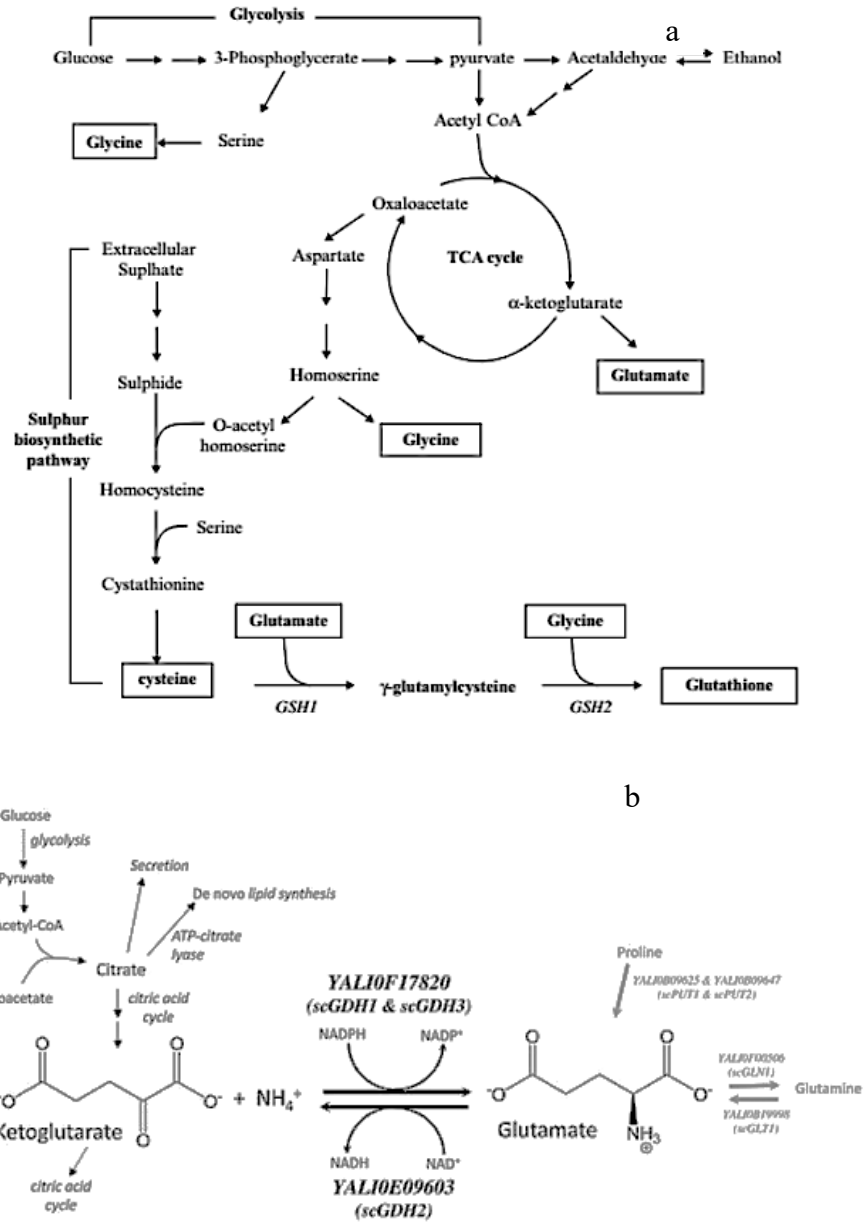


Figure 3. Sulphur metabolism and glutathione pathway in the yeast. a: Degradation sulphide in the yeast and glutathione production. *GSH1*, glutamyl syntetase, *GSH2*, glutathione synthetase. b: reversible glumatate pathway. *GDH1*, glutamate dehydrogenase 1, *GDH2*, glutamate dehydrogenase2 (adapted from Bachhawat et al., 2009)

1.7 γGC production in the yeast using genetic tools

Studies on this subject have begun and focused on increasing GSH production. The Table regarding GSH production in yeast using genetic tools is given below (**Table 1**).

Table 1. Directly and indirectly increasing γGC production in the yeasts by using metabolic and strain engineering tools

Yeast	Engineering	Ref
Saccharomyces cerevisiae	Identification *GSH1*	[40]
Saccharomyces cerevisiae, Candida utilis	Overexpression *GSH1, CYS4, DEF1*	[43]
Saccharomyces cerevisiae	Overexpression *GSH1, GSH2, CYS3, CYS4, YAP1*	[44]
Pichia pastoris	*GSH1, GSH2*	[45]
Saccharomyces cerevisiae	Overexpression *GPX1, GPX2, GPX3*	[46]
Hansenula polymorpha	Overexpression *GSH2, MET4*	[47]
Saccharomyces cerevisiae	Regulation of *GSH1* by H_2O_2	[48]
Saccharomyces cerevisiae	Overexpression *MET14, MET16, APA1, MET3*	[49]
Saccharomyces cerevisiae		[50]
Saccharomyces cerevisiae		[51]
Saccharomyces cerevisiae		[31]
Yarrowia lipolytica	Overexpression *GSH1, GSH2*	[33]
Yarrowia lipolytica	Overexpression *GSH1, GSH2*	[52]
Hansenula polymorpha	Overexpression *GSH1, MET4*	[53]
Saccharomyces cerevisiae FC-3	Regulation of γGC and GSH by kinetic analyses	[54]
Saccharomyces cerevisiae BCRC 21727.	Overexpression *GSH1, GSH2*	[55]

These approaches primarly focused on precursor enhancement, process optimization for increased biomass, efficient biotransformation of precursor amino acids, or strains improvement via evolutionary and metabolic engineering.

The inital production improvement stems from optimizing media composition. Additionally, using chemically defined media not only enables industrial scale-up but also allows for the use of low-cost feedstocks, potentially reducing production costs and consequently, the product price. The second area focuses on bioprocess control to optimize γGC production, avoid by-product formation and achieve high biomass. An additional approach to raise the γGC yield concentrated on increasing precursors in the amino acid biosynthetic pathway for the GSH synthesis. High γGC titers can be attained by supplementing bioreactor feeding with a medium containing a mix of cysteine and glutamate, along with other process control. Finally, we would like to note the importance of evolutionary or metabolic strain engineering for γGC production.

2. *Yarowia lipolytica*

2.1 Taxonomy, morphology and growth conditions

Yarrowia lipolytica, previously known as *Candida, Endomycopsis*, or *Saccharomycopsis lipolytica*, is a member of the Ascomycota family. The general name "Yarrowia" refers to Dutch researcher David Yarrow, and the species name "lipolytica" refers to its ability to hydrolyze lipids [56,57]. *Y. lipolytica* is an obligate aerobe yeast. The optimum growth temperature is between 25 to 30 °C, with the temperature limit for most strains being 32 to 34 °C. The most strains are psychrotrophic as they exhibit growth at 4-5 °C. *Y. lipolytica* can grow over a wide pH range: most species can grow between pH 3.5 and 8.0, while a few can tolerate pHs as low as 2.0 and pHs as high as 9.7. Depending on where it exist, *Y. lipolytica* can tolerate high salt concentrations in saline environments containing 7.5% NaCl for most strains and 15% NaCl for a few. It is known that this yeast can adsorb heavy metals such as Fe, Ni, Cr, Cu, Cd and Zn. It has also been reported to be a potential microorganism for the bioremediation of wastes containing these metals [58]. *Y. lipolytica* can use a wide variety of substrates with hydrophilic or hydrophobic structures as carbon sources [59,60]. Water-soluable carbon sources are limited to a few sugras (such as glucose fructose, and mannose), glycerol, organic acids and alcohols. It was previously thought that *Y. lipolytica* could only utilize certain hexoses and not pentoses as the sole carbon source. However, recent experimentson xylose assimilation in some strains have disproved this believe. Similarly, the belief that lactose could not be a substrate for *Y. lipolytica* was changed by the *Y. lipolytica* Po1d strain, which was engineered to express β-galactosidase, an enzyme that hydrolyzes lactose to glucose and and galactose [61].

Y. lipolytica is capable of utilizing use acetic, lactic, propionic, malic, succinic, citric andoleic acids as the sole sources of carbon and energy, generally without requiring pH modification [62]. In citric or rubber acid medium with glucose *Y. lipolytica* presented diauxic growth, while propionic, butyric and sorbic acids resulted in inhibition of yeast growth in glucose medium [63].

Y. lipolytica has been shown to exhibit the potential to efficiently use acetate as the sole carbon source for growth in most strains. Concentrations of sodium acetate up to 0.4% resulted in only limited growth inhibition, while higher concentrations reduced the growth rate even further and caused suppression of growth at levels above 1.0%.

Ethanol up to 3% concentration as a carbon source was previously studied by Barth and Gaillardin and they reported that higher concentrations of ethanol are toxic to *Y. lipolytica* [59].

Glycerol is a carbon source for many yeasts under aerobic conditions and can be assimilated by the glycerol-3-phosphate or dihydroxyacetone pathways. Eukaryotes some yeasts are believed to assimilate glycerol through dihydroxyacetone. Crude glycerol was also used as carbon source to grow yeast and produce citric acid or biocompounds. High initial glycerol (40 g.L-1) leaded to up to 35 g/L excretion of citric acid under nitrogen limitation. A significant level of lipid production has also been recorded by this yeast using glycerol as a carbon source [64].

Wild-type *Y. lipolytica* isolates offer a high potential for the utilisation of solid and liquid wastes from industrial and agricultural sources, including various water-insoluble carbon sources such as triglycerides, fatty acids, and alkanes, especially crude glycerol obtained from biodiesel production processes [58].

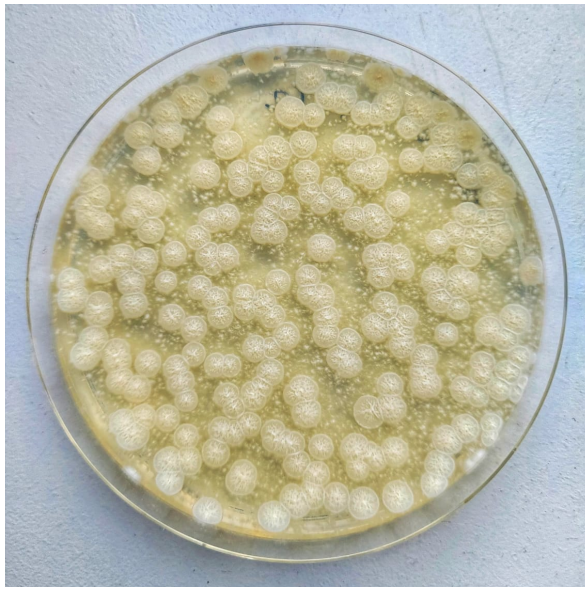


Figure 4. *Y. lipolytica* on YPD medium

This yeast, capable of degrading hydrophobic substrates, has served as a model for research on lipid metabolism, dimorphism , protein secretion and peroxisome formation [65,66]. *Y. lipolytica*, which is used in the industry for the microbial oil production containing citric acid and highly unsaturated fatty acids, also has the ability to synthesize many valuable metabolites such as erythritol, γ-decalactone, lipases, proteases and other hydrolytic enzymes [67-70]. *Y. lipolytica* is recognized as a safe microorganism, with several yeast based production processes receivied the GRAS (Generally Recognized as Safe) status from the FDA (Food and Drug Administraion, USA) since 2016. It is also

classified as Biosafety Level1 (BSL1) microorganism Washington DC Public Health Service. Aditionally IDF (International Dairy Federation) and the EFFCA (European Food and Feed Cultures Association) have identified it is a " microorganism documented for use in food" [71].

2.2 Genetics, its importance in metabolite engineering

Among non-conventional yeasts, the dimorphic *Y. lipolytica* possesses attractive features to be used as a robust cell factory for metabolic engineering of bioproduct production [72-75]. With the advent of metabolic engineering, more pathways were used for synthesis and productivity was increased [76-79].

Due to the development of molecular biology technologies in the 1980s, *Y. lipolytica* received attention as a heterologous protein expression host (in connection with new genetic systems including non-reversible auxotrophic strains, shuttle vectors, and transformation methods). The development in genetic engineering of *Y. lipolytica* has enabled a company to market cloning and gene expression tool kits YLEX (Eastern Biotech Co., Taiwan).

Both classical metabolic engineering methods (endogenous gene repression and deletion, heterologous gene repression) and more recently developed CRISPR-derived strategies (CRISPR Cas9 gene editing tools, gene activation, artificial chromosome design) have been used to transform the *Y. lipolytica* strain into an efficient cell factory. This tools can be used to redesigned metabolic pathways in order to synthesize a desired product. New approaches to engineering metabolic pathways are realized in the context of cofactor availability, oxidative environment and compartmentalization within different subcellular compartments all together modifying cellular behavior with an unknown impact on metabolic flux. Additionally, the resultant GM (genetically modified) strain can be further improved using multiomics technology for in silico modeling of genome-scale metabolic pathways to identify bottlenecks and limiting factors, indicating the next genetic engineering targets in a sustainable manner. Adaptive evolution strategy is an alternative to enhance evolution of GM strains cope with stresses under industrial growth conditions. Furthermore, they demand bioprocess engineering to design economically and environmentally production of cell factories.

The 20.5 Mb genome of E150, the first *Y. lipolytica* strain to be fully sequenced, assembled and annotated, consists of 6 chromosomes ranging in size from 2.6 to 4.9 Mb. This genome size is about twice that of in most other yeasts, included *S. cerevisiae* (12 Mb) [80]. A few other genomic features clearly highlight *Y. lipolytica* among all other hemiascomycetous yeasts. In particular, the G/C ratio, which averages 49% and about 53% across genes, and the percentage of intron-containing genes, which is 15%, are significantly higher compared to other yeasts (38%, 40% and 5% in *S. cerevisiae*, respectively) [81]. In contrast, although on the strong side of the range for hemiascomycetous yeasts, the gene number is not as high as one might infer from the large genome size. *Y. lipolytica* has a total gene count of 6703, which is more than the 5807 genes in *S. cerevisiae*, but less than the 6906 genes in *Debaryomyces hansenii*, both of which have genomes of around 12 Mb.

Genetic studies in *Y. lipolytica* have primarily been conducted in three popular genetic frameworks:

The strain W29 (French Wild-type strain ATCC20460™) is the most common strain in yeast collections world-wide isolated from sewage. It has become a kind of reference to compare the performance of new *Y. lipolytica* isolates or alternative *Yarrowia* strains for the growth or production of several compounds [82].

The first French/US *Y. lipolytica* strain inbreeding program aimed to develop new strains and create genetic maps by mating isolates of industrial interest, specifically W29 (MatA) and ATCC 18942 (MatA MatB). Both mating types undergo sporulation to form ascospores; multiple stages of genetic engineering allowed for the design of "sister" GM strains E129 and E150 of the compatible mating types [59]. The parent strain ATCC 18942, which naturally contains the Ylt1 retrotransposon, gave rise to the E129 (MatA, lys11-23, leu2-270, ura3-302, xpr-322 Lys-, Leu-, Ura-, Suc+, ΔAEP) and E150 (MatB, his1 , leu2-270, ura3-302, xpr-322 His-, Leu-, Ura-, Suc+, ΔAEP) strains. Both of these strains have multiple copies of the retrotransposon as well as solo zeta sequences, which can serve as dispersed targeting elements for further genetic engineering. E129 and E150 are both trioxotrophic strains carrying the ura3-302 allele, which results from the disruption of the *URA3* gene by a heterologous cassette expressing ScSUC2, allowing them to metabolize sucrose [83]. Additionally, these strains have a deletion of the major secreted protease AEP (alkaline extracellular protease repressed at neutral/alkaline pH), encoded by the *XPR2* gene, making them useful for heterologous protein production applications [59]. Strain E129 was one of the first strains applied to heterologous protein production.

The Po1 series derived from a series of backcrosses between W29 (MatA, Ura3-302, Ura-, Suc+), and CBS6142-2 (Po1a (MatA, leu2-270, ura3-302, Leu-, Ura-, Suc+), Po1d (MatA, leu2-270, ura3-302, xpr2-322, Leu-, Ura-, Suc+, ΔAEP), Po1e (MatA, leu2-270, ura3-302::URA3, xpr2-322, Leu-, Suc+, ΔAEP), Po1f (MatA, leu2-270, ura3-302, xpr2-322, axp1-2 Leu-, Ura-, Suc+, ΔAEP, ΔAXP), Po1g (MatA, leu2-270, ura3-302: :URA3, xpr2-322, axp1-2, Leu-, Suc+, ΔAEP, ΔAXP), Po1t (MatA, leu2-270, LEU2, ura3-302::URA3, xpr2-322, axp1-2, Suc+, ΔAEP, ΔAXP)) have been used for a number of studies, especially on heterologous protein production [84,85].

2.3 Main genetic tools used in heterologous protein production: vectors, selective markers, expression cassette

The vectors employed for transforming *Yarrowia* are shuttle vectors, similar to those used in other yeasts. These vectors are generally developed and maintained in *Escherichia coli* before being introduced into the target organism. They consist of plasmid backbone (bacterial component), a marker for expression or secretion in *Yarrowia*, an expression/secretion casette, and elements necessary for transformation and maintenance within yeast cells. These genetic elements of vector are illustrated in a virtual vector in **Figure 5** and described in more detail below. The shuttle vector is introduced into *E. coli* using conventional molecular biology techniques and then transferred to the target *Yarrowia* receptor strain of interest by using physical or chemical method.

Episomal vectors replicate independently within cells, unlike sequences integrated into the genome. No naturally occurring episomal plasmid has been found in *Y. lipolytica* so far. Therefore, synthetic episomal vectors have been developed by combining autonomously replicating sequences (ARS) and centromeres (CEN) from the *Y. lipolytica* genome [86,87]. These vectors are typically present in low copy numbers (1-3 copies per cell) and require constant selective pressure to be retained. As a result, episomal vectors are mainly used for transient protein expression, such as utilizing Cre recombination for selection marker rescue.

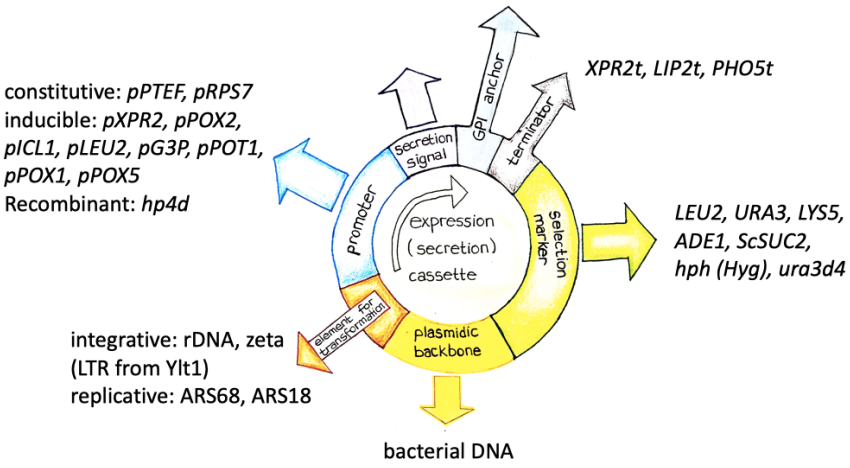


Figure 5. Scheme of a expression vector of heterologous proteins in *Yarrowia*

As previously mentioned, due to their stability in cells, integrated vectors are preffered for protein production in *Y. lipolytica*. Autocloning vectors have been designed to facilitate the targeted genomic integration cassette devoid of the bacterial part used for subcloning [88].

Le Dall et al., (1994) focus on the the rDNA cluster to perform multiplex integrations using the missense selection marker ura3d4 [89]. The other target includes an artificially created pBR322-based docking platform located at the URA3 locus of the Po1e and Po1g cell lines of this yeast.

A more natural placement platform is the zeta element, which represents an interesting situation. A strain originating from an American isolate (YB423), either directly or through breeding programs, contains the Ylt retrotransposon with long terminal repeats (LTRs) known as zeta elements. These are present in multiple copies throughout the genome and can serve as good sites for integration of plasmids which are engineered to carry homologous sequences. Examples of such strains are the widely used E129 and E150 strains studied by Madzak et al., [90]. Barth and Gaillardin (1996) reviewed the inbreeding programs. Surprisingly, in the zeta-negative W29 strains and derivatives like the Po1 series which is a frequently used host for proteins expression cassettes carrying zeta sequences are integrated into the genome via NHEJ (Non Homolous End Joining) as random insertion [59]. Other than random integration into zeta-free strains, this method is an alternative for conducting random mutagenesis studies [91]. It is however generally advantageous to perform targeted integration due to the undesired

effects of random insertional mutagenesis/increased probability for homogeneity among transformants. Bordes et al., (2007) created a zeta docking site by targeted integration into the LEU2 locus of a zeta-free strain to improve transformation efficiency and minimise clonal differences [92]. Juretzek et al., (2001) used ura3d4 to target various integration sites such as rDNA, zeta elements, and the gene-specific xpr2 locus; they observed that the missing marker identifies multicopy integration events regardless of the integration target [87]. Recently, Holkenbrink and colleagues (2018) identified 11 intergenic regions that allow high gene expression levels without affecting cell growth [93]. Deletion of the KU70 (a key protein involved in the NHEJ pathway) component of the NHEJ recombination machinery allowed the homologous integration sequences of the integration cassette to be shortened by up to 50 bp, thus simplifying the construction and manipulation of integration vectors [94].

Selection reagents re employed to identify successful transformations and to maintain episomal vectors within cells by applying selective pressure. For *Y. lipolytica*, common selection markers; hygromycin B, phleomycin, nourseothricin, and zeocin [95,96]. Auxotrophic markers used in *Y. lipolytica* typically orginate from essential amino acids biosynthesis pathways, such as leucine (*LEU2*), and lysine (*LYS5*) genes, or nucleobasis like uracil (*URA3*) and adenine (*ADE1*) [90]. As previously noted a promoter-defective allele of URA3 (ura3d4) requires multiple copies to restore a viable genotype, leading to multi-copy integration [87,89]. The *SUC2* gene from *S. cerevisiae*, which encodes invertase that enables growth on sucrose, has been used as a catabolic marker for *Y. lipolytica* [83]. By disrupting the *EYK1* gene (YALI0F01606g) from *Y. lipolytica*, which encodes an erythrulose kinase essential for growth in erythritol-based media [79], an auxotrophic strain was generated for complementation with an integrant carrying the functional version of this gene as a marker [97]. Traditional multistep genome editing often involves the sequential use of multiple selection markers and/or the rescue of these markers using systems like Cre-lox, which has been applied to this yeast [98].

2.4 Basic components for the expression cassette

In *Yarrowia* efficient expression of heterologous genes requires the use of sandwich vectors (cloning vectors) where the gene is positioned between yeast promoter and terminator sequences [99]. This combination forms the essential “expression casette”, which can optionally include a secretion signal (from a *Yarrowia* genom the heterologous gene itself) and a memvrane-targeting signal, such as a GPI (glycosylphosphatidylinositol) anchor for surface display.

In biology, a promoter is a piece of DNA that initiates the transcription of genes. Promoters are located close to the transcription start site of the relevant gene and on the same DNA strand as the gene. Promoters are located towards the 3' end of the opposite complementary DNA sequence before the gene sequence they initiate transcription. Promoters generally have different nucleotide lengths, but are between 100 and 1000 nucleotide pairs long. Selecting the right promoter is critical for the design of any engineering project. To produce a heterologous protein, the promoter must provide robustness and either constitutive expression or inducibility by a process-compatible inducer. When introducing or optimizing

a metabolic pathway, it is important that the expression of each gene can be fine-tuned as needed. The isolation and development of natural promoters has gained much attention in the yeast *Y. lipolytica*. Several natural promoters such as pFBA, pGPD, pTEF, and pFBAIN, have been characterised as constitutive promoters [74]. Other promoters, such as pLIP1 and pPOX1, were also isolated from genes involved in lipid metabolism (LIP1, encodes lipase) and β-oxidation (POX1, encodes peroxisomal acyl-CoA oxidase) respectively and the expression of these genes was triggered by the addition of oleic acid to the culture medium [100].

The strength of certain promoters, like pTEF, can be enhanced by inserting multiple copies of the upstream activation sequence (UAS) from pXPR2. A new UAS from pTEF was discovered and used to create a powerful hybrid promoter. A modular approach, combining UAS, a core promoter, and a TATA box, was employed to develop a series of hybrid promoters with increased effectiveness [28]. Among these, hp4d (which contains 4 copies of UAS1 from XPR2) has been commonly used for heterologous protein production [101], though it has been found to be growth phase-dependent rather than strickly constituve. Recently, a series of erythritol-inducible synthetic promoters, based on UAS combinations from pEYK1, with or without additional copies of UAS1B from XPR2, demonstrated up to 45-fold greater potency compared to pTEF [102]. Further modification of pEYK1 and pEYD1 have resulted in a range of promoters with varying strengths. However, it has been noted that increased promoter strength does not always correspond to improved productivity.

A terminator, or transcription terminator, is a nucleic acid sequence that signals the end of a gene during transcription in genomic DNA. The selection of an appropriate terminator is crucial [103]. Commonly used terminators in *Y. lipolytica* include XPR2t, minimal XPR2t, LIP2t, and PHO5t [88]. Additionally, synthetic terminators, which are shorter and provide comparable or superior performance to natural ones, have also been applied in this yeast [103].

2.5 Advanced cloning and genome editing techniques

In recent years, several multiple cloning technologies based on Golden Gate Assembly and Biobricks principles have been adapted for *Y. lipolytica* to improve recombinant protein production and introduce synthetic pathways for metabolite synthesis [104,74]. Additionally, the "EasyClone YALI" genetic toolbox employs CRISPR/Cas9 technology to achieve marker-free single or double gene knockouts and enable high gene expression at 11 specific intergenic sites [93].

2.6 Yarrowia lipolytica, an unconventional yeast, is a biofactory with metabolites produced intracellularly and extracellularly

The great potential of *Y. lipolytica* for industrial uses was realised more than 70 years ago. Initially, the production of biomass and metabolites was targeted and registered wild-type isolates or conventionally modified mutant strains were used [105-107, 58]. In the 1950s to 1970s, *Y. lipolytica* was used by the British Petroleum Company (BP, London, UK) to break down alkanes and produce single-cell protein (SCP) from crude oil, which was marketed as animal feed. In 1970, Pfizer Inc. (New

York City, USA) utilized *Y. lipolytica* for industrial citric acid production. Archer Daniels Co. (ADM, Chicago, USA) produced citric acid using *Y. lipolytica* with corn or rapeseed oil as the substrate [73]. Recent works on citric acid production with wild-type *Y. lipolytica* isolates have focused on obtaining new yeast strains capable of using glycerol as a substrate, in order to utilize biodiesel-derived crude glycerol waste [108]. Studies in this field have shown that citrate mutants increase the citric/isocitric acid ratio on the degradation of crude glycerol compared to wild-type strains [109]. Additionally, it has been suggested to use whey as a sustainable source basedon waste materials for lactose-positive *Y. lipolytica* strains that will produce citric acid [110]. The production of other organic acids, such as isocitric acid, succinic acid, and α-ketoglutaric acid, from wild-type or conventionally developed *Y. lipolytica* strains is also a significant area of research for industrial production platforms [58].

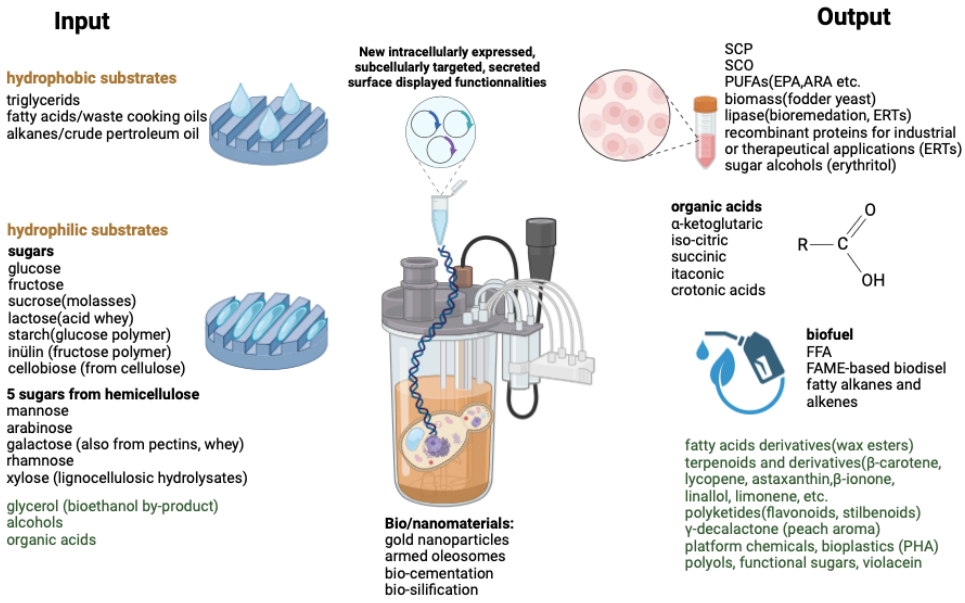


Figure 6. *Yarrowia lipolytica* use, substrate requirements and products diagram through conventional and bioengineering tools in biotechnology

3. Potential for γGC to be used as an additive in foods

3.1. Antioxidant potential of γGC

Oxidation is a series of reactions that reduce the likelihood of food acceptance by consumers by causing the production of low molecular weight, flavourless compounds, toxic compounds, lipid and protein dimers or polymers. Food oxidation can be reduced by shielding food from light and eliminating pro-oxidants such metals, free fatty acids, and oxidized substances. It is extremely difficult to remove all air and pro-oxidants, foods are fortified with antioxidants to slow down the oxidation process. Antioxidants significantly delay or inhibit the oxidation of oxidizable substrates even at low concentrations in foods with high lipid and protein content [111].

Oxygen acts on the fats, carbohydrates and proteins of the food, causing more or less noticeable quality decreases. This spontaneous interaction between and atmospheric oxygen and food components

is called "autoxidation" [112]. Oxidative degradation leads to several specific effects, including the development of rancid flavors and odors in fats, oils, and fat-containing foods. It causes pigment discoloration, the formation of toxic oxidation products, and the alteration or loss of taste and aroma. Additionally, oxidative damage results in texture deterioration and a reduction in nutritional value due to the breakdown of vitamins A, D, and E, as well as essential fatty acids, particularly linoleic acid.

Oxidation is classified as carbohydrate oxidation, protein oxidation and fat oxidation in foods [113].

Color and aroma changes occur with carbohydrate oxidation in foods. Color deterioration is usually manifested by the formation of brown, shaded, gray and yellow color [114]. Carbohydrate oxidation is mainly caused by Maillard and enzymatic reactions. Maillard is the most known non-enzymatic browning reactions and it takes place between reducing sugars and amino groups of amino acids. The negative effects of this reaction can be eliminated by adding sitric acid, ascorbic acid or other organic acids. Browning, which occurs when carbohydrates are oxidized by enzymes such as peroxidase or catalase, is associated with bad odor and bad taste. The only way to prevent such oxidation is to inactivate the enzyme by heat treatment [115].

Protein oxidation may not be immediately apparent in the foodstuff, producing off-flavors. Proteins are broken down by proteolytic enzymes and denatured by reactions such as heating and hydrolytic reactions. Especially color pigments bound to proteins change color by oxidizing very quickly [116]. This type of color change cannot be prevented by any food additive.

In lipid chemistry, oxidation is one of the most important reactions. This self-catalysed reaction chain consists of initiation, propagation and termination [117]. In addition, oxidation products catalyze the reaction.

The deterioration of the taste and aroma of food is accompanied by the presence of lipid hydroperoxides, carbonyl compounds, hydrocarbons, ketones and some other compounds that cause the rancidity of food and damage to the cells of the body. Degradation of oils is divided to four main groups.

Hydrolysis, formation of soap-like structure, taste and odor in food resulting from free fatty acids and glycerol formed. Rancidity: auto-oxidation of unsaturated fatty acids resulting in a bitter taste. Deversion: changing taste caused by oxidation of linoleic acids in vegetable oils, fish oils and other foods containing highly unsaturated fats. Polymerization: a flavour change in unsaturated fats caused by the breaking of the chain between two carbon atoms (C-C), the formation of oxygen bonds [118].

Initiation	$ROOH^{*} \rightleftharpoons RO^{\cdot} + H^{\cdot}$
	$ROOH \rightleftharpoons RO^{\cdot} + {}^{\cdot}OH$
	$2\,ROOH \rightleftharpoons RO^{\cdot} + H_2O + ROO^{\cdot}$
Propagation	$R^{\cdot} + O_2 \rightleftharpoons ROO^{\cdot}$
	$ROO^{\cdot} + R^1H \rightleftharpoons ROOH + R^{1\cdot}$
Termination	$ROO^{\cdot} + R^1OO^{\cdot} \rightleftharpoons ROOR^1 + O_2$
	$RO^{\cdot} + R^{1\cdot} \rightleftharpoons ROR'$

Oxygen is the primary factor responsible for initiating or speeding up oxidation, while light, temperature, metal ions like iron and copper, certain pigments, and the level of unsaturation further

accelerate the oxidation process. [119]. If these factors are eliminated, oxidation is also eliminated. However, this is not possible in practice. Therefore, it is very difficult to prevent autoxidation without adding any external substance. In cases where autoxidation cannot be prevented by physical and technological methods, antioxidants and synergists are used as additives [120].

Antioxidants are the most effective substances used in the production, storage, transport and marketing of vegetable and animal oils and fatty foods in the food industry to prevent the deterioration and bitterness of foods for a period of time by delaying the action of atmospheric oxygen at normal temperatures. They do not improve the quality of food and do not impart off-flavours or odours [112]. In order to increase or complement the effect of antioxidants, substances called “synergists” are often used. Combining substances has antioxidant effects, such as polyphenols and carotenoids, vitamin E, or vitamin C with commercial antioxidants, has been reported to be beneficial, as this approach can create a synergistic effect. This not only enhances efficacy but also reduces the need for higher amounts of food additives that may pose health concerns [121,122].

Antioxidants used in food products must be completely soluble in the water and oil phase of the product and must have high penetrating power into the product, must have low volatility, not add color or dye to the product, must be odorless and tasteless not be toxic and not have an effect on the skin, not be harmful when consumed with food, must be effective in small amounts, must be easy to obtain, must be inexpensive and be listed on the food label with the name, amount and purpose for which it is added [123].The use of antioxidants added directly to foods is limited. An antioxidant must be shown to be safe before it can be used in foods.

Table 2. Antioxidants naturally in foods and antioxidants used in food production

Major antioxidants in foods	
Phenolic compounds	tocopherols, polyphenols, phenolic acids, lignans
Ascorbic acid	ascorbic acid, sodium ascorbate, calsium ascorbate
Carotenoids	β-carotene, lycopene, lutein, capsanthin
Protein related compounds	cysteine, hypoxanthine, xanthine , glycine, methionine , histidine , tryptophan , proline, lysine, ferritin, transferritin, carnosine
Maillard reaction products	β-lactoglobulin, isomaltooligosaccharides
Phospholipids	phosphatidylethanolamine, phosphatidylcholine, phosphatidylinositol, phosphatidylserine
Sterols	β-sitosterol, stigmasterol, sitostenol
Antioxidants in food industry	
Antioxidants that bind and form complexes with free radicals	Butylated Hydroxyanisole (BHA), Butylated Hydroxytoluene (BHT), Tertiary butylhidroqumone (TBHQ) Gallic Acid Esters (Gallates) tocopherols amino acids, peptides, proteins
Reducing agents	ascorbic acid (vitamin C), sulphides, erythorbic acid, glucose oxidase
Sequesterants	citric acid, polyphosphates ecylenediaminetetraacetic acid (EDTA)

Hudson, (2012); Anwar et al., (2018)

Sulfur-containing compounds can act as antioxidants in foods by helping to prevent or reduce oxidation processes that can lead to spoilage of food products. Oxidation is a chemical reaction that can cause deterioration of flavors, colors, and nutritional value in foods [124]. Sulfur-containing antioxidants work by donating electrons to free radicals or chelating metal ions, thus preventing or slowing down oxidation reactions [125].

Sulphur hydride groups in amino acids, peptides and proteins are involved in anion, cation andfree radical reactions in vivo and in vitro. Negatively charged sulfur anions (-RS) have high chemical reactivity. Sulphur hydride groups are directly involved in numerous and various chemical reactions and biochemical processes, in addition to serving as precursors for disulphide (SS) bond that stabilize proteins. The antioxidant and detoxyfying effect of -SH containing amino acids such as methionine, cysteine, homocysteine and taurine stem from their ability to function as reducing agents, oxygen radicals scavengers, precursors of cellular GSH and inducing cellular detoxification.

In food applications, histidine has been identified as a potent antioxidant in herring oil emulsion by the oxygen consumption assay. Lysine, histidine, methionine, threonine have also been noted to slow the rate of oxygen uptake in the safflower oil-water emulsion. Amino acids containing thiols such as

cysteine, arginine, methionine, lysine and and tryptophan have been found to exhibit the strongest antioxidant activity under deep-frying conditions. By generating thiols and maillard peptides, l-cysteine increases the antioxidant activities of Maillard reaction products. Besides increase their potential for use in industrial flavor enhancers.

GSH, a powerful antioxidant naturally produced in the body, has gained attention for its potential benefits in the food industry. While it can be found naturally in foods, its use as an additive for various purposes such as antioxidant, preventing enzymatic browning, flavor enhancement, health supplement, preventing protein degredation, meat and sea food processing (lipid oxidation) has recently become widespread. Some studies on the use of GSH as an additive are listed in the **Table 3**.

The antioxidant effect of γGC in foods is primarily attributed to its ability to stimulate the synthesis and activity of GSH, the main antioxidant, and its participation in various redox reactions. How γGC contributes to the antioxidant properties of foods is described below:

GSH precursor: γGC is a precursor of GSH synthesis in cells. GSH contains cysteine and its synthesis is usually limited by the availability of cysteine. γGC helps to overcome this limitation by providing cysteine for GSH synthesis [5].

GSH Recycling: GSH plays a role in redox reactions by acting as a reducing agent. It can neutralise reactive oxygen species (ROS) and free radicals. After participating to these reactions, glutathione is oxidised (GSSG). γGC can promote the recycling of GSSG to its reduced form GSH, which is critical for the maintenance of cellular antioxidant capacity [126].

Enhancing antioxidant activity: by increasing the availability of GSH, γGC indirectly increases antioxidant activity in cells and tissues. This helps protect food products from oxidative degradation by minimising damage caused by free radicals and other pro-oxidant compounds [127].

Food preservation: In the food industry, γGC can be used for shelf life extension of products like meat [128] and beverages [129]. It helps to prevent discolouration, off-flavours and degradation of nutritional components due to oxidation.

Synergistic effects: γGC can work in synergy with other antioxidants and preservatives commonly used in food processing. This synergy may enhance the overall antioxidant effect by allowing the concentration of other potentially less desirable additives to be reduced [130]. The effectiveness of glutamyl cysteine as an antioxidant in foods may depend on factors such as food matrix, processing conditions and the presence of other antioxidants and pro-oxidants.

γGC's function as an antioxidant in food is mainly attributed to its ability to promote the production and activity of GSH, the primary antioxidant, as well as its involvement in a number of redox processes. However γGC contributes to the antioxidant properties of foods the effectiveness of glutamyl cysteine as an antioxidant in foods may depend on factors such as: food matrix, processing conditions and the presence of other antioxidants and the presence of pro-oxidants.

