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Molecular mechanisms controlling intracellular glutathione levels in baker's yeast *Saccharomyces cerevisiae* and a random mutagenized glutathione over-accumulating isolate

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Kerti Orumets

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LOODUS - JA TÄPPISTEADUSED B140

Rakusisesed glutatiooni taset kontrollivad molekulaarsed mehhanismid pagaripärmis *Saccharomyces cerevisiae* ja selle juhuslikul mutageneesil saadud glutatiooni üleakumuleerivas isolaadis

KERTI ORUMETS

TTÜ KIRJASTUS

CONTENTS

ABSTRACT

Glutathione (GSH) is the most abundant non-protein thiol compound found in eukaryotes. Besides its physiological importance in the cell it has also attracted attention for several industrial applications. Microbial production of GSH using genetically engineered yeast strains and precursor amino acid supplementation has potential to satisfy the increasing industrial demand of this tripeptide. In order to develop appropriate producer strains and production technologies, the molecular mechanisms controlling GSH accumulation need to be understood in detail. The metabolic analysis of random mutagenized GSH over-accumulating strains is one way of gaining novel ideas for the genetic and metabolic modifications necessary to enhance glutathione accumulation. This dissertation contains a study of the molecular mechanisms controlling intracellular GSH levels in both an industrial baker's yeast strain of *Saccharomyces cerevisiae* and a random mutagenized GSH over-accumulating isolate under various cultivation conditions. The results suggest that the over two-fold higher GSH accumulation in the mutant is not caused by mutations leading to a lack of feed-back inhibition on γ-glutamylcysteine synthetase (Gsh1p) and glutathione synthetase (Gsh2p) reaction steps, but rather due to increased biosynthesis of the major precursor amino acid cysteine through increased expression of cystathionine γ-lyase (*CYS3*) and cystathionine β-synthase (*CYS4*), as well as increased expression of *GSH1* and *GSH2*. It was shown that, in addition to the transcription of GSH biosynthesis genes *GSH1* and *GSH2*, *CYS3* and *CYS4* are also under the control of Yap1p, the oxidative stress responsive transcriptional activator. Thus, over-expression of a single gene, *YAP1*, allows one to improve two important prerequisites for high GSH accumulation in yeast – an increase in both the biosynthesis of cysteine as well as the throughput of the two enzymatic reactions of GSH biosynthesis. This finding may be applied for the construction of new genetically engineered yeast strains for industrial GSH production.

KOKKUVÕTE

Glutatioon (GSH) on eukarüootides levinud mittevalguline tioolühend. Lisaks füsioloogilisele tähtsusele rakkudes on GSH huvi pakkunud ka mitmetele tööstusvaldkondadele. GSH mikrobioloogiline tootmine, kasutades geneetiliselt modifitseeritud pärmi tüvesid ja eellas-aminohapete lisamist, on potentsiaalne meetod katmaks selle tehnoloogiliselt olulise tripeptiidi suurenenud tööstuslikku tootmisvajadust. Sobivate produtsent-tüvede ja tootmistehnoloogiate välja arendamiseks on vaja paremini mõista molekulaarseid mehhanisme, mis kontrollivad GSH akumulatsiooni rakkudes. Juhuslikul mutageneesil saadud glutatiooni üle-akumuleerivate tüvede ainevahetuse analüüs võiks olla üks võimalus GSH akumulatsiooni tõstmiseks vajalike geneetiliste ja metaboolsete modifikatsioonide leidmiseks. Antud doktoritöös uuriti võrdlevalt molekulaarseid mehhanisme, mis määravad erinevatel keskkonnatingimustel rakusisest GSH taset tööstuslikus pagaripärmis *Saccharomyces cerevisiae* ja selle juhuslikul mutageneesil selekteeritud GSH-d üle-akumuleerivas tüves. Tulemused näitasid, et üle kahe korra kõrgem GSH akumulatsioon mutandis ei olnud põhjustatud γ-glutamüültsüsteiin süntetaasi (Gsh1p) ja glutatioon süntetaasi (Gsh2p) reaktsioonide tagasisidestus-inhibitsiooni puudumisest, vaid pigem tsüsteiini, kui peamise limiteeriva eellas-aminohappe suurenenud biosünteesist, põhjustatuna vastavalt tsüstationiin γ-lüaasi (*CYS3*) ja tsüstationiin β-süntaasi (*CYS4*) ning *GSH1* ning *GSH2* geenide kõrgemast ekspressioonist. Samuti näidati, et lisaks GSH biosünteesis osalevatele geenidele (*GSH1* ja *GSH2*), kontrollib oksüdatiivse stressi transkriptsiooni aktivaator Yap1p ka geenide *CYS3* ja *CYS4* ekspressiooni. Seega, *YAP1* üles-ekspresseerimine võimaldab täita kahte olulist tingimust, mis on vajalikud kõrgeks GSH akumulatsiooniks pärmis, s.o. suurendada nii tsüsteiini biosünteesi, aga ka GSH biosünteesis osalevate reaktsioonide läbilaskvust. Antud teadmisi on võimalik ära kasutada GSH biotehnoloogiliseks tootmiseks sobilike uute geneetiliselt modifitseeritud pärmitüvede konstrueerimisel.

LIST OF PUBLICATIONS

The following publications form the basis of this dissertation and are reproduced in the appendices with permission from the publishers.

I Nisamedtinov I, Kevvai K, Orumets K, Rautio J, Paalme T. **Glutathione accumulation in ethanol-stat fed-batch culture of *Saccharomyces cerevisiae* with a switch to cysteine feeding.** *Applied Microbiology and Biotechnology*, 87(1):175-83, (2010)

II Nisamedtinov I, Kevvai K, Orumets K, Arike L, Sarand I, Korhola M, Paalme T. **Metabolic changes underlying the higher accumulation of glutathione in *Saccharomyces cerevisiae* mutants.** *Applied Microbiology and Biotechnology*, 89(4): 1029-1037, (2011)

III 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.** *Biotechnology Journal*, 7(4):566-568, (2012)

ADDITIONAL PUBLICATIONS

A Nisamedtinov I, Orumets K, Kevvai K, Viiard E, Sarand I, Paalme T. **Multilevel control of GSH accumulation in mutant and wild-type strains of *Saccharomyces cerevisiae* under conditions of smooth cysteine addition.** *Chemical Engineering Transactions: IBIC 2010 2nd International Conference on Industrial Biotechnology*, Editors Enrico Bardone and Aurelio Viglia, 91-96, (2010)

B Nisamedtinov I, Lindsey GG, Karreman R, Orumets K, Koplimaa M, Kevvai K, Paalme T. **The response of the yeast *Saccharomyces cerevisiae* to sudden *versus* gradual changes in environmental stress monitored by expression of the stress response protein Hsp12p.** *FEMS Yeast Research*, 8(6):829-838, (2008)

SUMMARY OF AUTHOR'S CONTRIBUTION

I, II In Publication I and Publication II the author carried out all cultivation experiments, was responsible for the deteremination of intracellular thiols by UPLC®, interpreted the data, and was involved in the writing of the manuscript.

III In Publication III, the author carried out all cultivation experiments, was responsible for the deteremination of intracellular thiols by UPLC®, interpreted the data, and wrote the manuscript.

LIST OF PRESENTATIONS

I Orumets K, Kevvai K, Nisamedtinov I, Tamm T, Paalme T. **The effect of *YAP1* over-expression on glutathione accumulation in *Saccharomyces cerevisiae*.** *Poster presentation at FEMS 2011, 4th Congress of European Microbiologists*, 26-30 June, 2011, Geneve, Switzerland.

II Orumets K, Kevvai K, Nisamedtinov I, Tamm T, Paalme T. **Up-regulation of the genes encoding enzymes in the glutathione and cysteine biosynthesis pathways in *YAP1* over-expressing strain correlates to enhanced glutathione accumulation.** *Poster presentation at the ESF-EMBO Symposium "Glutathione and Related Thiols in Living Cells"*, 4-9 September 2011, Sant Feliu de Guixols, Spain.

III Orumets K, Nisamedtinov I, Kevvai K, Rautio J, Paalme T. **Metabolic changes underlying the higher accumulation of glutathione in *Saccharomyces cerevisiae*.** *Oral presentation at FoodBalt 2010*, 29-30 October 2010, Tallinn, Estonia.

IV Orumets K, Nisamedtinov I, Kevvai K, Rautio J, Paalme T. **Glutathione accumulation in mutant and wild-type strains of *Saccharomyces cerevisiae* under conditions of smooth cysteine addition.** *Poster presentation at the 4th Conference on Physiology of Yeast and Filamentous Fungi (PYFF4)*, 1-4 June 2010, Rotterdam, The Netherlands.

V Orumets K, Nisamedtinov I, Kevvai K, Rautio J, Paalme T. **Glutathione accumulation in cysteine-stressed *Saccharomyces cerevisiae*.** *Poster presentation at MICROBIAL STRESS —- from Molecules to Systems*, 7-10 May 2009, Semmering, Austria.

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This work is dedicated to my lovely parents.

Kallid, ema ja isa, see töö on teile!

ACRONYMS

BLAST	basic local alignment search tool
CaSR	calcium sensing receptor
$EtOH_s$	concentration of ethanol set-point
GRP	grape reaction product
HIV	human immunodeficiency virus
UPLC®	trademark name for ultra high pressure liquid chromatography
iTRAQ	isobaric tags for relative and absolute quantitation
KEGG	Kyoto encyclopedia of genes and genomes
LC	liquid chromatography
mRNA	messenger ribonucleic acid
MS	mass-spectrometry
PCR	polymerase chain reaction
pJET1.2	Fermentas brand sequencing primers
PPO	polyphenol oxidases
ROS	reactive oxygen species
SNpc	*substantia nigra pars compacta*
TRAC	TRanscript Analysis with the aid of Affinity Capture
UV	ultraviolet

CHEMICALS AND ENZYMES

ACT1	actin, Yeast: YFL039C
Ald4p	mitochondrial aldehyde dehydrogenase product, Yeast: YOR374W
Ald6p	cytosolic aldehyde dehydrogenase product, Yeast: YPL061W
ATP	adenosine-5'-triphosphate, KEGG: C00002
CYS3	cystathionine γ-lyase, Yeast: YAL012W
Cys3p	cystathionine γ-lyase product, Yeast: YAL012W
CYS4	cystathionine β-synthase, Yeast: YGR155W
Cys4p	cystathionine β-synthase product, Yeast: YGR155W
CDNB	1-chloro-2,4-dinitrobenzene, PubChem: 6
Cbf1p	dual function helix-loop-helix protein product, Yeast: YJR060W
Crm1p	chromosome region maintenance protein 1, Yeast: YGR218W
CTA1	catalase A, Yeast: YDR256C
Cta1p	catalase A product, Yeast: YDR256C
CTT1	cytosolic catalase T, Yeast: YGR088W
Ctt1p	cytosolic catalase T product, Yeast: YGR088W

DNA	deoxyribonucleic acid, PubChem: 44135672
DTNB	5, 5′-dithiobis-(2-nitrobenzoic acid), PubChem: 6254
DUG1	Cys-Gly metallo-di-peptidase, Yeast: YFR044C
Dug1p	Cys-Gly metallo-di-peptidase product, Yeast: YFR044C
DUG2	component of glutamine amidotransferase, Yeast: YBR281C
Dug2p	component of glutamine amidotransferase product, Yeast: YBR281C
DUG3	component of glutamine amidotransferase, Yeast: YNL191W
Dug3p	component of glutamine amidotransferase product, Yeast: YNL191W
ECM38	γ-glutamyltranspeptidase, Yeast: YLR299W
Ecm38p	γ-glutamyltranspeptidase product, Yeast: YLR299W
γGT	γ-glutamyltranspeptidase, EC: 2.3.2.2
γGCS	γ-glutamylcysteinyl synthetase, EC: 6.3.2.2
GLR1	cytosolic and mitochondrial glutathione oxidoreductase, Yeast: YPL091W
Glr1p	cytosolic and mitochondrial glutathione oxidoreductase product, Yeast: YPL091W
GPX1	phospholipid hydroperoxide glutathione peroxidase, Yeast: YKL026C
Gpx1p	phospholipid hydroperoxide glutathione peroxidase product, Yeast: YKL026C
GPX2	phospholipid hydroperoxide glutathione peroxidase, Yeast: YBR244W
Gpx2p	phospholipid hydroperoxide glutathione peroxidase product, Yeast: YBR244W
GRX1	glutathione-dependent disulfide oxidoreductase, Yeast: YCL035C
Grx1p	glutathione-dependent disulfide oxidoreductase product, Yeast: YCL035C
Grx2p	cytoplasmic glutaredoxin product, Yeast: YDR513W
Grx3p	hydroperoxide and superoxide-radical responsive glutathione-dependent oxidoreductase product, Yeast: YDR098C
Grx4p	hydroperoxide and superoxide-radical responsive glutathione-dependent oxidoreductase product, Yeast: YER174C
GRX5	mitochondrial hydroperoxide and superoxide-radical responsive glutathione-dependent oxidoreductase, Yeast: YPL059W
Grx5p	mitochondrial hydroperoxide and superoxide-radical responsive glutathione-dependent oxidoreductase product, Yeast: YPL059W
GSH	γ-L-glutamyl-L-cysteinyl-glycine, PubChem: 124886
gshA	glutamate-cysteine ligase, E.coli.: b2688
gshB	glutathione synthetase, E.coli.: b2947
GSH1	γ-glutamylcysteine synthetase, Yeast: YJL101C
Gsh1p	γ-glutamylcysteine synthetase product, Yeast: YJL101C
GSH2	glutathione synthetase, Yeast: YOL049W
Gsh2p	glutathione synthetase product, Yeast: YOL049W
GSSG	glutathione disulfide, PubChem: 65359
GST	glutathione S-transferase
GS-X	GST S-conjugates
GTT1	endoplasmic reticulum associated glutathione S-transferase, Yeast: YIR038C
Gtt1p	endoplasmic reticulum associated glutathione S-transferase product, Yeast: YIR038C
GTT2	glutathione S-transferase, Yeast: YLL060C