Table 3. GSH applications in some food products

Aim of effect on product	Product		Ref
Aroma	Beer	Improving the anti-aging properties of beer by increasing the yeast's (*S. cerevisiae*)production of sulfur dioxide and GSH	[131]
	Wine	High-GSH producing yeasts obtained by genetic improvement strategies for novel wine strains	[132]
	Food packaging	Improving method for the production of food packaging film with enhanced GSH stability	[133]
	Yogurt	Investigating the industrial production potential of two liver-protective factors (GSH and S-adenosine-L-methionine) in yoghurt form through a new pathway	[134]
	Beef extract	Investigating flavor characteristics of GSH in raw and cooked foodstuffs.	[135]
	Beef soup	To evaluate the flavor and consumer acceptability of bef soup with GSH /MSG added	[136]
	Rice batter	Improvement in the bread-making guality of gluten-free rice batter by adding GSH	[137]
	Beef soup	Impacts of GSH maillard reaction products on sensory characteristics and consumer acceptability of beef soup	[138]
	Sparkling wine	Effect of GSH addition in sparkling wine	[139]
	Sparkling wine	To test effect of GSH during bottle storage of sparkling wine	[140]
	Wine	Detection of GSH and its precursor γGC in wine and model wine supplemented with oenological inactive dry yeast preparations	[141]
	White wine	To test effect of GSH addition on volatile profile of Trebbiano and Bombino Bianco wine	[142]
antioxidant	White wine	Browning susceptibility of white wine and antioxidant effect of GSH	[143]
	White wine	Investigation the antioxidant action of GSH and the ascorbic acid/ GSH pair in a model white wine	[130]
	White wine	Determination of the GSH effect by addition at harvest on Sauvignon Blanc wines	[144]
	Linseed oil	To test the influence of GSH on the oxidation of fats and fatty acids	[145]
	Corn oil	Inhibition of corn oil oxidation by N-acetyl cysteine and GSH	[146]
	Butter	Inhibition of butter oxidation by N-acetyl-cysteine and GSH	[147]
Functional food	GSH-enriched biomass	GSH-enriched baker's yeast: production, bioaccessibility and intestinal transport assays.	[148]

3.2 Potantial as an aroma enhancer

Recently, the effect of volatile thiol compounds containing sulfur on aroma has been widely investigated. The thiols 3-mercapto-hexan-1-ol (3MH) and 4-mercapto-4-methylpentan-2-one (4MMP), which form the basis of acetate formation especially in wines, contribute to the characteristic aroma formation of wine [149]. With the understanding of the importance of these thiol compounds, their precursors became the subject of research. The reason was investigated, especially since the amount of thiol in fruit juice and fermented product has a significant difference. The precursors of thiol compounds include cysteine S-conjugates (Cys-3MH and Cys-4MMP), glutathione S-conjugates (G-3MH and G-4MMP), Cysteinyl-glycine S-conjugate to 3MH (CysGly-3MH), sulfonic acids, has been studied previously [150]. The three known biosynthetic pathways—cysteine S-conjugates, glutathione S-conjugates, and the hexenal pathway—fail to account for the total production of thiols in wine. When the theoretical amount of thiols produced by these pathways is estimated from precursor concentrations, it falls significantly short of the actual thiol levels detected in the corresponding wines. Therefore CysGly-3MH and Cys-4MMP precursor compounds were also investigated in Savignon blanc wine [151].

Glutamyl cysteine, glutamyl dipeptides and cysteine are also constituents of some food such as maize [152], legumes [153] and fruits [151]. Especially g-glutamyl derivatives exist naturally as flavor enhancers in garlic [154] and onion [155].

A yeast extract containing 1% or more of γGC by weight, based on solids; it has been subjected to processes that involve treating it with gammaglutamylpeptide to produce a product rich in cysteine, which can enhance the flavor of foods or beverages (beef flavor, dried bonito flavor, etc.) [156].

3.3 Potantial as an additive to produce functional food

A functional food can be in the form of a natural food, or have been fortified with a bioactive compound such that it is isolated or concentrated and potentially used as a dietary supplement. This component could be incorporated, removed or biologically modified into a food to obtain health benefits specially for healthy growth and development; support the regulation of metabolic processes; protect against oxidative stress; maintain cardiovascular system and gastrointestinal physiology, support cognitive performance and mental well-being as well as physical function [157]. In fact, Japan, the United States and European countries constitute the highest functional food consuming [158]. Functional food consumption is predicted by gender, age, education and health status [159].

In some cases, disease-associated cellular GSH depletion may be primarily due to a decrease in the expression or activity of the first biosynthetic enzyme of GCL [160]. Studies in rodent models have shown that GCL levels decrease with age, which correlates with an age-related loss of homeostatic GSH [161,162]. The second enzyme responsible for GSH synthesis,GS, is a much simpler homodimer, consisting of two identic catalytic subunits. It generally has a higher specific activity than GCL, so that cellular levels of γGC are negligible [163]. Many diseases [164-166] are related to reduced GCL activity

due to genetic or environmental factors that reduce GSH homeostasis to levels insufficient to protect against oxidative stress onset. This supports the theoretical potential of using γGC as a means of increasing intracellular GSH levels. Since cytosolic concentrations of γGC are on the order of 7 μM [163], unlike GSH, any passive flux of exogenous γGC will be directed into the cell [167]. If cellular GSH depletion occurs as a result of disruption of the regulatory control of GCL activity, NAC or other cysteine prodrugs would theoretically unexpectedly increase GSH levels above reduced homeostasis [168]. On the other hand, exogenous γGC taken intact should feed directly on the unregulated enzyme GS and potentially increase GSH levels above homeostatic levels [18].

Early rodent studies in mice showed that intraperitoneal administration of γGC could restore depleted GSH levels in organs [169]. More recently, γGC has been demonstrated to attenuate oxidative damage to neurons and astrocytes in vitro and to increase brain GSH in vivo [170]. Further in vivo studies in neuronal [171], cardiac [172] and liver [173] tissues have shown that extracellular addition of γGC ethyl ester also increases intracellular GSH concentrations [174]. In vitro studies using isolated mitochondria have shown that γGC can directly replace the role of GSH [12]. The same researchers have also found that γGC can take over the antioxidant and neuroprotective functions of GSH by acting as a cofactor for glutathione peroxidase-1 in a mouse model [6].

Dipeptides are not considered to be useful as an oral therapeutic because they are easily hydrolyzed by digestion or serum proteases, whereas the γ-glutamyl bonds present in γGC is resistant to hydrolysis by most proteases and aminoproteases. Animal safety studies have shown γGC to be borderline safe [175].

The oral therapeutic effect has also been investigated in humans. The change in GSH content, the cellular uptake of γGC and the bio-availability of lymphocytes were investigated after a single γGC oral dose. Administration of γGC was associated with a significant increase in lymphocyte GSH content. In a pharmacokinetic study, a gradual rise in lymphocyte GSH levels was noted following γGC administration, likely due to the progressive absorption of γGC from the gastrointestinal tract through the hepatic portal system and its subsequent distribution throughout the body via the circulatory system. This increase reaches Cmax levels in 2-3 hours. The decline in GSH levels towards homeostasis for 2-3 hours after Tmax is due to the slowing of cellular γGC synthesis resulting from the high GSH levels exerting a feedback inhibition on GCL activity. This needs to be considered in therapeutic applications and will probably require the use of delivery systems that can best control and maintain the desired effect of oral γGC formulations [2]. The mechanism by which γGC is taken up by intact cells is still unclear. However, the existence of a specific transporter has been suggested [176].

While there are limited studies published on the use of γGC in functional food production, there are studies on the using the GSH in functional food production. Bearing in mind the importance of γGC in terms of health, it has high potential in functional food production.

Considering all the features of the γGC, it is considered as a potential additive for use in the food field.

4.Objective of thesis

Taking into account previous studies and the therapeutic and biotechnological usefulness of γGC, the aims of this study :

The main aim of the present thesis was to design new *Y. lipolytica* strains that are high-yield γGC producers. In the first stage of this work, yeast was modified to increase γGC yield by overexpressing and deleting the genes (*GSH1, GSH2, MET4*) thought to be important in the GSH metabolic pathway. Then, we evaluated potential of γ-glutamylcysteine as antioxidant and antimicrobial biocompound. We tested the impact of γ-glutamylcysteine, which shows promise in antioxidant testing, versus commercial antioxidants by mixing it with sunflower oil, the most common industrial oil that is oxidation-prone. Finally, we examined the potential of γ-glutamylcysteine to be used as a substitute for sulfur by adding it to wine during maturation and determination its effect on wine aroma.

Chapter 2

Biosynthesis of the antioxidant γ-glutamyl-cysteine with engineered *Yarrowia lipolytica*

This chapter evaluated *Y. lipolytica* as a high-value γ-glutamylcysteine producer. Genes thought to be important in the γGC metabolic pathway in yeast *Y. lipolytica*, *GSH1, MET4, GDH, CYSTE, CYSTF* were analyzed. In this time-consuming and labor-intensive project, I contributed to the development of some new strains by overexpression of *GSH1, GSH2* and *MET4* genes (https://doi.org/10.1002/biot.202300564).

1.Introduction

Glutathione (γ-L-glutamyl-L-cysteinyl-glycine, GSH) is the most abundant intracellular non-protein thiol compound [9]. It has a major role in many cellular processes including maintenance of redox status, storage of cysteine, detoxification of toxic compounds or neutralization of reactive oxygen species (ROS) and other free radicals [5,177]. In such reactions, GSH releases an electron and the GSH radicals generated neutralize by pairs through the formation of a disulfide bound in the resulting glutathione disulfide (GSSG). Homeostasis of GSH is ensured either by de novo synthesis, recycling of GSSG and catabolism of GSH [9]. GSH is synthesized in two consecutive ATP-requiring steps, the first of which being γ-glutamylcysteine (γGC) formation by glutamylcysteine ligase (GCL) from cysteine and glutamate. Subsequently, glycine is linked to γGC through the action of glutathione synthase (GS) to yield GSH. The GCL enzyme is regulated by non-allosteric feedback inhibition exerted by GSH that compete with glutamate for binding at the enzyme active site [14,15]. GS shows higher activity than GCL and no inhibitory regulation (Anderson 1998). Consequently, intracellular γGC level is low as it is immediately converted into GSH by GS [3]. The rate of GSH synthesis is limited by both the GCL activity and the availability of cysteine which intracellular concentration is significantly lower than those of glutamate and glycine [5,16]. In the recycling pathway, GSSG is reduced by glutathione reductase in the presence of cofactor NADPH while γ-glutamyltranspeptidase (γ-GT) catalyzes the hydrolysis of GSH with the release of free cysteine (Lu 2009). Under normal physiological status, glutathione pool is mainly under a reduced form [17].

In mammals, the onset of aging is associated with a significant decrease in both GCL activity and cysteine availability [18]. This lower de novo GSH synthesis has been associated with the development of age-related diseases such as Alzheimer's (AD) [19] or Parkinson's diseases (PD) [20]. As cysteine availability is a rate limiting factor for GSH synthesis, several cysteine prodrugs such as N-acetylcysteine (NAC) or NAC ethyl ester have been used in some clinical practices [18,178]. However, the low level of GCL activity stated above still limit a de novo synthesis. Similarly, exogenous supply of GSH is also non effective in human therapy. Indeed, GSH has a half-life of 1.6 minutes in plasma [21] and the passive transport of GSH form plasma to cells is not thermodynamically favorable [1], without mentioning the activity of membrane bound γ-GT [18]. γGC has been proposed as an alternative drug to increase intracellular GSH level [22,23]. Recent studies demonstrated that its oral administration increases GSH level in lymphocyte cell of healthy human [24] while in mice with AD, it improved special memory and neuroapoptosis and oxidative stress [25]. γGC was also found able to reduce oxidative stress damage and neuroinflammation induced by soluble amyloid β-oligomers in human astrocytes [26] as well as in human endothelial cells (Nakamura et al., 2012); it can also alleviate insulin resistance and hepatic steatosis [28]. Beside this, γGC was show able to replace GSH as cofactor of glutathione peroxidase 1 (GPX1), a ubiquitous enzyme in many human tissues that protect cells from

oxidative stress [6]. All of these highlight that γGC promising to prevent or partly cure chronic diseases or age-related disorders.

γGC can be synthesized chemically although the synthesis at a large-scale is a complex process that requires several steps of protection, coupling and deprotection of the reactive sulfhydryl group of cysteine. Moreover, coupling the constitutive amino acids through the unconventional γ-glutamyl peptide occurs only at low yield (less than 10%) and requires the use of highly toxic solvents leading to environmental and safety concerns [50]. Fermentation and enzymatic production processes have been patented (WO2016/017631, WO2006/102722, WO/2003/046154) but the product has not been released on the market probably due to the high process costs [136]. Recently, an enzymatic process based on the hydrolysis of GSH using phytochelatin synthase like enzyme (NsPCS) has been developed at lab scale [179]. Although the reaction reached a conversion yield of 0.8, it requires pure GSH solution as well as the production, purification and immobilization of recombinant NsPCS witch can be fastidious and time consuming. We recently reported on the engineering of yeast *Y. lipolytica* for efficient GSH production [33]. The obtained GSH titer and productivity were among the highest reported in the literature. Therefore, the yeast was used herein as a chassis strain to produce γGC.

2. Materials and methods

2.1. Strains, media and culture conditions

The strains used are listed in Table 4 and Appendix 1. *Escherichia coli* was grown at 37°C in Luria-Bertani medium supplemented with ampicillin (100 mg/L) or kanamycin sulfate (50 mg/L) when required. Yeasts were grown at 28 °C in YPD (20 g/L Difco bacto peptone, 10 g/L Difco yeast extract, 20 g/L glucose) or in YNBD medium (1.7 g/L Difco yeast nitrogen base medium without amino acids and ammonium sulfate, 20 g/L glucose, 2 g/L Difco casamino acids, 1 g/L ammonium chlorides, 0.05M phosphate buffer pH 6.8) supplemented with leucine (0.2 g/L) or uracil (0.1 g/L) to meet the requirements of auxotrophs. YNBDplus medium was constituted of YNB medium enriched with glutamate (0.1 g/L) or/and cysteine (0.1 g/L). YPDox was supplemented with H_2O_2 at concentration ranging from 0 to 24 mM. For solid media, agar (15 g/L) was added. All shake flask cultures were performed at least in triplicate. They were seeded at an initial optical density at 600 nm (OD600) of 0.2, with cells grown for 24 h in YPD medium and washed twice with phosphate buffer (50 mM, pH 6.8).

2.2. General molecular techniques, vectors and strains construction

Standard molecular genetic techniques were used Shambrook et al., (2001). Restriction enzymes and T4 DNA ligase were from New England BioLabs (MA, USA). PCR amplifications were made with GoTaq DNA polymerase (Promega, WI, USA) or Q5 High-Fidelity DNA polymerase (New England BioLabs) with primers listed in **Table 6**. PCR fragments were purified with a QiAgen Purification kit (Qiagen, Hilden, Germany) while DNA fragments were purified from the agarose gels using a Monarch® DNA Gel Extraction kit (BioLabs, New England, USA). GeneJET plasmid Miniprep Kit (Thermo Scientific) was used for plasmid extraction and purification. Preparation of yeast genomic DNA

and yeast cell transformation were as previously reported [180,181]. qPCR was as described elsewhere using primer listed in **Table 6** [33].

Table 4. *Y. lipolytica* strains used in this study

Y. lipolytica	Genotype	Origin, reference
RIY129	JMY2900, Po1d, URA3, LEU2	Lab stock
RIY176	Po1d, eyk1Δ Ura- Leu-	Trassaert et al., 2017
RIY441	RIY176, gsh2Δ Ura+ Leu-	This work
RIY448	RIY441, Ura- Leu-	This work
RIY454	RIY441, Ura+ Leu+	This work
RIY455	RIY441, pTEF-GSH1, Ura+ Leu+	This work
RIY445	RIY448, pTEF-GSH1, pTEF-GSH1, Ura+ Leu+	This work
RIY476	RIY445, Ura- Leu-	This work
RIY500	RIY476, pTEF-CYSE, Ura- Leu+	This work
RIY501	RIY476, pTEF-MET4, Ura- Leu+	This work
RIY502	RIY476, pTEF-GDH, Ura- Leu+	This work
RIY503	RIY476, pTEF-CYSF, Ura- Leu+	This work
RIY504	RIY500, pTEF-MET4, Ura+ Leu+	This work
RIY505	RIY502, pTEF-MET4, Ura+ Leu+	This work
RIY506	RIY503, pTEF-MET4, Ura+ Leu+	This work
RIY507	RIY500, Ura+ Leu+	This work
RIY508	RIY501, Ura+ Leu+	This work
RIY509	RIY502, Ura+ Leu+	This work
RIY510	RIY503, Ura+ Leu+	This work

Table 5. *E. coli* strains and plasmid used in this study

Strains (plasmid)	Genotype-Plasmid	Source/Reference
DH5α	Δ(lacZYA-argF)U169 recA1 endA1 hsdR17(rK- mK+) phoA supE44 λ– thi-1 gyrA96 relA1 F– φ80lacZΔM15	Promega
RIE110 (RIP110)	URA3ex	Fickers et al 2003
RIE111 (RIP111)	LEU2ex	Fickers et al 2003
RIE132 (RIP132)	Cre-EYK1	Vandemies et al 2017
RIE136 (RIP136)	pTEF expression vector, LEUex	Lab stock
RIE137 (RIP137)	pTEF expression vector, URAex	Lab stock
RIE210 (RIP210)	pTEF-GSH1, LEUex	Do and Fickers, 2020
RIE211 (RIP211)	pTEF-GSH1, URAex	Do and Fickers, 2020
RIE295 (RIP295)	pGEMTeasy, gsh2::URAex	This work
RIE310 (RIP310)	pTEF-GDH1, LEUex	Trotter et al., 2019
RIE316 (RIP316)	pGEMTeasy-MET4	This work
RIE317 (RIP317)	pGEMTeasy-CYSE	This work
RIE318 (RIP318)	pGEMTeasy-CYSF	This work
RIE319 (RIP319)	pTEF-MET4, URAex	This work
RIE320 (RIP320)	pTEF-MET4, LEUex	This work
RIE322 (RIP322)	pTEF-CYSE, LEUex	This work
RIE324 (RIP324)	pTEF-CYSF, LEUex	This work

Table 6. Primers used in this study

Name	Sequence 5'-3'	Restricition site, utilisation
GSH2pro_for	GAATAGCAGCTTTGCAACGCGAAG	
GSH2pro_rev	AGCAGGAGTTATCCGAAGCGATAATGGATTAGA TAGATAAGGTGTGGTTCAGTACATACAGTAC	
GSH2ter_for	GTTATCTAGGGATAACAGGGTAATCCGTCATTG GTTGTTTCGGAGACG	
GSH2ter_rev	GGTCTTCTCATCCTTGGGCTTC	
Auxo_fo	ATTATCGCTTCGGATAACTCCTGCT	
Auxo_rev	GATTACCCTGTTATCCCTAGATAAC	
GSH2_verif	GAGTCTTTGCGCCATCG	
URA-Fo	GGATGTTACCACCACCAAGG	
URA-Rev	GTTCTGGCCGTACAGACCTC	
pTEF-Fo	GGACCCAACCCCGGCG	
GSH1_rev	GCGCCTAGGCTACTCCTTCTCGTACTCAAAACC	
LoxP-Fo	GCATACATTATACGAAGTTATTCTGAATTC	
LoxR-Rev	GGGTAATTATCGCTTCGGATA	
Met4-Fo	GACGGATCCATGACTGACCGACTTTTCTTGGCCA ACCTCAATGCGATTGAAGGATCCTCAA	BamHI, internal BamHI removed
Met4-Rev	GCGTGTGGCCTAGGTTAGATTTGCTCAATCTTGG CGATGGGAG	AvrII, internal BglII removed
CystE_Fo	GCGGGATCCATGAGTCGCTGGATATACACG	BamHI
CystE-Rev	GCCCCTAGGCTAAAAATCCTCCAACTTGATCTCC CC	AvrII
CystF-Fo	GCGCAGATCTATGTCTCGAATTGGATCTGTGAC	BglII
CystF-Rev	GCGCCTAGGTTAATCCAGAACAACGTACTTTTG GAGAT	AvrII
Met4_verif	GACCCTAGGTTAGATCTGCTCAATCTTGGCGATG	
CystE_verif	CCAGAACTTACTCAAATGGCGATGGCC	
CystF_verif	GGGAATGGTGAGTCCCTTGGAC	
Gdh_verif	GGCACCCTTGACGAGAGAGGG	
qGsh11_Fo	TGACTTCGACGACATTCTGC	qPCR, GSH1
qGsh1_Rev	CACCCTTGGGCTCGTAATAA	qPCR, GSH1
qMet4_Fo	CCCAGGAGGAAATCCAGGCC	qPCR, MET4
qMet4_Rev	GAGCCACTTGTTCTCCATCTCCAG	qPCR, MET4
qGdh_Fo	CTTCCGTCAACCTGTCCATT	qPCR, GDH1
qGdh_Rev	GAAGGCGTAGCAGAATCGTC	qPCR, GDH1
qCyse_Fo	ATGTTTGTGGGCTCTTCCAC	qPCR, CYSTE
qCyse_Rev	TCCAACTTGATCTCCCCAAG	qPCR, CYSTE
qCysf_Fo	GGATCCGTGCTCCATTCTTA	qPCR, CYSTF
qCysf_Rev	ACCCTCCTTATCCAGCAGGT	qPCR, CYSTF

For the *GSH2* deletion, 1kb fragment (Pro_GSH2) upstream of the start codon and 1.2 kb fragment (Ter_GSH2) downstream of the stop codon of gene YALI0C17831g were PCR amplified using, respectively, primer pairs GSH2pro_for/GSH2pro_rev and GSH2ter_for/GSH2ter_rev and *Y. lipolytica* RIY129 genomic DNA as a template. The URA3 selection marker was amplified using primer pairs Auxo_fo/Auxo_rev and plasmid RIP110 as a template. The GSH2 disruption cassettes Pro_GSH2-URA3-Ter_GSH2 was obtained by overlapping PCR of Pro_GSH2, Ter_GSH2, and marker amplicons as templates and GSH2pro_for/GSH2ter_rev as primer pairs. The resulting 3.5 kb fragments was used for transformation of strain RIY176 and cloned into vector pGEMT-Easy vector to yield plasmid RIP295 (Table 4). Transformants were selected on YNBG and the correctness of the genotype was confirmed by analytical PCR using the primer pairs GSH2_verif/Auxo_rev. For marker rescue, the resulting strain

RIY441 was further transformed with replicative vector RIE132 as previously described [97]. The correctness of the resulting RIY448 was verified by PCR using primer URA-Fo/URA-Rev. Strain RIY441 was transform with plasmid RIP111 to confer prototrophy in the resulting RIY454 strain.

For constitutive overexpression of *GSH1* (YALI0E30129g), vector RIP210 (LEU2ex) and RIP211 (URA3ex) were Not1 digested, and the expression cassette was gel purified. Strain RIY441 was transform with RIP210 while strain RIY448 was transformed with both vectors. Transformants where selected on YNBD and correctness of the genotype was performed by analytical PCR using primer pairs pTEF_forw/GSH1_rev. The resulting strains were named RIY455 and RIY445, respectively (Table 4). The auxotrophic derivative of RIY445, namely RIY476 was obtained by transformation with vector RIP132 as previously described [97]. Marker excision was confirmed by analytical PCR using primers LoxP-Fo/LoxR-Rev.

Gene *MET4* (YALI0D04466g), *CYSE* (YALI0E08536g) and *CYSF* (YALI0F14047g) were PCR amplified using primer pairs Met4-Fo/Met4-Rev, CystE-Fo/CystE-Rev, CystF-Fo/CystE-Rev, respectively and cloned in pGEMT-easy vector to yield vectors RIP316, RIP317, RIP318. Correctness of the amplified sequences was confirmed by DNA sequencing using primer M13fo and M13rev. The MET4 coding sequence was released from RIP316 by BamHI/AvrII digestion and cloned at the corresponding sites of vector RIP136 and RIP137 to yield vectors RI320 and RIP319, respectively. The *CYSTE* and *CYSTF* coding sequences were released from RIP117 and RIP318 by BamHI/AvrII digestion and cloned at the corresponding sites of vector RIP136 to yield vector RIE322 and RIE324, respectively. Prior yeast transformation, pTEF based vectors were Not1 digested, and the corresponding expression cassette gel purified. Strain RIY476 was transformed with vector RIP310, RIP320, RIP322 and RIP324 after Not1 digestion and gel purification of the corresponding expression cassette to yield strain RIY500 to RIY503, respectively (**Table 4**). Strains RIY500, RIY502 and RIY503 were further transformed with purified expression cassette released from plasmid RIP319 to yield strains RIY504 to RIY506. Finally, strains RIY500 to RIY503 were transformed with vector RIP110 to yield the prototroph strains RIY507 to RIY510. Correctness of the genotype was verified by analytical PCR using primer pTEF-Fo and primer annealing in the coding sequence of the corresponding gene, namely Gdh_verif, Met4_verif, CystE_verif and CystF_verif. Auxothrophy was verified by PCR using primiers Auxo-fo/Auxo_rev.

2.3. Analytical Methods

The cell dry weight (gCDW/L) was used for biomass quantification. For cell extract preparation, cells from one ml of culture broth were collected by centrifugation at 5000 x g at room temperature for 10 minutes. The cell pellet was then washed twice with phosphate-buffered saline (PBS, pH 6.8) and re-suspended in 1 ml of the same buffer. The cell suspension was disrupted using a FastPrep-24 instrument (MP Biopmedicals, Eschwege, Germany, 4x2-minute, 6.5m/s) with 0.3 g of glass beads (acid-washed, Sigma). Cellular debris was then removed by centrifugation (10.000 x g for 10 min at 4°C). The total protein concentration in the cell extract was measured according to Bradford using the Coomassie

protein assay reagent (Thermo Scientific, Waltham/Massachusetts, USA). Protein concentration was calculated based on a calibration curve obtained with bovine serum albumin (BSA) standard solutions (Thermo Scientific, Waltham/ Massachusetts, USA). The γGC concentration in the cell extracts was determined using the Fluorometric Thiol Quantitation kit (Sigma-Alrich). Pretreatment of cell extracts with 10% 5- sulfosalicylic acid was carried out to precipitate all soluble proteins in the samples. Each measurement was performed in triplicate, and the means and standard deviations were then calculated. γGC titer was calculated in nmol per mg of protein in the cell extract (nmol/mg).

2.4. Characterization of γGC by UPLC-QTOF/MS

Yeast cell extract was deproteinized with 10% 5-sulfosalicylic acid as described above for Thiol quantitation. The concentration of the 2-50 µM standard solution of γGC, GSH, and GSSG was prepared in ultrapure water. Detection and quantification of the different molecules was performed using Agilent 1290 Infinity II HPLC system coupled to mass detector (Jet Stream ESI-qTOF 6530, Agilent) and separated on a C18 Acquity UPLC BEH column (2.1 × 50 mm × 1.7 µm; Waters) and 0.1% formic acid (solvent A)/acetonitrile acidified with 0.1% formic acid (solvent B) as mobile phase with constant flow rate at 0.3 ml min^{-1} and column temperature set at 40°C. First, gradient was kept at 100% A during 1.5 min before raising up to 100% B in 0.2 min and kept as such during 5 min before going back to initial ratio. MS spectra were recorded in positive mode with MS parameters set up as follows : capillary voltage: 3.5 kV; nebulizer pressure: 35 psi; drying gas:8 l min^{-1}; drying gas temperature: 300°C; flow rate of sheath gas: 11 l min^{-1}; sheath gas temperature: 350°C; fragmentor voltage: 175 V; skimmer voltage: 65 V; octopole RF: 750 V. MassHunter Qualitative Analysis software (Agilent) was used for data analysis. Confirmation of the different molecules was made based on retention time and accurate masses compared to commercial standards (Sigma Aldrich).

2.5. Characterization of γGC by nuclear magnetic resonance

Yeast cell extract was deproteinized in the same procedure as described above for thiol quantitation. The concentration of 2-50µM of pure γ-GC, GSH, and GSSG in phosphate buffer pH 6.8, 50mM were used as standards. Crude cell extracts from wild type (RIY129) and γGC producing strains (RIY445) were analyzed by NMR (700 MHz Bruker Avance III HD equipped with a helium-cooled triple nuclei (HCN) probe). NMR experiments were recorded at 298 K. The volume of 570 µl of cell lysate was added with 30 µl of D_2O for the internal frequency lock signal. For 1D 1H spectra, water suppression was achieved with the Bruker pulse sequence "ZGESGPPE" 256 transients were added prior to Fourier transform. The relaxation delay in between each transient was set to 1s. For 2D HH TOCSY, the Bruker pulse sequence "MLEVESGPPH" was used with a 256-time increment of 64 transient each. The spin-lock time and the relaxation time were respectively set to 80 ms and 1s.

2.6 Statistical analysis

Statistical signification of results was assessed by unpaired t-test or analysis of variance (ANOVA) followed by Dunnett's multiple comparison tests. Alpha value was set at 0.05. All statistical tests were performed using GraphPad Prism 9 software.

3. Results and Discussion

3.1. Construction of a γ-glutamyl-cysteine producer strain

In *Y. lipolytica*, GCL and GS are encoded by genes *GSH1* (YALI0E30129g) and *GSH2* (YALI0C17831g), respectively [33]. With the aims to construct a γGC producer strain, gene *GSH2* was first disrupted in strain RIY176. In the resulting prototroph strain RIY454 (Table 4), free thiol content was slightly increased as compared to the parental strain (94 and 71 nmol/mg, respectively. Fig 7).

In a second step, gene *GSH1* was overexpressed under the control of the strong constitutive promoter pTEF in one and two copies, to yield strains RIY455 and RIY445, respectively. As compared to the wild-type strain RIY129, expression level of *GSH1* was increased by 43 and 169-fold in strains RIY455 and RIY445, respectively (Appendix 2). As shown in Fig 7, the thiol titer was also significantly increased for the two later strains (249 and 283 nmol/mg) as compared to the parental strain RIY454 (94 nmol/mg, gsh2Δ, **Figure 7**). They are in the range of GSH titer obtained with strain RIY231 overexpressing GSH1 and GSH2 340 nmol/mg [33].

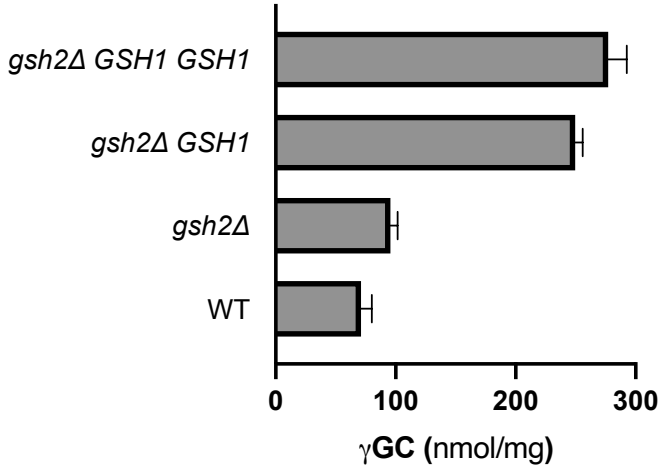


Figure 7. γ-glutamyl-cysteine intracellular concentration for the RIY129 wild-type strain (WT), RIY454 (gsh2Δ), RIY455 (gsh2Δ, *GSH1*) and RIY445 (gsh2Δ, *GSH1, GSH1*) after 24 h of YNBD medium.Data are the mean and standard deviation of triplicate experiments. γGC concentration is given in nmol of γGC per mg of protein in cell extract.

To evidence the synthesis of γGC in place of GSH in the constructed strains, the corresponding cell extract was analyzed by mass spectrometry. ESI-Q-TOF spectra obtained for RIY129 showed a molecular ion $[M+H]^+$ at m/z 308.0917 that correspond to GSH while such a specific molecular ion

could not be detected in the cell extract of strains disrupted for *GSH2* (RIY455, RIY445, **Table 7**, **Appendix 3-5**).

Table 7. Detected ions (m/z) for γ-glutamyl cysteine, γ-glutamylcystine (γ-glutamyl cysteine oxidized form), glutathione and glutathione disulphide (glutathione oxidized form) in cell extract of *Y. lipolytica* strain RIY129, RIY455 (GSH2Δ -pTEF-ylGSH1) and RIY445

	Calculated $[M+H]^+$	Measured $[M+H]^+$		
		RIY 129	RIY 455	RIY 445
γ-glutamylcysteine	251.0696	n.d.	251.0704	251.0701
Bis-γ glutamylcystine	499.1163	n.d.	499.1168	499.1166
Glutathione	308.0911	308.0917	n.d.	n.d.
Glutathione disulphide	613.1592	n.d.	n.d.	n.d.

Data are representative of measurements performed on two biological replicates. n.d.: no ion detected. Corresponding extracted ion chromatogram (EIC) and masse spectra are presented in Appendix 6.

By contrast, molecular ions $[M+H]^+$ characteristic of γGC and its oxidized form (m/z = 251.0696 and 499.1163, respectively) were detected for both cell extracts of strains RIY455 and RIY454. Such ions could not be detected in cell extract from strain RIY129. This suggests that gsh2Δ strains accumulate γGC instead of GSH. Cell extract of strains RIY445 were also analyzed by 2D TOSCY-NMR to highlight the specific γGC proton chemical shifts preliminary identified using pure γGC solution (**Table 8, Appendix 6**).

Furthermore, to confirm the presence of the dipeptide in the cell lysate of strain RIY445, an oxidation followed by addition of fresh reduced γGC peptide was conducted. For that purpose, the cell extract was left 48h at room temperature to oxidize the cysteine moiety (i.e. the conversion of γGC into bis-γ glutamylcystine). 1D 1H NMR spectrum effectively showed the disappearance of γGC characteristic signals at 8.11 and 2.52 ppm (Fig 8, red trace). An addition 100 µM of reduced γGC in cell extracts restored characteristic signals at 8.11 and 2.52 ppm (**Figure 8**, green trace). Form those analysis, it can be concluded that gsh2Δ, strains produce γGC instead of GSH.

Table 8. γGC peptide proton chemical shift (ppm) observed in the 2D TOCSY for cell lysate of strain

NHCO(Cys)	8.11
CHa(Cys)	4.39
CHa(Glu)	3.78
CH_2b(Cys)	2.93
CH_2g(Glu)	2.52
CH_2b(Glu)	2.16

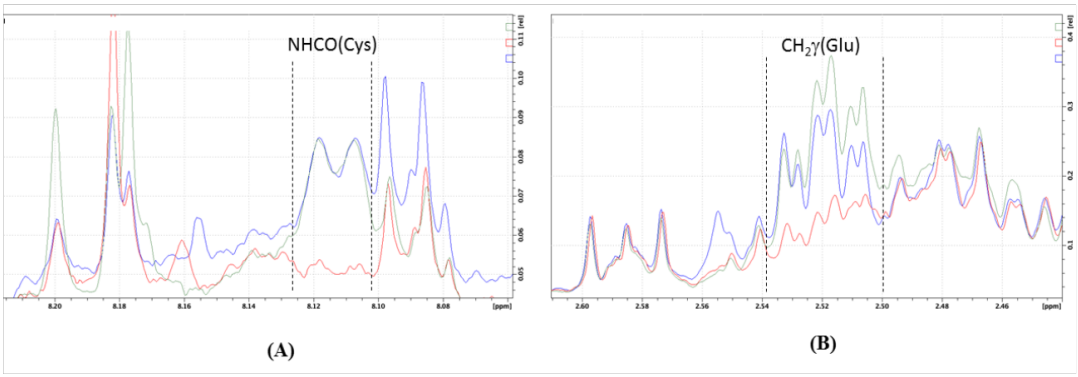


Figure 8. γ-glutamyl-cysteine 1H 1D spectra of cell extract of strain RIY445 (gsh2Δ, GSH1, GSH1) by NMR.Color code: blue, fresh cell extract from 48h culture; green, cell extract oxidized at RT for 48h; green, oxidized cell extract spiked with 100µM of γGC. A: NHCO signals, B: CH_2g signals.

3.2 γGC can substitute GSH in Y. lipolytica

In *S. cerevisiae*, *GSH2* encoding GS was shown as not essential for growth, although the growth of gsh2Δ mutant was significantly affected in minimal medium [40, 41]. Beside this, in *S. cerevisiae* mutant disrupted for GCL encoding gene, preincubation of cells with γGC prior exposure to H_2O_2 was found as efficient for cell survival as preincubation with GSH [7]. To assess if γGC can substitute GSH also in *Y. lipolytica*, strains RIY129 (WT) and RIY445 (gsh2Δ, pTEF-GSH1-GSH1) where grown in YPDox medium under oxidative stress in the presence of increasing concentration of oxygen peroxide known to trigger reactive oxygen species (ROS) formation. As shown in **Figure 9**, biomass reduction under increasing oxidative stress was not significantly different ($P<0.05$) for strain RIY129 and RIY445. This demonstrate that γGC can effectively substitute GSH in the gsh2Δ mutant strain.

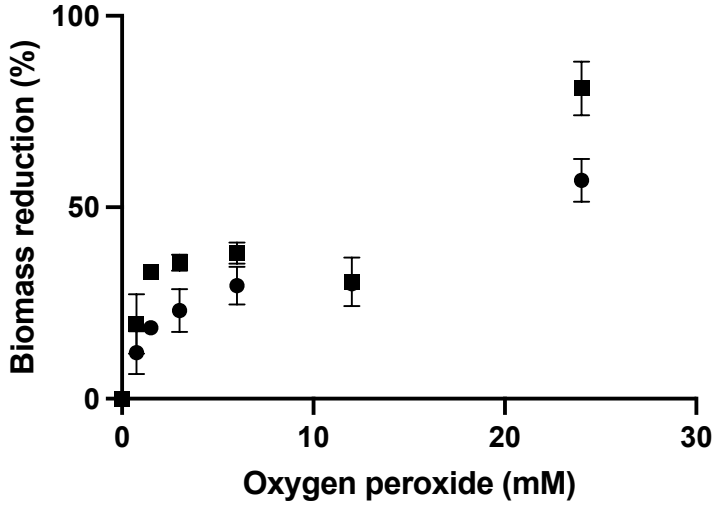


Figure 9. Biomass of strain RIY129 (WT) and RIY445 (gsh2Δ, GSH1, GSH1) after 24 h of growth in YNBD medium supplemented or not (w/o) hydrogen peroxide.

Biomass from culture in non-supplemented medium was used as a reference to calculate the decrease, in percent, of biomass obtained in H_2O_2 supplemented medium. Data are the mean and standard deviation of triplicate experiments.

3.3 γGC synthesis is limited by the availability of constitutive amino acid

Although γGC was significantly increased in strain RIY455 (gsh2Δ pTEF-GSH1, 249 nmol/mg) as compared to strain RIY454 (gsh2Δnmol/mg, **Figure 10**), no significant increase in γGC titre could be observed upon overexpression of *GSH1* in one (RIY445, 249 nmol/mg) or two copies (RIY455, 277 nmol/mg) (Fig.10). In *S. cerevisiae*, several strategies of constitutive amino acid supplementation have been developed with the aim to increase the GSH productivity [177]. To highlight any intracellular limitation in γGC constitutive amino acids in *Y. lipolytica*, the two strains were grown in YNBD minimal medium supplemented with glutamate and cysteine, alone or in combination. In the non-supplemented medium, γGC titer was lower as compared to the supplemented medium, especially for strain RIY445 (two copies of *GSH1*) grown on medium supplemented with both glutamate and cysteine (86 vs 239 nmol/mg protein, 2.8-fold increase). This clearly highlight that the intracellular availability of constitutive amino acids, especially cysteine, limits the γGC accumulation.

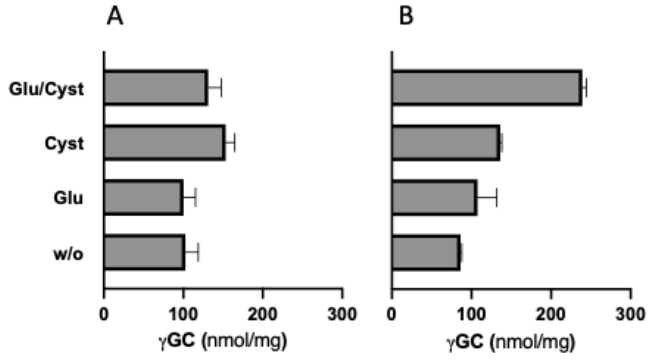


Figure 10. γ-glutamyl-cysteine intracellular concentration for strains RIY455 (panel A, gsh2Δ, GSH1) and RIY445 (panel B, gsh2Δ, GSH1, GSH1) after 24 h of growth in YNBD medium supplemented or not (w/o) with glutamte (glu) and/or cysteine (Cyst). Data are the mean and standard deviation of triplicate experiments. γGC concentration is given in nmol of γGC per mg of protein in cell extract.