Gtt2p	glutathione S-transferase product, Yeast: YLL060C
Opt1p	oligopeptide transporter (alias *HGT1*) product, Yeast: YJL212C
Hsp12p	12 kDa heat shock protein product, Yeast: YFL014W
Idp1p	mitochondrial isocitrate dehydrogenase product, Yeast: YDL066W
Idp3p	peroxisomal isocitrate dehydrogenase product, Yeast: YNL009W
Mae1p	mitochondrial malic enzyme product, Yeast: YKL029C
MET4	leucine-zipper transcriptional activator, Yeast: YNL103W
Met4p	leucine-zipper transcriptional activator product, Yeast: YNL103W
MET14	adenylylsulfate kinase, Yeast: YKL001C
MET16	3'-phosphoadenylsulfate reductase, Yeast: YPR167C
MET17	methionine and cysteine synthase, Yeast: YLR303W
NADPH	reduced dihydronicotinamide-adenine dinucleotide phosphate, PubChem: 5884
NADP+	oxidized dihydronicotinamide-adenine dinucleotide phosphate, PubChem: 5884
Rnr1p	major isoform of the large subunit of ribonucleotide-diphosphate reductase product, Yeast: YER070W
Rnr4p	ribonucleotide-diphosphate reductase, small subunit product, Yeast: YGR180C
SHR3	endoplasmic reticulum packaging chaperone, Yeast: YDL212W
SOD1	cytosolic copper-zinc superoxide dismutase, Yeast: YJR104C
Sod1p	cytosolic copper-zinc superoxide dismutase product, Yeast: YJR104C
SOD2	mitochondrial manganese superoxide dismutase, Yeast: YHR008C
Sod2p	mitochondrial manganese superoxide dismutase product, Yeast: YHR008C
STR2	cystathionine gamma-synthase, Yeast: YJR130C
STR3	peroxisomal cystathionine beta-lyase, Yeast: YGL184C
SUL1	high affinity sulfate permease, Yeast: YBR294W
TRR1	cytoplasmic thioredoxin reductase, Yeast: YDR353W
Trr1p	cytoplasmic thioredoxin reductase product, Yeast: YDR353W
TRR2	mitochondrial thioredoxin reductase, Yeast: YHR106W
Trr2p	mitochondrial thioredoxin reductase product, Yeast: YHR106W
TRX1	cytoplasmic thioredoxin isoenzyme, Yeast: YLR043C
Trx1p	cytoplasmic thioredoxin isoenzyme product, Yeast: YLR043C
Trx2p	cytoplasmic thioredoxin isoenzyme product, Yeast: YGR209C
TRX3	mitochondrial thioredoxin, Yeast: YCR083W
Trx3p	mitochondrial thioredoxin product, Yeast: YCR083W
YAP1	oxidative stress responsive transcriptional activator, Yeast: YML007W
Yap1p	oxidative stress responsive transcriptional activator product, Yeast: YML007W
Ycf1p	vacuolar glutathione S-conjugate transporter product, Yeast: YDR135C
ZWF1	glucose-6-phosphate dehydrogenase, Yeast: YNL241C
Zwf1p	glucose-6-phosphate dehydrogenase product, Yeast: YNL241C

THESIS

1

INTRODUCTION

Glutathione (GSH) is the most abundant non-protein thiol with different physiological functions in eukaryotic cells. Most of these functions have been related to its antioxidative properties caused by the thiol group in the cysteine moiety [1]. Due to the antioxidative properties of GSH there is an increasing interest for application of this tripeptide in several industrial areas, including cosmetics, pharmaceutical products, and foods.

Although GSH can be produced by chemical and enzymatic synthesis, microbial production using the yeasts *Saccharomyces cerevisiae* and *Candida utilis* is currently the most common method for the commercial production of GSH. Efficient GSH production using yeast relies on high biomass density cultivation with maximum possible GSH content at the point of biomass harvesting. The accumulation of GSH in yeast is tightly controlled at different levels. Thus, overcoming the molecular regulatory mechanisms which restrict GSH over-accumulation has become the major challenge to increase GSH concentration in yeast.

Different strategies have been proposed to increase intracellular GSH accumulation, including optimization of cultivation processes and improvement of producer strains. Strains obtained by both random mutagenesis as well as by genetic engineering techniques are used. Although random mutagenesis helps to improve the properties of yeast and usually results in highly specialized mutant strains, these mutants are often crippled due to wide range of other mutations which may result in severe growth defects. By contrast, the major advantage of genetic engineering is the directed modification of strains without the accumulation of such unfavorable mutations. However, despite the rapid development of "rational" metabolic engineering, the success of development of efficient producer strains is still often hampered by insufficient biochemical and genetic information concerning the metabolic pathways of interest. To overcome limitations of "rational" metabolic engineering, a bottom-up approach termed "inverse metabolic engineering" has been suggested as an alternative strategy [2]. Shortly, receiving information from the genetic and metabolic analysis of mutants obtained by random mutagenesis and mapping the metabolic changes which underlies the desired phenotype could assist in the generation of improved producer strains for industrial applications using genetic engineering techniques.

The primary aim of this dissertation is to gain better insights into the molecular mechanisms responsible for regulation of intracellular GSH concentrations in an industrial baker's yeast strain of *Saccharomyces cerevisiae* and a random mutagenized GSH over-accumulating isolate under conditions similar to an industrial yeast production process.

2

LITERATURE REVIEW

GLUTATHIONE WAS DISCOVERED in an ethanol extract from baker's yeast and was found to have the ability to hydrogenate sulfur, and was therefore initially named "philothion" [3]. In 1921, after the establishment of its molecular structure (see Figure 1) it was renamed "glutathione" [4].

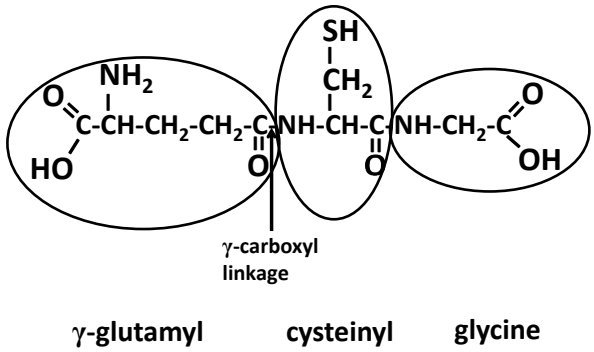

Figure 1. The chemical structure of GSH.

2.1 GLUTATHIONE AND ITS PHYSIOLOGICAL ROLES IN CELLS

Over 90% of GSH in eukaryotic cells is normally present in the free reduced form. The other forms include oxidized GSH (GSSG) or its disulfide complexes with other compounds, such as GS-S-CoA, GS-S-Cys, GS-S-protein.

Due to the relatively high concentration of GSH in cells (1-10 mM), low standard state redox potential (-240 mV) and high stability to proteases (resulting from the γ-glutamyl-cysteine linkage) it plays several physiological roles in cells. GSH is an important redox buffer, detoxifier and a key source of nitrogen and sulfur under conditions of their depletion in the environment. Consequently, the physiological functions of GSH become evident under adverse environmental conditions, such as oxidative stress, toxification with heavy metals and xenobiotics, and nutrient depletion [5–7]. As GSH is involved in protein folding and DNA synthesis, as well as in the transport of amino acids [8] it is an essential metabolite for the growth of eukaryotic cells. For example, knockout of the first enzyme in GSH biosynthesis leads to GSH auxotrophy in yeasts and embryonic lethality in mice [9].

2.1.1 *GSH as an intracellular redox buffer*

Cell redox homeostasis covers any process that maintains the redox environment of a cell. To protect against oxidant damage and stress, cells contain effective defence mechanisms including antioxidant enzymes and free radical scavengers [10]. A number of reports have highlighted that sulphydryl groups (-SH) play a key role in the function of many macromolecular structures including enzymes, transcription factors and membrane proteins as well as in response to oxidative stress to maintain the redox balance of the cells [11–13]. There are several mechanisms which regulate redox homeostasis in the yeast cells. These include the glutathione/glutaredoxin and thioredoxin systems (see Figure 2). Glutaredoxins and thioredoxins are small heat-stable oxidoreductases that contain two conserved cysteine residues in their active sites and function in many cellular processes, including defence of the cells against oxidative stress, deoxyribonucleotide synthesis, repair of oxidatively damaged proteins, protein folding and sulfur metabolism [7, 14]. However, despite a considerable functional overlap and similarity in the structure of glutaredoxins and thioredoxins, they are regulated differently.

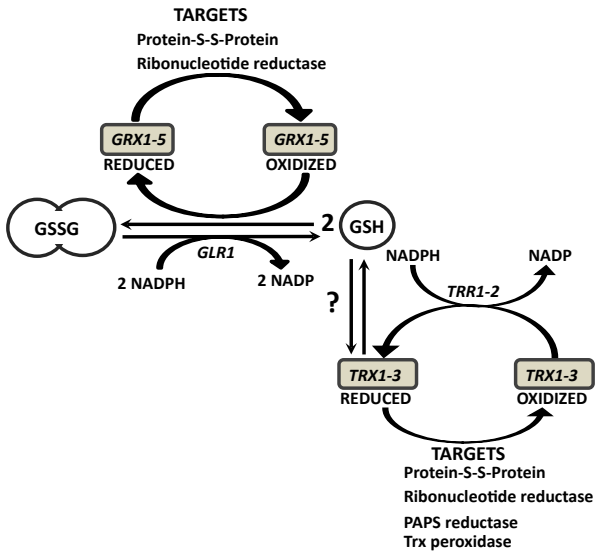

Figure 2. The glutathione/glutaredoxin and thioredoxin systems in yeast. Yeast contains five glutaredoxins (*GRX1*–*GRX5*) and three thioredoxins (*TRX1*–*TRX3*). *GLR1* — glutathione reductase; *TRR1*, *TRR2* — thioredoxin reductases; PAPS reductase (*MET16*) – 3'-phosphoadenosine 5'-phosphosulfate reductase; *TRX* peroxidase – thioredoxin peroxidase.

Yeast, as in some bacteria, plants, and mammals, have monothiol and dithiol glutaredoxins [15]. Monothiol glutaredoxins catalyze the reduction of protein–GSH mixed disulfides (Protein-S-S-G) only, while dithiol glutaredoxins reduce both the protein disulfides (Protein-S-S-Protein) and protein-GSH mixed disulfides. *Saccharomyces cerevisiae*

contains two dithiol glutaredoxins (Grx1p and Grx2p) and three monothiol glutaredoxins (Grx3p, Grx4p, and Grx5p). In addition to reduction of protein disulfides the dithiol glutaredoxins are also believed to act as hydrogen donors for ribonucleotide reductases (Rnr1p–Rnr4p), which play an essential role in DNA replication and repair. The role of GSH in the glutaredoxin cycle is to re-reduce the oxidized glutaredoxins. The enzyme glutathione reductase (Glr1p), in turn, maintains and restores physiological GSH/GSSG balance by reducing oxidized glutathione (GSSG) back to GSH in a NADPH dependent reaction. With this reaction NADPH is oxidized to $NADP^+$, which is further reduced back to NADPH by an action of $NADP^+$ dependent glucose-6-phosphate dehydrogenase (Zwf1p), the isocitrate dehydrogenases (Idp1p–Idp3p), the malic enzyme (Mae1p) and the aldehyde dehydrogenases (Ald4p–Ald6p) to maintain a normal redox status in the cells [16, 17]. Notably, it has been shown that *ZWF1* expression is inversely correlated to intracellular GSH levels which suggest that GSH replenishment may depend on *ZWF1* expression [18].

Similar to the glutathione/glutaredoxin system, the thioredoxin system helps to regulate the redox homeostasis in cells but without the direct involvement of GSH. The redox status of GSH may, however, provide a functional link between the glutathione/glutaredoxin and thioredoxin systems, as thioredoxins function along with Glr1p to maintain a high intracellular GSH/GSSG ratio [7]. *Saccharomyces cerevisiae* contains two cytoplasmic thioredoxins (Trx1p and Trx2p) and a mitochondrial thioredoxin Trx3p which all function to protect cells against reactive oxygen species (ROS) and oxidative stress [19]. Thioredoxins act as hydrogen donors for ribonucleotide reductases, thioredoxin peroxidases (*TRX* peroxidases, responsible for peroxide removal) and 3'-phosphoadenosine 5'-phosphosulfate reductase (PAPS reductase, converts 3'-phosphoadenosine 5'-phosphosulfate to sulfite in a sulfur assimilation pathway). Evidence suggests that the cytoplasmic thioredoxin system may have overlapping function with the glutathione/glutaredoxin system by reducing the protein disulfides [20, 21] while the mitochondrial thioredoxin system seems to be important in the defense against oxidative stress generated during respiratory metabolism [22]. The thioredoxin disulfides are reduced by NADPH and thioredoxin reductases (cytosolic Trr1p and mitochondrial Trr2p).

2.1.2 *GSH as the detoxificant*

GSH-dependent detoxification processes concern the elimination of toxic endogenous metabolites (*e.g.*, excess formaldehyde or methylglyoxal) as well as chemical substances that are foreign to a biological system (xenobiotics). The latter include, for example, fungicides, insecticides, drugs, carcinogens, mutagenic agents and heavy metals. Many xenobiotics have potential to react either spontaneously with the SH group of GSH or by the aid of glutathione S-transferases (GSTs) to form GSH S-conjugates (GS-X) which are then transported for sequestration to vacuoles by the GSH S-conjugate transporter (Ycf1p in yeast) [23]. It has been shown that strains lacking Ycf1p activity are sensitive to 1-chloro-2,4-dinitrobenzene (CDNB) and cadmium, indicating a function in the detoxification of S-conjugated xenobiotics [7, 24].

Glutathione S-transferases (GSTs) are detoxification enzymes that catalyse the conjugation of electrophilic substrates to GSH, thereby neutralizing their electrophilic sites and

raising the water solubility of the products before their removal from cells *via* glutathione conjugate pumps [25–27]. GST activity have been found to present in some bacteria, yeasts, plants, insects and in most mammalian tissues, especially in the liver, which plays a key role in detoxification [27, 28]. There are various classes of GSTs that differ in their specificity towards xenobiotics. Eukaryotes usually contain several GST paralogues with different catalytic activities and a wide range of cellular functions. *Saccharomyces cerevisiae* contains two GST isoenzymes (Gtt1p and Gtt2p) which have been shown to have GST activity with CDNB [28]. Notably, besides functioning in detoxification, Gtt1p and Gtt2p may also act in a similar way as heat shock proteins by increasing the stability of proteins under stress conditions [28]. Strains deleted for *GTT1* and *GTT2* are viable, but show no decreased sensitivity to ROS, indicating that they are not required for protection against oxidative stress [28].

In addition to the enzymatic redox balancing systems described in section 2.1.1, GSH also reacts non-enzymatically with a series of reactive oxygen species including $OH^{\bullet}$, HOCl, $RO^{\bullet}$, $RO_2^{\bullet}$ as well as with many nitrogen and carbon containing radicals through the formation of thiyl ($GS^{\bullet}$) species. $GS^{\bullet}$ species may generate $O_2^{-\bullet}$ which can be further neutralized by enzymes superoxide dismutase (Sod1p and Sod2p) and catalases Cta1p (mitochondrial catalase A) and Ctt1p (cytosolic catalase T) (see Figure 3A). The redox-active sulfhydryl group of GSH can also protect yeast cells by acting as a cofactor for glutathione peroxidases Gpx1p and Gpx2p (see Figure 3B). In fact, the latter are considered to be principal antioxidant enzymes for H_2O_2 detoxification due a higher affinity for H_2O_2 than catalases have [29].

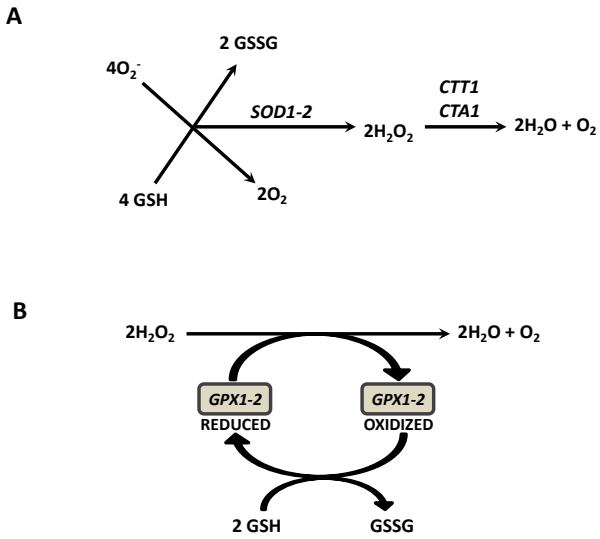

Figure 3. A: Removal of superoxide radicals by GSH and by superoxide dismutases and catalases. *SOD1*, *SOD2* — superoxide dismutases; *CTA1* — mitochondrial catalase A; *CTT1* – cytosolic catalase T. **B**: Protection of cells from peroxides by glutathione/-glutathione peroxidase system. *GPX1*, *GPX2* — glutathione peroxidases.

2.1.3 *GSH as the sulfur and nitrogen source*

Besides acting as the redox regulator and detoxifier GSH can also serve as an endogenous reserve of sulfur and nitrogen upon deprivation of these elements in cells. There is evidence that GSH levels are sensitive to the availability of nitrogen and sulfur sources [6, 30, 31]. During nitrogen starvation in *Saccharomyces cerevisiae* about 90% of the total cellular GSH can be degraded to provide the cells with glutamic acid which can further be converted to other nitrogen-containing monomers required for biosynthesis. At the same time, the nitrogen-response elements have been shown to be present in the promoters of both GSH biosynthesis genes (*GSH1* and *GSH2*) resulting in a transitory stimulation of GSH synthesis and consequently an increase in the cellular turnover of GSH upon nitrogen starvation [31]. Similarly to nitrogen starvation, *Saccharomyces cerevisiae* responds to sulfur deprivation by increasing the turnover rate of GSH and by channelling the liberated cysteine to protein synthesis as well as to the synthesis of other sulfur-containing compounds in the cell. Elskens *et al.* [30] showed that GSH in yeast was degraded until it reached the critical residual concentration of about 10% of its normal value under sulfur limitating growth conditions.

The hydrolysis of GSH in *Saccharomyces cerevisiae* to its constitutive amino acids glutamate, cysteine, and glycine occurs in two steps catalyzed by the vacuolar-membrane-bound enzymes, γ-glutamyl transpeptidase (Ecm38p) and cysteinylglycine dipeptidase (Dug1p) (see Figure 4). Ecm38p catalyzes the cleavage of the γ-glutamyl component of GSH to yield free glutamic acid and a dipeptide cysteinylglycine. Ecm38p is a heterodimeric glycoprotein with 64 kDa and 29 kDa subunits, with the active site on the smaller subunit [32–34]. Studies on the regulation of *ECM38* have indicated that it is dependent on the nitrogen regulatory circuit (ammonium ions cause repression and nitrogen starvation that leads to derepression), but also to a small extent by sulfur limitation [35–38]. Cysteinylglycine is hydrolysed by Dug1p [39], whose activity had previously been shown in *Saccharomyces cerevisiae* without identifying the open reading frame [34, 40]. An alternative pathway for GSH degradation requires the participation of three metalloproteins Dug1p, Dug2p and Dug3p [41], however, a recent study questions the hypothesis that these form a three protein complex and shows that Dug1p can also function in a Dug2p-Dug3p independent manner [39].

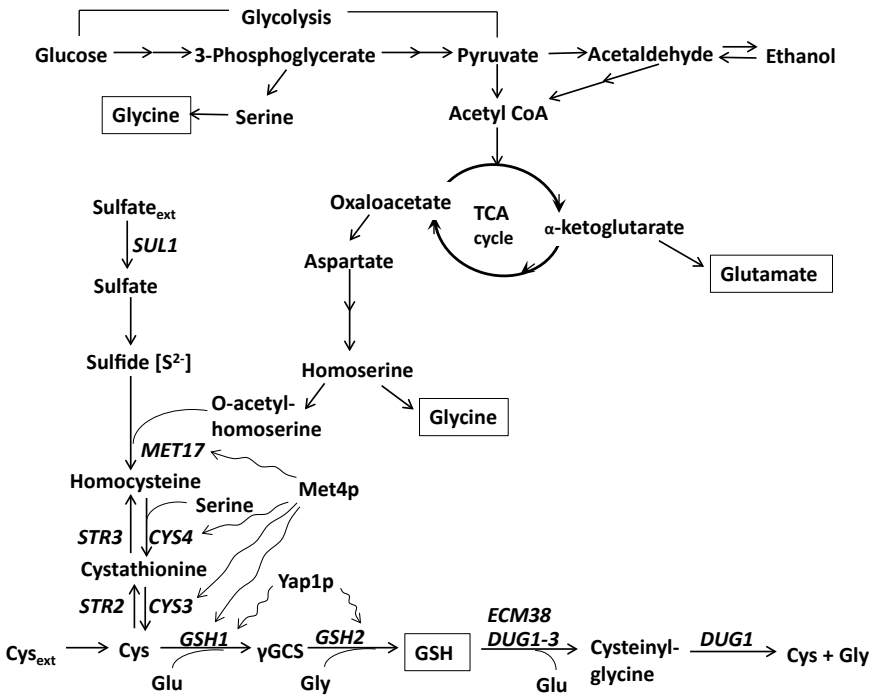

Figure 4. Schematic representation of the main pathways involved in the metabolism of GSH. *SUL1* -– high affinity sulfate permease; *MET17* -– homocysteine synthase; *CYS4* -– cystathionine β-synthase; *CYS3* -– cystathionine γ-lyase; *STR3* — cystathionine β-lyase; *STR2* -– cystathionine γ-synthase; *GSH1* — γ-glutamylcysteine synthetase; *GSH2* -– glutathione synthetase; *ECM38* — γ-glutamyltranspeptidase; *DUG2*, *DUG3* -– cysteinylglycine metallo-dipeptidases; *DUG1* -– L-cysteinylglycine dipeptidase; Met4p — leucine zipper transcriptional activator, Yap1p -– redox sensitive transcriptional activator. Modified from [42].

2.2 GLUTATHIONE BIOSYNTHESIS AND ITS REGULATION IN YEAST

Regulation of the GSH/GSSG balance in cells is extremely important. In one hand, its shortage results in severe oxidative stress related damages, while on the other hand, a very high concentration of GSH may become toxic, mostly due to indiscriminate glutathionylation of proteins and subsequent breakdown of metabolic processes [42]. For example, yeast over-expressing the GSH-transporter (Opt1p) was unable to tolerate fivefold higher than normal levels of intracellular GSH [43].

Although yeasts can transport GSH from the extracellular medium to some extent, the major factor determining GSH concentration in cells is *de novo* biosynthesis [42]. GSH is synthesized from three precursor amino acids (L-glutamate, L-cysteine, and L-glycine) over two consecutive ATP-dependent reactions, catalyzed by γ-glutamylcysteine syn-

thetase (Gsh1p) and GSH synthetase (Gsh2p) (see Figure 4). The concentration of GSH in cells, in addition to being dependent on the expression levels and activities of Gsh1p and Gsh2p, is also dependent on the energy source ATP as well as the intracellular concentration of the respective precursor amino acids [42].

The two GSH biosynthetic reactions (Gsh1p and Gsh2p) are specifically regulated at three different levels (see Figure 5): i) transcriptional level; ii) post-translational level and iii) substrate availability level.

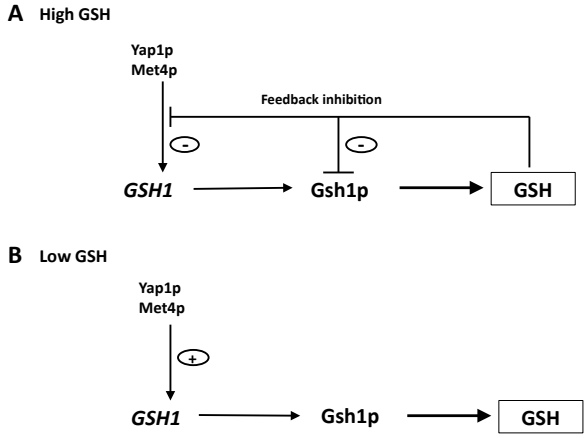

Figure 5. Model of the feedback control of GSH biosynthesis reactions. **A**: High concentrations of GSH inhibit both Gsh1p enzyme activity and Yap1p/Met4p-mediated expression of *GSH1* to prevent further synthesis of GSH. **B**: The absence of GSH relieves feedback inhibition and allows Yap1p/Met4p-mediated induction of *GSH1* expression leading to the synthesis of GSH. Modified from [44].

Transcriptional regulation of the *GSH1* gene to the availability of GSH is a complex process and mediated by the coupling of two transcriptional activators — redox sensitive Yap1p and Met4p, a principal factor mediating regulation of sulfur assimilatory pathways [45]. Yap1p (a member of the AP-1 family of transcription factors) is a key determinant in oxidative stress resistance in yeast, the activity of which is determined by its cellular localization between the nucleus and cytoplasm in response to the intracellular redox state and the respective conformational changes in its structure [46]. Under conditions of increased GSH concentrations (and the resulting reductive intracellular environment) Yap1p is deactivated *via* thioredoxin mediated reduction of this intramolecular disulfide bond and its following export from the nucleus to cystoplasm by Crm1p [47, 48]. In the absence of oxidative stress, Yap1p is exported from the nucleus *via* the nuclear export protein Crm1p. Under oxidative stress conditions an intramolecular disulfide bond is formed in Yap1p and this conformational change allows it to accumulate in the nucleus [49]. Studies have shown that both *GSH1* and *GSH2* gene promoters possess the Yap1p response element (YRE) sequence (TTA(C/G)TAA) [50, 51]. The transcriptional regulation of GSH biosynthesis by Yap1p has been suggested to be coupled to methionine availability

via the transcription factor Met4p, which governs the MET gene network in the biosynthesis of the sulfur-containing amino acids (methionine and cysteine) [45, 52]. Notably, Met4p seems to play only a secondary regulatory role in Yap1p mediated activation of *GSH1* by interacting with Cbf1p. The latter is a dual function protein which binds to the *GSH1* promoter and suppresses its expression by Yap1p. Upon GSH and cysteine depletion, Met4p interacts with Cbf1p to allow Yap1p mediated activation of *GSH1* expression. On the contrary, at higher GSH and cysteine concentrations Met4p becomes ubiquitinated and Cbf1p represses Yap1p activation of *GSH1* by binding to its promoter.

Post-translational regulation of GSH biosynthesis is a non-allosteric feedback inhibition of Gsh1p activity by increased GSH concentrations [53]. *In vitro* kinetic analyses with mammalian enzymes have established that the Gsh1p K_i value of 2 mM for GSH feedback inhibition, which is consistent with the physiological concentration of GSH in those cells [54]. The regulation of Gsh1p activity in response to GSH concentration (in addition to feedback repression of the *GSH1* expression), indicates a requirement for precise control of GSH biosynthesis in yeast which is necessary for retaining a proper intracellular redox balance. In this way, high concentrations of GSH prevent its futher accumulation by the feedback inhibition of both the activity of Gsh1p and *GSH1* gene expression. These feedback control mechanisms also allow for the up-regulation of GSH biosynthesis when its concentration in the cells becomes lower than the physiological demand.

In addition to transcriptional and post-translational feedback regulation of the Gsh1p and Gsh2p reactions, cysteine has been shown to be the major limiting precursor for GSH accumulation [55, 56]. Addition of cysteine to yeast culture has been shown to increase intracellular GSH levels [55–58]. Thus, the regulation of sulfur assimilation and cysteine biosynthesis pathways becomes also important from the perspective of GSH biosynthesis. The biosynthesis of cysteine starts from homocysteine and serine which combine to form cystathionine (see Figure 4). The reaction is catalysed by cystathionine β-synthase (Cys4p). In the second reaction cystathionine is cleaved at the γ position by cystathionine γ-lyase (Cys3p) to produce cysteine [59, 60]. Studies have revealed that the sulfur assimilation pathway leading to cysteine biosynthesis is under a tight transcriptional regulation by intracellular cysteine levels [60]. In yeast, the transcriptional activator Met4p is responsible for the expression of genes involved in the assimilation of inorganic sulfur and for the synthesis of sulfur-containing amino acids (*MET17*, *CYS3*, *CYS4*, *GSH1*). As mentioned above, Met4p activity is negatively regulated *via* ubiquitylation in response to increased intracellular cysteine levels [52, 60]. Thus, Met4p has an indirect effect on GSH biosynthesis by regulating the supply of its major limiting precursor cysteine [45].

2.3 PRACTICAL APPLICATIONS OF GLUTATHIONE

Besides the cellular physiological functions that GSH has, it is also used for different industrial applications. For example, GSH is widely used in the composition of pharmaceuticals and cosmetic products. It has also recently gained a lot of attention in the food industry with potential applications in baking and winemaking processes and as a flavor enhancer. Consequently, there is an increasing demand for the production of this tripeptide on an industrial scale. Presently, the estimated global annual production of

pure crystalline GSH and GSH-enriched yeast extract (15% GSH) in the world exceeds 200 and 800 tons, sold at a price of 300 and 150 USD/kg, respectively [61].