3.4 Increasing intracellular aminoacid in γGC producing strains

They are only few reports on the characterization of cysteine and glutamate synthesis in *Y. lipolytica*. Using a combined approach of transcriptomics and metabolite profiling, genes YALI0E08536g (*CYSE*) and YALI0F14047g (*CYSF*) were identified as putative cysteine synthase [182]. In the same study, homologous of gene *MET4* encoding a transcriptional activator of the sulfur amino acid pathway was identified as gene YALI0D04466g. We previously demonstrated that overexpression of *MET4* gene in *Ogataea (Hansenula) polymorpha* led to a significant increase in GSH productivity [53]. Regarding glutamate, gene YALI0F17820, the homologue of *GDH1* and *GHD3* in *S. cerevisiae*, has been characterized as coding for a glutamate dehydrogenase that convert α-ketoglutarate in glutamate [42].

With the aim to increase intracellular concentration of cysteine and glutamate and thus to further increase the γGC titer, those different genes were overexpressed alone or in combination under the control of the strong pTEF promoter in strain RIY476, an auxotroph derivative of strain RIY445 (gsh2Δ, pTEF-GSH1 in two copies). Overexpression of the corresponding gene was first checked by qPCR (data not shown). Cultures were then conducted for the different strains in YNBD medium and γGC titer was measured during the growth phase (i.e., 24h) and stationary phase (i.e., 48h). At 24h, the highest γGC titer was observed for strain that overexpressed *MET4* gene with a 1.7-fold increase as compared to the parental strain RIY445 (**Figure 11**; 277 and 464 nmol/mg, respectively).

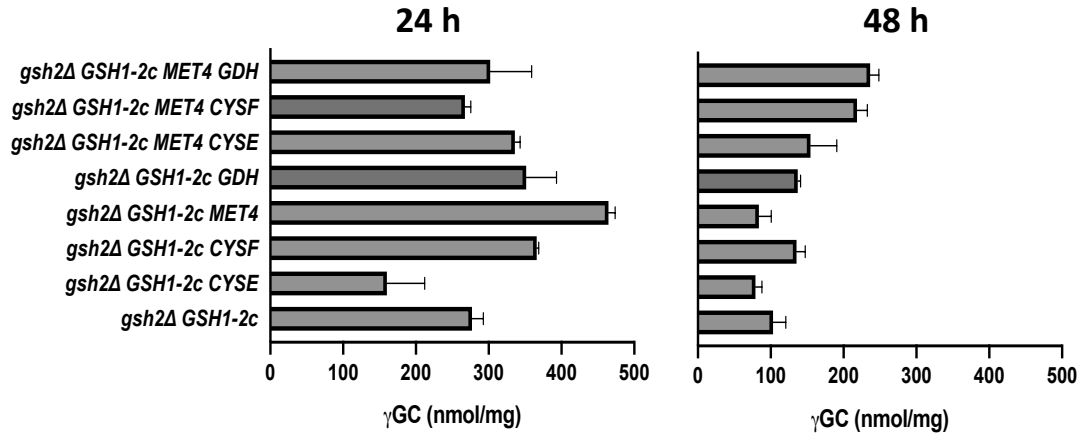


Figure 11. γ-glutamyl-cysteine intracellular concentration for strains RIY445 (gsh2D GSH1-2c), RIY507 (gsh2Δ GSH1-2c CYSE), RIY510 (gsh2Δ GSH1-2c CYSF), RIY508 (gsh2D GSH1-2c MET4), RIY509 (gsh2Δ GSH1-2c GDH), RIY504 (gsh2Δ GSH1-2c MET4 CYSE), RIY503 (gsh2Δ GSH1). Data are the mean and standard deviation of triplicate experiments. γGC concentration is given in nmol of γGC per mg of protein in cell extract. GSH1-2c denote that mutant strains contain 2 copies of pTEF-GSH1 expression cassette.

For strains that overexpress genes *CYSF* and *GDH1*, γGC titer was increased by 1.3-fold (i.e., 366 and 351 nmol/mg, respectively). Surprisingly, the biomass obtained at 24 h for strain overexpressing *GDH1* was 1.7-fold lower (i.e., 5.4 ± 0.3 gDCW/L) as compared to the mean biomass value of all tested strains (9.5 ± 0.5, data not shown). Overexpression of *CYSE* was detrimental for γGC production (i,e.,160 nmol/mg) but not for cell growth (i.e., 16.2 ± 0.3). Unexpectedly, overexpression of *MET4* in combination of *CYSE, CYSF* or *GDH* did not yielded to any significant increase in γGC titer. At 48 h, the γGC titers were significantly lower, especially for strain overexpressing *MET4* (7-fold reduction, 63 nmol/mg). γGC titer remained in the same range for both sampling time for strain overexpression *MET4-CYSF* (RIY503, 267 vs 218 nmol/mg) and *MET4-GDH* (RIY505, 302 vs 236 nmol/mg)

4. Conclusions

Interest in γGC as a therapeutic drug has been increasing over the recent years. Surprisingly reports on efficient producing strains or optimized processes are scarce as compared to those available for GSH. Only few processes for chemical or enzymatic γGC synthesis have been proposed but so far these are still with low productivity, low yield or high cost. In *S. cerevisiae*, mutants obtained by chemical

mutagenesis were found able to produce a mixture of γGC and GSH, with γGC titer of less that 5 mg/gDCW [55,54]. Herein, *Y. lipolytica* has been engineered to produce γGC and not GSH with titre of 464 nmol/mg, that corresponds to 93 mg/gDCW within 24 h.

Chapter 3

Determination of Antioxidant and Antimicrobial Properties of γGC and GSH

1. Introduction

Oxidation is an essential process for energy production and maintaining biological activities in living organisms. Free radicals and other reactive oxygen derivatives released by oxidation result in cell death and tissue damage. Oxidative stress has been associated with some diseases such as cancer, diabet, alzheimer, parkinson, cardiovascular and neurodegenerative diseases [183,184]. Almost all organisms possess antioxidant defence and repair systems that have evolved to protect against oxidative damage, yet these systems are insufficient to prevent damage completely. Antioxidant supplements or foods containing antioxidants can be used to reduce oxidative damage in cells.

Many biological compounds extracted from plants and microorganisms exhibit antioxidant activity. Among these compounds γ-glutamylcysteine (γGC) and glutathione (GSH) exist in eukaryotic cells. γGC is synthesized from cysteine and glutamic acid as substrates by the function of γ-glutamylcysteine synthetase. On the other hand, GSH is synthesized from γ-glutamylcysteine and glycine as substrates by the function of glutathione synthetase [16]. GSH is the most abundant cellular non-protein thiol compound with concentrations up to 10 mM. It plays a central role in cell biochemistry and physiology as a reducing agent, co-substrate or cofactor [9]. In cells with GSH deficiency, γGC can take over the biological role of GSH [12]. γGC has medical and biotechnological interest. It has been described as potentially effective against Alzheimer's disease and lifestyle-related diseases [170]. γGC can also reduce oxidative stress damage and neuroinflammation induced [185].

The measurement of γGC antioxidant activity is crucial in food science due to its significant implications for enhancing food quality, safety, and nutritional value. γGC, as a precursor to GSH, plays a vital role in mitigating oxidative stress by neutralizing reactive oxygen species (ROS), which can cause food spoilage and diminish shelf life [186]. Its direct antioxidant properties include scavenging free radicals, chelating metal ions, and reducing oxidative damage in food systems, which helps maintain the sensory and functional attributes of food products [187]. Additionally, because γGC can strengthen cellular antioxidant defence mechanisms and protect against oxidative stress-related health disorders such liver illnesses and dementia, its integration to functional foods or nutraceuticals is interesting [6]. Recent studies emphasize γGC's potential in formulating antioxidant-rich ingredients, such as peptides synthesized through enzymatic reactions, which exhibit robust ROS-scavenging activities and are suitable for food fortification [188]. The development of accurate methods to assess the antioxidant activity of γGC is important to optimise its use in foods and dietary supplements and to maintain its stability. Therefore, γGC represents a pivotal compound for advancing food science innovations that address consumer demands for healthier and more sustainable products.

Pathogenic microorganisms have been detrimental to humans throughout history. In 1928, this was partially prevented with the discovery of the first antibiotic. As the use of antibiotics has increased, antibiotic-resistant microorganisms have also developed. Therefore, there has been a need to find new antimicrobial compounds. Various natural antibiotic substances, especially plant extracts, have been

tested. The antimicrobials with antioxidant properties have also been investigated. Agents containing free sulfhydryl (-SH) group such as L-cysteine and N-acetylcysteine have been reported to have potential for use to produce antibacterial products [189,190]. Hamad et al., (2005) reported that microbial-derived GSH showed significant antibacterial activity against, Gram-positive strains, *Bacillus cereus* EMCC 1006, *Staphylococcus aureus* and *Streptococcus* mutans [191]. Besides bacteria, yeasts and moulds caused by environmental factors are often undesirable microorganisms in medicine, agriculture and food. Various techniques have been developed using essential oils and plant extracts to prevent the growth of fungi that cause disease in humans and plants and cause spoilage of food products [192].

γGC, a precursor to GSH, holds considerable promise for advancing scientific understanding of antimicrobial mechanisms due to its diverse biological activities. It has shown antioxidant, anti-inflammatory, and regulatory effects on oxidative stress, making it a candidate for mitigating pathogen-induced cellular damage. Recent studies have illustrated its role in reducing oxidative stress and inflammation in ethanol-induced hepatotoxicity and cadmium-induced neurotoxicity, which highlights its protective mechanisms against cellular apoptosis and inflammatory responses [193, 194].

The significance of γGC in microbial contexts is further highlighted by studies on GSH metabolism pathways in dental biofilm-associated bacteria, such as *Treponema denticola*, which generate toxic sulfur compounds associated with periodontal disease. The metabolism of γGC in these bacteria underscores its potential influence on microbial growth and pathogenesis, making it a candidate for exploring antimicrobial applications [195]. Moreover, its demonstrated stability under stress conditions, as seen in halophilic environments, suggests robustness for antimicrobial formulation [11].

This foundation can be leveraged to explore γGC's direct antimicrobial activities, whether by modulating microbial oxidative stress pathways or by disrupting biofilm formation. While existing research emphasizes its protective and regulatory roles, its capacity to influence microbial viability directly or indirectly positions it as an exciting target for antimicrobial studies.

The study of γGC as a compound with antimicrobial activity could significantly enhance scientific understanding and innovation in food science. γGC is known for its antioxidant properties and potential to modulate biological systems, which suggests it could serve as a natural antimicrobial agent. This could offer a dual benefit in food preservation by both inhibiting microbial growth and preventing oxidative spoilage, addressing key challenges in extending shelf life and maintaining food quality. Integrating such compounds into food systems aligns with the increasing consumer demand for natural additives, reducing reliance on synthetic preservatives. Moreover, exploring its mode of action, effectiveness across various food matrices, and interactions with other food components could pave the way for targeted applications in perishable goods, processed products, and minimally processed "clean-label" foods.

While specific literature directly linking γGC to antimicrobial activity in food systems is currently scarce, broader studies on its biochemical properties and potential roles in microbial inhibition could inspire interdisciplinary research. The relevance of such studies to food science could further be

bolstered by evaluating its synergistic effects with other preservatives or processing techniques, such as high-pressure food processing or fermentation. In addition, determining its safety and sensory impacts is critical for its practical application in commercial food systems.

In the present study antioxidant activity of γGC and GSH was determined by using DPPH and and ABTS methods. Also, it was interest to determined antimicrobial and antifungal activities of these biological compounds. The aim of this study to compare antioxidant and antimicrobial effect of γGC and GSH.

2.Materials and method

2.1 Determination of antioxidant capacity of γGC and GSH via DPPH

The radical scavenging activity of GSH and γGC was tested according to Blois et al (1958)[196] with some modification [197]. Briefly, solutions of GSH and γGC were prepared in water at concentrations ranging from 0.04 to 1.5 mM. 390 µl of DPPH solution (25 mg/L ethanol) was mixed to 100 µl of the different antioxidant solutions. After 30 minutes of incubation in the dark at room temperature (25°C). The results was measured at 517 nm absorbance by visible spectrophotometer (Perkin- Elmer, USA). A mixture of ethanol and DPPH was used as control. The scavenging activity was calculated according to Eq. (1). Results were evaluated in terms of IC50 (half-maximal inhibitory concentration).

DPPH radical scavenging activity (%) = [(Ab-As) / Ab] * 100 (1)

Ab is the blank absorbance and As is the absorbance of the sample.

IC_{50} was calculated for each sample on inhibition %.

2.2 Determination of antioxidant capacity of γGC and GSH via ABTS

Antioxidant activity assay was determined with a minor modification of Re et al. (1999) and Pellegrini et al., (2003)[198,199]. ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) method. $ABTS^+$ stock solution was prepeared by mixing 7mM ABTS and 2,45 mM potassium per sulphate ($K_2S_2O_8$) and incubated at 23 ˚C in the dark for 12-16 h. The stock solution was diluted with ethanol (80 %) to give absorbance 0.700±0.005 at 734 nm. The standart stock solution of γGC and GSH (0.04-1.5mM) was preapered in water. $ABTS^+$ (3.9 mL, absorbance 0.700±0.005) decoloration was performed by adding 0.1 mL of the test sample and mixed vigorously. The reaction kept in room temperature for 10 min and measured at 734 nm absorbance by visible spectrophotometer (Perkin-Elmer, USA).Total antioxidant activity was calculated according to Eq. (2):

Total antioxidant activity (TAA %) = [(O.D $ABTS^+$ - O.D sample) / O.D $ABTS^+$]* 100 (2)

IC_{50} was calculated for each sample on inhibition %.

2.3 Antimicrobial activity

For the disc diffusion method, Muller Hinton Agar (MHA), Yeast extract peptone dextrose agar (YPDA) and Potato Dextrose Agar (PDA) were used respectively for bacteria, and yeast and mould. For

the microdilution method, the broth used was Muller Hinton Broth (MHB). All media were autoclaved at 120°C for 20 minutes. All media were obtained from Merck & Co (Germany).

2.3.1 Microbial strains

In this study, the microbial activity of γGC and GSH was tested against four bacterial strains: *Escherichia coli* ATCC 11775 (NCTC 9001), and *Listeria monocytogenes* ATCC 11994 as Gram-negative strains; *Staphylococcus aureus* (Lab stock), and *Bacillus cereus* (Lab stock) as Gram-positive strains; yeast; *Candida albicans* (Lab stock) ; moulds; *Colletotrichricum acutatum* (Lab stock) and *Penicillum digitatum* DSM2750 . These strains were conserved in glycerol at -20°C.

2.3.2 Inoculum standardization

The bacterial inoculum was obtained by taking 2-3 colonies from 24-hour cultures which were aseptically collected and suspended in 0.9% sterile saline solution and shaken for 15 seconds, the turbidity was adjusted to 0.5 McFarland. The bacterial suspensions contain approximately 1-2 x 10^8 CFU/mL, while the yeast and mould suspension contains approximately 1-5 x 10^6 and 10^7 CFU/mL.

2.3.3 Disc agar diffusion method

The plates containing the agar medium MHA, YPDA and PDA were inoculated with impregnated swabs with bacterial and fungal suspensions, then the plates were dried for 10 min, and afterward, pre-impregnated 6 mm disc were disposed on the agar surface. Next, 100 µL of the 10 mM γGC and GSH were added to the discs. Ampicillin (AMP: antibiotic) 10 µg/disc, and fluconazole 10µg/ml (6 mm) were used as positive controls for bacteria and moulds. Finally, the plates were incubated for 24 hours at 37°C for bacteria, and at 30°C for 5 days for yeast and moulds. After incubation, the diameters of the inhibition zones were measured in mm by the transparent ruler.

2.3.4 Determination of the minimum inhibitory concentration

The minimum inhibitory concentration (MIC) were determined by microdilution assays according to Patel et al., 2017 [200]. First, 50 µL of the growth medium was added to each well of the microplate. Following that, 100 µL (10 mM) from each γGC and GSH (1/10 diluted in growth medium) were poured into the first well. After that, a micro-dilution was done by diluting the sample by a factor of 2 in each well, with the exception of the last well, which acted as the positive control for growth. Finally, the inoculation was carried out by depositing 50 µL of the microbial suspension (1/150 diluted in growth medium) whose turbidity was verified in the same way described previously. Then, the plates were incubated under agitation for 24 h at 37°C. To read the results, 20 µL of the 0.2% 2,3,5-triphenyltetrazolium chloride (TTC) indicator (Biokar, France) was added to each well, giving a pinkish coloration where there is growth due to the activity of the dehydrogenases after incubation for 2 hours. After 2 h of incubation, the MIC corresponds to the lowest concentration that does not produce a red color.

3. Results and Discussions

3.1 Comparative antioxidant activity analysis of γ-glutamyl cysteine and glutathione

DPPH, a stable free radical, is commonly employed to evaluate the free radical scavenging activity of compounds [201]. The effects of γGC and GSH on their reactions with the DPPH radical were analyzed, revealing a quantitative relationship between the absorbance of the DPPH solution and the concentration of GSH and γGC within the range of 0.04 to 1.5 µg/mL. (**Figure 12**).

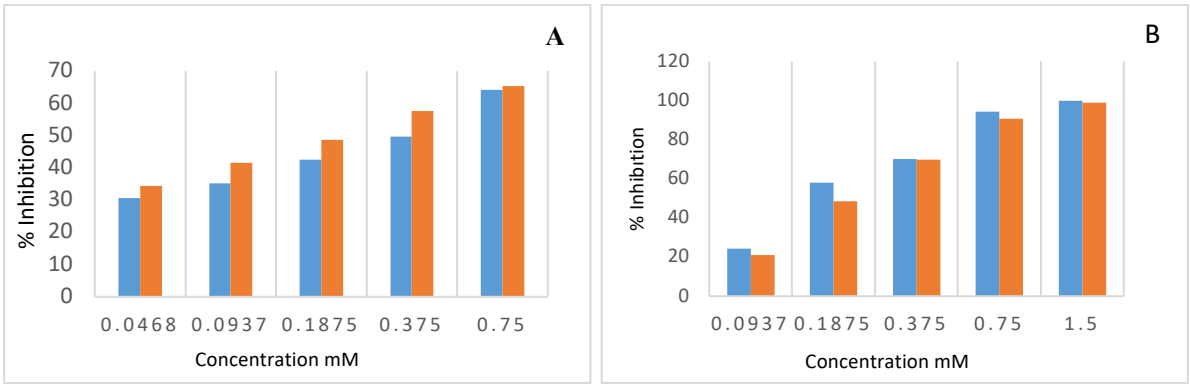


Figure 12. Antioxidant assay of γGC, γ-glutamylcysteine (blue column); GSH, glutathione (orange column). A: DPPH, 2,2-diphenyl-1-picrylhydrazyl radicals method; B: ABTS, 2,2′-azino-bis(3-ethylbenzthiozoline-6)-sulphonic acid method)($p < 0.05$)

The percentage inhibition was calculated as 64.22% for γGC and 65.34% for GSH, with the highest values observed at a concentration of 0.75 mM. The IC50, which represents the concentration of antioxidant needed to reduce the radical concentration by 50%, is inversely proportional to the antioxidant capacity of the compound.

In this study, the IC50 was determined based on percentage inhibition, yielding values of 0.29 mM for γGC and 0.36 mM for GSH. Compared to GSH, γGC exhibited higher antioxidant activity in the DPPH assay. In addition to the DPPH method, the IC_{50} value was calculated over the % inhibition value of antioxidant activity in the ABTS method. According to the ABTS result, the IC_{50} value was calculated as 0.19 and 0.22 for γGC and GSH, respectively. IC_{50} values revealed similar trends in both assays, despite with low variation. A difference can be observed between the two tests. This may be due to the type of solvent, as DPPH and ABTS model radicals are structurally different and it is not possible for the result to be the same in the two assays [202].

Literature corroborates these findings, underscoring γGC's potential in various contexts. Lin et al. (2020)[187] observed that γGC showed enhanced superoxide and DPPH radical scavenging activities compared to GSH ($p<0.05$), attributing this to structural differences and higher reactivity with free radicals in food and nutraceuticals [187]. Similarly, a study on sunflower oil demonstrated γGC's superior capacity to mitigate lipid oxidation, outperforming GSH under accelerated conditions [203].

Furthermore, computational studies emphasize the electron-donating capabilities of GSH's thiol group, a mechanism shared with γGC, though the latter's simpler structure may enhance reactivity [204].

In addition to previously discussed studies, Shcherbatykh et al. (2021) explored the antioxidant efficiency of sulfur-containing compounds, showing that GSH and γGC exhibit significant DPPH radical scavenging activity, with kinetic parameters favoring γGC due to enhanced reactivity [205]. Another study by Yang et al. (2019)[206] emphasized γGC's superior antioxidant and anti-inflammatory properties, especially under oxidative stress conditions like sepsis, where it performed better than GSH and N-acetyl-L-cysteine (NAC), highlighting its role in cellular glutathione synthesis and redox balance [206].

In oxidative stress models, γGC has also been identified as more effective in lipid oxidation prevention. For example, studies on sunflower oil demonstrated that γGC's antioxidant action exceeded GSH's under accelerated storage conditions, highlighting its potential for food industry applications [203]. Additionally, research into environmental stress on antioxidants has found that γGC's simpler dipeptide structure makes it more robust under diverse exogenous stress factors compared to GSH, which may degrade faster [207].

Findings align with established evidence suggesting that the higher radical scavenging efficiency of γGC could be due to enhanced accessibility or reactivity of its functional groups. Variations in IC50 values among assays reflect differences in radical types and assay mechanisms. These insights underline γGC's potential as a robust antioxidant for therapeutic and industrial applications, warranting further exploration into concentration effects and environmental stability.

These studies collectively affirm γGC's enhanced antioxidant activity due to its structural and functional properties, suggesting broader applications in medicine, nutraceuticals, and food preservation. The findings align well with IC50 data and inhibition rates observed in comparative DPPH and ABTS assays, underscoring γGC's utility as a potent antioxidant.

3.2 Evaluation of antimicrobial activity of γ-glutamyl cysteine and glutathione in vitro by agar diffusion assay

The antimicrobial activities of GSH, γGC, and AMP were evaluated against a range of microbial species, including Gram-negative bacteria, Gram-positive bacteria, yeast, and molds, as indicated by the inhibition zone diameters (mm). The result is shown in **Figure 13** and **Table 9**.

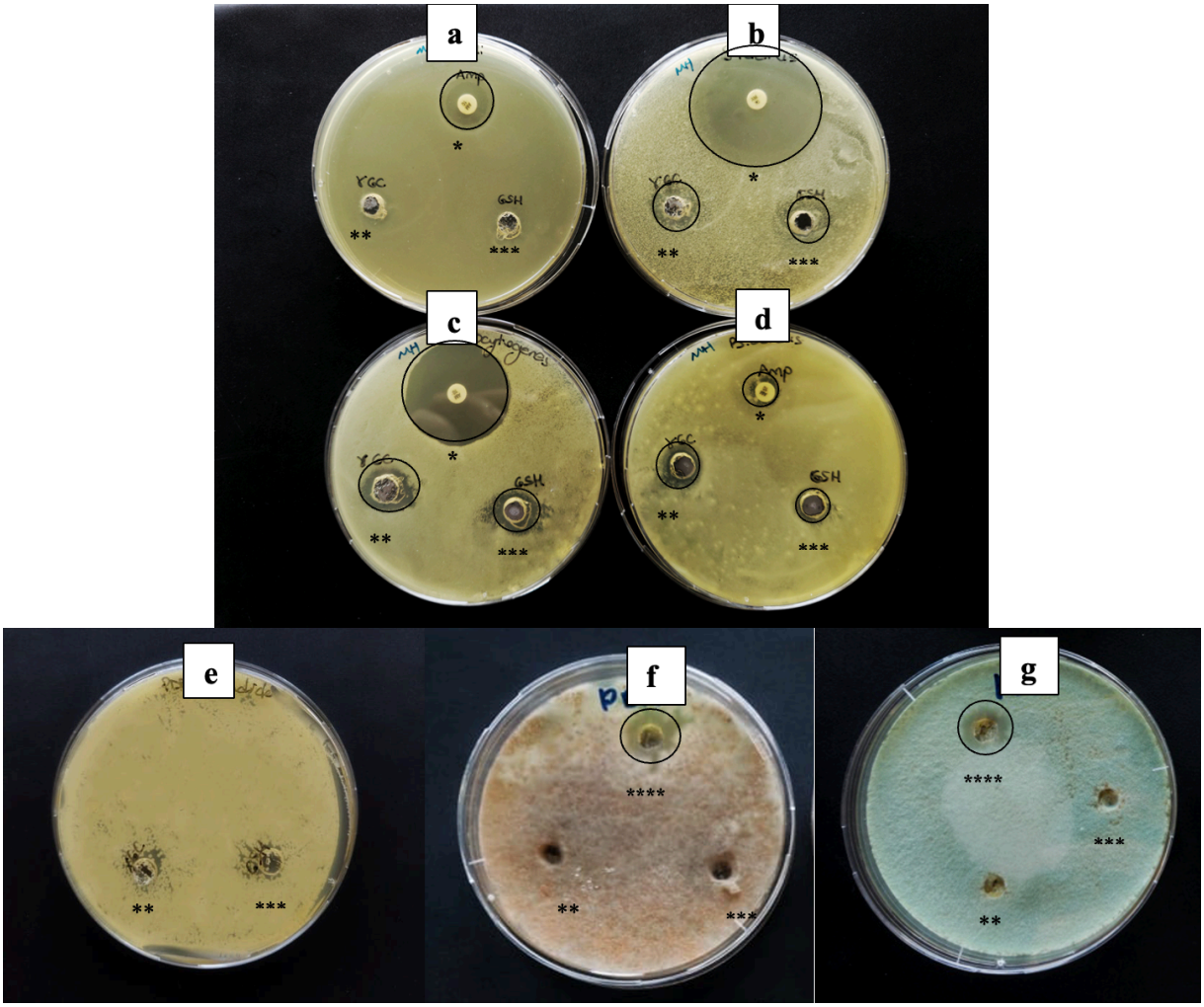


Figure 13. Impact of γGC and GSH on the growth of (a)*Escherichia coli* (b)*Staphylococcus aureus* (c)*Listeria monocytogenes* (d)*Bacillus cereus* on Mueller hinton agar and (e)*Candida albicans* (f)*Colletotrichum* sp. (g)*Penicillium digitatum* on PDA. *:Ampicillin (10µg/disc), **:γGC (10mM γGC=2.5 mg/ml), ***:GSH (10mM GSH=3mg/ml), ****:fluconazole (10µg/ml)

Table 9. Inhibition zone diameters of PRODUCT tested against microbial species

	Gram-negative bacteria		Gram-positive bacteria		Yeast	Mold	
Antimicrobial	***Escherichia coli***	***Listeria monocytogenes***	***Staphylococcus aureus***	***Bacillus cereus***	***Candida albicans***	***Penicillium digitatum***	***Colletotrichum* sp.**
	(mm)						
GSH	-	15.11±0.02	12.31±0.01	9.2±0.01	-	-	-
γGC	-	15.20±0.02	12.95±0.01	11.67±0.01	-	-	-
AMP	15.19±0.00	32.43±0.01	37.26 ± 0.01	9.42 ± 0.00	NA		

Two results (Mean values ± SD, n = 2) read from the same column and marked with the same letter do not differ significantly at a threshold α = 5 % (one-way ANOVA; Tukey's test). **NA:** Not Applicable

The results showed significant antibacterial activity, particularly against Gram-positive bacteria, with inhibition zones of 32.43 ± 0.01 mm and 37.26 ± 0.01 mm for *Listeria monocytogenes* and *Staphylococcus aureus*, respectively. Conversely, GSH and γGC exhibited moderate activity, with inhibition zones of 15.11 ± 0.02 mm and 15.20 ± 0.02 mm for *Listeria monocytogenes* and 12.31 ± 0.01 mm and 12.95 ± 0.01 mm for *Staphylococcus aureus* respectively. For *Bacillus cereus*, γGC (11.67 ± 0.01 mm) outperformed GSH (9.2 ± 0.01 mm), but both were considerably less effective than AMP. None of the antimicrobials showed significant activity against molds (*Penicillium digitatum* and *Colletotrichum acutatum*), highlighting limited antifungal potential.

When comparing γGC and GSH, γGC consistently yielded slightly larger inhibition zones. Antimicrobial activity can be related chemical reactivity of both compounds, as the γ-glutamyl structure in γGC and GSH has been shown to improve interactions with microbial enzymes or membranes. Similar observations have been made by Wang et al. (2019) [208], who reported that γ-glutamyl derivatives tend to exhibit greater antimicrobial activity than GSH alone due to their better stability and targeted mechanism of action.

Comparing these results with existing literature, GSH and γGC are reported to exert moderate antimicrobial activity primarily due to their ability to disrupt thiol-dependent enzymes and modulate oxidative stress within microbial cells. For instance, Liu et al. (2018)[209] observed that GSH exhibited inhibition zones ranging from 10–14 mm against Gram-positive bacteria at similar concentrations, aligning well with the current study's results for *Listeria monocytogenes* and *Staphylococcus aureus*. Similarly, the modest antifungal activity observed for γGC and GSH against *Candida albicans* in this study mirrors findings by Gupta et al. (2020)[210], who reported comparable inhibition zones (9–12 mm) for GSH-derived compounds.

However, neither γGC nor GSH demonstrated significant activity against Gram-negative bacteria (*Escherichia coli*) or molds (*Penicillium digitatum, Colletotrichum acutatum*), consistent with prior studies indicating that Gram-negative bacteria's outer membrane and fungal cell wall composition limit the efficacy of oxidative stress modulators [211]. This suggests that while γGC is marginally more effective than GSH, both compounds exhibit limited antimicrobial potency as standalone agents and require optimization or combination strategies for broader-spectrum applications.

3.3 Minimal Inhibitory Concentration

The minimum inhibitory concentration (MIC) analysis of γG and GSH revealed differential antimicrobial activities against Gram-positive and Gram-negative bacteria. The lowest amount that inhibits the growth of the microorganism is determined in μg/ml or mg/L. MIC for γGC and GSH tested against four bacteria are shown in **Table 10 and Figure 14.**

Table 10. MIC results of the γGC and GSH tested against microbial species

	Gram-negative bacteria		**Gram-positive bacteria**	
	Escherichia coli	***Listeria monocytogenes***	***Staphylococcus aureus***	***Bacillus cereus***
		(mM)		
γGC	10	5	10	5
GSH	-	5	10	10

5mM γGC= 1.25 mg/ml, 10mM γGC=2.5 mg/ml, 10mM GSH=3mg/ml

γGC demonstrated better efficacy than GSH, showing lower MIC values for *E. coli* (10 mM) and *B. cereus* (5 mM). For *L. monocytogenes*, both γGC and GSH exhibited the same MIC value (5 mM), while for *S. aureus*, γGC had an MIC of 10 mM, similar to GSH. These findings suggest that γGC is more effective against certain Gram-negative bacteria (*E. coli*), potentially due to its structural characteristics, which may improve its penetration through the outer membrane or enhance its reactivity with microbial components.

When compared to literature, the MIC results align with previous findings that thiol-based compounds like GSH and its derivatives have moderate antimicrobial activities. For instance, Wang et al. (2019) [208] reported that GSH displayed MIC values ranging from 8 to 16 mM against Gram-positive bacteria, depending on the microbial species and environmental conditions. Similarly, Miller et al. (2021) [211]found that γGC was more effective than GSH in their study, with MIC values of 5–10 mM for Gram-positive bacteria and 10–15 mM for Gram-negative strains. This superiority is attributed to the γ-glutamyl moiety, which enhances oxidative stress modulation and disrupts cellular redox balance more effectively than GSH.

Both compounds displayed lower efficacy against *S. aureus* compared to *L. monocytogenes* or *B. cereus*, a trend supported by Gupta et al. (2020)[210], who noted that some Gram-positive bacteria exhibit adaptive mechanisms against oxidative stress modulators. However, neither γGC nor GSH achieved the high efficacy observed with conventional antibiotics or other specialized antimicrobial agents, limiting their application as standalone therapies but highlighting their potential as adjunctive agents in combination therapies.

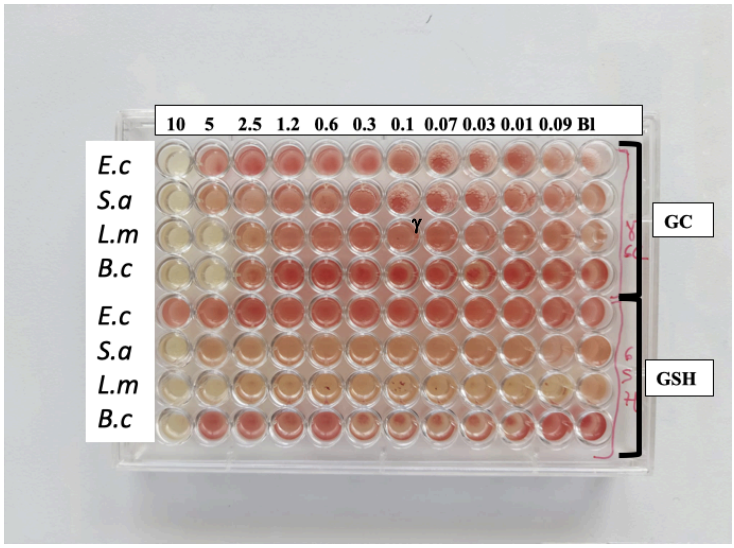


Figure 14. MIC results of γGC and GSH on the microplate. *E.c: E.coli S.a: Staphylococcus aureus, L.m: Listeria monocytogenes B.c: Bacillus cereus* in Mueller hinton agar. Bl: Blank

4. Conclusion

In this study, antioxidant activity of γGC and GSH was determined by two different methods. According to both methods, the antioxidant activity of γGC was higher than that of GSH. The antimicrobial activity of these two compounds was also tested against various microorganisms. Both *B. cereus* and *E. coli* indicated efficiency of γGC over GSH, but neither exhibited antifungal action. γGC has an antioxidant capacity as well as GSH and can be used as an effective antioxidant to protect the human body from free radicals and delay the progression of many chronic diseases. It also has the potential to be used as an antioxidant in foods.

Both compounds showed low antimicrobial activity, indicating that they cannot be effectively used as a stand-alone antimicrobial agent. This finding suggests that although the compound does not independently inhibit microbial growth, it may enhance the efficacy of established antimicrobial agents through complementary mechanisms. Further studies are needed to investigate the molecular basis of this synergistic interaction and to determine the optimum combinations and concentrations for effective application in antimicrobial formulations.

Chapter 4

Investigation of the antioxidant effect of two Thiols, γ-glutamylcysteine and glutathione, in sunflower oil under accelerated storage

1.Introduction

Based on its high content of unsaturated fatty acids, especially essential 9-cis and 12-cis-octadecadienoic acid, sunflower oil is considered as one of the highest quality vegetable oils for human nutrition [213]. Beside this, it is also one of the most prone to oxidation upon storage [214]. Compounds formed by lipid oxidation such as hydroxyperoxides, aldehydes, carbonyl compounds, hydrocarbons (alkane, alkene) formed during oil oxidation have adverse affects on human health but also alter the chemical and sensory properties of oils. Oxygen is mainly responsible of the initiation and acceleration of oils oxidation [215] although, temperature, light, degree of unsaturation and the presence of metal ions such as copper and iron also accelerate oxidation [216]. Therefore, it is challenging to avoid oil oxidation without adding any antioxidant substances.

Antioxidants are compounds that prevent or delay oxidative reactions based by a combination of scavenging free radicals, chelating prooxidative metals, quenching singlet oxygen among other [112]. Based of their lower cost and greater efficiency, antioxidants obtained by chemical synthesis, including butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and tertiary hydroquinone (TBHQ) are commonly utilized in the food industry [217]. However, they are also associated with issue in safety to use [218]. Consumers avoid the use of these products due to the fact that these chemically synthesized antioxidants have a harmful affect on human health. Therefore, the demand for natural antioxidants is constantly increasing.

In cells, different thiol compounds has antioxidants fuction prevent damage caused by free radicals and provide detoxification. Glutathione (GSH), cysteine (CYS), homocysteine (HCYS), N-acetylcysteine (NAC), captopril (CAP) and γ-glutamylcysteine (γGC) are the best known biological thiols [201]. γGC, a dipeptide composed of cysteine and glutamate is the cellular precursor of GSH. γGC and GSH are thought to protect cells against oxygen toxicity by destroying peroxides, disulfides, and other oxygen-generated species [129]. In particular, GSH is used in the cosmetic, medical and food industries as an active ingredient of these products to alleviate harmful oxidative processes and prevent the formation of toxic compounds such as radicals (superoxide, hydroxyl, peroxyl, alkoxyl) and non-radicals (hydrogen peroxide hydroperoxide etc.). It is also used to strengthen and repair skin whitening due to its anti-aging affect [188].

The oxidative stability of oils can be determined during storage and packaging under normal ambient conditions. However, oxidation takes a long time to occur, which makes it impractical for routine analysis. Therefore, accelerated oxidation test have been developped, notably by increasing storage temperature to 50 °C upon 15 days [219].

The objectives of this study were to evaluate the ability of GSH and its precursor γGC to prevent or at least reduce oxidation of sunflower oil and to compare thier antioxidant capacity between them and other used industrial antioxidants. For this purpose, sunflower oil was subjected to accelerated oxidation

under laboratory conditions and the affect of temperature, sun light and oxidation was monitored during storage period.

2.Material and methods

2.1. Materials and reagents

The refined organic sunflower oil used in this study was purchased from Beyorganic company located in Istanbul, Turkey. GSH, γGC, BHA, TBHQ and BHT were purchased from Sigma Aldrich Co. (St. Louis, MO, USA).

2.2. Oxidation of Sunflower oil in the presence of glutathione, γ-glutamyl cysteine and TBHQ

Accelerated oxidation test were performed according to the method of Papadopoulou and Roussis (Papadopoulou et al., 2008)[146]. Briefly, 50 ml of sunflower oil were stored in 100 ml flasks in the presence of GSH; 40 mg/L, γGC; 40 mg/L, TBHQ (T; 200 mg/L), γ-glutamylcystein+TBHQ (GCT; 40 mg/L+40 mg/L) and glutathione+TBHQ (GSHT; 40 mg/L+40 mg/L). Oil without additives was selected as a control (C; no additive). The experiment carried out with 3 repetition for each sample. Prior incubation, antioxidants were dispersed in the oil phase in an ultrasonic water bath (Bandelin, RK 1028H, Berlin) for 10 minutes. All samples were incubated at 50°C and in sun light conditions for 15 days. Analyzes were performed in 2 replications on days 0, 3, 6, 9, 12 and 15.

2.3. Determination of free radical scavenging activity for glutathione, γ-glutamyl cysteine, BHA, TBHQ, BHT

The radical scavenging activity of GSH and γGC and commercial antioxidants used in food, namely BHA, BHT, TBHQ were tested according to Blois et al (1958) with some modification (Blois, 1958). Briefly, solutions of GSH, γGC, BHA, TBHQ and BHT were prepared in ethanol at concentrations ranging from 0.04 to 1.5 mM. 390 µl of DPPH solution (25 mg/L ethanol) was mixed to 100 µl of the different antioxidant solutions. After, 30 minutes of incubation in the dark at room temperature (25°C). The results was measured at 517 nm absorbance by visible spectrophotometer (Perkin- Elmer, USA). For the reference sample, a mixture of ethanol and DPPH was used [220]. The scavenger activity was calculated according to Eq. (1):

DPPH radical scavenging activity (%) = [(Ab-As) / Ab] * 100 (1)

Ab is the blank absorbance and As is the absorbance of the sample.

IC_{50} was calculated for each sample on inhibition.

2.4. Determination of peroxide value, free fatty acids and p-Anisidine value

To determine the peroxyde value (PV), 1 g of sunflower oil was dissovled by gentle mixing in 25 mL of chloroform-acetic acid solution (2:3 v/v) mixture. One ml of saturated potassium iodide solution was then added and the mixture was incubated in the dark for 5 minutes. It was then diluted by adding 75

mL of distilled water. One mL of 1% starch solution was added and the final solution was titrated with 0.01N sodium thiosulfate ($Na_2S_2O_3$). The PV values were calculated according to AOCS, 2003, cd 8-53 method using Eq. (2):

PV= (V×N×1000 meq g O_2/kg) /m (2)

V: Spent sodium thiosulfate solution, mL N: Normality of sodium thiosulfate solution m: sample weight, g.