2.3.1 *Applications of GSH for the treatment of medical disorders*

The deficiency of GSH in humans has been shown to be associated with a number of medical disorders caused by oxidative stress, poisoning or compromised immune system (see Figure 6). These disorders include neurodegenerative diseases, cancer, cataracts, liver cirrhosis, pulmonary diseases, gastrointestinal and pancreatic inflammations and hemolytic anemia [62–64].

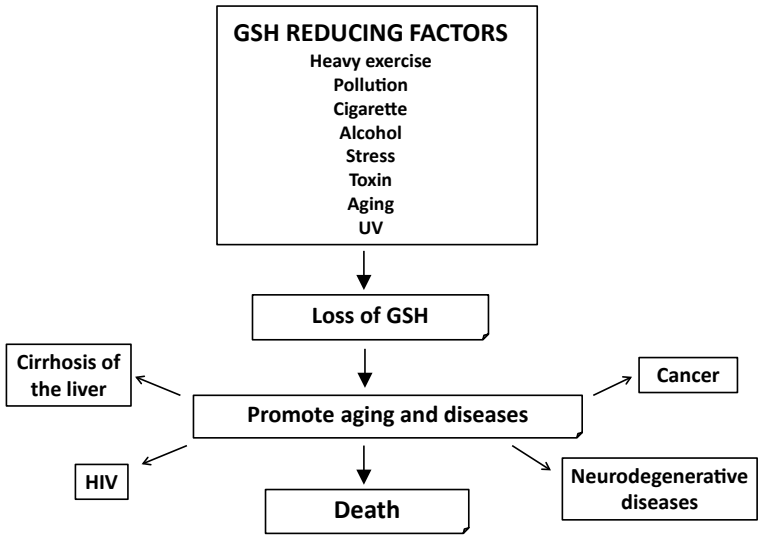

Figure 6. Lower glutathione levels are implicated in many diseases associated with oxidative stress, poisoning, compromised immune system and aging.

The major cause for several neurodegenerative diseases is the aging related free radical dependent oxidative damages to the brain cells. Therefore, age dependent changes in the levels of GSH in the brain are assumed to play a role in the development of those diseases. Studies have shown that of all the neurodegenerative diseases, evidence for a dysfunction in GSH metabolism is strongest in Parkinson's disease [65, 66]. Parkinson's disease is a neurodegenerative disease which is characterized by the loss of dopaminergic neurons in the brain structure *substantia nigra pars compacta* (SNpc). This leads to a severe depletion of dopamine in the *striatum* and deficits in motor function, which are the well-known symptoms of this disease. A number of studies have found that GSH levels are specifically decreased in the SNpc of the patients with Parkinson's disease, suggesting the depletion of GSH has an important effect in the development of this disease [64, 66].

The increased concentration of free radicals in cells can result in mutations in DNA, which in turn, can lead to the development of cancer. Studies of chemically induced

cancers in animal models show that epithelial cells are primary sites of mutations and transformation [67, 68]. The lack of GSH or GST enzyme activity, and therefore, an inability to detoxify carcinogens is associated with an increased risk toward a variety of cancers (*e.g.*, lung, colon and bladder cancer). Thus, limitation of GSH supply, decreased GSH synthetic rates or increased GSH degradation can reduce physiological protection against carcinogenesis.

GSH also helps the liver (the most concentrated source of GSH in animals) to detoxify numerous natural and synthetic acidic toxins (*e.g.*, cigarette smoke, exhaust from motor vehicles *et cetera*), chemicals (*e.g.*, acetaminophen – an active ingredient in pain relief medication), but also heavy metal ions (copper, cadmium, zinc, silver) [64, 69]. GSH binds to toxins, forming a water-soluble complex which is ultimately excreted in the urine as waste. GSH is either the cause or effect of a number of pathologies. For example, a partial GSH deficiency of genetic origin or as a consequence of drug use generally leads to impaired liver and kidney functions and also reduced resistance to different stress or infectious agents. Numerous studies have demonstrated that patients with compromised liver function due to alcohol abuse have significant reduction of GSH in their liver [64, 69, 70]. Chronic ethanol feeding leads to the selective reduction of mitochondrial GSH due to the partial inactivation of a specific mitochondrial membrane transport protein that translocates GSH from the cytoplasm to the mitochondria. This selective depletion of mitochondrial GSH sensitizes hepatocytes to oxidative stress and the decrease in the mitochondrial GSH pool starts to play a role in alcoholic liver cirrhosis [64, 70].

GSH is also necessary for supporting the immune system and is particularly needed when the host is immunologically challenged by bacteria, parasites or viruses. More specifically, GSH is required for replication and activation of the T-lymphocytes and polymorphonuclear leukocytes. Human immunodeficiency virus (HIV)-1 protease activity is regulated through cysteine modification, and there are relationships between GSH levels and HIV-infected patients. Decreased GSH levels lead to a series of downstream signal transduction events that allow HIV expression [63, 69, 71].

GSH can be supplemented either orally or by an intravenous administration. Intravenous administration has demonstrated numerous beneficial effects in treatment of cancer, heavy metal overload and Parkinson's disease [72–74], but has also induced a rapid reduction in acute pain from a rheumatoid arthritis and decreased the toxicity of several chemotherapy regimens [75]. Nevertheless, several studies have reported a remarkable increase in hepatic GSH content could not be achieved with GSH infusion [76, 77]. These findings suggest that the observed positive effects of GSH are rather related to the increase in the extracellular antioxidant capacity than due to intracellular GSH.

Despite numerous GSH-containing biosupplements on the market the information about the bioavailability of orally administered GSH is still controversial. Also, little is known about the average daily intake of GSH from food, the concentration of GSH in various food sources, or the importance of dietary GSH in prevention of diseases. The estimated daily intake from food has been suggested to be ca 150 mg of GSH per day [78]. Because of its distinctive structure (γ-glutamyl peptide bond in glutamylcysteine moiety) GSH is resistant to most gastric proteases but may be partially inactivated by gastric peptidases [79]. The transit of orally administered GSH to tissues is thought to occur *via* absorption from the intestinal lumen, export from enterocytes into the blood and uptake

from the plasma into the cells [80, 81]. Such gastrointestinal transport of GSH appears to be *via* non-energy-requiring, sodium-independent, carrier-mediated diffusion [82] . A major problem with reliable determination of bioavailability of GSH is related to its low concentrations in the plasma due to rapid relocation to different organs (kidneys, liver). For example, the serum half-life of GSH after intravenous administration was shown to be less than 2 minutes [83, 84]. Various epithelial cells, such as enterocytes, alveolar cells, endothelial cells and retinal pigmented epithelial cells are capable of uptaking exogenous GSH and this allows GSH concentrations to be maintained better than by *de novo* synthesis alone. Thus, increasing plasma GSH concentrations by oral administration has been shown to increase the availability of GSH for transport into these tissues [80, 81]. However, most cells do have rather low GSH transport capacity and thus the absorption of GSH is rather poor.

To enhance the bioavailability of orally administered GSH different formulations, such as liposomal glutathione, glutathione in association with either ascorbic acid, α-lipoic acid, or with selenium are being explored [42, 85]. Glutathione analogues and precursors are also being developed for avoiding the problem of poor absorption and transport. For instance, N-acetyl cysteine is an acetylated analogue of cysteine that can cross the cell membrane and be rapidly deacetylated inside the cell, allowing the released cysteine to increase intracellular GSH concentration by *de novo* synthesis. GSH deficiency can also be improved using GSH esters, like GSH monoethyl esters. Due to high hydrophobicity, these esters can cross the cell membrane more easily and GSH can be liberated in the cells by the activity of esterases [42]. In general, the results of studies to date on GSH bioavailability have been conflicting and scientists are still arguing whether significant increase of plasma GSH can be achieved by oral or intravenous administration. Futher investigations are needed to answer these questions.

2.3.2 *Applications of GSH in cosmetics*

Application of GSH in the composition of different cosmetical products has grown rapidly during the last decade [63, 86, 87]. GSH is used as a component in emulsifiers, oily substances, and moisturizers, primarily to enhance the whitening effect on skin and also to remove or prevent pimples and pimple marks. In addition, GSH is used in the composition of anti-aging and anti-sun skin products [42].

Aging is related to accumulation of oxidative damage in the cells causing declines in cellular function. Since GSH plays a central role in maintaining the oxidative balance of cells, a number of researches have studied the changes in the cellular GSH/GSSG ratio during aging [64, 88, 89]. These studies have shown a consistent decrease in the GSH/GSSG ratio at higher ages. For example, Jones *et al.* [89] showed that the GSH/GSSG ratio remained constant until the age of 45 years and declined linearly thereafter, suggesting that GSH metabolism fails to keep up with oxidizing events and stress in the beginning of late middle age. Therefore, GSH has gained attention in cosmetic industry as a possible component fighting against skin aging and wrinkles.

2.3.3 *Applications of GSH in the food industry*

GSH has found application as an ingredient in a variety of food products, including baked goods, beverages, breakfast cereals, cheeses, condiments, dairy product analogs, fats and oils, sauces and meat. The safety of GSH has been proved with the results of published toxicological and clinical studies [90–92].

Fresh bread is prized for its taste, aroma, appearance and texture. In recent years techniques to improve the bread texture have gained increased attention. Depending on the type of baked product the dough requires a certain combination of strength, extensibility and tolerance. These characteristics depend mostly on flour quality, water absorption and dough mixing conditions. Upon mixing flour with water, the cysteine sulfhydryl groups of different cereal proteins, in particular gluten proteins (monomeric gliadin and polymeric glutenin), can oxidize and form disulfide (S-S) bonds between each other [93] (see Figure 7A). This interaction increases dough strength and decreases its extensibility. During further mixing of the dough these linkages are broken mechanically to provide the extensibility needed for moulding. The process is, however, reversible and the gluten matrix reforms during the later stages of proofing and baking. This can be detrimental to the quality of a number of products, for example pizzas, tortillas, crackers and hard biscuits. Reducing agents are a type of dough conditioners used to break the disulfide crosslinks between gluten subunits chemically and to reduce the average molecular weight of glutenin protein aggregates. Both cysteine and GSH have been shown to induce the reduction of the disulfide crosslinks in the dough which is why they are often used with high-strength flour and high-speed processes to reduce mix time, lower energy input, improve machinability as well as the bread loaf volume [93, 94]. Notably, while the number of reduced disulfide cross-links is directly proportional to that of cysteine molecules added, considerably less GSH is required to have the same effect. This is because of the wheat glutathione reductase in flour which converts GSH disulfide to GSH with free SH groups that can participate in further disulfide reduction reactions (see Figure 7B).

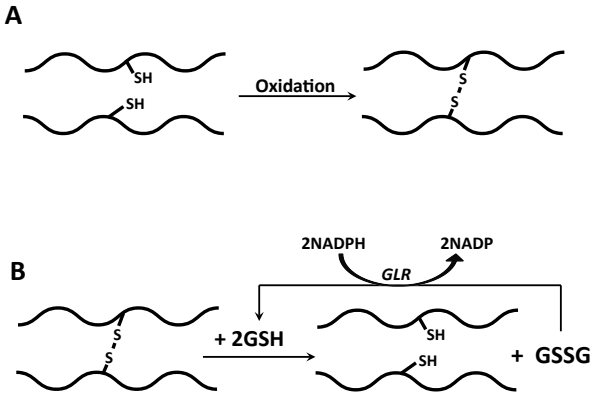

Figure 7. The role of GSH in dough reduction chemistry. **A**: Dough mixing induces oxidative formation of disulfide bridges between gluten molecules; the dough extensibility decreases. **B**: GSH reduces the disulfide bridges and becomes oxidized; wheat glutathione reductase (Glr1p) converts the oxidized GSH back to a reduced form.

Another food manufacturing area where the application of GSH has gained increased interest is in the production of fermented beverages, and in particular, winemaking. The quality of wine depends on its numerous constituents, the presence/absence and the concentration of a wide range of compounds. The production of wines, especially white wines, involves a great effort to avoid extensive contact of some of these compounds with oxygen. The most susceptible compounds to oxidation in wine are the grape phenols. The oxidation of these phenols can result in a decreased aroma loss and browning. After pressing of the grape must the hydroxycinnamates, especially caftaric acid, are the first phenolic compounds to be oxidized by the grape polyphenol oxidases (PPO). This oxidation leads to the formation of respecive o-quinones which can further polymerize and interact with various aroma compounds (thiols) resulting in browning of wines and aroma losses, respectively (see Figure 8). In addition, non-enzymatic oxidation of diphenols caffeic acid and catechin can also occur during wine aging [95]. GSH plays an important role in preventing the oxidative spoilage of white wines and can also improve their maturation potential. It interrupts the oxidation mechanism by trapping the caftaric acid in the form of 2-S-glutathionylcaftaric acid (see Figure 8), also known as grape reaction product (GRP) [96, 97]. GRPs are chemically stable, colorless and odorless compounds.

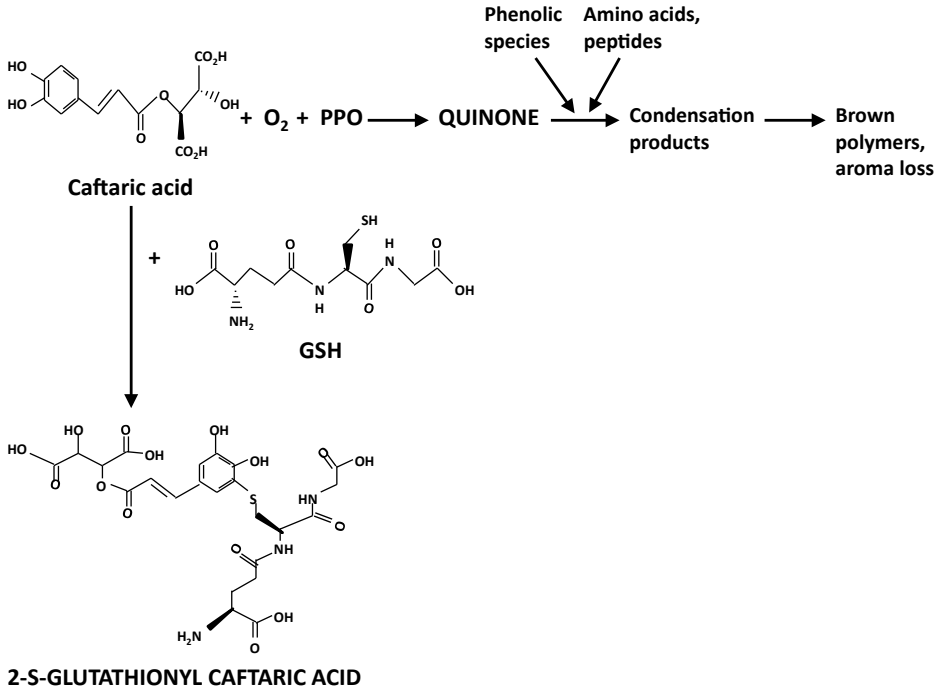

Figure 8. The role of GSH in wine reduction chemistry. Caftaric acid is oxidized by the grape polyphenol oxidases (PPO) to form o-quinones which can further be polymerized or interact with various aroma thiols, resulting in wine browning and aroma losses, respectively. GSH helps to limit phenol oxidation as it can react with caftaric acid, generating 2-S-glutahionyl caftaric acid.

Human sensory analyses have revealed that various extracellular calcium sensing receptor (CaSR) agonists enhance sweet, salty and umami tastes, although they have no taste themselves [86, 98]. These characteristics are introduced as "*kokumi*" taste and have been used for many years in traditional Japanese cuisine. *Kokumi* itself is a term used in the flavour industry to describe characteristics such as continuity, mouthfulness, richness and thickness. GSH is known as a typical *kokumi* taste substance (flavour enhancer) and it is abundantly present in food-grade yeast extract, which is commercially available and has been used to make foods taste savory and hearty [86, 98]. Although GSH itself is tasteless, it synergistically enhances the savoury taste several times in the presence of small amounts of umami taste substances such as monosodium glutamate or 5'mononucleotides [99]. Sensory analyses have shown that the *kokumi* taste intensity brought by GSH was much more significant when it was mixed into a solution such as chicken consommé or beef soup as a result of the coexistence of various flavors that taste sweet, salty and umami [98, 99].

2.4 METHODS FOR THE INDUSTRIAL PRODUCTION OF GLUTATHIONE

With the discovery of the presence of GSH in eukaryotic organisms, solvent extraction of GSH from animal or plant tissues was initially exploited as a preparative approach for GSH production. However, the relatively low intracellular content of GSH made the end product expensive which hampered the practical application of this method. Thus, other methods had to be considered for industrial GSH production. The three methods currently applied for GSH production on an industrial scale are: i) chemical synthesis; ii) *in vitro* enzymatic synthesis and iii) *in vivo* synthesis using producer microorganisms.

2.4.1 *Chemical synthesis*

More than 70 years ago Harington and Mead [100] used a modified version of benzylcarbonato method for a chemical synthesis of GSH. This method used N-benzylcarbonato amino acid derivatives to block the reactive amino terminus and a α-COOH ester of glutamate residue in order to bring the γ-COOH group into reaction. First, N-benzylcarbonylcystine was converted into the acid chloride and the latter coupled with glycine ester. The product, upon treatment with phosphonium iodide in acetic acid, was converted into cysteinylglycyl ester (I) which was conveniently isolated as the hydroiodide (see Figure 9). N-benzylcarbonylglutamic acid was then converted into the anhydride which with sodium methoxide in methyl alcohol yielded the α-monomethyl ester of N-benzylcarbonylglutamic acid. Treatment with phosphorus pentachloride converted the latter into the corresponding acid chloride (II) which was coupled with cysteylglycyl ester (III). The ester groups were then removed from III by a very careful hydrolysis in alkaline aqueous dioxan solution and the resulting acid was treated with phosphonium iodide under the usual conditions. This procedure yielded a tripeptide (IV) which proved to be identical to GSH. Although the process was commercialized in the 1950s, it was not favoured for industrial production because of its complexity. Moreover, the end product was an optically inactive (racemic) mixture of the D- and L-isomers. As only the L-isomer is physiologically active, an optical resolution was required to separate the two isomers which increased the process cost even higher. Because of this, chemical synthesis is nowadays almost never applied for the industrial production of GSH.

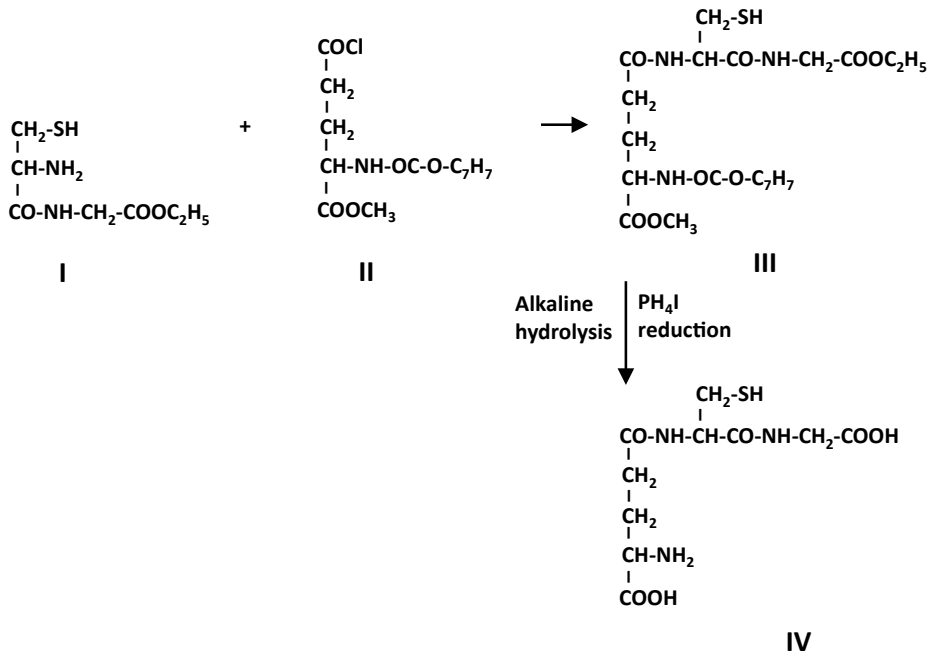

Figure 9. Chemical synthesis of GSH as devised by [100].
I — N-benzylcarbonylcystylglycine ethyl ester;
II — α-L-methyl N-benzylcarbonylglutamic chloride;
III — α-methyl N-benzylcarbonylglutamatocysteylglycine ethyl ester;
IV — GSH

2.4.2 *Enzymatic synthesis*

One of the well studied approaches for GSH production is *in vitro* enzymatic synthesis. Enzymatic methods (Table 1) use either free or immobilized enzymes in a bioreactor systems with added precursor amino acids as well as ATP and Mg^{2+}. The latter is a cofactor for the two GSH biosynthetic reactions. Enzymes from various microorganisms (*Saccharomyces cerevisiae*, *Escherichia coli*, *Proteus mirabilis*, *Proteus vulgaris*) are used. Depending on the nature of the enzyme (*i. e.*, the microorganisms where the enzymes are derived from), the process conditions may vary. For example, in case of yeast derived enzymes (Gsh1p and Gsh2p) and optimal temperature 30°C and pH 7.5 or temperature 35°C and pH 7.3 have been used [101, 102]. With *Escherichia coli* enzymes (*gshA* and *gshB*) a process at 37°C and pH 7.0 have been preferred [103].

Table 1. Various processes for the enzymatic production of GSH by microbial enzymes. Modified from [104].

Enzyme	ATP regeneration	GSH $mg \cdot L^{-1}$	Cysteine yield %	Ref.
Immobilized crude enzymes of Gsh1p, Gsh2p from *Saccharomyces cerevisiae* 500	Immobilized carbamylphosphokinase from *Streptococcus faecalis* R 600	325	58.6	[101]
Immobilized Gsh1p, Gsh2p from *Saccharomyces cerevisiae*		2517	82	[102]
Crude enzymes of *gshA*, *gshB* from *Escherichia coli* B ATCC 23226	Acetate kinase prepared from *Escherichia coli* B ATCC 23226	46.3	3	[103]
Cell-free extract from *Proteus mirabilis*	Dried yeast	1320	61.4	[105]
Crude *gshA*, *gshB* enzymes from *Proteus vulgaris* modified by N-ethylmaleimide		480	–	[106]

The requirement for ATP makes the enzymatic process difficult to scale-up, because it is economically impractical to add it on an industrial scale. For economic feasibility highly efficient ATP regeneration systems would be necessary. These systems can be briefly defined as the systems in which ATP-requiring reactions are coupled with ATP-producing reactions. Several ATP regeneration systems have been proposed [107, 108].

The advantage of enzymatic production is that a relatively high GSH concentrations (up to 9 $g \cdot L^{-1}$) without excess process-related by-products can be achieved [104]. This remarkably reduces the cost of the downstreaming processes. The drawbacks are high cost of the precursors and the need for ATP regeneration which adds complexity to the process setup. Because of this, enzymatic GSH production is, at the present day, less preferred than microbial production.

2.4.3 *Microbial production*

In vivo synthesis using different microorganisms followed by the extraction and purification of the product is currently the most common method for the commercial production of GSH [104]. Several microorganisms accumulate GSH and can thus be applied for GSH

production, however, the yeasts *Saccharomyces cerevisiae* or *Candida utilis* are by far the most widely used ones. The advantages of application of these yeasts are that they can naturally accumulate high intracellular concentrations of GSH. Furthermore, yeasts are fast growing organisms, can be grown to high cell densities on inexpensive substrates and are easy to handle on a large scale. Efficient GSH production using yeast cells relies on high-density cultivation of biomass with maximum GSH content at the time of biomass harvesting. One of the major drawbacks in using yeast as a cell factory for industrial GSH production is that the synthesis and intracellular accumulation of GSH are rigorously controlled by means of various complex molecular mechanisms.

The concentration of GSH in wild-type *Saccharomyces cerevisiae* strains are reported to occur within the range of 0.1–1.0% of the dry weight (dwt) of cells [35, 42]. This variation can be caused by several factors, such as growth conditions and the characteristics of individual strains. Different strategies have been proposed to increase GSH accumulation in yeast. To overcome substrate limitations, modulation of precursor amino acids, particularly cysteine, in the cultivation media has been suggested [55–58]. This can be combined with application of the producer strains with increased GSH biosynthetic capacity, obtained either by random mutagenesis or by means of genetic engineering techniques (see below in Section 2.5). Most studies to date have proposed a shot-wise cysteine addition using concentrations which are toxic to the cells and cause rapid growth inhibition [55–58, 104]. This may bring several negative impacts to industrial application, *e.g.*, increased process cost, inconsistent GSH accumulation and impact on the process waste water (which is a great challenge to yeast industries).

The extraction of GSH from yeast biomass is another important process step from an overall production efficiency point of view. Extraction with hot water (80-90°C) is commonly used in industrial GSH production [104]. Application of different solvents has also been studied. For example, Xiong et al. [109] described the extraction of GSH using 25% (v/v) ethanol at room temperature for 60 minutes. Such extraction process has many advantages, such as lesser consumption of energy and non-destruction of the cells resulting in a less protein extraction which reduce the complexity and cost of the purification process. On the other hand, considering that the solvent extraction does not have significant advantages with regards to extraction yield the cost of the process and environmental effect are definitely the limitations of solvent extraction.

Alternatively, induced biological excretion of GSH from cells can be used. This approach also reduces the feedback inhibition of intracellular GSH and overcome problems related to the toxicity of high intracellular GSH concentrations. Endogenously produced glutathione in the yeast cytosol has been shown to be secreted at low levels under normal growth conditions, which is then taken up again by the glutathione transporter (Opt1p). Deletion of Opt1p leads to threefold higher levels of GSH in the extracellular medium [110]. A recent genome wide study has tried to identify genes, which upon disruption, would lead to increased GSH secretion into the intracellular medium. Perrone et al. [110] were able to identify gene disruptants falling under 10 different functional classes that have lead to GSH over-excretion (2-37 fold) in the medium during the stationary growth phase. However, the exact mechanisms of GSH secretion are still not understood.

Another possible way to increase GSH secretion from the cells is by modulating the fermentation conditions. For instance, a low pH fermentation (pH 1.5) is one of the con-

ditions that has proved to lead to increased secretion of GSH [111]. Low concentrations of surfactants added to fermentation have also been used to achieve higher levels of glutathione in the extracellular medium without affecting significantly the growth and viability of the cells [42]. Glutathione secreted into the medium has been found to be predominantly in the reduced form at a GSH:GSSG ratio of 25–50:1 [42].

2.5 STRAIN IMPROVEMENT TECHNIQUES FOR ENHANCED GSH ACCUMULATION

Improving production efficiency by reducing the production cost is important to decrease the price of GSH. Considering that one of the major premises for the efficient microbial production of GSH is its high intracellular accumulation, overcoming the molecular regulatory mechanisms which restrict its over-accumulation has become a major challenge. Thus, a significant amount of research is currently being focused on producer strain improvement. This can be accomplished by means of genetic (metabolic) engineering or by random mutagenesis.

2.5.1 *Genetic engineering*

To improve GSH production, different genetically engineered microorganisms (*Saccharomyces cerevisiae*, *Escherichia coli*, *Lactococcus lactis*) have been used [112–115]. Early studies on the properties of Gsh1p and Gsh2p in yeast (or the same enzymes in other organisms) showed that these two enzymes are feedback-inhibited by GSH and GSSG, respectively [116]. Thus, overexpression of *GSH1* and *GSH2* genes have been the first attempts to improve GSH biosynthesis in the cells. For example, Gushima et al. [117] studied GSH biosynthesis in an *Escherichia coli* strain transformed with recombinat plasmids for enzymes *gshA* and *gshB* and observed a simultaneous increase in the activities of *gshA* (10.0-fold) and *gshB* (14.5-fold). Although the intracellular GSH concentration in such *Escherichia coli* cells increased only 1.3-fold compared with the wild type, the strain was an excellent GSH biosynthesis system, producing up to 5 $g{\cdot}L^{-1}$ of extracellular GSH in the presence of the three precursor amino acids. Similar work was done in *Saccharomyces cerevisiae*, where the expression of Gsh1p and Gsh2p increased by 1,039 times and 33 times, respectively, and intracellular GSH content increased twofold [118]. Grant et al. [119] over-expressed Gsh1p and Gsh2p in *Saccharomyces cerevisiae* and observed nearly 70% increase in intracellular GSH levels when cells were grown in a medium supplemented with appropriate concentrations of precursor amino acids. Notably, in most reported studies, the overexpression of either *GSH1* or *GSH2* (or both) in yeast have not led to more than twofold increase in intracellular GSH levels [42, 118, 119]. This can be due to: (1) feedback inhibition on GSH biosynthetic reactions, (2) excretion of excess GSH from the cells or (3) degradation of GSH by γ-glutamyltranspeptidase (γGT) (Ecm38p in yeast). An alternative strategy to avoid GSH degradation is to use yeast strains in which γGT has been deleted or naturally defective [37]. The reports on the exact effects of γGT deletion on GSH yields are scarce. However, an extremely high intracellular concentration of GSH (140 mM) upon addition of 5 mM L-cysteine was achieved in *Lactococcus lactis* expressing the genes *gshA* and *gshB* from *Escherichia coli* [115]. This is the highest intracellular GSH content achieved in a bac-

terium to date and could be explained by the fact that *Lactococcus lactis* is naturally not capable of synthesizing GSH and consequently lacks γGT activity as well as the feedback inhibition on biosynthetic reactions.