The amount of free fatty acids (FFA) in oil samples was calculated as oleic acid. To determine the FFA, 1g of oil sample was dissolved in ethanol-diethyl ether (1:1 v/v) solution. The resulting solution was then titrated by 0.1 N KOH in ethanol in the presence of phenolphthalein. Fatty acidity content was calculated according to the AOCS Ca 5a-40 method [221] using Eq. (3):

% FFA = V x N x Ma /m (3)

V: 0.1 N spent ethanolic potassium hydroxide solution, mL N: Normality of ethanolic potassium hydroxide solution, Ma: Molecular weight of oleic acid, m: Sample weight, g

The p-anisidine value (P-Av) was determined according to the modified AOCS 1990 method. First 0.5 g of oil sample was dissolved in 10 ml of hexane was measured at a wavelength of 350 nm with Lambda 25 UV-VIS (Perkin- Elmer, USA) spectrophotometer. Then 2.5 ml of the solution was taken and 0.5 ml of p-anisidine solution (0.25 g/100 ml of acetic acid) was added, and absorbance was measured at 350 nm. Finally, the P-Av was calculated according to Eq. (4):

P-Av= 10*(1.2 x A2- A1)/m (4)

A2 last reading, A1: first reading, m: sample quantity.

2.5. Measurement of Colour (L, a*, b*) and calculation of total oxidation (Totox) value*

Colour measurement in oil was performed by Chroma meter CR-400(Conica Minolta, Japan) colourimeter. In the CIELAB colour space *L**, *a**, and *b** values indicate lightness, green to red, and blue to yellow, respectively. The Totox value in oil was calculated on PV and P-Av according to theEq. (5):

Totox =(2*PV) + p-AV (5)

2.6. GC-MS analysis of Fatty Acids

For determination of fatty acid composition, the methylesters were prepared by cold transmethylation with potassium hydroxide according to IUPAC 2.301-2.302 method [222]. Briefly, 10 mg oil was dissolved in 10 ml hexane solvent. Then 0.5 ml of 2N KOH was added to the oil sample and left in the dark for 2 hours. Then, the upper phase was collected and filtered with a 0.45 μm filter before being analysed by gas chromatography-mass spectrometry (Agilent- 7890B GC -7010B MS) with an autosampler (Gerstel, Germany) equipped with the flame ionization detector (FID). A capillary DB-WAX column (Agilent J&W; 60m x 0.25 μm x 0.25μm) was used. The oven temperature was held at 50ºC for 1 min, raised to 200ºC at a rate of 25°C/min held for 10 min and then to 230ºC at a rate of 3

ºC/min held this temperature for 25 min. The injector (250 ºC) and detector temperatures (300ºC) were set. The sample size was 1µl and the the flow rate of helium carrier gas was 1 mL/min. The split used was 1:40. The identification of fatty acid was determined by checking with the retention times of known fatty acid standards and given as a percentage of the total.

2.7. Statistical analysis

The results of the analyzes were evaluated by analysis of variance (ANOVA), and Tukey tests. "Rstudio 2022.02.03 version "agricolae" package was used for statistical analysis. Principle component analysis was done by Xlstate 2023 software. Results were evaluated in biplot.

3.Results and Discussions

3.1 Antioxidant properties

Figure 15 presents the antioxidant properties using DPPH assay of the γGC and GSH, along with the reference standards TBHQ, BHA, and BHT. DPPH is a stable free radical widely used for screening compounds with free radical scavenging abilities [195].

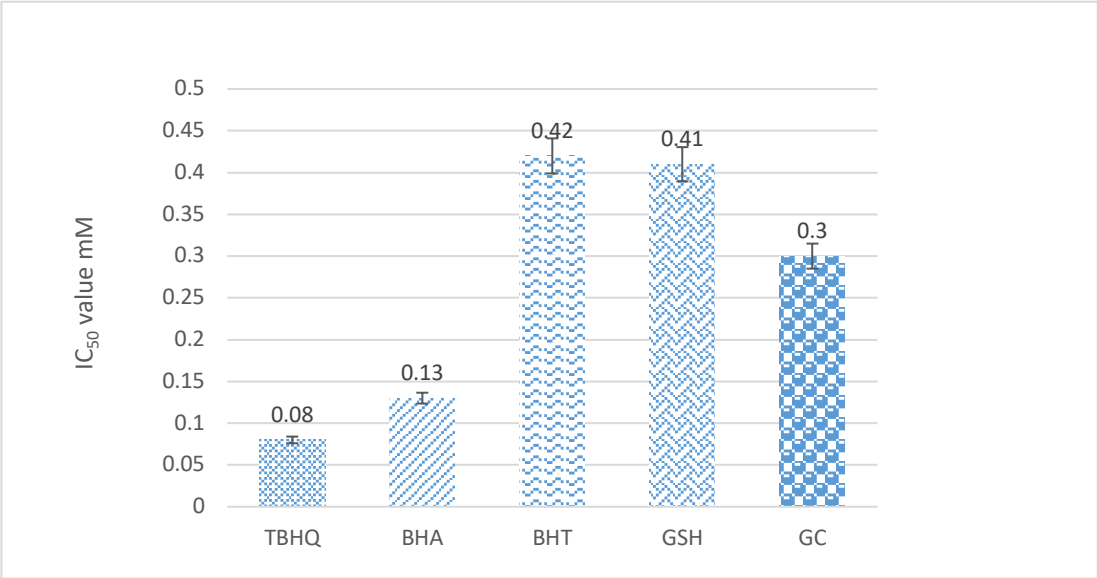


Figure 15. DPPH radical-scavenging activity of GC (γGC) and GSH compared with BHA, BHT, and TBHQ at different concentrations. Mean ± SD, n=3

In the present study, IC_{50} values were found in the range of 0.08 to 0.42 mM. It was observed that the DPPH radical-scavenging activity of the γGC and GSH were found to be higher than the TBHQ and BHA, but lower than the BHT (**Figure 15**). Their scavenging activity of DPPH radicals decreased in the following line TBHQ>BHA>γGC>GSH>BHT. Superoxide anion and hydroxyl groups are two of the most important free radicals. Superoxide anions are produced by adding an electron to molecular oxygen and are harmful reactive oxygen species because they damage cellular components in biological systems [223]. It was noted that the presence of -SH groups in the medium significantly eliminated the superoxide anion and hydroxyl groups. Since γGC and GSHS contain -SH groups, it has a powerful scavenging affect on free radicals [224].

Many studies regarding DPPH radical-scavenging activity in the literature are on plant extracts and oils comparing with synthetic antioxidants. Although there are not many studies on DPPH scavenging activity of γGC and GSH in oil, [196] compared hydroalcoholic extracts from *Aegopodium podagraria L.* with ascorbic acid and GSH. It was stated that the inhibition concentration of GSH was around 65% for 0.7 mM. In present study, it was found 64.2 % for 0.75mM GSH. In another study, the synergistic effect of GSH (50-200 μM) with various flavones was studied using DPPH. It was noted that inhibition % increased when GSH amount raised [225].

TBHQ IC_{50} value was determined as 6.87 μg/ml and 29.81 μg/ml in two studies [226,227]. In the present study, the IC_{50} value corresponds to 13μg/ml (0.08 mM) for TBHQ. Chen et al (2014) compared rosemery extract with commercial antioxidants in their study, the IC_{50} value was ordered as TBHQ>BHA>BHT [228]. Our finding was also in agreement with the results reported by previous studies [229,230]. The maximum limit of commercial antioxidants usage is determined as 200 mg/L by the Food Drug Administration (FDA). Although the determined IC_{50} values of these antioxidants are low, the limit used is high considering their negative effects on human health [231].

3.2. Determination of PV and FFA and P-Av in accelerated oxidation of sunflower oil

The degree of oxidation in oils is usually characterised by the peroxide value that relates to hydroperoxides, the primary oxidation products that are unstable and readily decompose to form mixtures of mainly volatile aldehyde compounds. It is known that oils are rapidly oxidized by heat, light and air, thus increasing the PV [232]. The affect of antioxidants on peroxide value in the sunflower oil samples is in shown in **Figure 16**. Results show that PV increases linearly for all samples with storage time. The increase in PV accelerated after 3rd day. As a result, the control sample had the highest PV till the end of storage time 4.67– 168.33 meq O_2/kg. When the samples are compared in terms of PV, the order was C>GSH>γGC>GSHT>GCT>T. There are significant differences between groups, particularly at the end of oxidation ($p<0.05$). As mentioned above, γGC, a dipeptide, is the precursor of GSH. According to the DPPH result, γGC, which showed higher activity than GSH, showed a stronger antioxidant affect against GSH during accelerated oxidation. Crapiste et al (1999) measured the peroxide value of sunflower oil before and after storage. The values obtanied indicated that while the peroxide value was 3.36 meq/kg at the beginning, it reached 90 meq/kg in 30 days at 47°C [233]. These results are in line with the peroxide value (168.33 meq/kg, at 50°C, 15 days) recorded in the present study corraborating the impact of temperature. In this study, we aimed to highlight any synergistic affects of γGC and GSH with TBHQ, as antioxidant in sunflower oil that will allow subsequent utilisation of lower concentration of TBHQ. According to the results, values close to each other were GCT, 19.33 meq O_2/kg and GSHT, 27.33 meq O_2/kg ($p<0.05$), TBHQ was recorded when 200 mg was used (15 meq O_2/kg ($p<0.05$)). This means that it showed a positive synergistic affect with the synthetic antioxidant. GSH was compared with synthetic antioxidant, and its antioxidant affect was investigated by measuring

the peroxide value in butter. GSH effect showed results close to BHA at the end of storage [147]. There are also studies close to our results to prevent the increase of PV in sunflower oil [234-236].

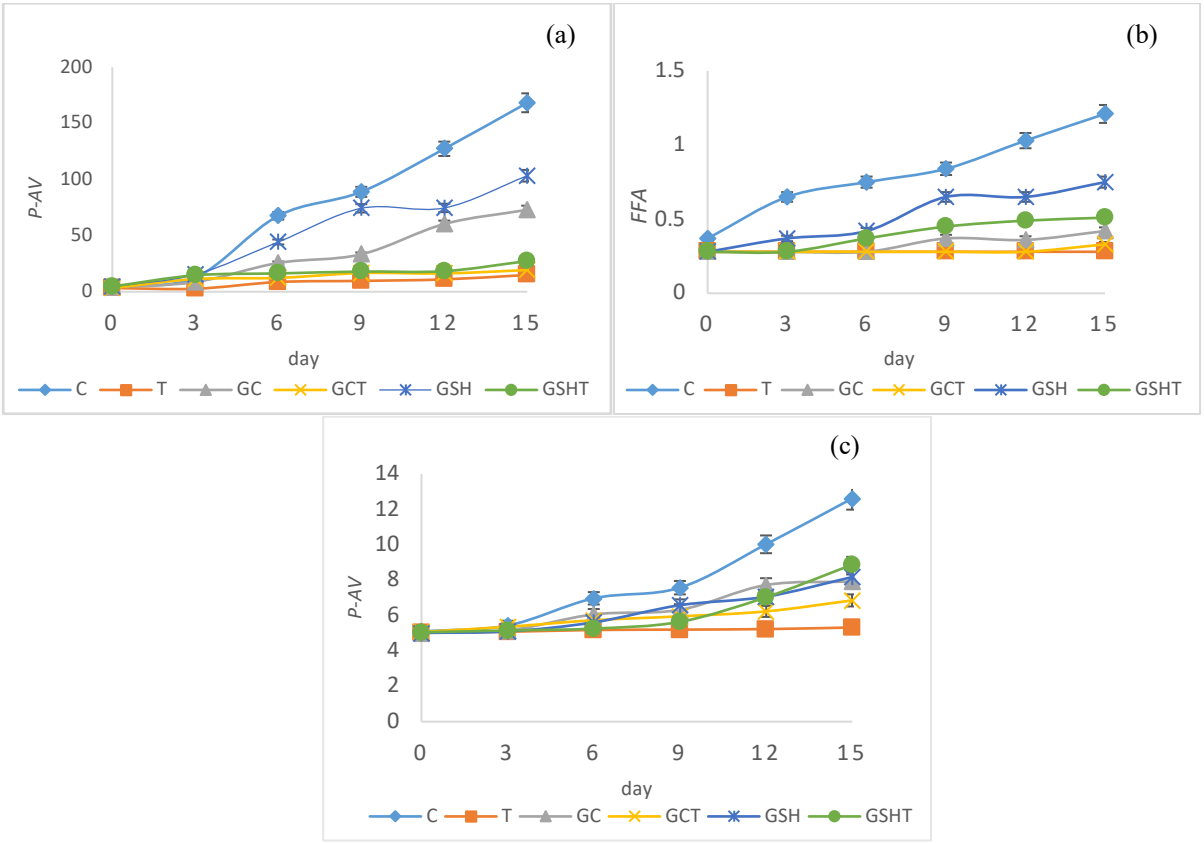


Figure 16. Affect of adding TBHQ, GC, and GSH on peroxide (a), FFA (b) and p-anisidine value (c) in sunflower oil under accelerated oxidation conditions. C (control), T (TBHQ), GC (γ-glutamyl cysteine), GCT (γ-glutamyl cysteine+TBHQ), GSH (glutathione), GSHT (glutathione+TBHQ). Mean ±SD, n=3

FFA refers to the free fatty acids and is expressed in mg of potassium hydroxide or sodium hydroxide required to neutralize 1 gram of fat. FFA is an important quality index for oil and is constantly used as a shelf-life monitoring parameter in oil. An increase in FFA means a decrease in stability to oxidation. This is one of the important indicators that the oil will start to become rancid [237]. The concentration of FFA calculated as % oleic acid are shown in **Figure 16.**

According to the FFA data, the best result during the storage period belongs to the oil sample containing TBHQ. A significant difference was not observed between groups till 1the 6th day ($p<0.05$). At the end of storage time, FFA of sunflower oil sample C, TBHQ, γGC, GCT, GSH, GSHT were 1.21±0.16, 0.28±0.00, 0.42±0.00, 0.33±0.08, 0.75±0.16, 0.51±0.08 (% oleic acid) respectively. According to Duncan's multiple comparison, there was no significant difference between T, γGC, GCT, but there was a statistical difference between these groups with GSH and GSHT. This shows that γGC has antioxidant affects close to TBHQ. In addition, when we examined the combination of γGC and GSH with TBHQ, GCT showed the highest antioxidant result. Generally FFA result increased in parallel with PV results [238]. FFA measurement by the accelerated oxidation method in sunflower oil has been measured in many studies. While FFA TBHQ value (0.28 oleic acid %) in this study had a similar to the

literature [228], GCT results showed higher antioxidant activity than the literature compared to plant-derived antioxidants. GCT results in this study showed higher antioxidant activity than literature [239-240].

The oxidative degradation of oils begins with the formation of primary compounds such as hydroperoxides. They react to undesirable secondary oxidation products, such as aldehydes, alkanes, esters, alcohols etc. Secondary oxidation products are determined by P-Av in oils and fats [241]. P-Av results are shown in **Figure 16**. The P-Av results were parallel to the PV results and increased on the 3rd day, with the highest value at the end of storage.

The data presented in **Figure 16** showed that sample C has the highest secondary oxidation products. A statistically significant difference was observed in P-Av between the γGC and GSH at the end of storage. After 15 days the increase in P-Av value is in the order of C (7.95±0.68) > γGC (6.37±0.65) > GSH (6.26±0.55) > GSHT (6.17±1.14) > GCT (5.87±0.66) when all days are averaged. As a result of P-Av, lower value was recorded in all the doped groups than the control group. Although γGC and GSH values were close, there was a statistically significant difference between TBHQ and GSH groups, but no difference was observed between TBHQ and γGC groups ($p<0.05$). There is no difference between γGC and GCT. It is thought that if the γGC concentration is increased in oil, it will slow down the increase of P-Av without TBHQ.

Two major methods seperate the oxidation level; the peroxide number measures the hydroperoxide level, while the anisidine value measures the secondary oxidation level. In a similar study the ability of GSH and N-acetyl-cysteine for all conditions showed results close to the BHA sample in P-Av measurement. As a result N-acetyl-cysteine and GSH may be taken into account as antioxidants in corn oil during storage, cooking or frying [146].

When the two measurements are made together and applied to the formula, the Totox level is revealed and the oxidation level is determined. According to many studies, the maximum levels are 5 meq/kg for peroxide level, 20 for anisidine and 26 for totox [242]. Totox value results shown in **Figure 17.**

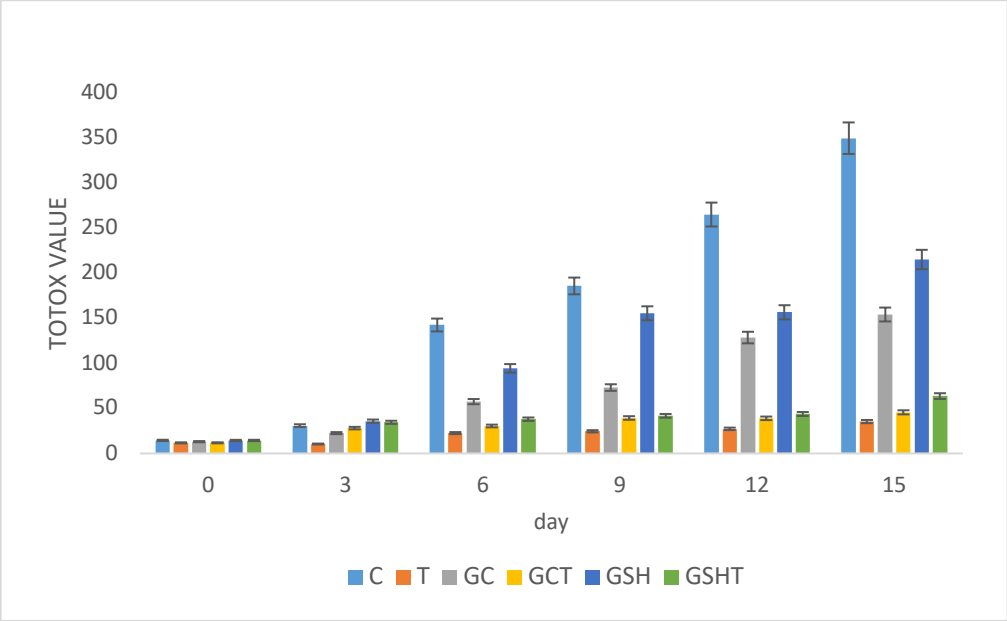


Figure 17. Comparison of p-Anisidine value of different treatments of sunflower oil during 15 days at 50˚C. C (control), T (TBHQ), GC (γ-glutamyl cysteine), GCT (γ-glutamyl cysteine+TBHQ), GSH (glutathione), GSHT (glutathione+TBHQ). Mean ±SD, n=3

At the end of the storage period, the totox value of all groups was above 26 meq O_2/kg. However, when the antioxidant groups were compared with TBHQ protected oil, a rapid increase was observed in the total oxidation value at the end of the 3rd day in parallel with the PV and P-Av. In the end of storage time totox value of C, T, γGC, GCT, GSH, GSHT determined (349.28±6.79) meq O_2/kg, (35.32±1.22) meq O_2/kg, (153.92±4.71) meq O_2/kg, (45.52±3.99) meq O_2/kg, (214.84±2.34) meq O_2/kg, (63.54±0.56) meq O_2/kg respectively. In a study in which lemon peel was used as an antioxidant, the totox value was compared with the oil sample containing BHT and the totox value was found higher than in the study compared to data given by Okhli et al., (2020)[243]. In another study researchers reported that using of fennel seed extract at 100-800 ppm in soybean oil reduced totox values in oil samples. The extract at levels of 300 and 400 ppm showed higher antioxidant activity than BHT and BHA [244].

3.3. Colour properties of the samples

The colour attributes of the oil are the main criteria affecting consumer acceptance. The colour data of the samples are displayed in **Figure 18**. As a measure of the colour (*L**, brightness/darkness and *b**, blueness/yellowness) of the oil. All treatments showed darkening over time (ie, decreasing *L**). *L** value was 54.1±0.14 at the beginning of the storage process for all samples. Then *L** decreased to 49.98, 53.70, 50.68, 51.71, 50.80 and 51.64 after 15 days under heating and light conditions for C, T, γGC, GCT, GSH and GSHT, respectively. At the same time, the initial *b** value of 3.6 increased to 5.68, 4.13, 5.04, 4.92, 5.10 and 5.19 for C, T, γGC, GCT, GSH and GSHT, respectively. The yellow colour of these oils is defined by increasing of *b** over time during storage, because of the natural carotenoids in the sunflower oil.

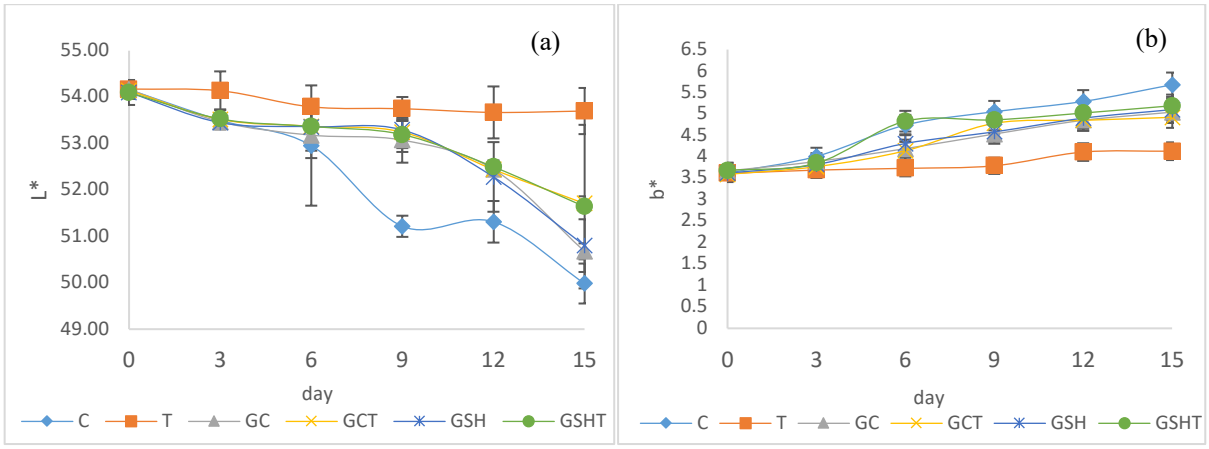


Figure 18. Changes in the *L** (a) and *b** (b) value in sunflower oil during 15 days at 50˚C. C (control), T (TBHQ), GC (γ-glutamyl cysteine), GCT (γ-glutamyl cysteine+TBHQ), GSH (glutathione), GSHT (glutathione+TBHQ). Mean ±SD, n=3

This study states that significant difference between the *b** value of γGC and GSH samples. However there was no difference for *L** value ($p>0.05$). In the control sample, while the *L** value decreased dramatically, the *b** value increased. Colour value is an important criterion especially in frying oils [234]. A colour change was observed in frying oil during oxidation in a study. In another study, it was observed that the *L** value decreased rapidly and the *b** value increased significantly [245].

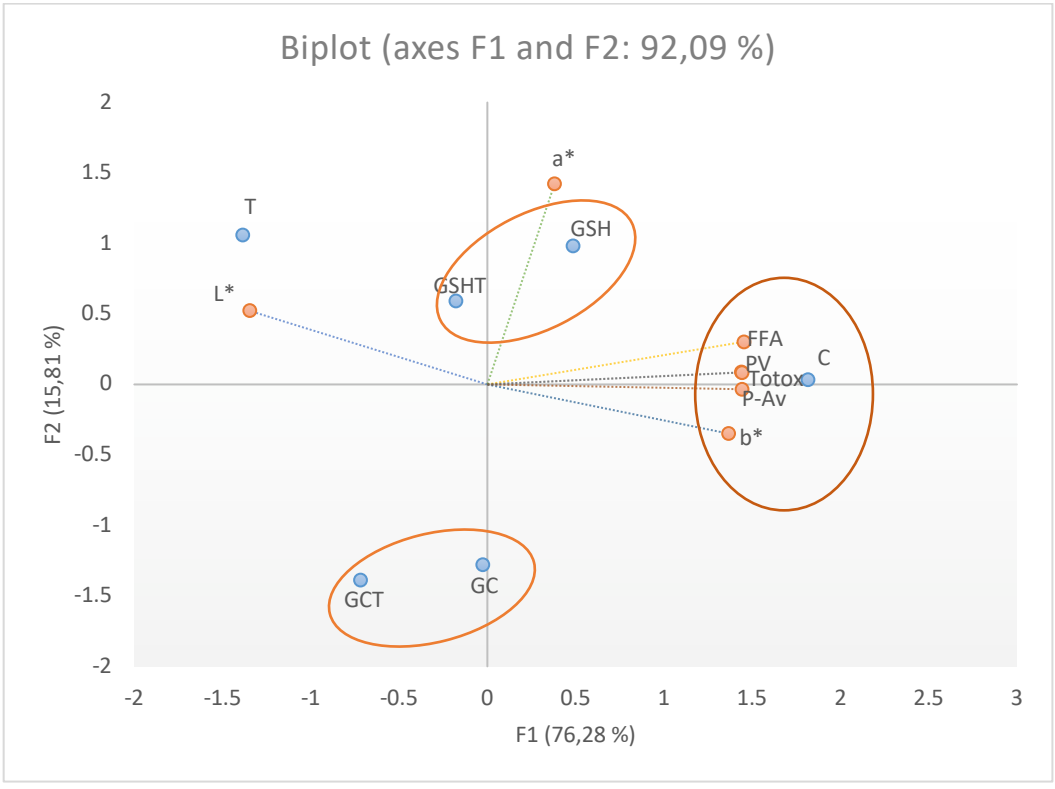


Figure 19. Principle component analysis according to the sunflower oil samples based composition of oils et the end of storage. C, GSH (40 mg/L), GC (40 mg/L) and TBHQ (200 mg/L), GCT (40 mg/L+40 mg/L) and GSHT (40 mg/L+40 mg/L)

Principal component analysis (PCA) was utilized to demonstrate a better explanation of the chemical composition of the sunflower oil with different additives. **Figure 19**. shows the correlation biplot for the composition of oil samples. The plot indicates that the first two components (F1 and F2) account for 92.09 percent of the required information regarding the differences between oils oxidation profile. Control sample replaced in the first region of the coordinate system, GSH alone fell in the same region as the C, GSHT found its place in the 2nd region with T. GCT and T added oil groups fell into separate regions on the graph, although they had the lowest value when looking at oxidation criteria. This indicates that they have close affects on oxidation, especially in maintaining the *L** value. Additionaly there was positive correlation between PV and FFA (r=0.94), *b** and P-Av (r=0.92), P-Av and FFA (r=0.93) while *L** and *b** showed negative correlation (r=0.93).

3.4. Fatty acid profile

Refined sunflower oil contains approximately 15% saturated, 85% unsaturated fatty acids and forms of 14-43% oleic and 44-75% linoleic acids in unsaturated fatty acid content. Fatty acid composition of oil samples shown in **Table 11**.

Table 11. Fatty acid composition of sunflower oil by GC/MS

	C16:0 (Palmitic acid)				**C18:0 (Stearic acid)**				**C18:1 (Oleic acid)**				**C18:2 (Linoleic asit)**			
	IS		ES		IS		ES		IS		ES		IS		ES	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
C	6.72^{a}	±0.97	7.61^{ab}	±0.05	2.84^{a}	±0.45	3.2^{a}	±0.02	31.65^{b}	±0.03	32.45^{a}	±0.08	55.96^{a}	±0.28	55.6^{ab}	±0.37
T	7.48^{a}	±0.06	7.46^{c}	±0.03	3.16^{a}	±0.03	3.17^{a}	±0.05	32.01^{a}	±0.1	31.93^{b}	±0.11	56.39^{a}	±0.25	56.39^{a}	±0.35
γGC	7.50^{a}	±0.07	7.51^{bc}	±0.09	3.16^{a}	±0.02	3.20^{a}	±0.02	32.05^{a}	±0.09	32.18^{ab}	±0.27	56.27^{a}	±0.17	55.83^{ab}	±0.43
GCT	7.48^{a}	±0.03	7.49^{bc}	±0.08	3.19^{a}	±0.02	3.19^{a}	±0.04	31.88^{a}	±0.05	31.89^{b}	±0.19	56.15^{a}	±0.01	56.17^{a}	±0.3
GSH	7.46^{a}	±0.05	7.66^{a}	±0.00	3.19^{a}	±0.02	3.25^{a}	±0.00	32.01^{a}	±0.11	32.47^{a}	±0.00	56.24^{a}	±0.27	55.35^{b}	±0.00
GSHT	7.44^{a}	±0.02	7.48^{bc}	±0.02	3.17^{a}	±0.02	3.18^{a}	±0.01	31.87^{a}	±0.00	32.08^{a}	±0.09	56.18^{a}	±0.02	56.36^{a}	±0.06

IS: initial storage, ES: end of storage, C (control), T (TBHQ), γGC (γ-glutamyl cysteine), GCT (γ-glutamyl cysteine+TBHQ), GSH (glutathione), GSHT (glutathione+TBHQ). Mean ±SD, n=3

Adding 200 mg TBHQ appears benificial in shielding the oil from oxidation, based on comparing the results before and after storage for all fatty acids. The level of oleic acid is the measure of oxidation. When γGC was compared to GSH, it was shown that γGC inhibited oleic acid growth ($p>0.05$). Palmitic acid and linoleic acid showed no appreciable change before and after storage. The results show lower levels of stearic, palmitic and linoleic fatty acids compared to test conducted with sunflower oil and higher levels of oleic acid [246,247].

Loh et al (2006) investigated the effect of synthetic antioxidants (100-750 mg/L) on palm oil during a 5-week storage period. They determined the synthetic antioxidants effect on fatty acids to be in order of vitamin E<BHT<TBHQ<BHA<PG (Propyl gallate) [207]. Considering the IC_{50} values in our current study, we predict that increasing the amount of γGC and GSH in future studies may decrease the fatty acid composition.

4. Conclusion

In this study, the antioxidant affect of two thiols, γGC and GSH, in sunflower oil under accelerated storage was studied. The findings of this study demonstrated that GSH and γGC worked affectively as antioxidants in sunflower oil when it was stored. The antioxidant of γGC, the precursor of GSH, was shown to be more important than GSH. When combined with the synthetic antioxidant, the use of γGC and GSH produced a synergistic affect. This indicates that the synthetic antioxidant level in sunflower oil can be reduced by using GSH and especially γGC as a curative strategy. It is estimated that these two compounds, which are affective even at low concentrations, will give affective results alone like TBHQ when the amount in the oil is increased.

Chapter 5

Evaluating the potential of gamma-glutamylcysteine and glutathione as substitutes for SO_2 in white wine

1.Introduction

Due to its antimicrobial and antioxidant properties, the colorless and pungent gas sulfur dioxide (SO_2) is a widely used preservative in the wine industry (from must to wine production) [248]. Moreover, it prevents both enzymatic and non-enzymatic processes [249]. More SO_2 is specifically utilized in the manufacture and storage of white wines to prevent oxidation reactions that alter the chemical composition and result in browning, which degrades the wine's sensory value.

Despite its antioxidant benefits, the European Union (EU) restricts the use of SO_2 in wine due to health concerns. The maximum limits for total SO_2 in white and rosé wines are 200 mg/l and 150 mg/l for red wines, according to European Union regulations (EU Regulations No 2019/934) [250]. Furthermore, for organic wines, they are lower: white and rosé: maximum 150 mg/l total SO_2; red: maximum 100 mg/l total SO_2. In recent years, the presence of SO_2 in wine has raised concerns about its adverse effects on health such as individuals with asthma, chronic lung disease. As a result, enological research is seeking for approaches to reduce or possibly replace SO_2. The majority of studies on substitutes for SO_2 has been focused on those with oxidative and antimicrobial characteristics [251,252].

Due to these factors, research has been conducted to determine whether SO_2 levels can be decreased or whether additives such as tannin, lysozyme enzyme, and ascorbic acid can be utilized as alternatives in wine production for a long time [253]. Recent research has focused on the antioxidant ‘’glutathione (GSH)’’, which has attracted interest due to its potential application as a SO_2 substitute in wine. Two methods have been used to assess the GSH effect in wine: adding it directly to the must or wine, or fermenting the wine using yeasts rich in GSH [129]. However, research on GSH supplementation is more prevalent.

γ-glutamyl cysteine (γGC, $C_8H_{14}N_2O_5S$) is a dipeptide comprised of cysteine and glutamate that forms in most living cells via glutamyl cysteine ligase (GCL) catalysis. Following the addition of glycine, glutathione synthetase converts it to GSH [254]. Studies on glutathione production by recombinant microorganisms have become more extensive as bioengineering has advanced, and studies on the recombinant production of γGC, its precursor, have gained interest in recent years.

The antioxidant activity of GSH is attributed to the presence of cysteine and the -SH functional group amino acids that it contains [255]. As γGC contains the amino acid cysteine, it is hypothesized that it will have an antioxidant effect similar to GSH, despite the lack of research in this field in the literature.

The above-mentioned reasons have led to the focus on γGC. Therefore, the present study aimed to evaluate the antioxidant capacity and potential of γGC and GSH as possible alternatives for SO_2 in white wine. Moreover, the effect of these two compounds on the physical, chemical, aroma, and sensory characteristics of two-month-matured wine was investigated.

2. Materials and Methods

2.1 Reagents and material

The grapes utilized in this study were of the Narince (*Vitis vinifera* L., 1753) variety and were obtained from the Nevşehir region of Turkey. Narince is a unique and commercially important white wine grape which has a historical importance from the Hittite period. Narince grape produces straw yellow colored wines with floral and citrus aromas. Narince wine has good potential for aging due to its medium- to full-bodied properties with good acidity character balanced by moderate alcohol level [256]. The yeast, *Saccharomyces cerevisiae* SAUVY^{TM}, used in the study was from Lallemend Co. (France). GSH, γGC (Glu-Cys), and all other chemicals were purchased from Sigma Aldrich Co. (St. Louis, MO, USA).

2.2 Winemaking, preparation and maturation of wine samples

The must obtained by pressing grape berries was stored at 10 °C for 24 hours. Then must was pre-clarified by filtration using a filter press to obtain higher yields of clear juice. Before being added to the must, the Sauvy yeast was rehydrated according to the manufacturer's (Lallemand Oenology, France) specifications. Subsequently, *S. cerevisiae* SAUVY^{TM} was dosed to the must at a concentration of 25 g/hL. The experiments were conducted in parallel sets with 3 L of must in 5 L glass drums. The fermentation was conducted at 18 °C. The following parameters were used to monitor the fermentation over the course of the 12-day process: total acidity using the NaOH standardization method [257], brix using a hand refractometer (KEM Kyoto, RA-130, Japan), density using a portable density meter (Mettler Toledo Densito 30PX, USA), and pH using a digital pH meter (Mettler Toledo S400, USA).

During alcoholic fermentation, total yeast (TY) and lactic acid bacteria (LAB) were enumerated on days 0, 4, 8, and 12. Potato dextrose agar (PDA, Merck, Germany) supplemented with oxytetracycline (100 mg/L, Sigma-Aldrich, USA) and De Man, Rogosa, and Sharpe agar (MRS, Merck, Germany) supplemented with cycloheximide (100 µg/mL, Sigma-Aldrich, USA) were used to enumerate TY and LAB, respectively.

Following the fermentation process, the wine was divided into four groups. For the first three groups, 30 mg/L of γGC, GSH, and sulfur dioxide (SO_2) were added to the wines separately. The fourth group, established as the control (C), proceeded with no additives. Prior to analysis, all wine samples had been maturated in 300-mL glass bottles at 10°C for two months. All experiments were set-up in duplicate.

2.3 Chemical analysis and alcohol determination

Density, dry matter, total acidity, pH monitored during fermentation process. At the end of the two-month maturation period, volatile acidity, free sulfur dioxide, and total sulfur dioxide were determined. All chemical analyses were conducted in accordance with the International Organization for Vine and Wine's International Methods of Wine and Must Analysis [257].

Using an Anton Paar Alcolyzer (DMA 4500M-Alcolyzer ME, Austria) equipment, the amount of ethyl alcohol in wines was determined by means of NIR (near infrared) spectroscopy. The alcohol content is represented as a percentage (%) of the total volume [257].

2.4 Determination of total phenolic compounds

The total phenol content of wine samples was determined using a modified version of the Folin-Ciacalteu colorimetric method as described by Nardini and Garaguso (2018)[258]. Using a spectrophotometer (Perkin Elmer Lambda 25-UV/VIS, USA), the absorbances were measured at 765 nm following a 30-minute dark incubation period. The calibration curve was prepared with a standard solution of gallic acid at several concentrations (0, 50, 100, 150, 250, and 500 mg/L). The results were expressed as mg gallic acid equivalents (GAE) per liter. All measurements were performed as triplicate.

2.5 Determination of organic acids and reducing sugars

Total and reducing sugars were determined by the Luff-Schoorl method [259]. Organic acids (tartaric, malic, and citric) and glycerol were quantified by HPLC (Shimadzu, LC-20AT, Kyoto, Japan) system. Wine samples was filtered via 0.45 μm PTFE filter (Sartorius, Germany) before injection. The system was composed of a quaternary pump, a column temperature control oven (CTO-10AS), an auto sampler unit (SIL- 20A), a degasser module (DGU-20A5), and a photodiode array detector (SPD-M20A). The analysis was performed at 210 nm wavelength using an Aminex HPX-87H (300 x 7.8 mm, Bio-Rad, USA) column.

Compounds were eluted using a 5 mM H_2SO_4 solution at a flow rate of 0.5 mL/min. HPLC analyzes were performed as triplicate.

2.6 Determination of browning and Measurement L, a*, b*, Hue, Chroma by CIELAB*

To determine the degree of enzymatic browning, a 10 ml sample was centrifuged at 4°C at 1000 rpm x g for 10 minutes. The supernatant (5 mL) is then combined with 5 mL of 95% ethyl alcohol and centrifuged under the same conditions. The absorbance of the resulting solution was measured at 420 nm against a blank (95 % ethanol) in a spectrophotometer (Perkin Elmer Lambda 25-UV/VIS, USA).
The color values (*L*, a*, b**) of the wines were directly measured by the ColorQuest XE (3A/SB, USA) model HunterLab device. *L** indicates the degree of light value and ranges from 0 (black) to 100 (white). The *a** value indicates green (-*a**) to red (+*a**), and the *b** value indicates blue (-*b**) to yellow (+*b**). The Chroma and Hue values are computed using the formulas described below.

$\text{Chroma} = (a^{*2}+b^{*2})^{1/2}$ $\text{Hue} = \tan^{-1}(b^*/a^*)$

2.7 Sensory evaluation of wines

Sensory evaluation of wine samples carried out after 2-month storage. The rating test, as outlined by Meilgaard et al. (2007)[260], was conducted by a panel of ten panelists comprising five males and five females, with ages ranging from 25 to 55. The Ethics Committee of Çukurova University's Food

Engineering Department authorized permission and ethical approval for this study to conduct sensory panel research, and each panelist provided informed consent prior to the sensory test. The panelists were asked to score each attribute from low to high using a 9-point hedonic scale (1: dislike extremely; 5: neither like nor dislike; 9: like extremely). The wine samples were judged based on their colour (light yellow-green, gold, and amber), odour (flowery, herbal tea, lavender, citrus, tropical fruits, tree fruits, honey, and herbaceous), and flavour (sourness, bitterness, sweetness, body, and harmony) criteria. During the sessions, International Standard Organization (ISO) wine glasses covered with glass petri dishes were used to serve the wine (20 mL at 20 ºC) in a random order.

2.8 Determination of aroma compounds by GC/FID/MS

Aroma compounds were determined with the Solid Phase Micro Extraction (SPME) technique by the "Agilent 7890B" GC equipped with a flame ionization detector (FID) and "Agilent 7010B" MS systems. Three mL of sample with 4-nonanol (41.57 ug/5 ul as internal standard) in a 20 mL vial is maintained at 40 °C for 10 minutes, and then aroma compounds were adsorbed for 30 minutes using a Solid Phase Micro Extraction (SPME) apparatus with a 50/30 µm Divinylbenzene/Carboxene/Polydimethylsiloxane (DVB/CAR/PDMS, 2 cm) coated fiber. The fiber was then injected into the DB-Wax (60 m x 0.25 mm i.d. x 0.25 m, J&W Scientific-Folsom, USA) capillary column following desorbing for 5 minutes. The injection temperature was set to 250 °C. The column temperature was increased by 2 °C per minute to 90 °C after 4 minutes of holding at 40 °C, then to 130 °C by increasing 3 °C per minute, and finally to 240 °C by increasing 4 °C per minute and holding at this temperature for 15 minutes. Helium was used as the carrier gas at a flow rate of 1 mL/min. The electron energy was 70 eV, the mass range was 30–600 m/z, the scan rate was 1.0 scans, the interface temperature was 250°C, and the source temperature was 120°C. The split ratio is 1:10. The aroma compounds were identified by comparing their retention index and mass spectra on the DB-Wax column with those of a commercial spectra database (W10N14, NIST11, NBS 75k) and the instrument's internal library, which was compiled from prior experimental studies [261,262]. Using an n-alkane series (C8-C26), retention indices of the compounds were computed. Following the identification of aroma compounds, the internal standard procedure was used to quantify the aroma compounds [263,264]. The ratio of peak area was corrected using response factors for each compound, which were determined using the intensity ratio of each compound to the internal standard.

2.9 Statistical analysis

The data obtained from the analyses were evaluated using a one-way analysis of variance (ANOVA) with the use of IBM SPSS Statistics software, version 23.0, developed by IBM Corporation in Armonk, NY, USA. Duncan's multiple-range tests were applied to assess the statistically significant differences between the mean values, with a significance level of $p < 0.05$. XLSTAT (2023) software was used to create a biplot graph for principle component analysis (PCA).

3.Results and Discussion

3.1 Monitoring alcohol fermentation and determination of microbial population

The growth of yeast and LAB were followed during fermentation. However, LAB and moulds can not grow in the presence of SO_2 depending on the concentration, yeasts are not generally effected. LAB in the wine are desirable to conduct malolactic fermentation. LAB are also sensitive to SO_2. Enumeration of yeasts and LAB during SO_2 free alcohol fermentation is shown in **Figure 20**.

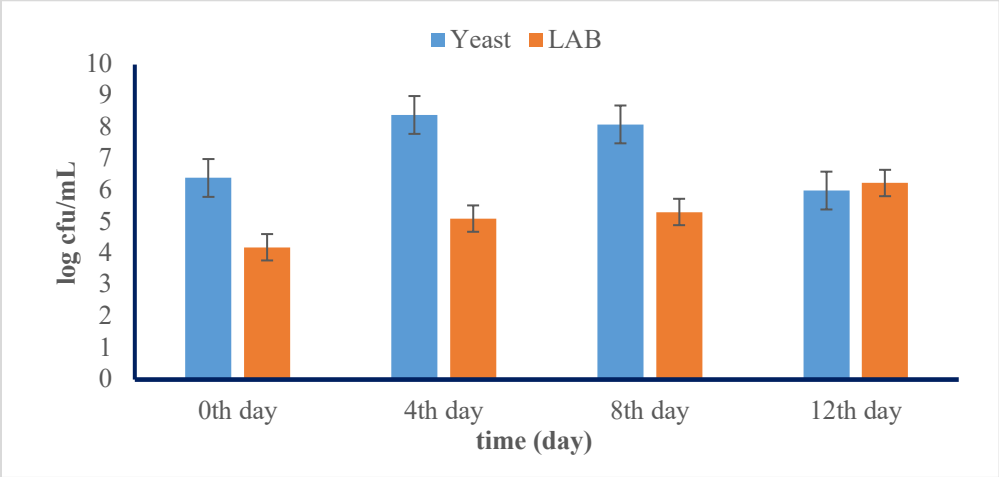


Figure 20. Total yeast and LAB growth during alcohol fermentation with must without SO_2. The first coumn indicates total yeast and second column total LAB (n=2, p<0.05).
* Different letters within the columns of the same microbial group represent statistically significant differences ($p < 0.05$) among the different times of fermentation.

Saccharomyces cerevisiae is the key microorganism in wine due to its satisfactory fermentative capacity, high ethanol productivity, rapid growth, easy adaptation, high tolerance to SO_2, and production of numerous sensorially active compounds (i.e., esters, higher alcohols, volatile acids, etc.) [265]. Fermentation started with 6.4 log cfu/mL. While the number of yeast increased until the 8th day of fermentation, it then started to decrease and reached 6 log cfu/mL at the end of fermentation ($p < 0.05$). Yeast growth demonstrated the same trend with the study of Çelik et al. (2019)[266], which produced white wine from Narince grapes using autochthonous and commercial *S. cerevisiae* strains.

High SO_2 supplementation (>20 mg/mL) has been shown to decrease LAB growth [267]. However, in this investigation, in which SO_2 was not employed, the LAB population was 4.2 log cfu/mL at the start of fermentation and progressively rose over the course of the following days ($p < 0.05$). At the end of the fermentation, it reached 6.24 log cfu/mL. In other studies, the population of LAB generally starts at 3 log cfu/mL on the first day of fermentation and then increases, as in this study [268-270]. Subsequently, the initiation of malolactic fermentation occurs at the level of a population of LAB reaching 6 log cfu/mL [271]. Although a slightly higher LAB number was determined in SO_2-free wine in this study, these findings were consistent with the previous studies reported above.

3.2 Composition of SO_2- free Wine

The quality of a wine is determined by its chemical composition, which includes parameters such as ethanol content, residual sugars, total and volatile acidity, organic acids, color, phenolic compounds, and aroma compounds. **Table 12** describes the physico-chemical composition of the must and wines that were produced.

Glucose and fructose, which are referred to as reducing sugars, constitute the primary sugars found in grapes. The reducing sugar content in grape must is between 150 and 250 g/L [272]. Sugars, being fundamental nutrients for yeasts during the process of fermentation, are a crucial factor that influences the final form of the product [273]. The Narince grape, which is widely recognized as a prominent white wine grape variety in Turkey, is predominantly grown in the Nevşehir and Tokat districts. The current study involved conducting fermentation using Narince grape-must characterized by a dry matter content of 24.8%, total acidity of 5.66 g/L tartaric acid, pH of 3.03, and sugar content of 230.5 g/L.

The reducing sugar level in wine samples was determined to be 3.62 g/L. There was no statistically significant difference observed in the sugar content of the wines among the C, SO_2, γGC, and GSH groups. According to the EU regulation 753/2002, wines are classified as dry (<4g/L), medium dry (<12g/L), medium (<45g/L), sweet (>45g/L) based on sugar content. The total sugar content of the wines was C, SO_2, γGC, GSH; 3.86, 3.77, 3.79, 3.80 respectively.

The alcohol content of all samples was determined as 12.5 % by volume. The alcohol content of wine produced from the Narince grape often ranges within the range of 10% to 12% [256,266].

The component glycerol, which is produced by yeasts during the fermentation of alcohol, contributes to the sweetness, smoothness, and complexity of wine [274]. Additionally, glycerol plays a significant role in enhancing the feeling of fullness of the body in wine [275]. The quantity of glycerol is dependent on the sugar concentration present in the must as well as the temperature at which fermentation occurs. The glycerol yield can be improved by elevated levels of sugar content and fermentation temperature [276]. In this context, the amount of glycerol was consistent with the initial sugar level, as seen in **Table 12.** It was determined that the corresponding glycerol concentrations for C, SO_2, γGC, and GSH were 7.12 g/L, 6.62 g/L, 6.85 g/L, and 6.80 g/L, respectively. The control sample exhibited a statistically significant difference when compared to the wine with additives ($p<0.05$).

Typically, the concentration of glycerol in white wines is approximately 7 g/L [276]. The glycerol contents of white wines produced from must with 20.4 brix ranged from 5.93 to 7.37 g/L in a study by [256] that used the same grape variety (Narince) and fermentation temperature (18 °C) as our investigation. In another comparative study [277], glycerol content was varied from 7.06 to 9.30 g/L in white wines produced using the Malvar grape variety, fermented at 20 °C, and including 230 g/L reducing sugar in must. As a result, the recorded amount of glycerol in the current investigation was consistent with previously published studies on white wines.

Organic acids, which are abundant in fermented foods, are compounds that contribute to food quality and organoleptic properties. Organic acids in wine serve to impart physicochemical and microbiological stability [278]. The aroma of wine is also influenced by organic acids [279]. The main organic acids found in wine are tartaric, malic, citric, succinic, and acetic acids [280].

Tartaric acid is a critical factor in determining the overall acidity of wine and plays a significant role in preserving its chemical stability, color, and flavor. The concentration of tartaric acid may decrease due to the occurrence of precipitation in the form of tartaric crystals during the process of fermentation [281]. The amounts of tartaric and malic acid in the control, SO_2, γGC, and GSH wine samples were 3.32 g/L, 3.41 g/L, 3.40 g/L, 3.57 g/L and 2.18 g/L, 2.45 g/L, 2.20 g/L, and 2.20 g/L, respectively. Among the volatile acids, citric acid records the lowest concentration. Between wine samples, there was no significant difference between γGC and GSH sample. The highest concentration was determined in (0.63±0.02) SO_2 sample.Volatile acids are chemical compounds that are produced through the process of alcohol fermentation, with acetic acid being a particularly significant example. When the wine samples were examined, the γGC sample had the lowest volatile acid in terms of acetic acid, with 0.52 g/L, and the control sample had the highest (0.6 g/L). The difference between the volatile acid values was significant ($p<0.05$) (**Table 12**).

SO_2 present in wine can be categorized into two distinct groups: bound and free forms. Bound SO_2 contains aldehydes, ketones and phenolic derivatives, while free SO_2 is H_2SO_3, HSO_3^- and SO_3^{2-}. The International Organization of Vine and Wine (OIV) has been gradually decreasing the permissible total sulfur dioxide (SO_2) in wines due to the health issues linked to its usage [282]. Wines with a high sugar content have a higher SO_2 limit because they deteriorate quicker. The established threshold for demi-sec wines is 275 mg/L. The free and total sulfur content of the wines was determined to be 19.20;57.06, 13.86;40.00, 13.33;33.06, 6.40;12.80 mg/L in the following order: SO_2, GSH, γGC, C ($p<0.05$). The quantity of sulfur dioxide (SO_2) is a subject of significant concern. A study was conducted to evaluate the sulfur dioxide (SO_2) content of 316 wine samples. The recorded values, ranging up to a maximum of 340 mg/L, were then compared based on the countries of origin and the varietals of wine. German (113.85 mg/kg) and Italian (113.58 mg/kg) wines had lower SO_2 levels than Chinese (193.17 mg/kg) and Korean (340 mg/kg) wines, whereas white (122 mg/kg) wines have higher SO_2 levels than red (71.13 mg/kg) wines. World-International Organization of Vine and wine (OIV) limited SO_2 in wine as 300 mg/L (>4g/L containing reducing substances), 400 mg/L (certain sweet white wines) [283].

3.3 Determination of total fenolic compounds in wine samples

Phenolics are the most important secondary metabolites found in plants, influencing wine quality. Phenolics are divided into two groups: flavonoids and non-flavonoids. The compounds are present in grape skin, seeds and juice. These compounds vary from grape type, geographical location, fermentation conditions and post-fermentation processes. Since phenolic compounds have an effect on the color and organoleptic properties of wine, they should be preserved until consumption. Total phenol content was

determined to be 192.23, 205.57, 215.22, and 218.43 GAE/L in mature wine for C, SO_2, GSH, and γGC wines, respectively. While there is no statistically significant difference between γGC and GSH, it is worth noting that these two groups have a higher value than wine containing SO_2.

The total phenolic levels were comparable to those reported by Vaimakis and Rousis (1995) [284] who reported total phenolic values in white wine with adding GSH ranging from 110-150 mg GAE/L. In present study GSH and γGC wine samples had higher phenol content. However, according to Bayram and Kayalar (2018)[285], who worked with Narince grapes harvested from two different localities (Emirseyit and Erbaa), the total phenolic content (443 and 403 GAE/L for Erbaa and Emirseyit wines) was higher than this study. According to the findings of Jakabová et al. (2021), the concentrations of total phenolic substances in conventional and SO_2-free wines were 273 and 285 mg GAE/L, respectively. The white wine with the addition of GSH, total phenol content was measured in the range of 241-449 mg caffeic acid by El-Hosry et al., (2009) [142]. In addition to preventing phenolic substance loss, glutamyl cysteine and GSH consumption is important for consumer health.

Table 12. Compositon of Narince grape must and white wine

Composition of must					
Density (g/cm^3)	1.125±0.02				
Brix (%)	24.8±0.00				
pH	3.03±0.05				
Total acidity (g/L)	5.66±0.05				
Total sugar (g/L)	230.5±0.02				
Composition of wine					
Density (g/cm^3), 20°C	1.002±0.001				
Alcohol (%volume), 20°C	12.50±0.03				
Total acidity (g/L)[1]	6.73±0.17				
pH	3.03±0.06				
Composition of wine samples after storage					
	Control	SO_2	γGC	GSH	
Glycerol (g/L)	7.12±0.19^a	6.62±0.14^b	6.85±0.15^{a,b}	6.80±0.08^b	*
Reducing sugar (g/L)	3.62±0.00	3.62±0.00	3.62±0.03	3.62±0.00	ns
Total Sugar (g/L)	3.86±0.00^a	3.77±0.00^c	3.79±0.00^b	3.80±0.05^b	*
Tartaric acid (g/L)	3.32±0.08^b	3.41±0.12^{a,b}	3.4±0.06^b	3.57±0.00^a	*
Malic acid (g/L)	2.18±0.06^b	2.45±0.03^a	2.2±0.00^b	2.20±.00^b	***
Citric acid (g/L)	0.61±0.1^b	0.63±0.02^a	0.6±0.01^{b,c}	0.60±0.06^c	**
Volatile acidity (g/L)[2]	0.60±0.00^a	0.55±0.00^{b,c}	0.52±0.01^c	0.56±0.01^b	***
Free SO_2 (mg/L)	6.40±0.5^c	19.20±0.5^a	13.33±0.5^b	13.86±0.5^b	***
Total SO_2 (mg/L)	12.8±1.6^d	57.06±1.6^a	33.06±1.6^c	40±1.6^b	***
Colour					
*L**	88.45±0.15^d	89.32±0.08^c	90.39±0.29^b	90.8±0.08^a	***
*a**	-0.46±0.01^d	-0.14±0.01^a	-0.22±0.01^c	-0.20±0.00^b	***
*b**	9.72±0.08^c	11.54±0.06^a	11.39±0.08^b	11.47±0.02^{a,b}	***
Chroma (C)	9.73±0.08^c	11.54±0.06^a	11.39±0.08^b	11.47±0.02^{a,b}	***
Hue (°)	92.71±0.12^a	90.70±0.04^c	91.14±0.08^b	90.00±0.00^b	***

Data shown with different letters (a, b, c) within lines are significantly different by Duncan ($p<0,05$), *=$p<0,05$, **=$p<0,01$, ***=$p<0,001$ level. C: Control SO_2: Sulphur dioxide, γGC: gamma glutamyl cysteine and GSH: glutathione. 1= as tartaric acid, 2= as acetic acid.

3.4 Browning and color determinations in white wines

Browning consists of enzymatic and non-enzymatic reactions. A green-brown colour is observed as a result of the oxidation of phenolic substances in wine. This is particularly undesirable in young wines. Browning is usually determined by measuring the colour at 420 nm. This parameter is related to the decrease in SO_2 concentration in wine. The OD_{420} values obtained in this investigation indicated that SO_2 (0.031±0.004) and γGC (0.032±0.001) exhibited the lowest values. A low OD_{420} value indicates a reduced degree of browning. The OD_{420} absorbance values of GSH and control samples were determined to be 0.034±0.001 and 0.042±0.002, respectively (**Figure 21**). SO_2 and γGC were identified as the most efficacious additives in minimizing browning, as indicated by the results. All three groups differed significantly from the control group ($p<0.05$). It is worth noting that γGC has a greater browning reducing impact than GSH. While no reported data exists regarding the impact of γGC on browning in wines, the SO_2 and GSH values were consistent with those reported in the literature [286]. According to El-Hosry et al., (2009)[142] wine the addition GSH, resulted in a considerable decrease in browning degree even on day 0. Consequently, it has been demonstrated that GSH significantly affects the stability of wine when exposed to oxidation. Although the components responsible for browning have not been identified in white wines, diphenols that cause browning have not been identified; diphenol compounds are known to be the most susceptible to browning [287]. Consequently, protecting phenolic substances is critical for maintaining color stability [288].

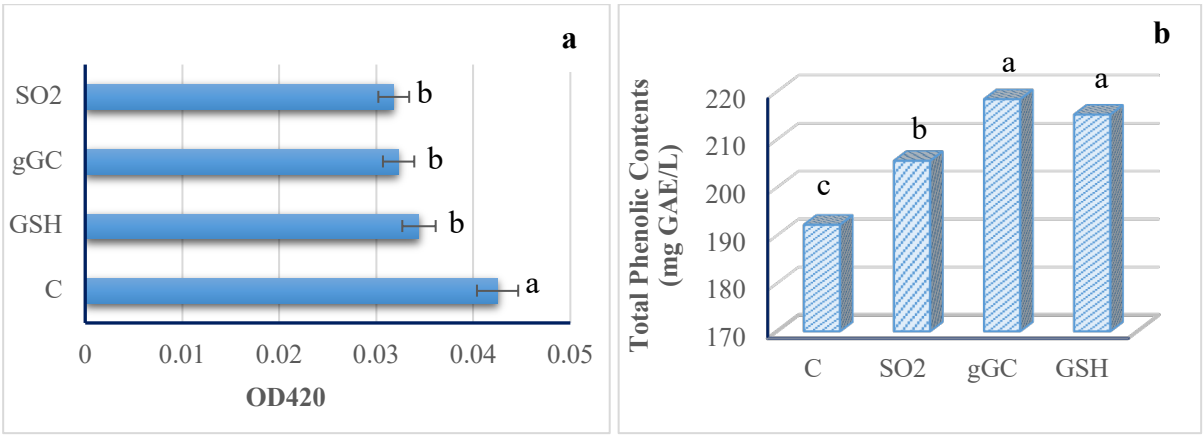


Figure 21. The influence of SO_2, gGC, and GSH additions to white wine during maturation on browning degree (a) and total phenolic content (b)Abbrevations: C (control, without additives), SO_2 (30 mg/L), gGC (gamma glutamyl cysteine, 30 mg/L), GSH (glutathione 30 mg/L)

Table 12 demonstrate changes in the color of the wines as a measure of the color (L^*: brightness/darkness, a^*: greenish/redness and b^*: blueness/yellowness) of the samples. In the experiments, the L^* values were ranked from highest to lowest as GSH, γGC, SO_2, and C, respectively. The L^*, a^*, and b^* values were determined to be 90.80, -0.20, and 11.47 in the GSH-added sample; 90.39, -0.22, and 11.39 in the γGC-added sample; 89.32, -0.14, and 11.54 in the SO_2-added sample; and 88.45, -0.46, and 9.72 in the control sample.

When the results of all the experiments are compared, the L^* value is lower and the b^* value is higher than the values reported in the literature [289]. The color results in this study indicated that all produced wines, including the control group, had a clear pale yellow color, which is comparable to the study of Cosme et al. (2019)[290], who reported that the color of the white wines changed from a clear pale yellow to light salmon.

The application of principal component analysis (PCA) was employed to provide a more comprehensive elucidation of the chemical composition of Narince wines based on different additions. The total variance explained by two principal components was 97.43%, with the first component (F1) comprising 69.00% and the second component (F2) 28.43 % (**Figure 22**). γGC and GSH treatments were seperated along PC2 (upper right quadrant) and displayed a positive correlation with each other. These two treatments were characterized with high L^* and total phenolic compounds values. This indicates that GSH and γGC were successful in preserving the level of phenolic substances. The wine containing SO_2 is located in the bottom right quadrant and is explained by its high organic acid (tartaric, malic, and citric acid) contents, consistent with the results given in **Table 12**. As can be seen from the **Figure 22**, control wine sample was located in the bottom left quadrant and characterized with high sugar and glycerol content.

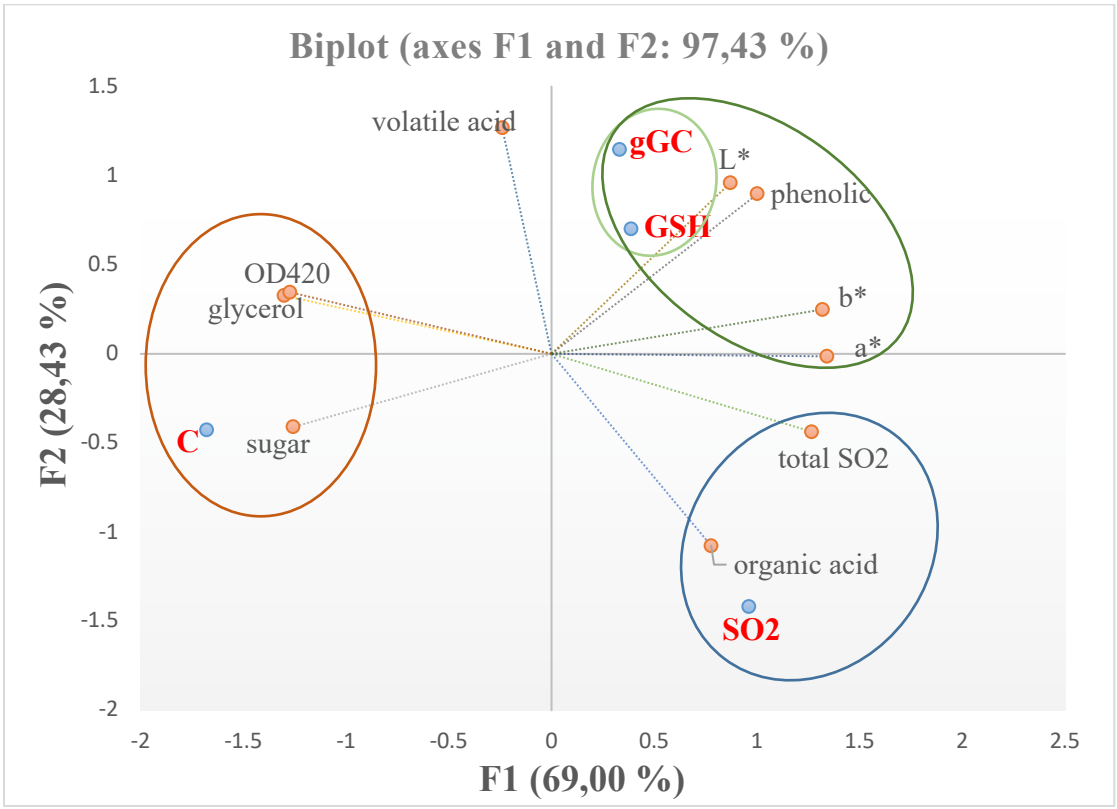


Figure 22. Bi-plot of PCA based on the chemical composition of Narince wine samples. (C: control = without additives, SO_2: 30 mg/L, gGC: gamma-glutamylcysteine = 30 mg/L, GSH: glutathione = 30 mg/L)

3.5 Determination of aroma compounds by SPME GC/MS

The volatile components of SO_2, γGC, GSH added and C wines are shown in **Table 13**. A total of 34 volatile components were determined by the SPME method. **Table 13** lists the total volatile organic compounds (VOC) that were detected: eleven higher alcohols, twelve esters, seven volatile acids, three carbonyl compounds, one alkane, and one thiol. This is also illustrated in the PCA biplot in **Figure 23**.

Higher alcohols were the most abundant compounds in total and contributed to the aroma along with other flavor-active compounds. They can also promote ester production. The samples exhibited significant variations in the total amount of higher alcohols ($p<0.05$). The cumulative concentrations of higher alcohols in the C, SO_2, γGC, and GSH experiments were 149.890, 161.250, 168.783, and 170.339 mg/L, respectively. The two most abundant compounds in total VOCs in all samples were 2-phenyl ethanol and isoamyl alcohol. The presence of higher alcohols has a positive effect on wine up to 300 mg/L, while over 400 mg/mL has a detrimental effect [291]. One of the most common higher alcohols in wine, 2-phenyl ethanol, contributes to the wine's aroma with a sweet and flowery character. In this study, the highest concentration of phenyl ethanol was found in the control wine, followed by SO_2-added wine. The main higher alcohol found in this study was isoamyl alcohol (2-methyl-1-butanol). It was found in high and close concentrations in both GSH (125.795 mg/L) and γGC (124.664 mg/L)-added wines. Butanediol gives a buttery and creamy aroma that varies by wine type and has a direct effect on wine aroma. The amount of 2,3-butanediol showed a higher concentration in C (1.016 mg/L) and SO_2 (0.965 mg/L) in comparison to the GSH (0.918 mg/L) and γGC (0.852 mg/L) added wines. The amount of 1-hexanol also showed significant differences among the four samples ($p<0.05$). 1-hexanol affects wine with a resiny and flowery aroma.

Esters are aroma compounds that are present in the highest amounts in wine VOCs and give a characteristic fruity taste to the wine. The most common esters in wine are acetates. Isoamyl acetate, also known as 1-butanol-3-methyl acetate, is an ester formed from acetic acid and isoamyl alcohol. It is known for having a strong banana flavor. Compared to the SO_2 group, the GSH and γGC samples have greater levels of isoamyl acetate. The results for all samples had lower concentration as stated in the [266,291] 3-methyl-propanoate (isoamyl propionate) is a carboxylic ester that has a sweet and bitter taste. The comparison test revealed that there was a difference with treatment C regarding the amount of isoamyl propionate, but not between treatments SO_2, GSH, or γGC. Ethyl butyrate, ethyl hexanoate, ethyl decanoate, and ethyl octanoate are derived from the condensation reaction between organic acids and alcohol. They enhance the wine's fragrance by imparting a fruity flavor [292].

Esters are produced by yeast during fermentation and contribute to the characteristic flavor of wines. The large group (12 individual compounds) of volatile organic compounds (VOCs) in wine samples consisted of quantified ethyl esters (ethyl butyrate, ethyl decanoate, ethyl hexanoate, ethyl heptanate, ethyl acetimidate and ethyl octanoate) and acetates (isoamyl acetate, isoamyl propanate, hexyl acetate, hexyl 2-metil propanate, 1-3 propanediol diacetate and phenylethyl acetate). Isomethyl acetate and

phenylethyl acetate contribute to fruit jam aroma in wine [293]. The threshold values of isoamyl acetate (3-methyl butyl acetate), ethyl hexanoate, and ethyl octanoate are 30 μg/L, 50 μg/L, and 20 μg/L, respectively [291]. According to the results obtained, these compounds are above the threshold value. However, the results in this study were lower than those in Bayram et al. (2018)[285].

According to the total ester content, the highest group was GSH (10.485 mg/L), followed by γGC (9.722 mg/L), C (9.487 mg/L), and the lowest was SO_2 (8.976 mg/L).

Volatile acids are related to negative characteristics in wine, such as rancid and fatty, but they are also important for the aromatic equilibrium of wine [294]. All samples included a low amount of total volatile acid concentration, as in other studies such as Selli et al. (2006)[295]. It was noteworthy that C wine (4.145 mg/L) contained a smaller amount of volatile acids than the others.

1-alkyl thiols, sweets, and tropical factors decreased from a peak at 1-heptane thiol. Up to 2 mg/L of decan compound has a tropical aroma feature; over 4 mg/L, it can produce unwanted fishy aromas [296]. 1-Decanethiol was only detected in wine supplemented with GSH at a concentration of 1.47 μg/L.

Table 13. Volatile composition of wine from Narince grape with different additives

Volatile compounds (μg/L)						
Higher alcohols	RI	C	SO_2	γGC	GSH	F
1-Propanol	1037	1082.92±3.3^{c}	1026.76±2.68^{d}	1193.93±4.22^{a}	1157.55±6,32^{b}	***
Isobutyl alcohol	1085	9824.06±15.89^{b}	10,626.05±19.44^{a}	8988.51±10.9^{c}	8975.82±0.87^{c}	***
1-butanol	1165	606.18±0.08^{b}	521.54±8.87^{c}	636.17±11.09^{a}	618,99±7.84^{a,b}	***
Isoamyl alcohol	1210	102,109.28±21.2^{c}	112,865.66±612.12^{b}	124,664.86±277.2^{a}	125,795.3±462.4^{a}	***
1-hexanol	1370	1786.61±0.55^{b}	1961.89±6.94^{a}	1498.26±5.57^{c}	1550.16±8.71^{c}	***
(Z)3-Hexzen-1-ol	1401	118.36±0.19^{a}	103.28±2.94^{c}	116.02±0.73^{a}	109.5±1.76^{b}	***
2,3 butanediol	1495	1016.91±2.14^{a}	965.37±0.09^{b}	852.56±0,08^{d}	918.79±1.82^{c}	***
1,2,3 butanetriol	2062	295.81±0,66^{c}	321.01±4.96^{b}	290.76±2.45^{c}	467.66±2.33^{a}	***
1-octyn-3-ol	1719	1850.65±0.6^{c}	2205.66±6.59^{b}	3162.22±16.99^{a}	3315.32±18.1^{a}	**
2-phenyl ethanol	1916	29,526±390.3^{a}	29,323.97±88.34^{a}	25,889.43±3,48^{b}	25,910.2±3,01^{b}	**
2 nonen-1-ol	1804	1673.69±4.65^{a}	1329.61±43.68^{c}	1491.04±1.47^{b}	1520.51±0.41^{b}	***
Sum		149,890.47	161,250.8	168,783.76	170,339.8	
Esters						
Isoamyl acetate	1119	1951.46±10,5^{b}	1830.56±9,67^{c}	1946.76±15,3^{a,b}	2054.36±49,5^{a}	**
Ethyl hexanoate	1241	1185.95±7,66^{b}	1109.55±2,98^{c}	1134.55±5,22^{b,c}	1324.95±44,34^{a}	**
Isoamyl propionate	960	700.4±0,01^{b}	1342.46±0,27^{a}	1340.24±0,08^{a}	1332.56±0,08^{a}	**
Hexyl acetate	1250	266.7±2,09^{b}	202.1±0,01^{c}	596.7±1,91^{a}	286.7±8,99^{a}	**
Hexyl 2 methyl propanate	2891	1870.12±0,37^{b}	2142.56±0,01^{a}	1612.7±0,33^{d}	1824.64±0,03^{c}	***
Ethyl octanoate	1430	1164.4±12,13^{b}	369.25±0,38^{c}	816.8±0,78^{c}	1383.65±16,5^{a}	**
Ethyl decanoate	1635	336.5±0,95^{b}	342.62±4,57^{a}	123.75±2,63^{c}	167.12±0,15^{c}	***
Ethyl butyrate	1037	813.02±0,01^{b}	716.46±0,08^{c}	902.2±0,46^{a}	844.34±0,04^{a}	***
1,3-propanediol diacetate	1189	153,68±0,79^{b}	126,65±7,71^{c}	192,27±8,58^{a}	180,33±8,34^{a}	**
Phenylethyl acetate	1785	712.25±0,06^{a}	615.5±0,51^{b}	401.25±1,15^{d}	506.5±1,62^{c}	***
Ethyl acetimidate	1091	210.2±0,03^{c}	300.56±0,17^{b}	348.43±0,25^{a}	307.5±0,02^{b}	**

Ethyl heptanate	1511	122.8±0,06^{c}	170.24±0,04^{b}	306.54±0,42^{a}	292.36±0,0^{a}	**
Sum		9487.48	8976.51	9722.19	10,485.01	
Volatile acids						
Proponioic acid	1538	33.93±0,75^{b}	44.71±2,70^{a}	34.59±0,4^{b}	34.08±2,15^{b}	*
Decanoic acid	2183	372.4±0,01^{b}	876.62±1,02^{a}	169.24±0,05^{c}	153.44±0,29^{c}	**
Nonanoic acid	2158	212.5±0,04^{c}	141.56±0,06^{d}	365.52±0,95^{b}	762.84±0,01^{a}	***
Butryic acid	1628	573.24±0,13^{a}	342.86±0,28^{c}	361.2±0,01^{b,c}	389.02±0,1^{b}	**
Octanoic acid	2060	1163.3±0,25^{c,d}	1452.04±1,23^{b}	1191.44±1,35^{c}	1833.23±0,43^{a}	***
Hexanoic acid	1840	1790.2±0,01^{b}	3345.06±0,49^{a}	3350.45±0,33^{a}	1800.4±0,0^{b}	**
Oxalecetic acid	2263	0,89±0,04^{c}	1,48±0,35^{b}	1,54±0,0^{a}	1,57±0,41^{a}	**
Sum		4145.16	6204.33	5473.78	4974.58	
Carbonyl compounds						
Acetaldehyde	500	5050.24±0,15^{a}	4700.5±0,0^{b,c}	4650.04±0,01^{c}	4750.22±0,02^{b}	**
Cathinone	1749	1155.3±0,09^{a}	1070.68±1,19^{b}	930.66±0,17^{c}	935.54±0,01^{c}	ns
3-hydroxy butanal	1325	150.45±0,01^{b}	192.08±0,01^{a}	153.44±0,02^{b}	151.24±0,06^{b}	*
Sum		6355.9	5963.26	5734.14	5837	
Thiol derivative						
10-Azido-1-decanethiol	1671	nd	nd	nd	1.47	

C (Control, without additives), SO_2 (30 mg/L), γGC (gamma glutamyl cysteine, 30 mg/L), GSH (glutathione 30 mg/L). Data shown with different letters (a, b, c) within lines are significantly different by Duncan ($p<0,05$), *=$p<0,05$, **=$p<0,01$, ***=$p<0,001$ level, ns=not significant, nd=not detected

Figure 23 displays the correlation biplot illustrating the relationship between volatile chemicals and the wine samples. All 34 VOCs were analyzed using PCA to determine how the samples differed from one another in terms of volatile content. With a total variance of 81.16%, the first two principal components, PC1 (51.54%) and PC2 (29.63%), were expressed.

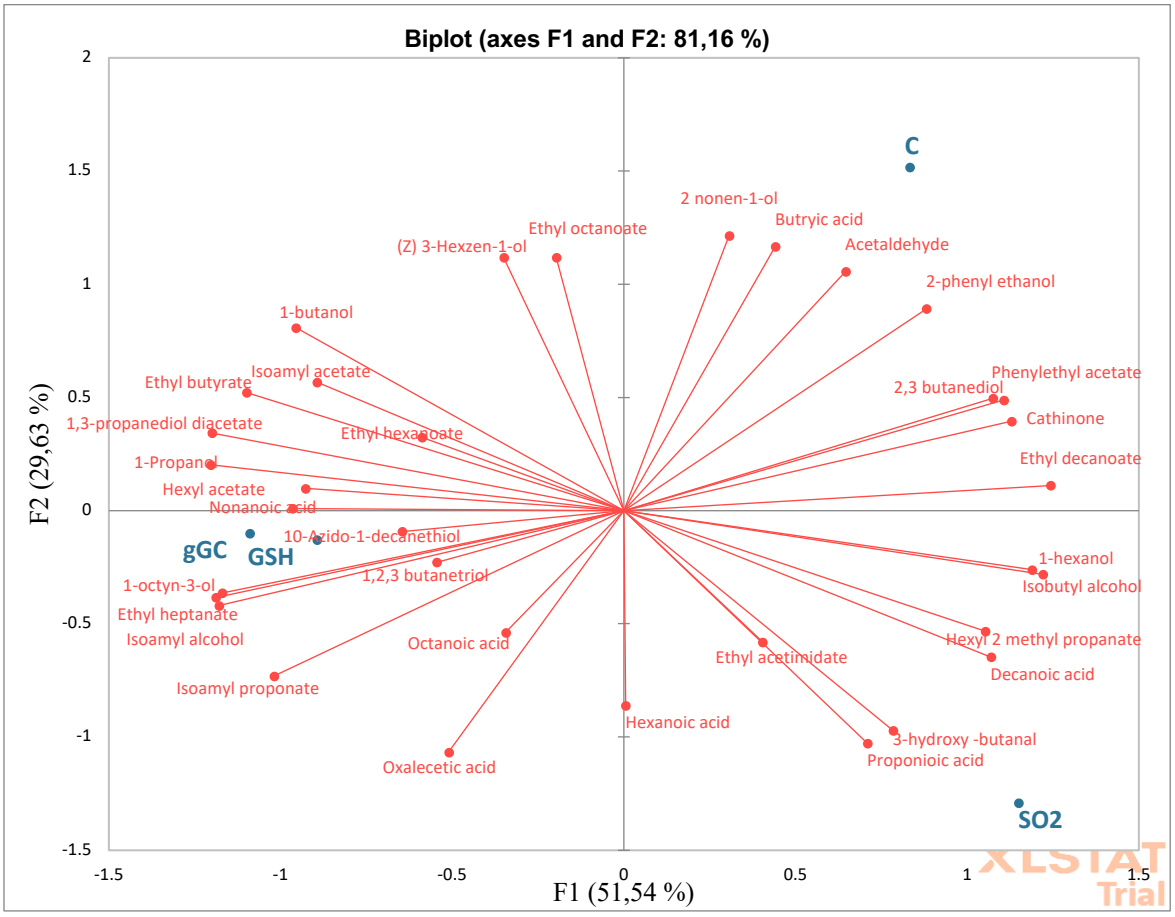


Figure 23. Principle component analysis of quantified volatile compounds of four wine samples C (Control, without additives), SO_2 (30 mg/L), gGC (gamma glutamyl cysteine, 30 mg/L), GSH (glutathione 30 mg/L)

The total variance explained by two principal components was 81.16%, with the first component (F1) comprising 51.54% and the second component (F2) 29.63 % (Figure 23). As can be seen from Figure 23, four samples were distributed among three distinct quadrants. However, γGC and GSH were included in the same quadrant (bottom left). Control wine was separated in upper right quadrant and associated mainly with 2-phenyl ethanol and 2-nonen-1-ol and 2,3-butanediol as higher alcohols; acetaldehyde and cathinone as carbonyl compounds; phenylethyl acetate and ethyl decanoate as esters; butyric acid as volatile acid group. The wines with GSH and γGC were located close to each other (left bottom quadrant) in the bi-plot graph, and they were correlated to the esters and thiol compounds. The SO_2 group wines separated in bottom right quadrant and explained mainly by volatile acids as propionic acid, decanoic acid and hexanoic acids. There was positive correlation between higher alcohols and organic acids (r=0.80, data not shown), alkane and higher alcohols (r=0.98, data not shown). According

to the VOCs groups, higher alcohols, organic acids and alkanes were close to each other while stay far from esters and thiols.

3.6 Sensory analysis of wine

The odor and flavor profiles of wine samples reported on the spider web diagram are shown in **Figure 24**.