Very recently, Hara et al. [120] showed that engineering of sulfur metabolism in *Saccharomyces cerevisiae* proved to be a more valuable method to boost GSH production than the simple addition of cysteine to a yeast culture. They enhanced the GSH productivity by increasing the supply of cysteine by metabolically engineering sulfur metabolism. GSH was produced by mutant strains which over-expressed adenylylsulfate kinase (*MET14*) and PAPS reductase (*MET16*), the genes involved in sulfate assimilation pathway. As the result the intracellular GSH content increased up to 1.2 and 1.4-fold higher than that of the parental strain, respectively. Such metabolic engineering of a sulfate assimilation reactions allowed to avoid cell growth inhibition related to externally added cysteine toxicity and increase the overall process yield.

2.5.2 *Random mutagenesis*

In addition to genetically engineered strains, several mutation strategies have been described in the literature for the isolation of GSH over-accumulating variants. The physical or chemical mutagenesis methods used included UV, X-radiation, γ-radiation, and *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine treatment, while resistance to highly toxic compounds such as ethionine, 1,2,4-triazole and sodium cyanide was used to screen for GSH overproducers [104]. Notably, random mutagenesis has resulted in selection of much more efficient GSH producers than genetic engineering. For example, Lai *et al.* [121] were able to select a mutant which accumulated GSH in fourfold higher concentrations (32-34 mg/g dry cell weight) than the respective wild-type strain. The mutant qualified for use in industrial fermentation because it was stable and retained the ability to produce GSH with high yield even after thirty generations and after three-years storage. Although GSH over-accumulation has been related to mutations in the *GSH1* gene and the resulting disruption or release of the feedback inhibition of GSH on Gsh1p, there have been no reports confirming this hypothesis to date.

The major problem with random mutagenesis is that the mutations are uncontrolled and may cause various undesired side-effects, such as growth inhibition *et cetera* [122, 123]. This usually hampers an efficient application of many selected GSH overproducing mutants. Nevertheless, the metabolic analysis of such mutants, in order to reveal biological mechanisms which underlie the higher GSH accumulation, could assist in the generation of improved GSH accumulating yeast strains by means of genetic engineering.

3

AIMS OF THIS DISSERTATION

The general aim of the present study is to gain insight into the molecular mechanisms responsible for regulation of intracellular GSH levels in an industrial baker's yeast strain of *Saccharomyces cerevisiae* and a random mutagenized glutathione over-accumulating isolate of this strain under conditions similar to an industrial yeast production process. Specifically, the aims are as follows:

I To develop strategies for addition of precursor amino acids for the commercial production of GSH enriched yeast.

II To identify dominating molecular mechanisms which limit GSH accumulation in *Saccharomyces cerevisiae*.

III To identify molecular mechanisms which explain the higher observed GSH accumulation in the random mutagenized glutathione over-accumulating isolate and use inverse metabolic engineering to construct a new GSH producer strain.

4

MATERIALS AND METHODS

More detailed descriptions of the materials and methods applied are available in the publications. The following sections are provided to make this material more accessible.

4.1 YEAST STRAINS

A commercial baker's yeast strain *Saccharomyces cerevisiae* LYCC7048 (Lallemand Yeast Culture Collection, Lallemand, Inc.) was kindly provided by Lallemand, Inc. (Montreal, Canada). The same yeast strain was used to produce a UV-mutagenized GSH over-accumulating mutant (LYCC6267-56267) (Dr. Matti Korhola, Alkomohr OY, Helsinki, Finland). The mutant was selected for its resistance to 1,2,4-triazole and azide after UV mutagenesis as described by Hamada et al. [124] and accumulated GSH in the concentrations that were more than twofold higher than in its wild-type parent strain. *YAP1* over-expressing mutant (S288C *+pRS426-YAP1*) was constructed using the *Saccharomyces cerevisiae* laboratory strain S288C (*MATa/MATα ura3-52/ura3-5 trp1Δ63/+ leu2Δ1/+*) (Publication III).

4.2 CULTIVATION METHODS AND MEDIA

A series of ethanol-stat fed-batch cultivations ($\mu \approx \mu_{crit}$, $EtOH_s$ = 0.2 $g \cdot L^{-1}$) with a switch to continuous addition of GSH precursor amino acids (cysteine and glycine) were carried out in a 7 L "BioBench" fermenter (Applikon, The Netherlands). The cultivation system was equipped with an ADI 1030 bio-controller, the cultivation control software "BioXpert", the necessary bio-sensors (pO_2, pH, temperature, ethanol, CO_2), two variable speed pumps (for main feeding medium and for the one containing precursor amino acids, respectively) and a fixed speed pump for titration. Fed-batch cultivations were performed using mineral feeding media with and without the precursor amino acids, respectively (Publication I). The concentrations of precursor amino acids cysteine and glycine in the feeding media were 56, 112, and 225 $\mu mol \cdot g^{-1}$ of sucrose, respectively.

Continuous cultivation experiments with GSH over-accumulating mutant and its wild-type parent strain were carried out in the chemostat (D = 0.1 h^{-1}) and turbidostat cultivation conditions (OD_{set} = 20 U at 600 nm), using a 1 L Biobundle fermenter (Applikon, The Netherlands) equipped with the bio-sensors, pumps and software as described above. Chemostat cultivations (D = 0.07 h^{-1}) were also used to study the GSH and cysteine accu-

mulation in the *YAP1* over-expressed mutant. Both chemostat and turbidostat cultivations were carried out using defined mineral feeding media (Publication II).

4.3 ANALYTICAL METHODS

Culture samples (6-7 mL) were collected rapidly into 15 mL tubes on ice; 1 mL aliquots (with the biomass dry weight $\approx$ 20 mg) were distributed into six Eppendorf microcentrifuge tubes and centrifuged to wash and recover the biomass. The biomass was washed once with ice-cold Rnase-free (dimethyl pyrocarbonate-treated) water. The washed biomass samples and the culture supernatant were snap frozen in liquid nitrogen and stored at -80°C until required. Total processing of the samples did not take more than 5 minutes. All analyses were performed in triplicate. The exact concentration of biomass was determined by weighing the cell pellets after drying in the oven at 105°C for 24 hours.

Intracellular thiols (cysteine, γGCS and GSH) were determined by UPLC® after extraction with 0.1 N formic acid (70°C, 1 h) and derivatization with 5, 5′-dithiobis-(2-nitrobenzoic acid) (DTNB) (Publication I). The concentration of cysteine in the cultivation medium was expressed as the sum of the concentration of reduced and oxidized cysteine, determined simultaneously by the two UPLC®s using derivatization with DTNB and an AccQFluor kit (Waters, USA), respectively.

Expression levels of the selected genes involved in GSH and sulfur metabolism (*GSH1*, *GSH2*, *ECM38*, *SUL1*, *MET17*, *CYS3*, *CYS4*, *STR2*, *STR3*, *YAP1*, and *MET4*) were monitored by a solution hybridization method (TRanscript Analysis with the aid of Affinity Capture (TRAC)) using a pool of oligonucleotide probes designed for the respective target genes, separable and quantifiable by capillary electrophoresis. Details about the exact procedure of the transcript analysis is described in Publication I.

The comparative analysis of expression of proteins in the mutant and its parent strain was carried out using labeling with iTRAQ 4plex reagents (Applied Biosystems, Foster City, CA), followed by analysis with LC-MS/MS. The data generated was compared against *Saccharomyces cerevisiae* sequence data (KEGG Genes Database 20/04/2010) using the MassMatrix search tool. Details about the exact procedure of the analysis is described in Publication II.

In order to determine the possible mutations in *GSH1*, the gene was amplified by PCR in two fragments ($\approx$ 1,200 base pairs) and cloned into a pJET1.2 vector (Fermentas, Lithuania). Four clones per fragment were sequenced using a BigDye kit (Applied Biosystems, USA) plasmid specific primers flanking the cloning site. The genomic sequences obtained were analyzed using BLAST.

4.4 SYSTEM EQUATIONS AND DATA PROCESSING

The specific rates of Gsh1p and Gsh2p reactions ($Q_{Gsh1p}$and Q_{Gsh2p}, $\mu mol \cdot g^{-1} \cdot h^{-1}$) were calculated using the following equations.

For fed batch cultures:

$$Q_{Gsh2p} = \frac{d[GSH_X \cdot X \cdot V]}{dt[X \cdot V]} \tag{4.1a}$$

$$Q_{Gsh1p} = Q_{Gsh2p} + \frac{d[\gamma GCS_X \cdot X \cdot V]}{dt[X \cdot V]} \tag{4.1b}$$

For continuous cultures:

$$Q_{Gsh2p} = \frac{\frac{dGSH_X}{dt} + F_{out} \cdot X \cdot GSH_X}{X \cdot V} \tag{4.2a}$$

$$Q_{Gsh1p} = Q_{Gsh2p} + \frac{\frac{d\gamma GCS_X}{dt} + F_{out} \cdot X \cdot \gamma GCS_X}{X \cdot V} \tag{4.2b}$$

where GSH_X and γGCS_X are the concentrations of glutathione and γ-glutamylcysteine in the biomass ($\mu mol \cdot g^{-1}$); F_{out} is the culture outflow from the fermenter ($L \cdot h^{-1}$); V is the current fermenter volume (L); X is the biomass concentration in the fermenter ($g_{dwt} \cdot L^{-1}$); t is time (h).

5

RESULTS AND DISCUSSION

The results of this dissertation are presented and discussed in four sections. Each section presents the results of one complete study.

5.1 SUPPLEMENTATION OF GSH PRECURSOR AMINO ACIDS

Continuous feeding of cysteine and glycine was applied in an ethanol-stat fed-batch culture of commercial baker's yeast *Saccharomyces cerevisiae* LYCC7048. Glutamate, a highly abundant amino acid in cells, was excluded from the feeding media. This feeding strategy was applied to decrease the inhibiting effect of cysteine on biomass growth that ocurrs with shot-wise addition. In addition, this experiment allowed us to study the kinetics of glutathione accumulation in response to precursor amino acid supplementation (Publication I). The culture at near-maximum respiratory growth rate ($\mu \approx \mu_{crit}$) was grown overnight in fed-batch fermenter, and a shift from a basic mineral medium to one containing precursor amino acids was carried out at a biomass concentration of ≈ 40 $g_{dwt} \cdot L^{-1}$.

In all three experiments the shift to a feeding medium enriched with cysteine and glycine caused an immediate increase in the synthesis rates of γGCS (Q_{Gsh1p}) and GSH (Q_{Gsh2p}) (see Figure 10), suggesting that GSH accumulation was limited by the availability of cysteine. This effect was more pronounced at higher cysteine to sucrose ratios in the feed. The highest specific accumulation rate of GSH in all experiments was observed during the first 2 hours following the media shift during which time we observed the maximum GSH concentration of 35 – 45 $\mu mol \cdot g^{-1}$. Maximum GSH yields, based on cysteine consumption, were similar in all experiments (0.35–0.4 $mol \cdot mol^{-1}$). Notably, both the GSH yield as well as the specific synthesis rates of γGCS and GSH started to decrease when the concentration of intracellular GSH was higher than 30 $\mu mol \cdot g^{-1}$, suggesting that an onset of feed-back inhibition on GSH biosynthesis.

The added cysteine had an effect on the yeast growth rate and biomass yield in a concentration-dependent manner, *i. e.*, being negligible at a ratio of 56 $\mu mol \cdot g^{-1}$ sucrose and progressively inhibiting at the two higher concentrations (112 and 225 $\mu mol \cdot g^{-1}$ sucrose) (see Figure 10). We show that the inhibition of growth under conditions of increased intracellular free cysteine can be related to accumulation of H_2S. Notably, a remarkable down-regulation in the expression of homocysteine synthase (*MET17*) which is involved in homocysteine biosynthesis from sulfide (see Figure 4) was observed when cysteine supplementation was started (see Figure 11). This down-regulation was accom-

panied by shunting the sulfide flux toward H_2S production resulting in a repression of respiration and a decrease in specific growth rate. H_2S as well as sulfide have been reported to inhibit cellular respiration *via* cytochrome c oxidase (the last enzyme in the respiratory electron transport chain of mitochondria, located in the mitochondrial membrane) activity inhibition [125–128]. Thus, from the process efficiency point-of-view the continuous addition of cysteine at its optimized concentration (50 – 60 $\mu mol \cdot g^{-1}$ sucrose) in the feeding would be preferred over shot-wise supplementation.