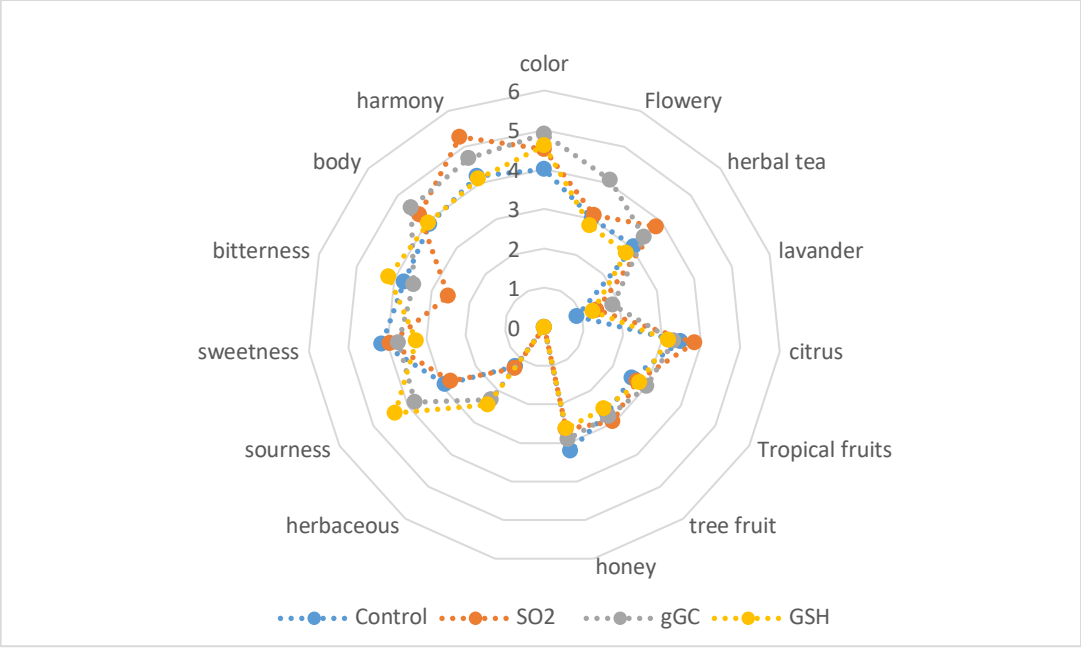


Figure 24. Sensory profile of wine samples. C (Control, without additives), SO_2 (30 mg/L), gGC (gamma-glutamylcysteine, 30 mg/L), GSH (glutathione 30 mg/L)

On a 9-point scale, the color value indicates the colors yellow-green, gold, and amber from low to high. As a result, all wine samples were in the yellow-gold range. The corresponding color scores for C, SO_2, γGC, and GSH were 4.00, 4.50, 4.90, and 4.60, respectively.

In an evaluation of the wines based on their odor profiles, tropical fruit, flowery, and citrus characteristics predominated. SO_2 obtained the highest value in the citrus profile (3.83), whereas γGC had the highest values for flowery and tropical fragrances (4.09 and 3.00, respectively).

The last criterium was about evaluation of taste. The judges determined that GSH possessed the highest values for sourness and bitterness, scoring 4.37 and 4.15, respectively. In contrast, sample C exhibited the highest sweetness with a score of 4.14. In the assessment of the wines' body properties, the control sample exhibited the lowest value (3.9) compared to the γGC-added wine, which exhibited the maximum value (4.54). The wines were generally ranked in the following order of appreciation: SO_2, γGC, GSH, and C.

Although the addition of γGC has not been tried in wine before, the citrus, exotic fruit, honey, and caramel properties of GSH-added wine [297] showed statistically significant differences among themselves, while a decrease in citrus flavor was observed in wine.

According to Cruege et al. (2002)[298], adding 10 mg/L GSH to the wine during the rapid oxidation test can maintain the color stability and stop the smells from eroding. Additionally, it was also observed that the addition of N-acetyl cysteine and 20 mg/L GSH prevented the loss of significant volatile compounds [299]. The decline in the amount of linalool and alpha-terpineol in muscat wines were inhibited with the addition of and N-acetyl cysteine [129].

The affect of GSH on wine depends on the grape variety, fermentation and maturation. Although the tasters in this study did not assert an adverse impact, there have been studies that have assessed both the positive and negative impacts of GSH on wine. Low amounts of GSH have been shown to preserve flavor components, whilst high levels have been shown to generate sulfide off-flavors in wine [300].

4.Conclusion

This study examined substitutive additions for SO_2 in wine, particularly γGC and GSH. The findings demonstrate that using γGC and GSH as substitutes in wine has an effect on the overall composition, aroma components, and organoleptic qualities of the wine. There was no major difference in proximate composition of wines supplemented with γGC, GSH and SO_2. Furthermore, it has been proven that the inclusion of γGC and GSH in wine proved to be efficacious in protecting phenolic compounds. γGC added wine obtained a value similar to SO_2 added wine in terms of browning. The utilization of γGC and GSH did not result in any detrimental impact on the aroma of the wine. Consuming wines that are made without the addition of sulfur dioxide not only reduces the potential negative effects associated with sulfur dioxide, but also provides a source of γGC or GSH for the human body. Substituting γGC for SO_2 in wine production is recommended based on the positive results obtained in this study.

Further studies should be conducted to assess the ideal usable level of γGC in wines and its synergistic effect with SO_2, evaluate the role of γGC in alcoholic fermentation, investigate its different concentrations, and clarify its impact on the chemical and sensory composition of red and white wines.

Chapter 6

General conclusions and perspectives

1.Conclusions

In this study the metabolic engineering of the unconventional yeast *Y. lipolytica* for efficient γGC synthesis was investigated. The yeast was first transformed into a γGC producer by disruption of the *GSH2* gene encoding GSH synthase and constitutive expression of the glutamylcysteine ligase encoding *GSH1*. Genes involved in cysteine and glutamate anabolism, namely *MET4, CYSE, CYSF* and *GDH1*, were then overexpressed to increase their intracellular availability. With such a strategy, a γGC titre of 464 nmol mg-1 protein (93 mg gDCW-1) was achieved within 24 h of cell growth.

In the second part of the study, antioxidant and antimicrobial properties of γGC and GSH were tested. Antioxidant was measured by DPPH and ABTS methods. According to DPPH test, IC50 values were 0.29 mM and 0.36 mM for γGC and 0.19 mM and 0.22 mM for ABTS, respectively. The antimicrobial properties of the two compounds were determined against gram negative and gram positive bacteria, yeasts (*Candida albicans*) and moulds (*Penicillium digitalatum* and *Colletetrichum acutatum*) by agar diffusion and micro-dilution methods. According to agar diffusion test, both γGC and GSH were ineffective against *E. coli* growth, while the zone of inhibition for *Listeria monoycytogenes*, *Staphylococcus aureus* and *Bacillus cereus* was 15.20, 15.11, 12.95, 12.31 and 11.67, and 9.2 mm for γGC and GSH, respectively. The minimum inhibition concentration was determined as 10 mM, 5 mM, 10 mM, 5 mM, 5 mM for γGC and 5 mM, 5 mM, 10 mM, 10 mM, 10 mM for GSH in *E. coli, Listeria monoctogenes, Staphylococus aureus* and *Bacillus cereus*, respectively. It has no effect on *E. coli.* Both compounds had no inhibitory effect on yeast and mould growth.

In the last part of the study, we chose two food products, sunflower and wine, which are prone to oxidative degradation, to see the antioxidant effect of γGC and GSH in foods.

The antioxidant effect of two thiols, γGC and GSH, in sunflower oil under accelerated storage was studied. Oil samples was stored at 50°C for a period of 15 days. TBHQ was used as a positive control while no additive oil selected as a negatif control. Oxidation level indicators such as peroxide (PV), free fatty acidity (FFA), p-anisidine (p-AV) but also total oxidation (Totox), colour (*L*, a*, b**) and fatty acid profile were determined. At the end of storage, oxidation in sunflower oil was substantially reduced by 40 mg/L of γGC. Analysis with 2,2-diphenyl-1-picrylhydrazyl (DPPH) resulted in the following order of IC_{50}; T (0.08±0.01), BHA (0.13±0.03), γGC (0.3±0.01), GSH (0.41±0.00), BHT (0.42±0.02). The samples resistance to the generation of primary and secondary oxidation products was T>GCT>γGC>GSHT>GSH>C for up to 15 days under storage conditions. The fatty acid profile analysed by GC/MS further demonstrated that these thiols outperformed the control group in terms of performance. Findings demonstrated that γGC, precursor of GSH, has stronger antioxidant activity than GSH. As a result, it is recommended to be explored as a potential source of antioxidants in applications for the food industry to prevent lipid oxidation.

White wine, which produced from Narince grapes, had a density of 1.002 g/cm^3, an alcohol content of 12.5%, a pH value of 3.03, and a total acidity of 6.73 g/L. Wines were maturated for two months following the separate addition of SO_2, γGC, and GSH at the concentration of 30 mg/L. Also, the wine without additives was used as control. As a result, the protective properties of γGC and GSH additives on phenolic compounds and decelerating the browning degree in white wine were eluciated. At the end of the maturation period, γGC (218,43 GAE/L), GSH (215,22 GAE/L) treatments had the highest amount of phenolic compounds, followed by SO_2 (205,57 GAE/L) and control (192,23 GAE/L). Furthermore, γGC (OD_{420} 0.032) added wine displayed almost the same level of browning with the SO_2 (OD_{420} 0,031) added wine. As a result, γGC is more efficient than GSH and may be used as a substitute for SO_2.

2.Perspectives

The following suggestions can be given for further studies based on the results of this thesis:

Enhanced Strain Optimization and Metabolic Engineering: While the study successfully engineered *Y. lipolytica* for γGC production, there is still room for optimization. Future research could focus on increasing the yield and efficiency of γGC production through advanced metabolic engineering techniques, such as CRISPR-based gene editing, adaptive laboratory evolution, or systems biology approaches. Additionally, exploring co-culture systems or synthetic microbial consortia could enhance the overall production process by leveraging the metabolic capabilities of multiple organisms.

Industrial Scale-Up and Process Optimization: Scaling up the production of γGC from laboratory to industrial scale presents several challenges and opportunities. Future research should focus on optimizing fermentation conditions, such as pH, temperature, and nutrient availability, to maximize γGC yield in large-scale bioreactors. Moreover, developing cost-effective downstream processing methods for the extraction and purification of γGC will be critical for its commercial viability.

Comprehensive Toxicological and Regulatory Assessment: Before γGC can be widely adopted in food, beverage, or pharmaceutical applications, comprehensive toxicological studies are necessary to ensure its safety for human consumption. This includes long-term studies on its metabolic fate, potential allergenicity, and interaction with other food components. Additionally, engaging with regulatory agencies to establish safety guidelines and obtain approval for γGC as a food additive will be crucial.

Expansion of Antioxidant Applications: The superior antioxidant properties of γGC, as demonstrated in sunflower oil and wine, suggest that it could have broader applications in various industries. Future studies could explore the use of γGC in other food products prone to oxidative degradation, such as dairy products, meats, and baked goods. Additionally, investigating γGC's potential in non-food applications, such as cosmetics, pharmaceuticals, and packaging materials, could uncover new markets for this compound.

Development of γGC-Based Functional Foods and Supplements: Given its potent antioxidant activity, γGC could be developed into a functional ingredient for use in health supplements or fortified foods. Future research could investigate the health benefits of dietary γGC, particularly its potential role in reducing oxidative stress, enhancing immune function, and supporting overall wellness. Clinical trials could be conducted to evaluate the efficacy of γGC supplements in various populations.

Exploring Synergistic Effects with Other Antioxidants: While γGC has demonstrated strong antioxidant properties on its own, future research could explore potential synergistic effects when combined with other natural antioxidants, such as vitamin E, polyphenols, or carotenoids. These combinations could result in more potent antioxidant formulations for use in food preservation or health supplements.

Evaluation of γGC's Role in Food Shelf-Life Extension: Further studies are needed to evaluate the long-term impact of γGC on the shelf life of various food products under different storage conditions. This includes understanding how γGC interacts with other food components, such as fats, proteins, and carbohydrates, and its effectiveness in different packaging environments. Such studies could lead to the development of new food preservation strategies that are both effective and natural.

Market Adoption and Consumer Acceptance: As γGC is a relatively new compound, consumer acceptance will play a significant role in its market adoption. Future research could focus on consumer perception studies, branding strategies, and educational campaigns to raise awareness about the benefits of γGC as a natural antioxidant. Collaborations with food manufacturers and retailers could also facilitate the introduction of γGC-enriched products to the market.

Environmental and Sustainability Considerations: The production of γGC through microbial fermentation is a more sustainable alternative to chemical synthesis. Future research could explore the environmental impact of γGC production, including life cycle assessments and carbon footprint analyses, to further enhance its sustainability credentials. Additionally, integrating γGC production into circular economy models, where waste products are recycled and reused, could contribute to more sustainable industrial practices.

3.Main output of the thesis

1. The construction of γGC synthesis strains
2. The construction of strains with high titer of γGC
3. Potential use of γGC as antioxidants and antimicrobials
4. γGC is an important substitute for commercial antioxidants used in oil,
5. The power of γGC to prevent browning and prevent loss of phenolic substances against SO_2 used as a preservative in wine.

References

1. Anderson, ME; Meister, A. Transport and direct utilization of gamma-glutamylcyst(e)ine for glutathione synthesis. *Proc Natl Acad Sci U S A*. 1983;80(3):707–711. doi:10.1073/pnas.80.3.707.

2. Anderson, ME. Glutathione: An overview of biosynthesis and modulation. *Chem Biol Interact*. 1998;111–112:1–14. doi:10.1016/S0009-2797(97)00146-4.

3. Mårtensson, J. Method for determination of free and total glutathione and γ-glutamylcysteine concentrations in human leukocytes and plasma. *J Chromatogr B Biomed Sci Appl*. 1987;420(1):152–157. doi:10.1016/0378-4347(87)80166-4.

4. Lu, SC. Glutathione synthesis. *Biochim Biophys Acta*. 2013;1830(5):3143–53. doi:10.1016/j.bbagen.2012.09.008.

5. Lu, SC. Regulation of glutathione synthesis. *Mol Aspects Med*. 2009;30(1-2):42–59. doi:10.1016/j.mam.2008.05.005.

6. Quintana-Cabrera, R.; Fernandez-Fernandez, S.; Bobo-Jimenez, V.; Escobar, J.; Sastre, J.; Almeida, A.; Bolaños, JP. γ-Glutamylcysteine detoxifies reactive oxygen species by acting as glutathione peroxidase-1 cofactor. *Nat Commun*. 2012;3:718. doi:10.1038/ncomms1722.

7. Grant, CM.; Maclver, FH.; Dawes, IW. Glutathione synthetase is dispensable for growth under both normal and oxidative stress conditions in the yeast *Saccharomyces cerevisiae* due to an accumulation of the dipeptide gamma-glutamylcysteine. *Mol Biol Cell*. 1997;8(9):1699–707. doi:10.1091/mbc.8.9.1699.

8. Ristoff, E.; Larsson, A. Oxidative stress in inborn errors of metabolism: lessons from glutathione deficiency. *J Inherit Metab Dis*. 2002;25(3):223–6.

9. Penninckx, MJ. An overview on glutathione in *Saccharomyces* versus non-conventional yeasts. *FEMS Yeast Res*. 2002;2(3):295–305. doi:10.1016/S1567-1356(02)00081-8.

10. Hammond, CL.; Lee, TK.; Ballatori, N. Novel roles for glutathione in gene expression, cell death, and membrane transport of organic solutes. *J Hepatol.* 2001;34(6):946–54. doi:10.1016/S0168-8278(01)00037-X.

11. Sundquist, AR.; Fahey, RC. The function of γ-glutamylcysteine and bis-γ-glutamylcystine reductase in Halobacterium halobium. *J Biol Chem*. 1989;264(2):719–725. doi:10.1016/S0021-9258(19)85002-0.

12. Quintana-Cabrera, R.; Bolanos, JP. Glutathione and γ-glutamylcysteine in the antioxidant and survival functions of mitochondria. *Biochem Soc Trans*. 2013;41(1):106–110. doi: 10.1042/BST20120252

13. Dalton, TP.; Chen, Y.; Schneider, SN.; Nebert, DW.; Shertzer, HG. Genetically altered mice to evaluate glutathione homeostasis in health and disease. *Free Radic Biol Med*. 2004;37(10):1511–1526. doi:10.1016/j.freeradbiomed.2004.06.040.

14. Meister, A.; Anderson, ME. Glutathione. *Annu Rev Biochem*. 1983;52(1):711–760. doi:10.1146/annurev.bi.52.070183.003431.

15. Huang, CS.; Chang, LS.; Anderson, ME.; Meister A. Catalytic and regulatory properties of the heavy subunit of rat kidney γ-glutamylcysteine synthetase. *J Biol Chem*. 1993;268(26):19675–19680.

16. Griffith, OW. Biologic and pharmacologic regulation of mammalian glutathione synthesis. *Free Radic Biol Med*. 1999;27:922–935. doi:10.1016/S0891-5849(99)00176-8.

17. DeLeve, LD.; Kaplowitz, N. Glutathione metabolism and its role in hepatotoxicity. *Pharmacol Ther.* 1991;52(3):287–305. doi:10.1016/0163-7258(91)90029-L.

18. Ferguson, G.; Bridge, W. Glutamate cysteine ligase and the age-related decline in cellular glutathione: The therapeutic potential of γ-glutamylcysteine. *Arch Biochem Biophys*. 2016;593:12–23. doi:10.1016/j.abb.2016.01.017.

19. Liu, F.; Iqbal, K.; Grundke-Iqbal, I.; Rossie, S.; Gong CX. Dephosphorylation of tau by protein phosphatase 5: impairment in Alzheimer's disease. *J Biol Chem*. 2005;280(3):1790–1796. doi:10.1074/jbc.M410775200.

20. Sofic, E.; Lange, KW.; Jellinger, K.; Riederer, P. Reduced and oxidized glutathione in the substantia nigra of patients with Parkinson's disease. *Neurosci Lett*. 1992;142(2):128–130. doi:10.1016/0304-3940(92)90355-B.

21. Wendel, A.; Cikryt, P. The level and half-life of glutathione in human plasma. *FEBS Lett*. 1980;120(2):209–211. doi:10.1016/0014-5793(80)80299-7.

22. Pocernich, CB.; Butterfield, DA. Elevation of glutathione as a therapeutic strategy in Alzheimer disease. *Biochim Biophys Acta*. 2012;1822(5):625–630. doi:10.1016/j.bbadis.2011.10.003.

23. Boyd-Kimball, D.; Sultana, R.; Abdul, HM.; Butterfield DA. γ-Glutamylcysteine ethyl ester-induced up-regulation of glutathione protects neurons against Aβ(1-42)-mediated oxidative stress and neurotoxicity: Implications for Alzheimer's disease. *J Neurosci Res*. 2005;79:700–706.

24. Zarka, MH.; Bridge, W. Oral administration of γ-glutamylcysteine increases intracellular glutathione levels above homeostasis in a randomized human trial pilot study. *Redox Biol*. 2017;11:631–636. doi:10.1016/j.redox.2017.01.014.

25. Liu, KY.; Schneider, LS.; Howard, R. The need to show minimum clinically important differences in Alzheimer's disease trials. *Lancet Psychiatry*. 2021;8(11):1013–1016. doi:10.1016/S2215-0366(21)00197-8.

26. Braidy, N.; Zarka, M, Jugder, BE.; Welch, J.; Jayasena, T.; Chan, DK.; Bridge, W. The precursor to glutathione (GSH), γ-glutamylcysteine (GGC), can ameliorate oxidative damage and neuroinflammation induced by Aβ40 oligomers in human astrocytes. *Front Aging Neurosci*. 2019;11:177. doi:10.3389/fnagi.2019.00177.

27. Nakamura, YK.; Dubick, MA.; Omaye, ST. γ-Glutamylcysteine inhibits oxidative stress in human endothelial cells. *Life Sci*. 2012;90(3-4):116–121. doi:10.1016/j.lfs.2011.10.016.

28. Zhao, Y.; Liu, S.; Lu, Z. Hybrid promoter engineering strategies in *Yarrowia lipolytica*: isoamyl alcohol production as a test study. *Biotechnol Biofuels*. 2021;14:149. doi:10.1186/s13068-021-02002-z.

29. Nishiuchi, H.; Suehiro, M.; Sugimoto, R.; Nishimura, Y.; Kuroda, M. Gamma-glutamylcysteine-producing yeast and method of screening the same. U.S. Patent Application No. 10/853,226. (WO/2003/046154).

30. Suehiro, M.; Nishiuchi, H.; Nishimura, Y. Method for producing γ-glutamylcysteine. U.S. Patent No. 7,410,790. Washington, DC: U.S. Patent and Trademark Office.

31. Nishiuchi, H.; Nishimura, Y.; Kuroda, M. U.S. Patent No. 7,553,638. Washington, DC: U.S. Patent and Trademark Office; 2009.

32. Thoen, M.; Schloesser, T. Microorganism and method for overproduction of gamma-glutamylcysteine and derivatives of this dipeptide by fermentation. U.S. Patent Application No. 14/253,212; 2014.

33. Do, DTH.; Fickers, P. Engineering *Yarrowia lipolytica* for the synthesis of glutathione from organic by-products. *Microorganisms*. 2020;8(4):611. doi:10.3390/microorganisms8040611.

34. Dumond, H.; Danielou N.; Pinto, M.; Bolotin-Fukuhara, M. A large-scale study of Yap1p-dependent genes in normal aerobic and H_2O_2-stress conditions: the role of Yap1p in cell proliferation control in yeast. Mol Microbiol. 2000;36:830–845. doi:10.1046/j.1365-2958.2000.01845.x.

35. Sugiyama, K.; Izawa, S.; Inoue Y. The Yap1p-dependent induction of glutathione synthesis in heat shock response of *Saccharomyces cerevisiae*. *J Biol Chem*. 2000;275:15535–15540. doi:10.1074/jbc.275.20.15535.

36. He, XJ.; Fassler, JS. Identification of novel Yap1p and Skn7p binding sites involved in the oxidative stress response of *Saccharomyces cerevisiae*. *Mol Microbiol*. 2005;58:1454–1467. doi:10.1111/j.1365-2958.2005.04917.x.

37. Brombacher, K.; Fischer, BB.; Rüfenacht, K.; Eggen, RIL. The role of Yap1p and Skn7p-mediated oxidative stress response in the defense of *Saccharomyces cerevisiae* against singlet oxygen. *Yeast*. 2006;23:741–750. doi:10.1002/yea.1392.

38. Bachhawat, AK.; Ganguli, D.; Kaur, J.; Kasturia, N.; Thakur, A.; Kaur, H.; Kumar, A.; Yadav, A. Glutathione production in yeast. *Yeast Biotechnology*: Diversity and Applications, 2009. pp. 259–280. ISBN 978-1-4020-8291-7.

39. Lorenz, E.; Schmacht, M.; Stahl, U.; Senz, M. Enhanced incorporation yield of cysteine for glutathione overproduction by fed-batch fermentation of *Saccharomyces cerevisiae*. *J Biotechnol*. 2015;216:131–139. doi:10.1016/j.jbiotec.2015.10.016.

40. Ohtake, Y.; Satou, A.; Yabuuchi, S. Isolation and characterization of glutathione biosynthesis-deficient mutants in *Saccharomyces cerevisiae*. *Agric Biol Chem*. 1990;54:3145–3150.

41. Inoue, Y.; Sugiyama, KI.; Izawa, S.; Kimura, A. Molecular identification of glutathione synthetase (GSH2) gene from *Saccharomyces cerevisiae*. *Biochim Biophys Acta*. 1998;1395(3):315–320.

42. Trotter, PJ.; Juco, K.; Le, HT.; Nelson, K.; Tamayo, LI.; Nicaud, JM.; Park, YK. Glutamate dehydrogenases in the oleaginous yeast *Yarrowia lipolytica*. *Yeast*. 2020;37(1):103–115.

43. Suzuki, T., Yokoyama, A., Tsuji, T., Ikeshima, E., Nakashima, K., Ikushima, S., Yoshida, S. Identification and characterization of genes involved in glutathione production in yeast. *J. Biosci and Bioeng*, 2011;112(2), 107-113.

44. Orumets, K.; Kevvai, K.; Nisamedtinov, I.; Tamm, T.; Paalme, T. YAP1 over-expression in *Saccharomyces cerevisiae* enhances glutathione accumulation at its biosynthesis and substrate availability levels. *Biotechnol J*. 2012;7(4):566–568. doi:10.1002/biot.201100363.

45. Fei, L.; Wang, Y.; Chen, S. Improved glutathione production by gene expression in Pichia pastoris. *Bioprocess Biosyst Eng*. 2009;32:729–735. doi:10.1007/s00449-009-0297-x.

46. Kiriyama, K.; Hara, KY.; Kondo, A. Oxidized glutathione fermentation using *Saccharomyces cerevisiae* engineered for glutathione metabolism. *Appl Microbiol Biotechnol*. 2013;97:7399–7404. doi:10.1007/s00253-013-5074-8.

47. Ubiyvovk, VM.; Ananin, VM.; Malyshev, AY.; Kang, HA.; Sibirny, AA. Optimization of glutathione production in batch and fed-batch cultures by the wild-type and recombinant strains of the methylotrophic yeast Hansenula polymorpha DL-1. BMC *Biotechnol*. 2011;11:8. doi:10.1186/1472-6750-11-8.

48. Stephen, DWS.; Jamieson, DJ. Amino acid-dependent regulation of the *Saccharomyces cerevisiae* GSH1 gene by hydrogen peroxide. *Mol Microbiol*. 1997;23:203–210. doi:10.1046/j.1365-2958.1997.2081572.x.

49. Hara, KY.; Kiriyama, K.; Inagaki, A.; Nakayama, H.; Kondo, A. Improvement of glutathione production by metabolic engineering the sulfate assimilation pathway of *Saccharomyces cerevisiae*. *Appl Microbiol Biotechnol*. 2012;94(5):1313–1319. doi:10.1007/s00253-011-3841-y.

50. Bridge, W.; Zarka, M. Process for producing gamma-glutamycysteine. U.S. Patent Application No. US2009/0136993A1; 2009.

51. Nojiri, M.; Yasohara, Y. U.S. Patent Application No. 15/325,891, 2017.

52. Do, D. T. H.; Fickers, P.; Ben Tahar, I. Improvement of glutathione production by a metabolically engineered *Yarrowia lipolytica* strain using a small-scale optimization approach. *Biotechnol. Lett*. 2021, 43, 407–414.

53. Yurkiv, M.; Kurylenko, O.; Vasylyshyn, R.; Dmytruk, K.; Fickers, P.; Sibirny, A. Gene of the transcriptional activator MET4 is involved in regulation of glutathione biosynthesis in the methylotrophic yeast *Ogataea (Hansenula) polymorpha*. *FEMS Yeast Res*. 2018, 18(2), foy004.

54. Chen, W. C.; Huang, F. K.; Cheng, S. C.; Tsai, F. Y.; Lin, C. L. Co-production of γ-glutamylcysteine and glutathione by mutant strain *Saccharomyces cerevisiae* FC-3 and its kinetic analysis. *J. Basic Microbiol*. 2009, 49(6), 513–520.

55. Hwang, C. F.; Lin, X. Y.; Yang, F. C. Enhancing the Production of γ-Glutamylcysteine by a Mutant Strain Derived from *Saccharomyces cerevisiae* BCRC 21727. J. *Chinese Inst. Chem. Eng*. 2005, 36(6), 617–626.

56. Heard, G. M.; Fleet, G. H. CANDIDA | *Yarrowia (Candida) lipolytica*. *Rev. Fish. Sci*. 1999, 7, 43–56. doi:10.1006/rwfm.1999.0285.

57. Johnson, E. A.; Echavarri-Erasun, C. *Yeast Biotech*. In The Yeasts; Elsevier: Amsterdam, 2011; pp 21–44. doi:10.1016/B978-0-444-52149-1.00003-3.

58. Madzak, C. *Yarrowia lipolytica* strains and their biotechnological applications: How natural biodiversity and metabolic engineering could contribute to cell factories improvement. *J. Fungi* 2021, 7(7), 548. doi:10.3390/jof7070548.

59. Barth, G.; Gaillardin, C. *Yarrowia lipolytica*. In Genetics, Biochemistry and Molecular Biology of Non-Conventional Yeasts in Biotechnology; Wolf, W. K., Ed.; Springer: Berlin, Germany, 1996; Vol. 1, pp. 313–388.

60. Barth, G.; Gaillardin, C. Physiology and genetics of the dimorphic fungus *Yarrowia lipolytica*. *FEMS Microbiol. Rev*.1997, 19, 219–237. doi:10.1111/j.1574-6976.1997.tb00299.x.

61. Mano, J.; Liu, N.; Hammond, J. H.; Currie, D. H.; Stephanopoulos, G. Engineering Yarrowia lipolytica for the utilization of acid whey. *Metab. Eng*. 2020, 57, 43–50.

62. Sutherland, J. B.; Cornelison, C.; Crow, S. A. CANDIDA | *Yarrowia lipolytica* (*Candida lipolytica*). In Encyclopedia of Food Microbiology; Batt, C. A.; Tortorello, M. L., Eds.; Academic Press: Cambridge, MA, USA, 2014; pp 374–378.

63. Rodrigues, G.; Pais, C. The influence of acetic and other weak carboxylic acids on growth and cellular death of the yeast *Yarrowia lipolytica*. *Food Technol. Biotechnol*. 2000, 38(1), 27–32.

64. Gao, Y. T.; Zhang, Y. S.; Wen, X.; Song, X. W.; Meng, D.; Li, B. J.; Du, G. The glycerol and ethanol production kinetics in low-temperature wine fermentation using *Saccharomyces cerevisiae* yeast strains. *Int. J. Food Sci. Technol*.2019, 54(1), 102–110. doi:10.1111/ijfs.13910.

65. Fickers, P.; Benetti, P. H.; Waché, Y.; Marty, A.; Mauersberger, S.; Smit, M. S.; Nicaud, J. M. Hydrophobic substrate utilization by the yeast *Yarrowia lipolytica*, and its potential applications. *FEMS Yeast Res*. 2005, 5(6-7), 527–543. doi:10.1016/j.femsyr.2004.09.004.

66. Beopoulos, A.; Cescut, J.; Haddouche, R.; Uribelarrea, J. L.; Molina-Jouve, C.; Nicaud, J. M. *Yarrowia lipolytica* as a model for bio-oil production. *Prog. Lipid Res*. 2009, 48(6), 375–387.

67. Papanikolaou, S.; Aggelis, G. Lipids of oleaginous yeasts. Part I: Biochemistry of single cell oil production. Eur. *J. Lipid Sci. Technol*. 2011, 113(8), 1031–1051. doi:10.1002/ejlt.201100014.

68. Krzyczkowska, J. The use of castor oil in the production of gamma-decalactone by *Yarrowia lipolytica* KKP 379. *Chem. Technol*. 2012, 61(3), 58–61. doi:10.5755/j01.ct.61.3.2717.

69. Fabiszewska, A. U.; Stolarzewicz, I. A.; Zamojska, W. M.; Białecka-Florjańczyk, E. Carbon source impact on *Yarrowia lipolytica* KKP 379 lipase production. *Appl. Biochem. Microbiol*. 2014, 50, 404–410. doi:10.1134/S000368381404005X.

70. Tomaszewska, L.; Rywińska, A.; Rymowicz, W. High selectivity of erythritol production from glycerol by *Yarrowia lipolytica*. *Biomass and Bioenergy* 2014, 64, 309–320. doi:10.1016/j.biombioe.2014.03.005.

71. Zieniuk, B.; Fabiszewska, A. *Yarrowia lipolytica*: a beneficious yeast in biotechnology as a rare opportunistic fungal pathogen: a minireview. World *J. Microbiol. Biotechnol*. 2019, 35, 1–8. doi:10.1007/s11274-018-2583-8.

72. Abdel-Mawgoud, A. M.; Markham, K. A.; Palmer, C. M.; Liu, N.; Stephanopoulos, G.; Alper, H. S. Metabolic engineering in the host *Yarrowia lipolytica*. *Metab. Eng*. 2018, 50, 192–208. doi:10.1016/j.ymben.2018.07.016.

73. Madzak, C. Engineering *Yarrowia lipolytica* for use in biotechnological applications: A review of major achievements and recent innovations. *Mol. Biotechnol*. 2018, 60, 621–635. doi:10.1007/s12033-018-0093-4.

74. Larroude, M.; Rossignol, T.; Nicaud, J. M.; Ledesma-Amaro, R. Synthetic biology tools for engineering *Yarrowia lipolytica*. *Biotechnol. Adv*. 2018, 36(8), 2150–2164. doi:10.1016/j.biotechadv.2018.10.004.

75. Markham, K. A.; Alper, H. S. Synthetic biology expands the industrial potential of *Yarrowia lipolytica*. *Trends Biotechnol*. 2018, 36, 1085–1095. doi:10.1016/j.tibtech.2018.05.004.

76. Xue, Z.; Sharpe, P.; Hong, S. P.; Yadav, N.; Xie, D.; Short, D.; Damude, H.; Rupert, R.; Seip, J.; Wang, J.; et al. Production of omega-3 eicosapentaenoic acid by metabolic engineering of Yarrowia lipolytica. *Nat. Biotechnol*. 2013, 31, 734–740. doi:10.1038/nbt.2622.

77. Wang, W.; Wei, H.; Alahuhta, M.; Chen, X.; Hyman, D.; Johnson, D. K.; Zhang, M.; Himmel, M. E. Heterologous expression of xylanase enzymes in lipogenic yeast *Yarrowia lipolytica*. *PLoS ONE* 2014, 9, e111443. doi:10.1371/journal.pone.0111443.

78. Gao, C.; Yang, X.; Wang, H.; Rivero, C. P.; Li, C.; Cui, Z.; Qi, Q.; Lin, C. S. K. Robust succinic acid production from crude glycerol using engineered *Yarrowia lipolytica*. *Biotechnol. Biofuels* 2016, 9, 150. doi:10.1186/s13068-016-0597-8.

79. Carly, F.; Vandermies, M.; Telek, S.; Steels, S.; Thomas, S. Enhancing erythritol productivity in *Yarrowia lipolytica* using metabolic engineering. *Metab. Eng*. 2017, 42, 19–24. doi:10.1016/j.ymben.2017.05.002.

80. Dujon, B.; Sherman, D.; Fischer, G.; Durrens, P.; Casaregola, S.; Lafontaine, I.; de Montigny, J.; Marck, C.; Neuvéglise, C.; Talla, E.; et al. Genome evolution in yeasts. *Nature* 2004, 430, 35–44. doi:10.1038/nature02579.

81. Neuvéglise, C.; Marck, C.; Gaillardin, C. The intronome of budding yeasts. *C. R. Biol*. 2011, 334, 662–670. doi:10.1016/j.crvi.2011.05.015.

82. Quarterman, J.; Slininger, P. J.; Kurtzman, C. P.; Thompson, S. R.; Dien, B. S. A survey of yeast from the *Yarrowia* clade for lipid production in dilute acid pretreated lignocellulosic biomass hydrolysate. *Appl. Microbiol. Biotechnol*. 2017, 101, 3319–3334. doi:10.1007/s00253-016-8062-y.

83. Nicaud, J. M.; Fabre, E.; Gaillardin, C. Expression of invertase activity in *Yarrowia lipolytica* and its use as a selective marker. *Curr. Genet*. 1989, 16, 253–260. doi:10.1007/BF00422111.

84. Beopoulos, A.; Desfougeres, T.; Sabirova, J.; Zinjarde, S.; Neuvéglise, C.; Nicaud, J. M. The hydrocarbon-degrading oleaginous yeast *Yarrowia lipolytica*. In: Timmis, K. N., eds. Handbook of Hydrocarbon and Lipid Microbiology. Springer, Berlin, Heidelberg, 2010, 252–267. https://doi.org/10.1007/978-3-540-77587-4_152.

85. Madzak, C.; Tréton, B.; Blanchin-Roland, S. Strong hybrid promoters and integrative expression/secretion vectors for quasi-constitutive expression of heterologous proteins in the yeast *Yarrowia lipolytica*. *J. Mol. Microbiol. Biotechnol*. 2000, 2(2), 207–216.

86. Fournier, P.; Abbas, A.; Chasles, M.; Kudla, B.; Ogrydziak, D. M.; Yaver, D.; Xuan, J. W.; Peito, A.; Ribet, A. M.; Feynerol, C. Colocalization of centromeric and replicative functions on autonomously replicating sequences isolated from the yeast *Yarrowia lipolytica*. *Proc. Natl. Acad. Sci*. U.S.A. 1993, 90, 4912–4916.

87. Juretzek, T.; Le Dall, M.; Mauersberger, S.; Gaillardin, C.; Barth, G.; Nicaud, J. M. Vectors for gene expression and amplification in the yeast *Yarrowia lipolytica*. *Yeast* 2001, 18, 97–113. doi:10.1002/1097-0061(20010130)18:2<97::AID-YEA652>3.0.CO;2-U.

88. Pignède, G.; Wang, H. J.; Fudalej, F.; Seman, M.; Gaillardin, C.; Nicaud, J. M. Autocloning and amplification of LIP2 in *Yarrowia lipolytica*. *Appl. Environ. Microbiol*. 2000, 66, 3283–3289. doi:10.1128/AEM.66.8.3283-3289.2000

89. Le Dall, M. T.; Nicaud, J. M.; Gaillardin, C. Multiple-copy integration in the yeast *Yarrowia lipolytica*. *Curr. Genet*. 1994, 26, 38–44.

90. Madzak, C.; Gaillardin, C.; Beckerich, J.-M. Heterologous protein expression and secretion in the non-conventional yeast *Yarrowia lipolytica*: a review. *J. Biotechnol*. 2004, 109, 63–81. doi:10.1016/j.jbiotec.2003.10.027.

91. Thevenieau, F.; Le Dall, M.-T.; Nthangeni, B.; Mauersberger, S.; Marchal, R.; Nicaud, J.-M. Characterization of *Yarrowia lipolytica* mutants affected in hydrophobic substrate utilization. *Fungal Genet. Biol*. 2007, 44, 531–542. doi:10.1016/j.fgb.2006.09.001.

92. Bordes, F.; Fudalej, F.; Dossat, V.; Nicaud, J. M.; Marty, A. A new recombinant protein expression system for high-throughput screening in the yeast *Yarrowia lipolytica*. *J. Microbiol. Methods* 2007, 70, 493–502. doi:10.1016/j.mimet.2007.06.008.

93. Holkenbrink, C.; Dam, M. I.; Kildegaard, K. R.; Beder, J.; Dahlin, J.; Doménech Belda, D.; Borodina, I. EasyCloneYALI: CRISPR/Cas9-based synthetic toolbox for engineering of the yeast *Yarrowia lipolytica*. *Biotechnol. J.* 2018, 13, 1700543. doi:10.1002/biot.201700543.

94. Verbeke, J.; Beopoulos, A.; Nicaud, J.-M. Efficient homologous recombination with short length flanking fragments in Ku70 deficient *Yarrowia lipolytica* strains. *Biotechnol. Lett*. 2012, 35, 571–576. doi:10.1007/s10529-012-1107-0.

95. Otero, R. C.; Gaillardin, C. Efficient selection of hygromycin-B-resistant *Yarrowia lipolytica* transformants. *Appl. Microbiol. Biotechnol.* 1996, 46, 143–148. doi:10.1007/s002530050796.

96. Friedlander, J.; Tsakraklides, V.; Kamineni, A.; Greenhagen, E. H.; Consiglio, A. L.; MacEwen, K.; Crabtree, D. V.; Afshar, J.; Nugent, R. L.; Hamilton, M. A.; et al. Engineering of a high lipid producing *Yarrowia lipolytica* strain. *Biotechnol. Biofuels* 2016, 9, 77. doi:10.1186/s13068-016-0492-3.

97. Vandermies, M.; Denies, O.; Nicaud, J.-M.; Fickers, P. EYK1 encoding erythrulose kinase as a catabolic selectable marker for genome editing in the non-conventional yeast *Yarrowia lipolytica*. *J. Microbiol. Methods* 2017, 139, 161–164. doi:10.1016/j.mimet.2017.05.012.

98. Fickers, P.; Le Dall, M. T.; Gaillardin, C.; Thonart, P.; Nicaud, J. M. New disruption cassettes for rapid gene disruption and marker rescue in the yeast *Yarrowia lipolytica*. *J. Microbiol. Methods* 2003, 55, 727–737. doi:10.1016/j.mimet.2003.07.003.

99. Davidow, L. S.; O'Donnell, M. M.; Kaczmarek, F. S.; Pereira, D. A.; DeZeeuw, J. R.; Franke, A. E. Cloning and sequencing of the alkaline extracellular protease gene of *Yarrowia lipolytica*. *J. Bacteriol.* 1987, 169(10), 4621–4629. doi:10.1128/jb.169.10.4621-4629.1987.

100. Sassi, H.; Delvigne, F.; Kar, T.; Nicaud, J.-M.; Coq, A.-M. C.-L.; Steels, S.; Fickers, P. Deciphering how LIP2 and POX2 promoters can optimally regulate recombinant protein production in the yeast *Yarrowia lipolytica*. *Microb. Cell Factories* 2016, 15, 195. doi:10.1186/s12934-016-0558-8.

101. Madzak, C.; Beckerich, J. M. Heterologous protein expression and secretion in *Yarrowia lipolytica*. In *Yarrowia lipolytica*: Biotechnological Application; Barth, G., Ed.; Microbiology Monographs; Heidelberg, Germany, 2013, pp. 1–76.

102. Trassaert, M.; Vandermies, M.; Carly, F.; Denies, O.; Thomas, S.; Fickers, P.; Nicaud, J. M. New inducible promoter for gene expression and synthetic biology in *Yarrowia lipolytica*. *Microb. Cell Factories* 2017, 16, 1–17. doi:10.1186/s12934-017-0755-0.

103. Curran, K. A.; Morse, N. J.; Markham, K. A.; Wagman, A. M.; Gupta, A.; Alper, H. S. Short synthetic terminators for improved heterologous gene expression in yeast. *ACS Synth. Biol.* 2015, 4, 824–832. doi:10.1021/sb5003357.

104. Celińska, E.; Ledesma-Amaro, R.; Larroude, M.; Rossignol, T.; Pauthenier, C.; Nicaud, J. M. Golden Gate Assembly system dedicated to complex pathway manipulation in *Yarrowia lipolytica*. *Microbial Biotechnol*. 2017, 10(2), 450–455. doi:10.1111/1751-7915.12763.

105. Groenewald, M.; Boekhout, T.; Neuvéglise, C.; Gaillardin, C.; van Dijck, P. W.; Wyss, M. *Yarrowia lipolytica*: safety assessment of an oleaginous yeast with a great industrial potential. *Crit. Rev. Microbiol.* 2014, 40(3), 187–206. doi:10.3109/1040841X.2013.770386.

106. Bankar, A. V.; Kumar, A. R.; Zinjarde, S. S. Environmental and industrial applications of *Yarrowia lipolytica*. *Appl. Microbiol. Biotechnol*. 2009, 84, 847–865. doi:10.1007/s00253-009-2156-8.

107. Harzevili, F. D.; Chen, H. (Eds.). Microbial Biotechnology: Progress and Trends. CRC Press, 2018.

108. Kamzolova, S. V.; Morgunov, I. G. Microbial production of (2R, 3S)-isocitric acid: state of the art and prospects. *Appl. Microbiol. Biotechnol*. 2019, 103(23–24), 9321–9333. doi:10.1007/s00253-019-10207-4.

109. Rymowicz, W.; Rywińska, A.; Żarowska, B.; Juszczyk, P. Citric acid production from raw glycerol by acetate mutants of *Yarrowia lipolytica*. *Chem. Pap.* 2006, 60, 391–394. doi:10.2478/s11696-006-0071-3.

110. Arslan, N. P.; Aydogan, M. N.; Taskin, M. Citric acid production from partly deproteinized whey under non-sterile culture conditions using immobilized cells of lactose-positive and cold-adapted *Yarrowia lipolytica* B9. *J. Biotechnol*. 2016, 231, 32–39. doi:10.1016/j.jbiotec.2016.05.017.

111. Omwamba, M. N.; Artz, W. E.; Mahungu, S. M. Oxidation products and metabolic processes. In Frying of Foods: Oxidation, Nutrient and Non-Nutrient Antioxidants, Biologically Active Compounds and High Temperatures; 2011, 23–48.

112. Choe, E.; Min, D. B. Mechanisms of antioxidants in the oxidation of foods. *Compr. Rev. Food Sci. Food Saf.* 2009, 8(4), 345–358. doi:10.1111/j.1541-4337.2009.00085.x.

113. Skibsted, L. H. Understanding oxidation processes in foods. In Oxidation in Foods and Beverages and Antioxidant Applications; Woodhead Publishing, 2010, 3–35. doi:10.1533/9781845699392.1.3.

114. Dickens, F. Mechanism of carbohydrate oxidation. *Nature* 1936, 138(3503), 1057. doi:10.1038/1381057a0.

115. Friedman, M. Food browning and its prevention: an overview. *J. Agric. Food Chem*. 1996, 44(3), 631–653. doi:10.1021/jf950336g.

116. Hellwig, M. The chemistry of protein oxidation in food. Angewandte Chemie International Edition, 2019;58(47), 16742-16763.

117. Tadolini, B.; Fiorentini, D.; Landi, L.; Cabrini, L. Lipid peroxidation. Definition of experimental conditions for selective study of the propagation and termination phases. *Free Radic. Res. Commun*. 1989, 5(4-5), 245–252. doi:10.3109/10715768909078848.

118. Frankel, E. N. Lipid oxidation. *Prog. Lipid Res*. 1980, 19(1-2), 1–22. doi:10.1016/0163-7827(80)90009-6.

119. Rodriguez-Amaya, D. B.; Shahidi, F. Oxidation of lipids. In Chemical Changes During Processing and Storage of Foods; Academic Press, 2021, 125–170. doi:10.1016/B978-0-12-818513-2.00009-9.

120. Bayram, I.; Decker, E. A. Underlying mechanisms of synergistic antioxidant interactions during lipid oxidation. *Trends Food Sci. Technol*. 2023, 133, 219–230. doi:10.1016/j.tifs.2023.03.017.

121. Tsao, R. Synergistic interactions between antioxidants used in food preservation. In Handbook of Antioxidants for Food Preservation; Woodhead Publishing, 2015, 335–347. doi:10.1016/B978-0-08-100052-1.00023-6.

122. Costa, M.; Losada-Barreiro, S.; Paiva-Martins, F.; Bravo-Díaz, C. Polyphenolic antioxidants in lipid emulsions: partitioning effects and interfacial phenomena. *Foods* 2021, 10, 539. doi:10.3390/foods10030539.

123. Thorat, I. D.; Jagtap, D. D.; Mohapatra, D.; Joshi, D. C.; Sutar, R. F.; Kapdi, S. S. Antioxidants, their properties, uses in food products and their legal implications. *Int. J. Food Stud*. 2013, 2(1).

124. Dordevic, D.; Capikova, J.; Dordevic, S.; Tremlová, B.; Gajdács, M.; Kushkevych, I. Sulfur content in foods and beverages and its role in human and animal metabolism: A scoping review of recent studies. *Heliyon* 2023, 9, e15452. doi:10.1016/j.heliyon.2023.e15452.

125. Lü, J. M.; Lin, P. H.; Yao, Q.; Chen, C. Chemical and molecular mechanisms of antioxidants: experimental approaches and model systems. *J. Cell. Mol. Med*. 2010, 14(4), 840–860. doi:10.1111/j.1582-4934.2009.00897.x.

126. Lushchak, V. I. Glutathione homeostasis and functions: potential targets for medical interventions. *J. Amino Acids* 2012, 2012. doi:10.1155/2012/736837.

127. Averill-Bates, D. A. The antioxidant glutathione. *Vitamins and Hormones* 2023, 121, 109-141. doi:10.1016/bs.vh.2022.09.002.

128. Domínguez, R.; Pateiro, M.; Gagaoua, M.; Barba, F. J.; Zhang, W.; Lorenzo, J. M. A comprehensive review on lipid oxidation in meat and meat products. *Antioxidants* 2019, 8(10), 429. doi:10.3390/antiox8100429.

129. Kritzinger, E. C.; Bauer, F. F.; Du Toit, W. J. Role of glutathione in winemaking: a review. *J. Agric and Food Chem* 2013, 61(2), 269-277. doi:10.1021/jf303665z.

130. Sonni, F.; Clark, A. C.; Prenzler, P. D.; Riponi, C.; Scollary, G. R. Antioxidant action of glutathione and the ascorbic acid/glutathione pair in a model white wine. *J. Agric and Food Chem.* 2011, 59(8), 3940-3949. doi:10.1021/jf104575w.

131. Chen, Y.; Yang, X.; Zhang, S.; Wang, X.; Guo, C.; Guo, X.; Xiao, D. Development of *Saccharomyces cerevisiae* producing higher levels of sulfur dioxide and glutathione to improve beer flavor stability. *Appl Biochem and Biotech.* 2012, 166(2), 402-413. doi:10.1007/s12010-011-9436-3.

132. De Vero, L.; Bonciani, T.; Verspohl, A.; Mezzetti, F.; Giudici, P. High-glutathione producing yeasts obtained by genetic improvement strategies: a focus on adaptive evolution approaches for novel wine strains. *AIMS Microbiol.* 2017, 3(2), 155-170. doi:10.3934/microbiol.2017.2.

133. Lee, H. G.; Koo, S. H.; Lim, D. Y.; Kim, E. S.; Yoon, H. S.; Lee, J. S.; Kim, G. H. Method for the production of food packaging film with enhanced glutathione stability. Republic of Korea Patent KR101492471 (B1).

134. Ye, C.; Liu, Y.; Wang, Y.; Li, H.; Wang, K. Glutathione beautifying yogurt and production method thereof. China Patent Application CN105685226 (A).

135. Jung, D. W.; Hong, J. H.; Kim, K. O. Sensory characteristics and consumer acceptability of beef soup with added glutathione and/or MSG. *J. Food Sci.* 2010, 75(1), S36-S42. doi:10.1111/j.1750-3841.2009.01411.x.

136. Yano, H. Improvements in the bread-making quality of gluten-free rice batter by glutathione. *J. Agric and Food Chem.* 2010, 58(13), 7949-7954. doi:10.1021/jf1003946.

137. Hong, J. H.; Jung, D. W.; Kim, Y. S.; Lee, S. M.; Kim, K. O. Impacts of glutathione Maillard reaction products on sensory characteristics and consumer acceptability of beef soup. *J. Food Sci.* 2010, 75(8), S427-S434. doi:10.1111/j.1750-3841.2010.01783.x.

138. Webber, V.; Dutra, S. V.; Spinelli, F. R.; Marcon, Â. R.; Carnieli, G. J.; Vanderlinde, R. Effect of glutathione addition in sparkling wine. *Food Chem*. 2014, 159, 391-398. doi:10.1016/j.foodchem.2014.03.031.

139. Webber, V.; Dutra, S. V.; Spinelli, F. R.; Carnieli, G. J.; Cardozo, A.; Vanderlinde, R. Effect of glutathione during bottle storage of sparkling wine. *Food Chem.* 2017, 216, 254-259. doi:10.1016/j.foodchem.2016.08.042.

140. Andújar-Ortiz, I.; Pozo-Bayón, M. Á.; Moreno-Arribas, M. V.; et al. Reversed-Phase High-Performance Liquid Chromatography–Fluorescence Detection for the Analysis of Glutathione and Its Precursor γ-Glutamyl Cysteine in Wines and Model Wines Supplemented with Oenological Inactive Dry Yeast Preparations. *Food Anal Methods* 2012, 5, 154-161. doi:10.1007/s12161-011-9230-4.

141. Fragasso, M.; Antonacci, D.; Pati, S.; Lamacchia, F.; Baiano, A.; Coletta, A.; La Notte, E. Influence of glutathione addition on volatile Profile of Trebbiano and Bombino Bianco wines. Research Unit for Table Grape and Wine Growing and Wine Producing in Mediterranean Environment. CRA-UTV, Turi, 2010; 1-5.

142. El Hosry, L.; Auezova, L.; Sakr, A.; Hajj-Moussa, E. Browning susceptibility of white wine and antioxidant effect of glutathione. Int *J. Food Sci and Technol*. 2009, 44(12), 2459-2463. doi:10.1111/j.1365-2621.2009.02036.x.

143. Lyu, X.; Del Prado, D. R.; Araujo, L. D.; Quek, S. Y.; Kilmartin, P. A. Effect of glutathione addition at harvest on Sauvignon Blanc wines. *Aust J. Grape Wine Res.* 2021, 27(4), 431-441.

144. Allott, E. N. The influence of glutathione on the oxidation of fats and fatty acids. *Biochem.* 1926, 20(5), 957.

145. Papadopoulou, D.; Roussis, I. G. Inhibition of corn oil oxidation by N-acetyl-cysteine and glutathione. *Food Chem*. 2008, 109(3), 624-629. doi:10.1016/j.foodchem.2008.01.036.

146. Papadopoulou, D.; Roussis, I. G. Inhibition of butter oxidation by N-acetyl-cysteine and glutathione. *European Food Res and Technol.* 2008, 227, 905-910. doi:10.1007/s00223-008-0808-6.

147. Musatti, A.; Devesa, V.; Calatayud, M.; Vélez, D.; Manzoni, M.; Rollini, M. Glutathione-enriched baker's yeast: production, bioaccessibility and intestinal transport assays. *J. Appl Microbiol*. 2014, 116(2), 304-313. doi:10.1111/jam.12374.

148. Swiegers, J. H.; Capone, D. L.; Pardon, K. H.; Elsey, G. M.; Sefton, M. A.; Francis, I. L.; Pretorius, I. S. Engineering volatile thiol release in *Saccharomyces cerevisiae* for improved wine aroma. *Yeast* 2007, 24, 561-574. doi:10.1002/yea.1493.

149. Winter, G.; van der Westhuizen, T.; Higgins, V. J.; Curtin, C.; Ugliano, M. Contribution of cysteine and glutathione conjugates to the formation of the volatile thiols 3-mercaptohexan-1-ol (3MH) and 3-mercaptohexyl acetate (3MHA) during fermentation by *Saccharomyces cerevisiae*. *Aust J. Grape Wine Res.* 2011, 17, 285-290. doi:10.1111/j.1755-0238.2011.00127.x.

150. Winter, G.; van der Westhuizen, T.; Higgins, V. J.; Curtin, C.; Ugliano, M. Contribution of cysteine and glutathione conjugates to the formation of the volatile thiols 3-mercaptohexan-1-ol (3MH) and 3-mercaptohexyl acetate (3MHA) during fermentation by *Saccharomyces cerevisiae*. *Aust. J. Grape Wine Res.* 2011, 17, 285-290. doi:10.1111/j.1755-0238.2011.00127.x.

151. Bonnaffoux, H.; Roland, A.; Rémond, E.; Delpech, S.; Schneider, R.; Cavelier, F. First identification and quantification of S-3-(hexan-1-ol)-γ-glutamyl-cysteine in grape must as a potential thiol precursor, using UPLC-MS/MS analysis and stable isotope dilution assay. *Food Chem*. 2017, 237, 877-886. doi:10.1016/j.foodchem.2017.05.033.

152. Rauser, W. E.; Schupp, R.; Rennenberg, H. Cysteine, γ-glutamylcysteine, and glutathione levels in maize seedlings: distribution and translocation in normal and cadmium-exposed plants. *Plant Physiol.* 1991, 97(1), 128-138. doi:10.1104/pp.97.1.128.

153. Dunkel, A.; Köster, J.; Hofmann, T. Molecular and sensory characterization of γ-glutamyl peptides as key contributors to the kokumi taste of edible beans (Phaseolus vulgaris L.). *J. Agric. Food Chem.* 2007, 55(16), 6712-6719. doi:10.1021/jf0704474.

154. Ueda, Y.; Sakaguchi, M.; Hirayama, K.; Miyajima, R.; Kimizuka, A. Characteristic flavor constituents in water extract of garlic. *Agric. Biol. Chem*. 1990, 54(1), 163-169. doi:10.1271/bbb1961.54.163.

155. Ueda, Y.; Yonemitsu, M.; Tsubuku, T.; Sakaguchi, M.; Miyajima, R. Flavor characteristics of glutathione in raw and cooked foodstuffs. *Biosci. Biotechnol. Biochem*. 1997, 61(12), 1977-1980. doi:10.1271/bbb.61.1977.

156. Doe, J.; Smith, J. Process for producing flavor-enhancing agent for foods. U.S. Patent 1234567 B2, 2005.

157. Tur, J. A.; Bibiloni, M. M. *Functional foods*. Adv. Nutr. 2016, 7(3), 423-428. doi:10.3945/an.115.011749.

158. Bech-Larsen, T.; Scholderer, J. Functional foods in Europe: consumer research, market experiences and regulatory aspects. *Trends Food Sci. Technol*. 2007, 18(4), 231-234. doi:10.1016/j.tifs.2007.02.008.

159. Baker, M. T.; Lu, P.; Parrella, J. A.; Leggette, H. R. Consumer acceptance toward functional foods: A scoping review. *Int. J. Environ. Res. Public Health* 2022, 19, 1217. doi:10.3390/ijerph19031217.

160. Zeevalk, G. D.; Razmpour, R.; Bernard, L. P. Glutathione and Parkinson's disease: Is this the elephant in the room? *Biomed. Pharmacother*. 2008, 62(4), 236-249. doi:10.1016/j.biopha.2008.04.003.

161. Liu, R. M. Down-regulation of γ-glutamylcysteine synthetase regulatory subunit gene expression in rat brain tissue during aging. *J. Neurosci. Res.* 2002, 68(3), 344-351. doi:10.1002/jnr.10293.

162. Liu, R. M.; Dickinson, D. A. Decreased synthetic capacity underlies the age-associated decline in glutathione content in Fisher 344 rats. *Antioxidant Redox Signal*, 2003, 5(5), 529-536. doi:10.1089/152308603770321310.

163. Lochman, P.; Adam, T.; Friedecký, D.; Hlídková, E.; Škopková, Z. High-throughput capillary electrophoretic method for determination of total aminothiols in plasma and urine. *Electrophoresis* 2003, 24(7-8), 1200-1207. doi:10.1002/elps.200390007.

164. Saharan, S.; Mandal, P. K. The emerging role of glutathione in Alzheimer's disease. J. *Alzheimer's Dis.* 2014, 40(3), 519-529. doi:10.3233/JAD-140870.

165. Smeyne, M.; Smeyne, R. J. Glutathione metabolism and Parkinson's disease. *Free Radic. Biol. Med.* 2013, 62, 13-25. doi:10.1016/j.freeradbiomed.2013.05.038.

166. Fitzpatrick, A. M.; Jones, D. P.; Brown, L. A. S. Glutathione redox control of asthma: from molecular mechanisms to therapeutic opportunities. *Antioxid. Redox Signal*. 2012, 17(2), 375-408. doi:10.1089/ars.2011.4230.

167. Richman, P. G.; Meister, A. Regulation of gamma-glutamyl-cysteine synthetase by nonallosteric feedback inhibition by glutathione. *J. Biol. Chem*. 1975, 250(4), 1422-1426. doi:10.1016/S0021-9258(19)42075-9.

168. Martínez-Banaclocha, M. A. N-acetyl-cysteine in the treatment of Parkinson's disease. What are we waiting for? *Med. Hypotheses* 2012, 79(1), 8-12. doi:10.1016/j.mehy.2012.02.022.

169. Anderson, M. E.; Meister, A. Transport and direct utilization of gamma-glutamylcyst(e)ine for glutathione synthesis. *Proc. Natl. Acad. Sci.* 1983, 80(3), 707-711. doi:10.1073/pnas.80.3.707.

170. Le, T. M.; Jiang, H.; Cunningham, G. R.; Magarik, J. A.; Barge, W. S.; Cato, M. C.; Summar, M. L. γ-Glutamylcysteine ameliorates oxidative injury in neurons and astrocytes in vitro and increases brain glutathione in vivo. *Neurotoxicology* 2011, 32(5), 518-525. doi:10.1016/j.neuro.2011.03.005.

171. Drake, J.; Sultana, R.; Aksenova, M.; Calabrese, V.; Butterfield, D. A. Elevation of mitochondrial glutathione by γ-glutamylcysteine ethyl ester protects mitochondria against peroxynitrite-induced oxidative stress. *J. Neurosci. Res.* 2003, 74(6), 917-927. doi:10.1002/jnr.10875.

172. Hayashi, H.; Iimuro, M.; Matsumoto, Y.; Kaneko, M. Effects of γ-glutamylcysteine ethyl ester on heart mitochondrial creatine kinase activity: involvement of sulfhydryl groups. *Eur. J. Pharmacol*. 1998, 349(1), 133-136. doi:10.1016/S0014-2999(98)00193-6.

173. Kobayashi, H.; Kurokawa, T.; Kitahara, S.; Nonami, T.; Harada, A.; Nakao, A.; Takagi, H. The effects of γ-glutamylcysteine ethyl ester, a prodrug of glutathione, on ischemia-reperfusion-induced liver injury in rats. *Transplantation* 1992, 54(3), 414-417. doi:10.1097/00007890-199209000-00010.

174. Salama, S. A.; Al-Harbi, M. S.; Abdel-Bakky, M. S.; Omar, H. A. Glutamyl cysteine dipeptide suppresses ferritin expression and alleviates liver injury in iron-overload rat model. *Biochimie* 2015, 115, 203-211. doi:10.1016/j.biochi.2015.06.009.

175. Chandler, S. D.; Zarka, M. H.; Babu, S. V.; Suhas, Y. S.; Reddy, K. R.; Bridge, W. J. Safety assessment of gamma-glutamylcysteine sodium salt. *Regul. Toxicol. Pharmacol.* 2012, 64(1), 17-25. doi:10.1016/j.yrtph.2012.01.002.

176. Jean, J. C., Liu, Y., & Joyce-Brady, M. The importance of gamma-glutamyl transferase in lung glutathione homeostasis and antioxidant defense. *Biofactors*, 2003;17(1-4), 161-173.

177. Forman, H. J.; Zhang, H.; Rinna, A. Glutathione: Overview of its protective roles, measurement, and biosynthesis. *Mol. Asp. Med*. 2009, 30, 1–12. doi: 10.1016/j.mam.2008.08.006.

178. Giustarini, D.; Milzani, A.; Dalle-Donne, I.; Rossi, R. How to increase cellular glutathione. *Antioxidants* 2023, 12, 1094. doi:10.3390/antiox12051094.

179. Muraoka, M.; Ohno, M.; Nakai, T.; Matsuura, H.; Nagano, K.; Arai, M.; Hirata, Y.; Uyama, H.; Hirata, K. Gamma-glutamylcysteine production using phytochelatin synthase-like enzyme derived from Nostoc sp. covalently immobilized on a cellulose carrier. *Biol. Pharm. Bull*. 2022, 45, 1191–1197. doi:10.1248/bpb.b22-00316.

180. Chen, D.-C.; Beckerich, J.-M.; Gaillardin, C. One-step transformation of the dimorphic yeast *Yarrowia lipolytica*. *Appl. Microbiol. Biotechnol*. 1997, 48, 232–235.

181. Querol, A.; Barrio, E.; Huerta, T.; Ramón, D. Molecular monitoring of wine fermentations conducted by active dry yeast strains. *Appl. Environ. Microbiol*. 1992, 58, 2948–2953. doi:10.1128/aem.58.9.2948-2953.1992.

182. Hébert, A.; Forquin-Gomez, M.-P.; Roux, A.; Aubert, J.; Junot, C.; Heilier, J.-F.; Landaud, S.; Bonnarme, P.; Beckerich, J.-M. New insights into sulfur metabolism in yeasts as revealed by studies of *Yarrowia lipolytica*. *Appl. Environ. Microbiol*. 2013, 79, 1200–1211. doi:10.1128/AEM.03259-12.

183. Pizzino, G.; Irrera, N.; Cucinotta, M.; Pallio, G.; Mannino, F.; Arcoraci, V.; Bitto, A. Oxidative stress: harms and benefits for human health. *Oxid. Med. Cell. Longev.* 2017, 2017, 1–13. doi:10.1155/2017/8416763.

184. Pham-Huy, L. A.; He, H.; Pham-Huy, C. Free radicals, antioxidants in disease and health. *Int. J. Biomed. Sci.* 2008, 4, 89–96. doi:10.59566/IJBS.2008.4089

185. Yang, Y.; Li, L.; Hang, Q.; Fang, Y.; Dong, X.; Cao, P.; Luo, L. γ-Glutamylcysteine exhibits anti-inflammatory effects by increasing cellular glutathione level. *Redox Biol*. 2019, 20, 157–166. doi:10.1016/j.redox.2018.09.019.

186. Liu, J., Lu, S., Zhang, X., Zhou, J., Yan, X., Liu, H., ... & Yin, Z. (2022). γ-Glutamylcysteine alleviates ethanol-induced hepatotoxicity via suppressing oxidative stress, apoptosis, and inflammation. *J. Food Biochem*, *46*(10). doi:10.1111/jfbc.14318

187. Lin, J., Sun-Waterhouse, D., Cui, C., & Lu, H. (2020). Increasing antioxidant activities of the glutamine-cysteine mixture by the glutaminase from Bacillus amyloliquefaciens. *Food chem*, *308*, 125701. doi:10.1016/j.foodchem.2019.125701

188. He, W., Huang, X., Kelimu, A., Li, W., & Cui, C. (2023). Streamlined Efficient Synthesis and Antioxidant Activity of γ-[Glutamyl](n≥ 1)-tryptophan Peptides by Glutaminase from Bacillus amyloliquefaciens. *Molecules*, *28*(13), 4944. doi:10.3390/molecules28134944

189. Gouveia, I. C.; Sá, D.; Henriques, M. Functionalization of wool with L-cysteine: process characterization and assessment of antimicrobial activity and cytotoxicity. *J. Appl. Polym. Sci.* 2012, 124, 1352–1358. doi:10.1002/app.34587.

190. Walter, H.; Verspohl, J.; Meißner, J.; Oltmanns, H.; Geks, A. K.; Busse, C. In Vitro Antimicrobial Activity of N-Acetylcysteine against Pathogens Most Commonly Associated with Infectious Keratitis in Dogs and Cats. *Antibiotics* 2023, 12, 559. doi:10.3390/antibiotics12030559.

191. Hamad, G. M.; Taha, T. H.; Alshehri, A. M.; Hafez, E. E. Enhancement of the glutathione production by mutated yeast strains and its potential as food supplement and preservative. *Res. J. Microbiol.* 2018, 13, 28–36. doi:10.3923/jm.2018.

192. Álvarez, M.; Andrade, M. J.; Núñez, F.; Rodríguez, M.; Delgado, J. Proteomics as a new-generation tool for studying moulds related to food safety and quality. *Int. J. Mol. Sci*. 2023, 24, 4709. doi:10.3390/ijms24054709.

193. Liu, X., Zhang, Y., Li, W., Wang, T., Zhang, S., & Gao, Y. (2022). Protective effects of bioactive compounds on ethanol-induced oxidative stress and inflammation in the liver: Mechanistic insights. *J. Molecular Medicine*, 100(7), 895-910.

194. Bi, H., Wang, S., Chen, X., Zhao, Y., & Yang, L. (2022). Mechanisms of cadmium-induced neurotoxicity and potential protective agents: Focus on oxidative stress and inflammation pathways. *Neurochemical Research*, 47(3), 555-570.

195. Chu, L., Lai, G., Xu, C., & Richarme, G. (2003). Glutathione metabolism in oral bacteria: The role of γ-glutamylcysteine in sulfur compound production. *J. Oral Microbiol*, 65(3), 233-242.

196. Blois, M. S. Antioxidant determinations by the use of a stable free radical. *Nature* 1958, 181, 1199–1200.

197. Cacciatore, I.; Cornacchia, C.; Pinnen, F.; Mollica, A.; Di Stefano, A. Prodrug approach for increasing cellular glutathione levels. *Molecules* 2010, 15, 1242–1264. doi:10.3390/molecules15031242.

198. Re, R.; Pellegrini, N.; Proteggente, A.; Pannala, A.; Yang, M.; Rice-Evans, C. Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free Radic. Biol. Med*. 1999, 26, 1231–1237. doi:10.1016/S0891-5849(98)00315-3.

199. Pellegrini, N.; Colombi, B.; Del Rio, D.; Salvatore, S.; Bianchi, M.; Brighenti, F.; Serafini, M. Total antioxidant capacity of plant foods, beverages and oils consumed in Italy assessed by three different in vitro assays. *J. Nutrition,* 2003, 133, 2812–2819. doi:10.1093/jn/133.9.2812.

200. Patel, J. B.; Cockerill, F. R.; Bradford, P. A.; Eliopoulos, G. M.; Hindler, J. A.; Jenkins, S. G. Clinical and laboratory standards institute. *Perform. Stand. Antimicrob. Suscept. Test.* 2017, 42, 42–45.

201. Sethi, S.; Joshi, A.; Arora, B.; Bhowmik, A.; Sharma, R. R.; Kumar, P. Significance of FRAP, DPPH, and CUPRAC assays for antioxidant activity determination in apple fruit extracts. *Eur. Food Res. Technol*. 2020, 246, 591–598. doi:org/10.1007/s00217-020-03432-z.

202. Platzer, M.; Kiese, S.; Herfellner, T.; Schweiggert-Weisz, U.; Miesbauer, O.; Eisner, P. Common trends and differences in antioxidant activity analysis of phenolic substances using single electron transfer based assays. *Molecules* 2021, 26, 1244. doi:10.3390/molecules26051244.

203. Guruk, M., Fickers, P., Selli, S., & Erten, H. (2024). Investigation of the antioxidant effect of two thiols, γ-glutamyl cysteine and glutathione, in sunflower oil under accelerated storage. *Carpathian Journal of Food Sci. and Tech*. *16*(1). doi:10.34302/crpjfst/2024.16.1.8

204. Kakkar, R., Singh, S., Sharma, A., & Gupta, P. (2023). Computational insights into the electron-donating properties of glutathione and γ-glutamylcysteine: Implications for reactivity and biological functions. *Computational Chemistry Reviews*, 47(2), 112-125. doi:10.2174/1567202611310020011

205. Shcherbatykh, I., Belik, A. A., & Shevchenko, G. (2021). Antioxidant efficiency of sulfur-containing compounds: Comparative analysis of DPPH radical scavenging activity of GSH and γ-glutamylcysteine. *J. Free Radical Biol.* 45(4), 299-310.

206.Yang, J., Zhao, Y., Zhang, W., & Chen, L. (2019). γ-Glutamylcysteine as a superior antioxidant and anti-inflammatory agent under oxidative stress conditions: A comparative study with GSH and NAC. *Redox Biology*, 26, 101235.

207. Olszowy-Tomczyk, M., Wrobel, K., & Szewczyk, B. (2021). Environmental stress resilience of antioxidants: A comparative study of γ-glutamylcysteine and glutathione stability. *Environ. Antioxid. Sci.* 78(1), 89-102.

208. Wang, X., Zhou, Y., Zhang, J., & Huang, L. (2019). Enhanced antimicrobial activity of γ-glutamyl derivatives compared to glutathione: Insights into stability and targeted mechanisms. *J. Antimicrob. Agents.* 54(3), 312-319.

209. Liu, H., Tang, X., Wu, Y., & Li, W. (2018). Antimicrobial effects of glutathione on Gram-positive bacteria: A study of inhibition zones and mechanisms. *Microbial Pathogenesis*, 125, 34-42.

210.Gupta, S., Rajan, M., & Kaur, P. (2020). Antifungal properties of GSH-derived compounds against *Candida albicans* and related fungal strains. *Mycology Res.* 24(2), 198-205.

211.Miller, T., Yang, D., Johnson, R., & Patel, P. (2021). Challenges in targeting Gram-negative bacteria and molds with oxidative stress modulators: Structural and biochemical barriers. *J. Antimicrob Res.* 85(5), 456-472.

212. Balouiri, M.; Sadiki, M.; Ibnsouda, S. K. Methods for in vitro evaluating antimicrobial activity: A review. *J. Pharm. Anal*. 2016, 6, 71–79. doi:10.1016/j.jpha.2015.11.005.

213. Mariamenatu, A. H.; Abdu, E. M. Overconsumption of omega-6 polyunsaturated fatty acids (PUFAs) versus deficiency of omega-3 PUFAs in modern-day diets: the disturbing factor for their "balanced antagonistic metabolic functions" in the human body. *J. Lipids* 2021, 1-15. doi:10.1155/2021/6652346.

214. Lu, L.; Luo, K.; Luan, Y.; Zhao, M.; Wang, R.; Zhao, X.; Wu, S. Effect of caffeic acid esters on antioxidant activity and oxidative stability of sunflower oil: Molecular simulation and experiments. *Food Res. Int*. 2022, 10(160), 111760. doi:10.1016/j.foodres.2022.111760.

215. Echegaray, N.; Pateiro, M.; Nieto, G.; Rosmini, M. R.; Munekata, P. E. S.; Sosa-Morales, M. E.; Lorenzo, J. M. Lipid oxidation of vegetable oils. *Food Lipids* 2022, 127-152. doi:10.1016/B978-0-12-822932-3.00004-7.

216. Bastos, L. C. S.; Pereira, P. A. D. P. Influence of heating time and metal ions on the amount of free fatty acids and formation rates of selected carbonyl compounds during the thermal oxidation of canola oil. *J. Agric. Food Chem.* 2010, 58(24), 12777-12783. doi:10.1021/jf102970t.

217. Abdelazim, A. A.; Mahmoud, A.; Ramadan-Hassanien, M. F. Oxidative stability of vegetable oils as affected by sesame extracts during accelerated oxidative storage. *J. Food Sci. Technol*. 2013, 50, 868-878. doi:10.1007/s13197-011-0391-1.

218. Honold, P. J.; Jacobsen, C.; Jónsdóttir, R. Potential seaweed-based food ingredients to inhibit lipid oxidation in fish-oil-enriched mayonnaise. Eur. *Food Res. Technol*. 2016, 242, 571-584. doi:10.1007/s00217-015-2564-0.

219. Bandyopadhyay, M.; Chakraborty, R.; Raychaudhuri, U. Antioxidant activity of natural plant sources in dairy dessert (Sandesh) under thermal treatment. *LWT-Food Sci. Technol*. 2018, 41(5), 816-825. doi:10.1016/j.lwt.2007.04.008.

220. Kamkar, A.; Javan, A. J.; Asadi, F.; Kamalinejad, M. The antioxidative effect of Iranian Mentha pulegium extracts and essential oil in sunflower oil. *Food Chem. Toxicol*. 2010, 48(7), 1796-1800. doi:10.1016/j.fct.2010.04.020.

221. Tyburczy, C.; Mossoba, M. M.; Rader, J. I. Determination of trans fat in edible oils: current official methods and overview of recent developments. *Anal. Bioanal. Chem*. 2013, 405, 5759-5772. doi:10.1007/s00216-013-6938-3.

222. Paquot, C. Standard methods for the analysis of oils, fats and derivatives. Elsevier; 2013. doi:10.1016/B978-0-08-022939-3.50008-7.

223. Yu, J.; Hu, Y.; Xue, M.; Dun, Y.; Li, S.; Peng, N.; Zhao, S. Purification and identification of antioxidant peptides from enzymatic hydrolysate of Spirulina platensis. *J. Microbiol. Biotechnol*. 2016, 26(7), 1216-1223. doi:10.4014/jmb.1512.12068.

224. Jie, Y.; Yuanliang, H.; Mingxiong, X.; Yaohao, D.; Shenao, L.; Nan, P. Purification and Identification of Antioxidant Peptides from Enzymatic Hydrolysate of Spirulina platensis. *J. Microbiol. Biotechnol.* 2016, 26(7), 1216-1223. doi:10.4014/jmb.1512.12068.

225. Pereira, R. B.; Sousa, C.; Costa, A.; Andrade, P. B.; Valentão, P. Glutathione and the antioxidant potential of binary mixtures with flavonoids: synergisms and antagonisms. *Molecules* 2013, 18(8), 8858-8872. doi:10.3390/molecules18088858.

226. Zheng, X.; Liu, B.; Li, L.; Zhu, X. Microwave-assisted extraction and antioxidant activity of total phenolic compounds from pomegranate peel. J. Med. *Plants Res*. 2011, 5(6), 1004-1011. doi:10.5897/JMPR10.601.

227. Gharib, F. A.; da Silva, J. T. Composition, total phenolic content and antioxidant activity of the essential oil of four Lamiaceae herbs. *Med. Aromat. Plant Sci. Biotechnol*. 2013, 7(1), 19-27. doi:10.3923/pjbs.2013.19.27.

228. Chen, X.; Zhang, Y.; Zu, Y.; Yang, L.; Lu, Q.; Wang, W. J. Antioxidant effects of rosemary extracts on sunflower oil compared with synthetic antioxidants. *Int. J. Food Sci. Technol*. 2014, 49(2), 385-391. doi:10.1111/ijfs.12356.

229. Laulloo, S. J.; Bhowon, M. G.; Hoolash, A. Influence of chemical refining processes on the total phenolics and antioxidant activity of sunflower oil. *Int. J. Nutr.* 2015, 1(2), 38. doi:10.14511/ijn.15002.

230. Prevc, T.; Šegatin, N.; Poklar Ulrih, N.; Cigić, B. DPPH assay of vegetable oils and model antioxidants in protic and aprotic solvents. *Talanta* 2013, 109, 13-19. doi:10.1016/j.talanta.2013.02.007.

231. Sharma, S.; Cheng, S. F.; Bhattacharya, B.; Chakkaravarthi, S. Efficacy of free and encapsulated natural antioxidants in oxidative stability of edible oil: Special emphasis on nanoemulsion-based encapsulation. *Trends Food Sci. Technol.* 2019, 91, 305-318. doi:10.1016/j.tifs.2019.06.007.

232. Gharby, S.; Harhar, H.; Guillaume, D.; Haddad, A.; Matthäus, B.; Charrouf, Z. Oxidative stability of edible argan oil: A two-year study. *LWT-Food Sci. Technol*. 2011, 44(1), 1-8. doi:10.1016/j.lwt.2010.05.007.

233. Crapiste, G. H.; Brevedan, M. I.; Carelli, A. A. Oxidation of sunflower oil during storage. *J. Am. Oil Chem. Soc.* 1999, 76(12), 1437. doi:10.1007/s11746-999-0247-9.

234. Erol, N. D.; Erdem, Ö. A.; Yilmaz, S. T.; Kayalar, H.; Cakli, S. Effects of the BHA and basil essential oil on nutritional, chemical, and sensory characteristics of sunflower oil and sardine (Sardina pilchardus) fillets during repeated deep-frying. *LWT Food Sci. Technol*. 2022, 163, 113557. doi:10.1016/j.lwt.2022.113557.

235. Naserzadeh, Y.; Mahmoudi, N. J. B. Chelating effect of black tea extract compared to citric acid in the process of the oxidation of sunflower, canola, olive, and tallow oils. *Int. Conf. Agric. Prod. Impact Infrastruct. Agric. Eng*. 2018, 12(5), 5. doi:10.1186/s42770-018-0004-1.

236. Saeed, A.; Shabbir, A.; Khan, A. Stabilization of sunflower oil by using potato peel extract as a natural antioxidant. *Biomass Conv. Bioref.* 2022, 5, 1-10. doi:10.1007/s13399-022-02131-1.

237. Frankel, E. N. Lipid Oxidation. Elsevier; 2015. doi:10.1016/B978-0-12-417224-0.00007-0.

238. Chong, Y. M.; Chang, S. K.; Sia, W. C. M.; Yim, H. S. Antioxidant efficacy of mangosteen (Garcinia mangostanaLinn.) peel extracts in sunflower oil during accelerated storage. *J. Food Biosci.* 2015, 12, 18-25. doi:10.1016/j.fbio.2015.07.005.

239. Iqbal, S.; Bhanger, M. Stabilization of sunflower oil by garlic extract during accelerated storage. *J. Food Chem.* 2007, 100(1), 246-254. doi:10.1016/j.foodchem.2005.08.011.

240. Mei, W. S. C.; Ismail, A.; Mohd Esa, N.; Akowuah, G. A.; Wai, H. C.; Seng, Y. H. The effectiveness of rambutan (Nephelium lappaceum L.) extract in stabilization of sunflower oil under accelerated conditions. *Antioxidants* 2014, 3(2), 371-386. doi:10.3390/antiox3020371.

241. Ramadan, M. F.; Mörsel, J. T. Oxidative stability of black cumin (Nigella sativa L.), coriander (Coriandrum sativum L.) and niger (Guizotia abyssinica Cass.) crude seed oils upon stripping. *Eur. J. Lipid Sci. Technol.* 2004, 106(1), 35-43. doi:10.1002/ejlt.200300073.

242. FAO/WHO FOOD STANDARDS PROGRAMME CODEX ALIMENTARIUS COMMISSION. (2015). Committee of Fats and Oils, Thirty-eighth Session CICG, Geneva, Switzerland 6-11 July 2015. doi:10.1007/s11306-016-0983-6.

243. Okhli, S.; Mirzaei, H.; Hosseini, S. E. Antioxidant activity of citron peel (Citrus medica L.) essential oil and extract on stabilization of sunflower oil. *Oils Fats Crops Lipids* 2020, 27, 32. doi:10.2516/oc.2020.1101.