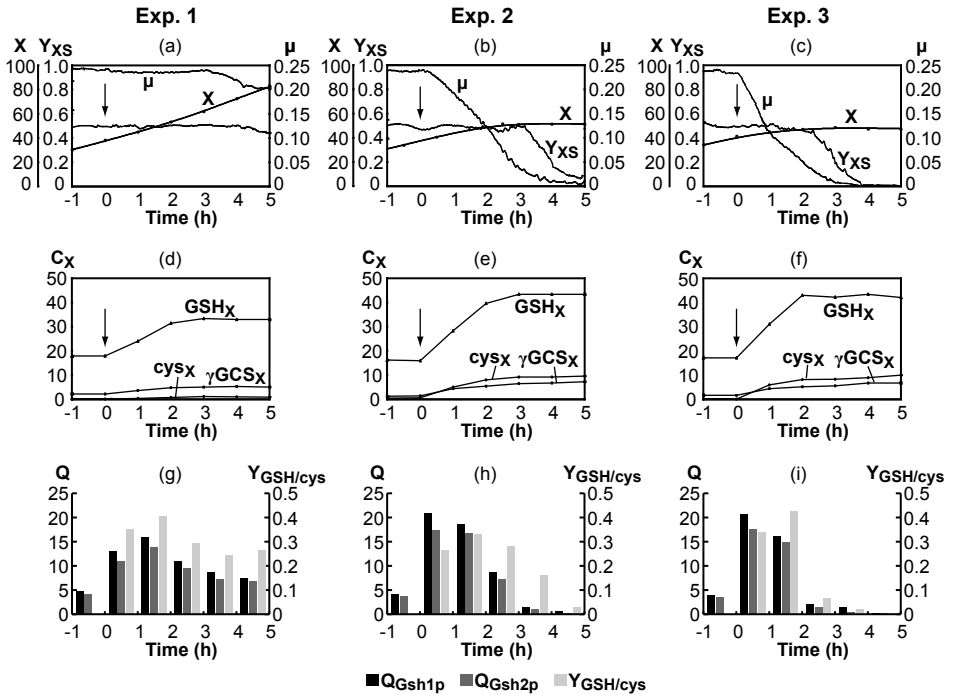

Figure 10. Ethanol-stat fed-batch cultivation of *Saccharomyces cerevisiae* LYCC7048 with a shift to a feeding medium containing cysteine and glycine: 56 $\mu mol \cdot g^{-1}$ sucrose in Exp. 1; 112 $\mu mol \cdot g^{-1}$ sucrose in Exp. 2; 225 $\mu mol \cdot g^{-1}$ sucrose in Exp. 3. X is the biomass concentration in the cultivation medium ($g \cdot L^{-1}$); Y_{XS} is the biomass yield based on substrate (sucrose) consumption ($g \cdot g^{-1}$); μ is the specific growth rate (h^{-1}); cys_X, γGCS_X, GSH_X are the concentration of cysteine, γGCS, and GSH in the cells, respectively ($\mu mol \cdot g^{-1}$); Q_{Gsh1p}, $Q_{Gsh2p}$are the hourly averages of specific γGCS and GSH synthesis rates, respectively ($\mu mol \cdot g^{-1} \cdot h^{-1}$); $Y_{GSH/cys}$ is the hourly average of cysteine accumulation yield into intracellular GSH ($mol \cdot mol_{cys}^{-1}$). The arrows denote the switch to the cultivation media containing cysteine and glycine. (Publication I)

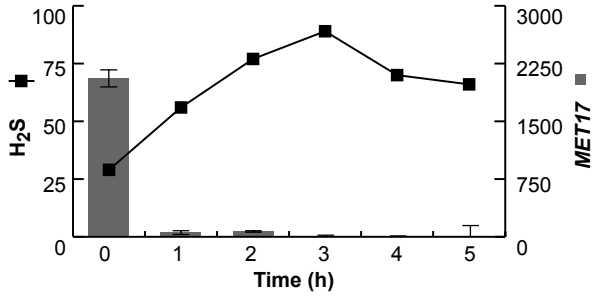

Figure 11. Expression of the gene *MET17* (in fluorescence intensity units) and the concentration of H_2S in culture medium ($\mu g \cdot L^{-1}$) in an ethanol-stat fed-batch cultivation of *Saccharomyces cerevisiae* LYCC7048 after a shift to a feeding medium containing cysteine and glycine (225 $\mu mol \cdot g^{-1}$ sucrose). (Publication I)

5.2 GSH ACCUMULATION IN THE MUTANT AND WILD-TYPE STRAIN

The intracellular concentrations of GSH and its precursors in the mutant and its parent strain were compared under different physiological conditions using different cultivation strategies: i) fully respiratory metabolism ($\mu < \mu_{crit}$) using chemostat cultivation; ii) near-overflow metabolism ($\mu \approx \mu_{crit}$) using ethanol-stat fed-batch cultivation and iii) fermentative metabolism ($\mu = \mu_{max}$) using turbidostat cultivation (Publication I and Publication II).

The concentration of GSH in the mutant (40 – 50 $\mu mol \cdot g^{-1}$) was more than twofold higher than in the wild-type parent strain under all cultivation conditions studied (Table 2). These results are well in accordance to what has been reported for industrial *Saccharomyces cerevisiae* strains used for biotechnological GSH production [104]. Notably, the concentrations of other intracellular thiols (cysteine and γGCS) were also markedly higher in the mutant. According to this, the corresponding calculated Gsh1p and Gsh2p reaction rates were more than two times greater than observed in the wild-type strain. It was not possible to draw clear conclusions about the relationships between the growth mode and GSH accumulation, except that at higher growth rates the specific reaction rates of Gsh1p and Gsh2p were higher. The results suggest that, besides a possible lack of feedback inhibition on the Gsh1p reaction, the higher GSH accumulation in the mutant may be caused also by both higher cysteine availability and higher fluxes through GSH biosynthetic reactions. This is also supported by the fact that with cysteine supplementation to the wild-type yeast the maximum observed GSH concentration was very similar to that in the mutant.

Table 2. Concentration of intracellular cysteine, γ-glutamylcysteine (γGCS) and glutathione (GSH) and the specific reaction rates of Gsh1p (Q_{Gsh1p}) and Gsh2p (Q_{Gsh2p}) determined for mutant and wild-type strain under three different growth conditions.

Parameter	Units	Value	
		Wild type	Mutant
Chemostat cultivation		$\mu = D = 0.1 \cdot h^{-1}$	
cysteine	$\mu mol \cdot g^{-1}$	0.4 ± 0.02	1.91 ± 0.18
γGCS	$\mu mol \cdot g^{-1}$	3.98 ± 0.64	28.2 ± 0.6
GSH	$\mu mol \cdot g^{-1}$	23.3 ± 1.6	50.1 ± 1.3
Q_{Gsh1p}	$\mu mol \cdot g^{-1} \cdot h^{-1}$	2.5 ± 0.3	7.5 ± 0.2
Q_{Gsh2p}	$\mu mol \cdot g^{-1} \cdot h^{-1}$	2.3 ± 0.1	4.8 ± 0.1
Ethanol-stat fed-batch cultivation		$\mu \approx \mu_{crit}$	
μ_{crit}	h^{-1}	0.190 ± 0.005	0.125 ± 0.005
cysteine	$\mu mol \cdot g^{-1}$	0.22 ± 0.11	0.87 ± 0.13
γGCS	$\mu mol \cdot g^{-1}$	2.8 ± 0.1	13.4 ± 0.5
GSH	$\mu mol \cdot g^{-1}$	12.7 ± 1.8	41.6 ± 2.9
Q_{Gsh1p}	$\mu mol \cdot g^{-1} \cdot h^{-1}$	2.9 ± 0.2	8.2 ± 0.7
Q_{Gsh2p}	$\mu mol \cdot g^{-1} \cdot h^{-1}$	2.3 ± 0.2	6.4 ± 0.3
Turbidostat cultivation		$\mu \approx \mu_{max}$	
μ_{max}	h^{-1}	0.293 ± 0.018	0.230 ± 0.007
cysteine	$\mu mol \cdot g^{-1}$	N/A	N/A
γGCS	$\mu mol \cdot g^{-1}$	0.8 ± 0.2	4.5 ± 0.7
GSH	$\mu mol \cdot g^{-1}$	12.8 ± 1.0	50.1 ± 1.8
Q_{Gsh1p}	$\mu mol \cdot g^{-1} \cdot h^{-1}$	3.7 ± 0.3	14.1 ± 2.7
Q_{Gsh2p}	$\mu mol \cdot g^{-1} \cdot h^{-1}$	3.7 ± 0.3	13.0 ± 2.4

5.3 METABOLIC CHANGES UNDERLYING GSH OVER-ACCUMULATION IN THE MUTANT

First, we aimed to identify the dominant regulatory mechanism (transcriptional, posttranslational, or substrate availability level) that prevents GSH from over-accumulation in wild-type yeast. Following this we studied whether these mechanisms are weakened or completely missing in the GSH over-accumulating mutant. For this, we conducted comparative fed-batch fermentations between the mutant strain and the wild type. In both fermentations we employed a switch to a feeding medium containing precursor amino acids cysteine and glycine at two different concentrations (56 and 112 $\mu mol \cdot g^{-1}$ sucrose, respectively) (Publication II). In contrast with the wild-type parent strain (*Saccharomyces cerevisiae* LYCC7048) cysteine addition to the mutant culture did not cause a

rapid increase in the specific rate of the Gsh1p reaction (see Figure 12), suggesting that feedback regulation mechanisms that prevent GSH from over-accumulating likely still function in the mutant.

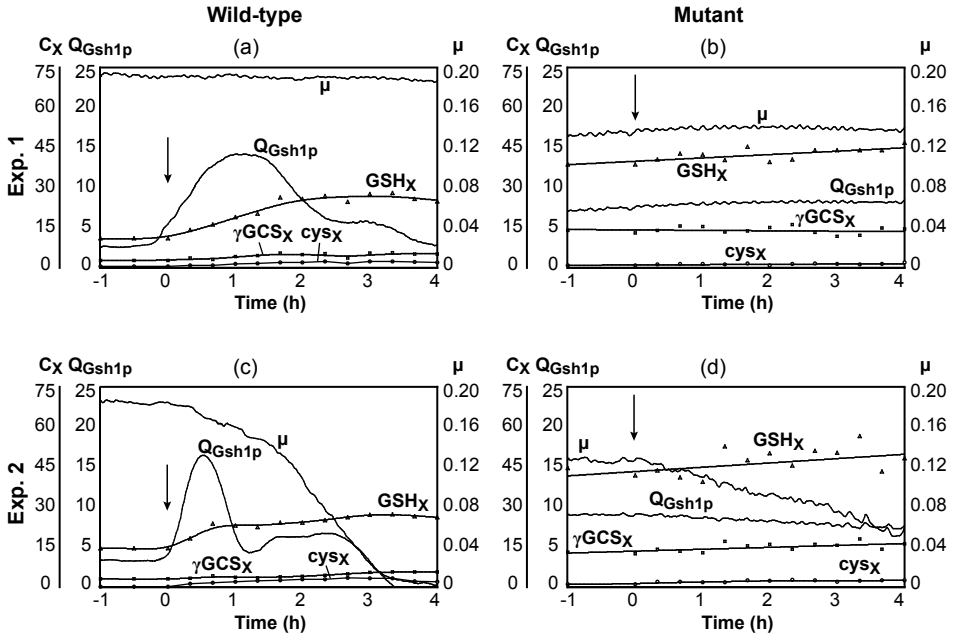

Figure 12. Ethanol-stat fed-batch cultivation of the wild-type strain and mutant with switch to feeding containing cysteine: 56 μmol·g^{-1} sucrose in Exp. 1 and 112 μmol·g^{-1} sucrose in Exp. 2. C_X is the concentration of cysteine (cys_X), γ-glutamylcysteine (γGCS_X), and glutathione (GSH_X) in the biomass (μmol·g^{-1}), $Q_{Gsh1p}$is the specific rate of the Gsh1p reaction (μmol·g^{-1}·h^{-1}), μ – specific growth rate (h^{-1}). (Publication II)

In order to study the GSH regulation mechanisms further, we performed a transcript analysis of *GSH1* and *GSH2* genes before and during cysteine addition (see Figure 13). The results do not show any remarkable down-regulation in the expression of *GSH1* and *GSH2* genes in the two strains. This suggests that, as with its parent strain, further accumulation of GSH in the mutant is controlled at the post-translational level, rather than at the transcriptional level. To check this, we performed a comparative *GSH1* sequence analysis in both strains, which did not indicate that any mutations disrupted or changed the feedback inhibition of GSH on Gsh1p in the mutant.

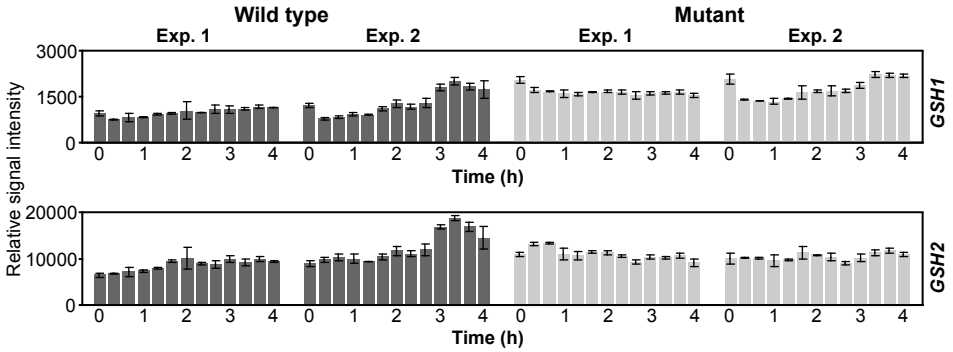

Figure 13. Expression of the target genes *GSH1* and *GSH2*, relative to an internal hybridization control and the expression of reference genes (*ACT1* and *SHR3*) after the switch to cysteine supplemented feeding medium: 56 $\mu mol_{cys} \cdot g^{-1}$ sucrose in Exp. 1; 112 $\mu mol_{cys} \cdot g^{-1}$ sucrose in Exp. 2. The means of duplicates are shown $\pm$ average absolute deviation. The numbers under the columns represent the cultivation time (in hours from the switch of the feeding media).

Thus, other factors are at play which cause $\approx$ 2.5 times higher GSH accumulation in the mutant strain. We then carried out a comparative analysis of the genes involved in the sulfate assimilation pathway, cysteine metabolism, and GSH metabolism, together with proteome analysis (Publication II). This transcript analysis revealed that the mRNA levels of genes involved in the cysteine and GSH biosynthesis pathways (*CYS3*, *CYS4*, *GSH1* and *GSH2*), but also the transcriptional activator *YAP1*, were significantly higher in the mutant (see Figure 14). Differences in the mRNA levels of *MET4*[1] and other genes, were not observed. These results are also in accordance with the quantitative proteome analysis (data not illustrated). A total of 660 proteins, including Gsh2p, Cys3p, and Cys4p were examined in both strains. While the majority of the proteins analyzed, including Gsh2p and Cys4p, did not show a statistically significant difference in their level of expression (*i. e.*, more than a 1.5-fold difference between the expression levels), Cys3p was expressed at a level more than two times higher in the mutant strain.