244. Mazaheri, K. M.; Bassiri, A.; Jalali, H. Evaluation of antioxidant properties of essential oil of fennel (Foeniculum vulgare) and its effect on the oxidative stability of soybean oil. *Int. J. Biomed. Eng.* 2014, 45(2), 131-139. doi:10.2139/ssrn.3038911.

245. Urbančič, S.; Kolar, M. H.; Dimitrijević, D.; Demšar, L.; Vidrih, R. Stabilisation of sunflower oil and reduction of acrylamide formation of potato with rosemary extract during deep-fat frying. *LWT Food Sci. Technol.* 2014, 57(2), 671-678. doi:10.1016/j.lwt.2014.01.022.

246. Akkaya, M. R. Prediction of fatty acid composition of sunflower seeds by near-infrared reflectance spectroscopy. *J. Food Sci. Technol*. 2018, 55(6), 2318-2325. doi:10.1007/s11483-018-1494-5.

247. Wang, D.; Fan, W.; Guan, Y.; Huang, H.; Yi, T.; Ji, J. Oxidative stability of sunflower oil flavored by essential oil from Coriandrum sativum L. during accelerated storage. *LWT Food Sci. Technol.* 2018, 98, 268-275. doi:10.1016/j.lwt.2018.08.021.

248. Giacosa, S.; Segade, S. R.; Cagnasso, E.; Caudana, A.; Rolle, L.; Gerbi, V. SO2 in wines: Rational use and possible alternatives. In Red wine technology; Academic Press: 2019; pp. 309-321. doi:10.1016/B978-0-12-814399-5.00021-9.

249. Oliveira, C. M.; Ferreira, A. C. S.; De Freitas, V.; Silva, A. M. Oxidation mechanisms occurring in wines. Food Research International 2011, 44(5), 1115-1126. doi:10.1016/j.foodres.2011.03.050.

251. EUR-Lex. (2019). Commission Delegated Regulation (EU) 2019/934 of 12 March 2019 supplementing Regulation (EU) No 1308/2013 of the European Parliament and of the Council as regards wine-growing areas where the alcoholic strength may be increased, authorised oenological practices and restrictions applicable to the production and conservation of grapevine products, the minimum percentage of alcohol for by-products and their disposal, and publication of OIV files. Available online: https://eur-lex.europa.eu/legal-content/en/TXT/?uri=CELEX%3A32019R0934. Accessed 26 Dec 2024.

252. Lisanti, M. T.; Blaiotta, G.; Nioi, C.; Moio, L. Alternative methods to SO_2 for microbiological stabilization of wine. *Compr. Rev. Food Sci & Food Saf.* 2019, 18(2), 455-479. doi:10.1111/1541-4337.12422.

253. Bağder Elmacı, S.; Gülgör, G.; Tokatlı, M.; Erten, H.; İşci, A.; Özçelik, F. Affectiveness of chitosan against wine-related microorganisms. Antonie Van Leeuwenhoek 2015, 107, 675-686. doi:10.1007/s10482-014-0362-6.

254. Bachhawat, A. K.; Yadav, S. The glutathione cycle: Glutathione metabolism beyond the γ-glutamyl cycle. *Iubmb Life* 2018, 70, 585-592. doi:10.1002/iub.1756

255. Elias, R. J.; Kellerby, S. S.; Decker, E. A. Antioxidant activity of proteins and peptides. Critical *Rev. in Food Sci and Nutr* 2008, 48, 430-441. doi:10.1080/10408390701425615

256. Arslan, E.; Çelik, Z. D.; Cabaroğlu, T. Effects of pure and mixed autochthonous *Torulaspora delbrueckii* and *Saccharomyces cerevisiae* on fermentation and volatile compounds of Narince wines. *Foods* 2018, 7, 147. doi:10.3390/foods7090147

257. OIV. OIV Statistical Report on World Vitiviniculture 2022.

258. Nardini, M.; Garaguso, I. Affect of sulfites on antioxidant activity, total polyphenols, and flavonoid measurements in white wine. *Foods* 2018, 7, 35. doi:10.3390/foods7030035

259. OIV. Compendium of International Methods of Analysis of Wines and Musts 2015.

260. Meilgaard, M.C., Carr, B.T., & Carr, B.T. (2007). Sensory Evaluation Techniques (4th ed.). CRC Press. doi:10.1201/b16452

261. Darıcı, M., & Cabaroglu, T. (2022). Chemical and sensory characterization of Kalecik Karası wines produced from two different regions in Turkey using chemometrics. *J. Food Process Preserv.* 46: e16278. doi:10.1111/jfpp.16278

262. Agirman, B., Yildiz, I., Polat, S., & Erten, H. (2024). The evaluation of black carrot, green cabbage, grape, and applejuices as substrates for the production of functional waterkefir-like beverages. *Food Sci Nutr*. 12, 6595-6611.doi:10.1002/fsn3.4293

263. Schneider, R.; Razungles, A.; Augier, C.; Baumes, R. Monoterpenic and norisoprenoidic glycoconjugates of Vitis vinifera L. cv. Melon B. as precursors of odorants in Muscadet wines. *J. Chromat A* 2001, 936, 145-157. doi:10.1016/S0021-9673(01)01150-5.

264. Eker, T.; Cabaroglu, T.; Darıcı, M.; Selli, S. Impact of kernel size and texture on the in vivo and in vitro aroma compounds of roasted peanut and peanut paste. *J. Food Comp and Analys*. 2023, 119, 105260. doi:10.1016/j.jfca.2023.105260.

265. Maicas, S. (2020). The role of yeasts in fermentation processes. *Microorganisms*, 8, 1442. doi:10.3390/microorganisms8081142

266. Çelik, Z. D.; Erten, H.; Cabaroglu, T. The influence of selected autochthonous *Saccharomyces cerevisiae* strains on the physicochemical and sensory properties of Narince wines. *Fermentation* 2019, 5, 70. doi:10.3390/fermentation5030070.

267. Capozzi, V.; Tufariello, M.; De Simone, N.; Fragasso, M.; Grieco, F. Biodiversity of oenological lactic acid bacteria: Species-and strain-dependent plus/minus affects on wine quality and safety. *Fermentation* 2021, 7, 24. doi:10.3390/fermentation7010024.

268. Lafon-Lafourcade, S.; Carre, E.; Ribéreau-Gayon, P. Occurrence of lactic acid bacteria during the different stages of vinification and conservation of wines. *Appl and Environmental Microbiol*. 1983, 46, 874-880. doi:10.1128/aem.46.4.874-880.1983.

269. Petri, A.; Pfannebecker, J.; Fröhlich, J.; König, H. Fast identification of wine related lactic acid bacteria by multiplex PCR. *Food Microbiol.* 2013, 33, 48-54. doi:10.1016/j.fm.2012.08.011.

270. Hasalliu, R.; Gozhdari, K.; Meta, F.; Kongoli, R. The assessment of initial number of yeasts and lactic acid bacteria in some Albanian grape varieties compared with other varieties used for wine production. *Alban J. Agricul Sci.* 2017, 16, 293-299.

271. Lonvaud-Funel, A. Lactic acid bacteria in the quality improvement and depreciation of wine. In Lactic Acid Bacteria: Genetics, Metabolism and Applications; Proceedings of the Sixth Symposium on Lactic Acid Bacteria: Genetics, Metabolism and Applications; Veldhoven, The Netherlands, 1999; 317-331. Springer Netherlands.

272. Alnuwaiser, M. A. Content of sugars in fresh grapes and raisins, and fresh and dried apricot: a comparative study. *Int J. Res Granthaalayah* 2017, 5, 177-186. doi:10.29121/granthaalayah.v5.i11.2017.2345.

273. Jakabová, S.; Fikselová, M.; Mendelová, A.; Ševčík, M.; Jakab, I.; Aláčová, Z.; Ivanova-Petropulos, V. Chemical composition of white wines produced from different grape varieties and wine regions in Slovakia. *Appl Sci*. 2021, 11, 11059. doi:10.3390/app112211059.

274. Gao, Y. T., Zhang, Y. S., Wen, X., Song, X. W., Meng, D., Li, B. J., ... & Du, G. (2019). The glycerol and ethanol production kinetics in low-temperature wine fermentation using *Saccharomyces cerevisiae* yeast strains. *Int J Food Sci Technol.*, *54*(1), 102-110. doi:10.1111/ijfs.13910

275. Zhao, X.; Procopio, S.; Becker, T. Flavour impacts of glycerol in the processing of yeast fermented beverages: a review. *J. Food Sci and Technol.* 2015, 52, 7588-7598. doi:10.1007/s13197-015-1977-y.

276. Ivit, N. N.; Longo, R.; Kemp, B. The Affect of Non-*Saccharomyces* and *Saccharomyces* Non-Cerevisiae Yeasts on Ethanol and Glycerol Levels in Wine. *Fermentation* 2020, 6, 77. doi:org/10.3390/fermentation6030077.

277. García, M., Esteve-Zarzoso, B., Cabellos, J. M., & Arroyo, T. (2020). Sequential non-*Saccharomyces* and *Saccharomyces cerevisiae* fermentations to reduce the alcohol content in wine. *Fermentation*, 6, 60. doi:10.3390/fermentation6020060

278. Robles, A.; Fabjanowicz, M.; Chmiel, T.; Płotka-Wasylka, J. J. Determination and identification of organic acids in wine samples. *Trends Anal Chem.* 2019, 120, 115630. doi:10.1016/j.trac.2019.115630.

279. Bae, H.; Yun, S. K.; Yoon, I. K.; Nam, E. Y.; Kwon, J. H.; Jun, J. H. Assessment of organic acid and sugar composition in apricot, plumcot, plum, and peach during fruit development. *J. Agricul and Food Chem* 2014, 87. doi:10.5073/JABFQ.2014.087.004.

280. Nascimento Silva, F. L.; Schmidt, E. M.; Messias, C. L.; Eberlin, M. N.; Sawaya, A. C. H. F. J. A. M. Quantitation of organic acids in wine and grapes by direct infusion electrospray ionization mass spectrometry. *Analytical Methods* 2015, 7, 53-62. doi:10.1039/C4AY00114A.

281. Ivanova-Petropulos, V.; Naceva, Z.; Sándor, V.; Makszin, L.; Deutsch-Nagy, L.; Berkics, B.; Kilár, F. J. E. Fast determination of lactic, succinic, malic, tartaric, shikimic, and citric acids in red Vranec wines by CZE-ESI-QTOF-MS. *Electrophoresis* 2018, 39, 1597-1605. doi:10.1002/elps.201700492.

282. Santos, M. C.; Nunes, C.; Saraiva, J. A.; Coimbra, M. A. Chemical and physical methodologies for the replacement/reduction of sulfur dioxide use during winemaking: Review of their potentialities and limitations. *Eur Food Res and Technol.* 2012, 234, 1-12. doi:10.1007/s00217-011-1614-6.

283. Kontaxakis, E.; Trantas, E.; Ververidis, F. Resveratrol: A fair race towards replacing sulfites in wines. *Molecules* 2020, 25, 2378. doi:10.3390/molecules25102378.

284. Vaimakis, V.; Roussis, I. G. Must oxygenation together with glutathione addition in the oxidation of white wine. *Food Chem.* 1996, 57(3), 419-422.

285. Bayram, M.; Kayalar, M. White wines from Narince grapes: Impact of two different grape provenances on phenolic and volatile composition. *OenoOne*. 2018, 52(2), 81-92. doi:10.20870/oeno-one.2018.52.2.2114.

286. Motta, S.; Tirelli, A.; Cravero, M. C.; Guaita, M.; Bosso, A. Effect of SO_2, glutathione and gallotannins on the shelf-life of a Cortese white wine bottled with different oxygen intakes. *OenoOne*. 2022, 56(4), 221-235. doi:10.20870/oeno-one.2022.56.4.7139.

287. Giménez, P., Anguela, S., Just-Borras, A., Pons-Mercadé, P., Vignault, A., Conals, J. M., Teissedre, P. L., & Zamora, F. (2022). Development of a synthetic model to study browning caused by laccase activity from *Botrytis cinerea*. *LWT-Food Sci and Technol.* 154, 112871. doi:10.1016/j.lwt.2021.112871

288. Milat, A. M., Boban, M., Teissedre, P. L., Šešelja-Perišin, A., Jurić, D., Skroza, D., & Mudnić, I. Affects of oxidation and browning of macerated white wine on its antioxidant and direct vasodilatory activity. *J. Funct Foods.* 2019, 59, 138-147. doi:10.1016/j.jff.2019.05.025.

289. Recamales, Á. F., Sayago, A., González-Miret, M. L., & Hernanz, D. The affect of time and storage conditions on the phenolic composition and colour of white wine. *Food Res Int.* 2006, 39(2), 220-229. doi:10.1016/j.foodres.2005.07.009.

290. Cosme, F., Andrea-Silva, J., Filipe-Ribeiro, L., Moreira, A. S. P., Malheiro, A. C., Coimbra, M. A., & Nunes, F. M. The origin of pinking phenomena in white wines: An update. In BIO Web Conf. 2019, 12, 02013. doi:10.1051/bioconf/20191202013.

291. Swiegers, J. H., Bartowsky, E. J., Henschke, P. A., & Pretorius, I. Yeast and bacterial modulation of wine aroma and flavour. *Aust. J. Grape Wine Res.* 2005, 11(2), 139-173. doi:10.1111/j.1755-0238.2005.tb00285.x.

292. Scutarașu, E. C., Luchian, C. E., Vlase, L., Nagy, K., Colibaba, L. C., Trinca, L. C., & Cotea, V. V. Influence Evaluation of Enzyme Treatments on Aroma Profile of White Wines. *Agronomy.* 2022, 12(11), 2897. doi:10.3390/agronomy12112897.

293. Antonelli, A., Castellari, L., Zambonelli, C., & Carnacini, A. Yeast influence on volatile composition of wines. *J. Agric. Food Chem*. 1999, 47(3), 1139-1144. doi:10.1021/jf9807317.

294. Belda, I., Ruiz, J., Esteban-Fernández, A., Navascués, E., Marquina, D., Santos, A., & Moreno-Arribas, M. V. Microbial contribution to wine aroma and its intended use for wine quality improvement. *Molecules.* 2017, 22(2), 189. doi:10.3390/molecules22020189.

295. Selli, S., Canbas, A., Cabaroglu, T., Erten, H., Lepoutre, J. P., & Gunata, Z. Affect of skin contact on the free and bound aroma compounds of the white wine of Vitis vinifera L. cv Narince. *Food Control.* 2006, 17(1), 75-82. doi:10.1016/j.foodcont.2004.09.005.

296. Sakoda, Y., & Hayashi, S. Relationship of odour and chemical structure in 1-and 2-alkyl alcohols and thiols. *Spec. Publ. R. Soc. Chem.* 2002, 277, 15-26.

297. Panero, L., Motta, S., Petrozziello, M., Guaita, M., & Bosso, A. Affect of SO2, reduced glutathione and ellagitannins on the shelf life of bottled white wines. *Eur. Food Res. Technol*. 2015, 240, 345-356. doi:10.1007/s00217-014-2334-5.

298. Lavigne-Cruege, V., & Dubourdieu, D. Role of glutathione on development of aroma defects in dry white wines. In 13th Int. Enology Symp., Trogus, H., Gafner, J. & Sutterlin, A. Eds. Int. Assoc. Enology: Montpellier, France, 2002; pp 331-347.

299. Papadopoulou, D., & Roussis, I. G. Inhibition of the decrease of volatile esters and terpenes during storage of a white wine and a model wine medium by glutathione and N-acetylcysteine. *Int. J. Food Sci. Technol.* 2008, 43(6), 1053-1057. doi:10.1111/j.1365-2621.2007.01562.x.

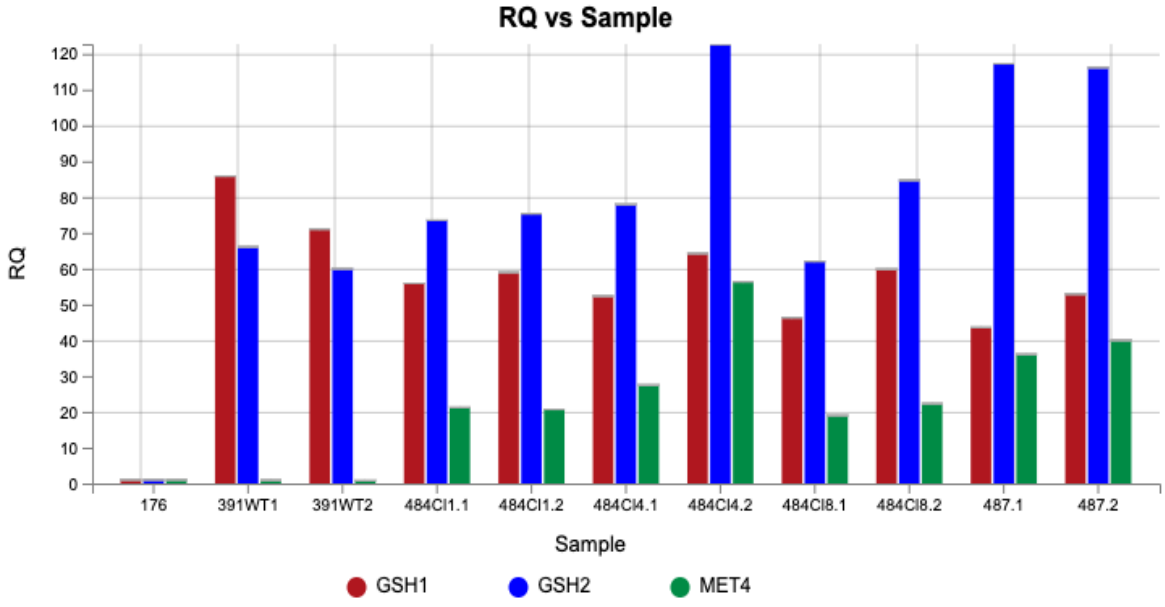
300. Wegmann-Herr, P., Ullrich, S., Schmarr, H. G., & Durner, D. Use of glutathione during white wine production–impact on S-off-flavours and sensory production. In BIO Web Conf. 2016, 7, 02031. doi:10.1051/bioconf/20160702031.

APPENDIX

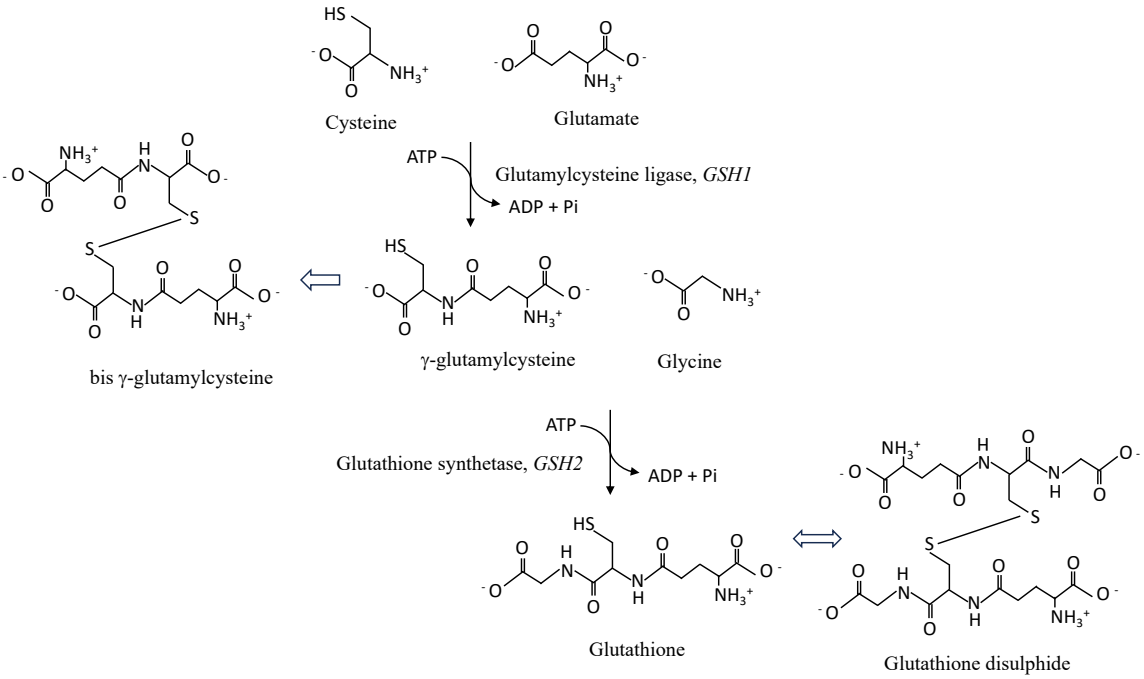
Appendix 1. *E. coli* strains and plasmid used in this study

Strains (plasmid)	Genotype-Plasmid	Source/Reference
DH5α	Δ(lacZYA-argF)U169 recA1 endA1 hsdR17(rK- mK+) phoA supE44 λ– thi-1 gyrA96 relA1 F– φ80lacZΔM15	Promega
RIE110 (RIP110)	URA3ex	(Fickers et al., 2003)
RIE111 (RIP111)	LEU2ex	(Fickers et al., 2003)
RIE132 (RIP132)	Cre-EYK1	(Vandermies et al., 2017)
RIE136 (RIP136)	pTEF expression vector, LEUex	Lab stock
RIE137 (RIP137)	pTEF expression vector, URAex	Lab stock
RIE210 (RIP210)	pTEF-GSH1, LEUex	(Do and Fickers, 2020)
RIE211 (RIP211)	pTEF-GSH1, URAex	(Do and Fickers, 2020)
RIE295 (RIP295)	pGEMTeasy, gsh2::URAex	This work
RIE310 (RIP310)	pTEF-GDH1, LEUex	(Trotter et al., 2020)
RIE316 (RIP316)	pGEMTeasy-MET4	This work
RIE317 (RIP317)	pGEMTeasy-CYSE	This work
RIE318 (RIP318)	pGEMTeasy-CYSF	This work
RIE319 (RIP319)	pTEF-MET4, URAex	This work
RIE320 (RIP320)	pTEF-MET4, LEUex	This work
RIE322 (RIP322)	pTEF-CYSE, LEUex	This work
RIE324 (RIP324)	pTEF-CYSF, LEUex	This work

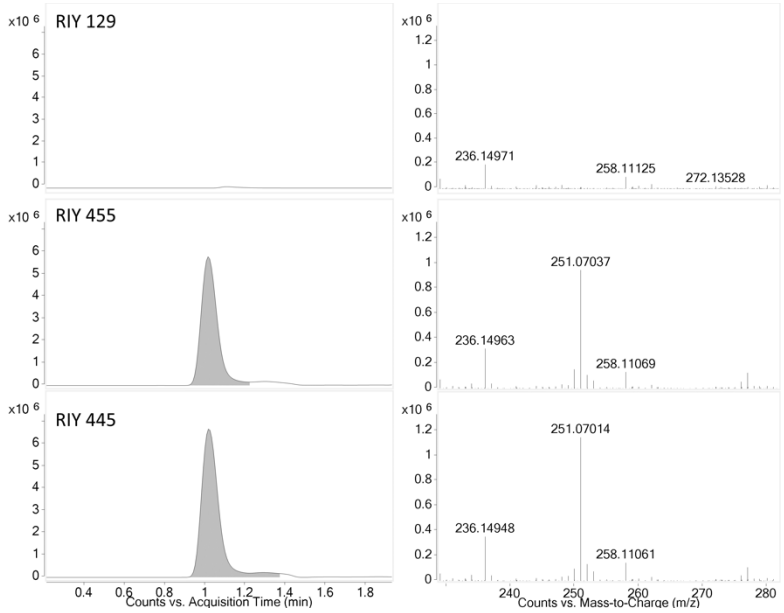
Appendix 2. qPCR for the genes *GSH1, GSH2, MET4*



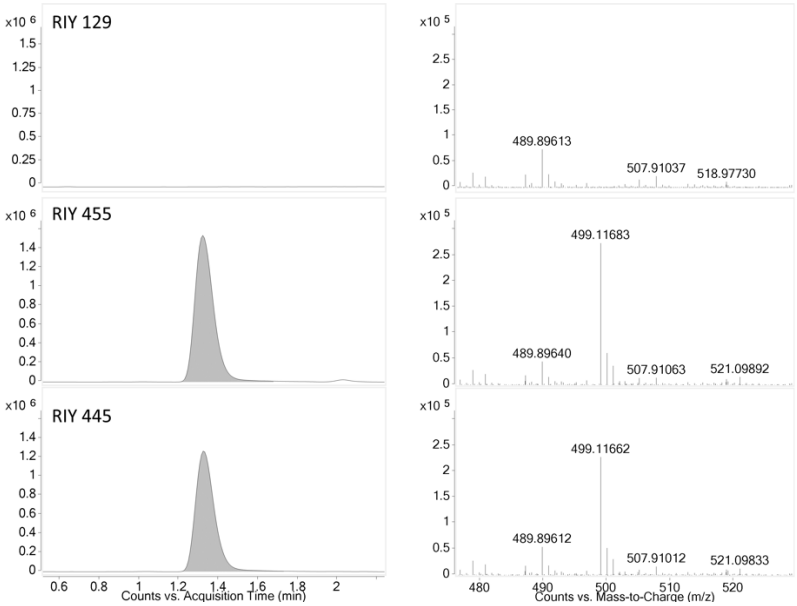
Appendix 3. The γ-glutamyl-cysteine and glutathione pathway (adapted from Copley and Dhillon, 2002)



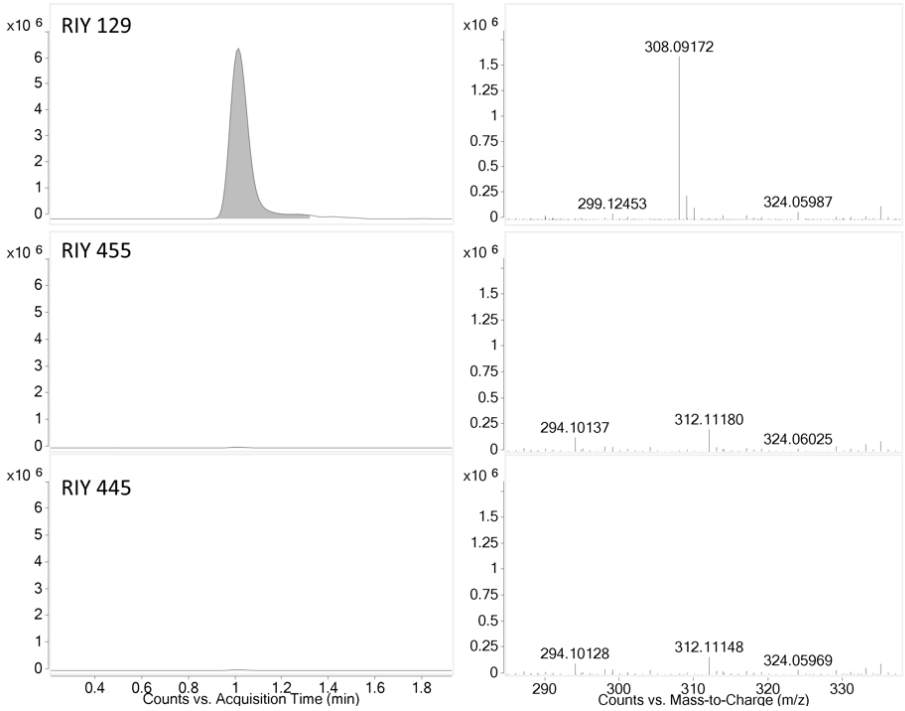
Appendix 4. Extracted ion chromatogram (EIC) (left panel) and masse spectra (right panel) of γ-glutamylcysteine ($[M+H]^+$; m/z 251.070) of deproteinized cell extract from strains RIY129 (wt), RIY455 (gsh2Δ, pTEF-ylGSH1) and RIY445 (gsh2Δ, pTEF-GSH1, pTEF-GSH1). γ-glutamylcysteine is eluted at 1.05 min.



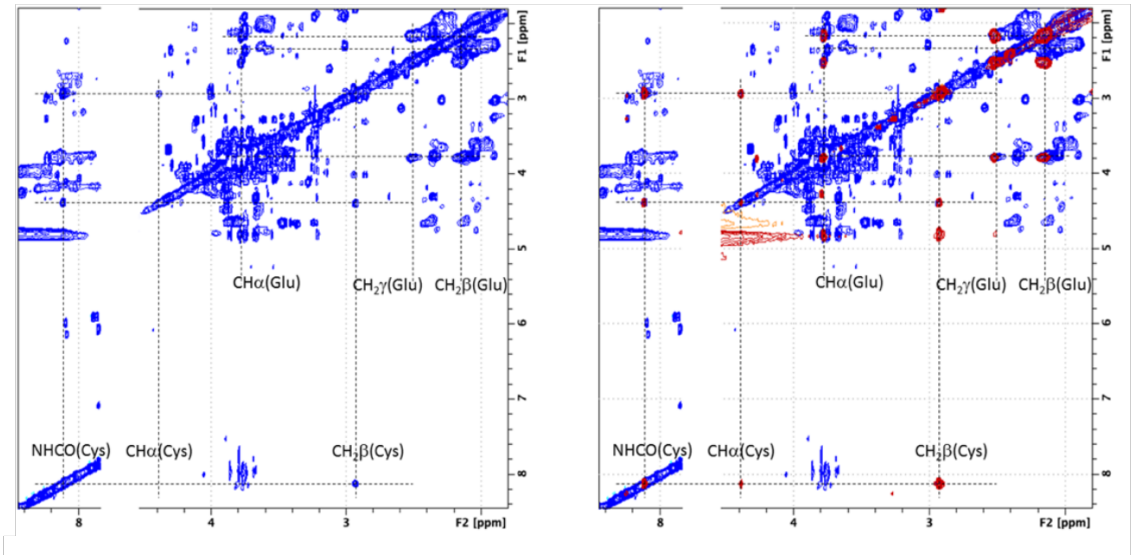
Appendix 5. Extracted ion chromatogram (EIC) (left panel) and masse spectra (right panel) of Bis-γ glutamylcystine ($[M+H]^+$; m/z 499.117) of deproteinized cell extract from strains RIY129 (wt), RIY455 (gsh2Δ, pTEF-GSH1) and RIY445 (gsh2Δ, pTEF-GSH1, pTEF-GSH1). Bis-γ glutamylcystine is eluted at 1.38 min.



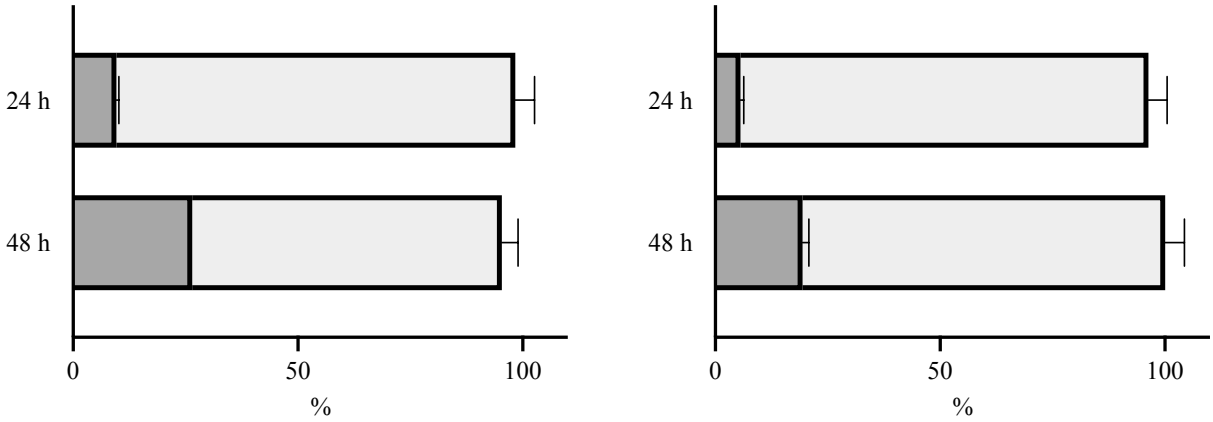
Appendix 6. Extracted ion chromatogram (EIC) (left panel) and mass spectra (right panel) of glutathione ($[M+H]^+$; m/z 308.092) of deproteinized cell extract from strains RIY129 (wt), RIY455 (gsh2Δ, pTEF-GSH1) and RIY445 (gsh2Δ, pTEF-GSH1, pTEF-GSH1). Glutathione is eluted at 1.15 min.



Appendix 7. Identification of characteristic γ-glutamylcysteine signals from cell extract of RIY445 strain (gsh2Δ, pTEF-GSH1, pTEF-GSH1) by NMR. (A) 2D TOCSY of cell extract from RIY445. (B) superposition of 2D TOCSY of RIY445 cell extract (in blue) and 50 mM pure γ-glutamylcysteine (in red). Correlations between connected protons are indicated with dash lines for γ-glutamylcysteine. Strains were grown in YPD for 48 h and cell extract was prepared as described in Materials and Methods.



Appendix 8. Relative abondance of intracellular γ-glutamyl-cysteine (light grey) and bis-γ glutamylcystine (dark grey) for strains RIY455 (gsh2□□ GSH1, left panel), RIY445 (gsh2□, GSH1-2c, right panel) after 24 h and 48 h of growth in YNBD medium. Data are the mean and standard deviation of duplicate experiments. Values are expressed as the percentage of the intracellular thiols contend determined based the signals obtained from extracted ion chromatogram. GSH1-2c denote that mutant strains contain 2 copies of pTEF-GSH1 expression cassette.



Appendix 9. Primers used in this study

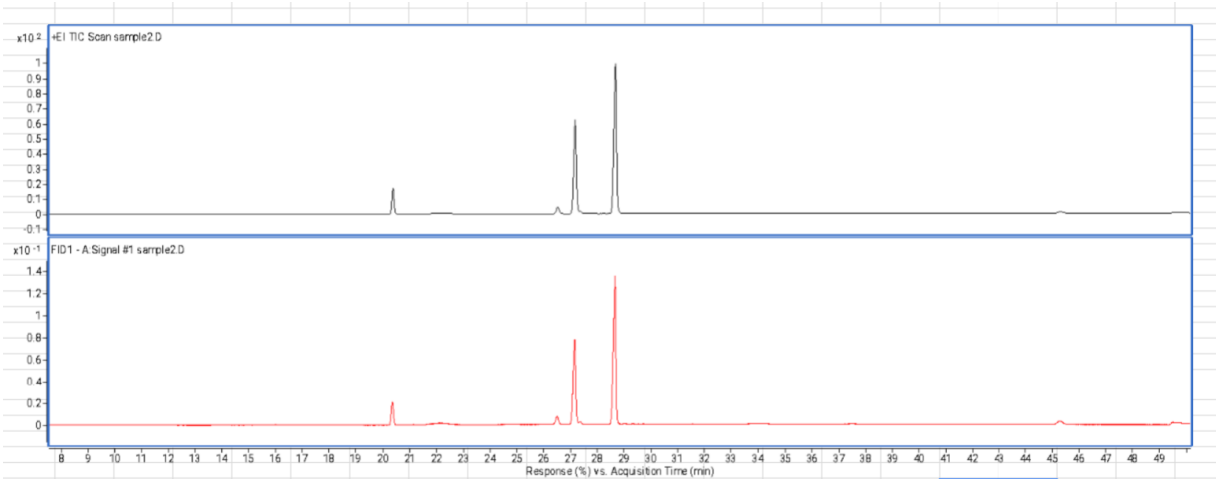
Name	Sequence 5'-3'	Restricition site, utilisation
M13fo	GTAAAACGACGGCCAGT	
M13rev	AACAGCTATGACCATG	
GSH2pro_for	GAATAGCAGCTTTGCAACGCGAAG	
GSH2pro_rev	AGCAGGAGTTATCCGAAGCGATAATGGATTAGA TAGATAAGGTGTGGTTCAGTACATACAGTAC	
GSH2ter_for	GTTATCTAGGGATAACAGGGTAATCCGTCATTG GTTGTTTCGGAGACG	
GSH2ter_rev	GGTCTTCTCATCCTTGGGCTTC	
Auxo_fo	ATTATCGCTTCGGATAACTCCTGCT	
Auxo_rev	GATTACCCTGTTATCCCTAGATAAC	
GSH2_verif	GAGTCTTTGCGCCATCG	
URA-Fo	GGATGTTACCACCACCAAGG	
URA-Rev	GTTCTGGCCGTACAGACCTC	
pTEF-Fo	GGACCCAACCCCGGCG	
GSH1_rev	GCGCCTAGGCTACTCCTTCTCGTACTCAAAACC	
LoxP-Fo	GCATACATTATACGAAGTTATTCTGAATTC	
LoxR-Rev	GGGTAATTATCGCTTCGGATA	
Met4-Fo	GACGGATCCATGACTGACCGACTTTTCTTGGCCA ACCTCAATGCGATTGAAGGATCCTCAA	BamHI, internal BamHI removed
Met4-Rev	GCGTGTGGCCTAGGTTAGATTTGCTCAATCTTGG CGATGGGAG	AvrII, internal BglII removed
CystE_Fo	GCGGGATCCATGAGTCGCTGGATATACACG	BamHI
CystE-Rev	GCCCCTAGGCTAAAAATCCTCCAACTTGATCTCC CC	AvrII
CystF-Fo	GCGCAGATCTATGTCTCGAATTGGATCTGTGAC	BglII
CystF-Rev	GCGCCTAGGTTAATCCAGAACAACGTACTTTTG GAGAT	AvrII
Met4_verif	GACCCTAGGTTAGATCTGCTCAATCTTGGCGATG	
CystE_verif	CCAGAACTTACTCAAATGGCGATGGCC	
CystF_verif	GGGAATGGTGAGTCCCTTGGAC	
Gdh_verif	GGCACCCTTGACGAGAGAGGG	
qGsh11_Fo	TGACTTCGACGACATTCTGC	qPCR, GSH1
qGsh1_Rev	CACCCTTGGGCTCGTAATAA	qPCR, GSH1
qMet4_Fo	CCCAGGAGGAAATCCAGGCC	qPCR, MET4
qMet4_Rev	GAGCCACTTGTTCTCCATCTCCAG	qPCR, MET4
qGdh_Fo	CTTCCGTCAACCTGTCCATT	qPCR, GDH1
qGdh_Rev	GAAGGCGTAGCAGAATCGTC	qPCR, GDH1
qCyse_Fo	ATGTTTGTGGGCTCTTCCAC	qPCR, CYSTE
qCyse_Rev	TCCAACTTGATCTCCCCAAG	qPCR, CYSTE
qCysf_Fo	GGATCCGTGCTCCATTCTTA	qPCR, CYSTF
qCysf_Rev	ACCCTCCTTATCCAGCAGGT	qPCR, CYSTF

Appendix 10. Relative quantification of γ-glutamylcysteine γGC) and bis-γ-glutamylcystine (bγGC) in culture supernatant of strain RIY445 (gsh2Δ, GSH1-2c) and RIY508 (gsh2Δ, GSH1-2c, MET4) after 24 h and 48 h of growth in YNBD. The values under γGC/X and bγGC/X correspond to the values of peak area of EIC for γ-GC (m/z 251.0696) and bγ-GC (m/z 499.1163) normalized to biomass values (X, gCDW/L), those under γGCtot correspond the sum of values under γGC/X and bγGC/X while those under the Ratio 48/24 correspond to the ratio of γGCtot values at 48 h and 24 h of growth in YNBD medium. Data are the mean and standard deviation (SD) of duplicate experiments.

		γ–GC/DO		βγ–GC/DO		γ–GCtot	Ratio 48/24
Strain	Time	Mean	SD	Mean	SD		
RIY445	24 h	799064	84367	713852	9754	1512916	1.39
RIY445	48 h	1212687	615099	895519	522723	2108206	
RIY508	24 h	1852194	1097011	1492133	236696	3344327	
RIY508	48 h	3610168	1654669	2075840	52603	5686009	1.71

Appendix 11. Sunflower oil with addition TBHQ, gamma glutamyl cysteine and glutathione

Appendix 12. Cromotogram of fatty acids (palmitic, stearic, oleic and linoleic acid) analysis by GC/MS.



Apendix 13. Winemaking, preparation and maturation of wine samples

Appendix 14. Sensory analysis of wine