These results suggest that the over-accumulation of GSH in the mutant was caused by the higher expression of *CYS3* and *CYS4*, leading to an increase in the rate of cysteine biosynthesis, and also by higher expression of *GSH1* and *GSH2*, leading to higher Gsh1p and Gsh2p levels and higher rates of their respective reactions. While the latter was most likely caused by higher levels of Yap1p transcriptional activator, it remained to be elucidated whether *CYS3* and *CYS4* and the resulting increased cysteine biosynthesis were Yap1p-dependent.

1 *MET4* is a transcription factor which governs the MET genes network which is responsible for the assimilation of extracellular sulfate into methionine and cysteine

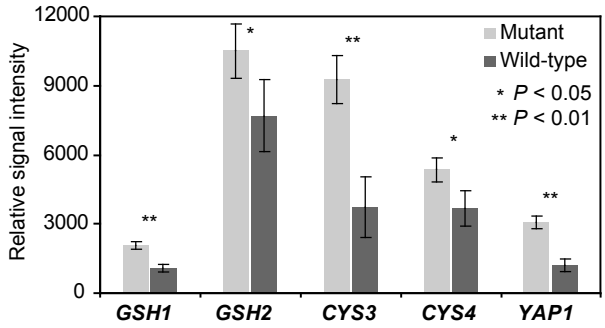

Figure 14. The initial expression levels of the genes of cysteine and GSH biosynthesis relative to an internal hybridization control and the expression of reference genes (*ACT1* and *SHR3*) in wild-type and mutant strains. The averages of two independent experiments are shown ± standard deviation. The mean values were compared using a two-sample Student's t-test. (Publication II)

5.4 OVER-EXPRESSION OF *YAP1* INCREASES BOTH CYSTEINE AVAILABILITY AND THE RATE OF GSH BIOSYNTHESIS

The mRNA analysis of selected genes in the GSH over-accumulating mutant showed that, in addition to higher expression of *GSH1* and *GSH2*, also *CYS3* and *CYS4* were more highly expressed along with higher *YAP1* expression. This suggests that, in addition to *GSH1* and *GSH2*, also *CYS3* and *CYS4*, currently known as the MET-genes, may be also Yap1p-controlled. To verify this hypothesis, a *YAP1* over-expressing mutant of laboratory strain *Saccharomyces cerevisiae* S288C was constructed and the accumulation of cysteine and GSH as well as the expression of the genes in the biosynthesis pathways of both compounds was observed in chemostat culture (Publication III). A comparative gene expression analysis showed that, in addition to *GSH1* and *GSH2*, the expression of which is well-known to be under control of Yap1p, also *CYS3* and *CYS4* have several fold higher expression levels than in the parent strain (see Figure 15). The resulting GSH concentration in the mutant was over twofold higher than that in the parent strain (32.5 ± 0.3 and 14.5 ± 0.1 $\mu mol \cdot g^{-1}$, respectively). Notably, the concentration of cysteine in the *YAP1* over-expressing mutant was also significantly higher than in the parent strain (17.7 ± 2.6 and 12.4 ± 1.0 $\mu mol \cdot g^{-1}$, respectively). This suggests a combined effect of *YAP1* over-expression on GSH accumulation *via* transcriptional activation of the biosynthesis genes of both GSH and its precursor cysteine. The over-expression of *YAP1* could thus be applied for the improvement of GSH producer strains.

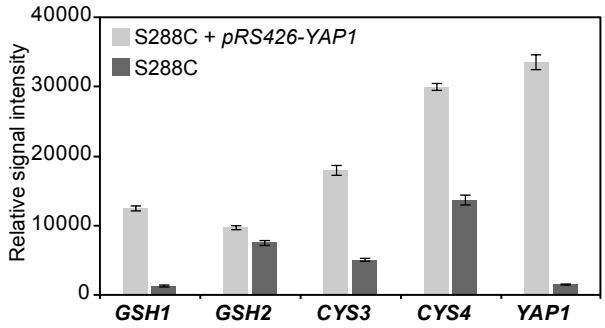

Figure 15. The expression levels of the key genes of cysteine and GSH biosynthesis relative to internal hybridization control and the expression of reference genes *ACT1* and *SHR3* (determined using TRAC) in wildtype and *YAP1* over-expressed strains. The average levels from two independent glucose-limited chemostat experiments are shown ± standard deviation. The mean values were compared using two-sample Student's t-test and $p < 0.01$ in all cases. (Publication III)

SUMMARY

6

CONCLUSIONS

THREE CONCLUSIONS RESULT FROM THIS DISSERTATION.

I Cysteine availability and the feedback inhibition of Gsh1p activity are the key factors that determine GSH concentration and restrict the over-accumulation of GSH in the industrial strain of *Saccharomyces cerevisiae* we studied.

II Higher GSH accumulation in the UV-mutant is related to the combined effects of higher expression of enzymes responsible for cysteine (Cys3p and Cys4p) and GSH (Gsh1p and Gsh2p) biosynthesis.

III The over-expression of a single gene *YAP1* allows one to satisfy two important premises for high GSH accumulation in yeast — i) increase cysteine biosynthesis by inducing the expression of *CYS3* and *CYS4*, and ii) increase the fluxes through GSH biosynthesis by inducing the expression of *GSH1* and *GSH2*.

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APPENDICES

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Glutathione accumulation in ethanol-stat fed-batch culture of *Saccharomyces cerevisiae* with a switch to cysteine feeding

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BIOTECHNOLOGICAL PRODUCTS AND PROCESS ENGINEERING

Glutathione accumulation in ethanol-stat fed-batch culture of *Saccharomyces cerevisiae* with a switch to cysteine feeding

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Abstract Shot-wise supplementation of cysteine to a yeast culture is a common means of promoting glutathione (GSH) production. In the present work, we study the accumulation kinetics of cysteine, γ-glutamylcysteine, and GSH and the expression of genes involved in GSH and sulfur metabolism in ethanol-stat fed-batch cultures as a result of switching to a medium enriched with cysteine and glycine. Supplementation in this fashion resulted in a rapid but short-term increase in the rate of GSH synthesis, while the expression of *GSH1* decreased. Expression of *GSH1* and GSH synthesis rate were observed to revert close to the base level after a few hours. These results indicate that, under such conditions, the control of GSH synthesis at higher concentrations occurred at the enzymatic, rather than the transcriptional level. The incorporation of cysteine into GSH was limited to ~40% of the theoretical yield, due to its requirement as a source of sulfur for protein synthesis under conditions whereby the sulfate assimilation pathway is down-regulated. This was supported by the expression profiles of genes involved in cysteine and homocysteine interconversion.

Keywords Glutathione · Cysteine · Ethanol-stat · Fed-batch · TRAC · *Saccharomyces cerevisiae*

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Introduction

The non-protein thiol glutathione (GSH) is a physiologically important tripeptide, which is synthesized in most eukaryotes (Meister and Anderson 1983; Pócsi et al. 2004) and in several prokaryotes (Fahey et al. 1978). Under standard conditions, GSH is required for protein and DNA synthesis and amino acid transport (Douglas 1987). The role of GSH under adverse conditions is primarily related to the defense of cells in response to a variety of environmental challenges, such as oxidative stress, toxification with heavy metals and xenobiotics, as well as nutrient depletion (Grant and Dawes 1996; Penninckx 2000; Soltaninassab et al. 2000; Grant 2001). In addition to its physiological role, GSH is also, due its antioxidative properties, widely used as a pharmaceutical compound and also has the potential to be used by the cosmetic and food industries (Sies 1999). Consequently, there is an increasing demand for the production of this tripeptide on an industrial scale. Although GSH can be produced by chemical or enzymatic synthesis, microbiological production using the yeasts *Saccharomyces cerevisiae* and *Candida utilis* is currently the most common method for

the commercial production of GSH (Murata and Kimura 1990; Li et al. 2004).

The concentration of GSH in wild-type *S. cerevisiae* strains has been reported to occur within the range of 0.1–1.0% of the dry weight (dwt) of cells (Li et al. 2004). This variation can be caused by several factors, such as growth conditions and the characteristics of individual strains. GSH is synthesized in two sequential ATP-dependent reactions, catalyzed firstly by γ-glutamylcysteine (γGCS) synthetase (Gsh1p) and secondly by GSH synthetase (Gsh2p). Analysis of GSH biosynthesis in different eukaryotes has suggested that cellular levels are controlled at both the transcriptional and post-translational levels (Richman and Meister 1975; Soltaninassab et al. 2000; Wheeler et al. 2002; Pócsi et al. 2004) and that the first reaction, catalyzed by Gsh1p, is the rate-limiting step (Huang et al. 1988; Meister 1988; Murata and Kimura 1990; Grant et al. 1997). Post-translational regulation is a non-allosteric feedback inhibition of Gsh1p activity by GSH (Soltaninassab et al. 2000). At the transcriptional level, the expression of *GSH1* is regulated by coupling of Met4p, a transcription factor which governs the *MET* gene network in the biosynthesis of the sulfur-containing amino acids (Thomas and Surdin-Kerjan 1997) and a redox-sensitive Yap1p transcription factor (Wheeler et al. 2002, 2003). It has been suggested that Met4p could be regulated in response to the availability of methionine and GSH (Wheeler et al. 2003). More recently, Menant et al. (2006) proposed that the intracellular cysteine, and not GSH or methionine, is responsible for Met4p regulation. In addition to transcriptional and post-translational regulation of the Gsh1p reaction step, cysteine has been shown to be the major limiting precursor for GSH accumulation (Alfafara et al. 1992a; Wen et al. 2004) and thus, the regulation of sulfur assimilation pathways also becomes important from the perspective of cysteine biosynthesis.

Efficient GSH production using yeast cells relies on high-density cultivation of biomass with maximum possible GSH content at the point of harvesting. Different cultivation conditions for increasing the intracellular GSH concentration using fed-batch cultures have been studied (Alfafara et al. 1992b; Sakato and Tanaka 1992; Wen et al. 2006; Zhang et al. 2007; Wang et al. 2007; Liang et al. 2008a, b). With few exceptions, these works rely on the addition of precursor amino acids of GSH, particularly cysteine, to the yeast culture. However, the primary aim of these studies was to determine the optimum conditions required to maximize the GSH content in yeast and not to investigate the metabolic changes resulting from increased cysteine concentration in cells. To the best of our knowledge, the kinetics of GSH accumulation under conditions of increased cysteine concentration in *S. cerevisiae* has not been studied to date. The aim of the present work is to describe the physiological capacity of baker's yeast to promote GSH synthesis in high biomass density fed-batch cultures with continuous cysteine and glycine addition.

Materials and methods

Yeast strain and cultivation media composition

A commercial *S. cerevisiae* baker's yeast (strain #7048, Lallemand Yeast Culture Collection, Lallemand, Inc.), kindly provided by AS Salutaguse Pärmitehas (Tallinn, Estonia) was used in all experiments. Fed-batch cultivations were performed using mineral media. The feeding media composition, described by van Hoek et al. (2000) and Walker (2004) was used with some modifications. Starting medium (1.9 L) comprised 17 g L^{-1} KH_2PO_4, 6 g L^{-1} $MgSO_4{\cdot}7H_2O$ and 7 g L^{-1} NH_4Cl. The basic feeding medium contained 400 g L^{-1} sucrose, 30 mg L^{-1} $CaCl_2{\cdot}2H_2O$, 150 mg L^{-1} $ZnSO_4{\cdot}7H_2O$, 150 mg L^{-1} $FeSO_4{\cdot}7H_2O$, 50 mg L^{-1} $CuSO_4{\cdot}5H_2O$, 40 mg L^{-1} $MnSO_4{\cdot}H_2O$, 10 mg L^{-1} $CoSO_4{\cdot}7H_2O$, 2 mg L^{-1} $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$, 250 mg L^{-1} myo-inositol, 10 mg L^{-1} thiamine, 10 mg L^{-1} Ca-pantothenate, 10 mg L^{-1} pyridoxine, 10 mg L^{-1} nicotinamide, and 5 mg L^{-1} biotin. Microelements and vitamins were sterilized separately by microfiltration and added aseptically to the autoclaved feeding medium prior to experimentation. The pH of the feeding medium was adjusted to pH 4.6, using 1 N H_2SO_4. Precursor amino acids (cysteine and glycine) containing feeding media (56, 112, and 225 μmol of each per gram of sucrose) were prepared by dissolving the amino acids in deoxygenated (flushed with N_2 for at least 20 min) basic feeding medium. Notably, glycine was included in equimolar concentrations with cysteine to prevent its possible limitation and any effects of this possible limitation on the experimental results.

Cultivation system and cultivation process routines

A series of fed-batch cultivations using different cysteine and glycine concentrations in the feeding (exp. 1, exp. 2, and exp. 3) and a reference experiment without cysteine addition were carried out in a 7 L "BioBench" fermenter (Applikon, The Netherlands), equipped with pO_2, pH, O_2, CO_2, and temperature sensors. Ethanol concentration was measured online using a "Chemgard" infrared gas monitor (Mine Safety Appliances, USA). Cultivation was controlled using an ADI 1010 bio-controller (Applikon, The Netherlands) and cultivation control software "BioXpert" (Applikon, The Netherlands). Variable speed pumps (Cole Parmer, USA) were used to supply the feeding media with or without the

added cysteine and glycine. The feeding media vessels were each placed on a balance (Sartorius, Germany) to precisely determine the feeding rates of substrate and precursor amino acids. The pH control ($pH_{set\text{-}point}$=4.6) was carried out by titration with 1 M NH_4OH, which also served as the nitrogen source. The dO_2 was controlled at min 20% of air saturation by adjusting the ratio of air and oxygen in the aeration gas mixture (aeration rate 10 L min^{-1}), while the reactor was stirred continuously at 800 rpm. One hundred milliliters of seed cream yeast (~20 g dwt) was inoculated into 1.9 L of starting medium. An in-house developed adaptive proportional–integral algorithm for controlling the ethanol concentration in the exhaust gas at a set-point of 40 ppm (corresponding to a concentration of 0.2 g L^{-1} in the culture medium) by adjusting the feeding rate was used to grow the cells at near-maximum respiratory growth rate $\mu \approx \mu_{crit}$ (ethanol-stat). In exp. 1, exp. 2, and exp. 3, the culture was grown overnight in fed-batch, and a shift from the basic feeding medium to one containing cysteine and glycine was carried out at a biomass concentration of ~40 g dwt L^{-1}, followed by cultivation a further 5 h as described above.

Analytical methods

Culture samples (6–7 mL) were collected and rapidly transferred into 15-mL tubes maintained on ice; 1-mL aliquots were distributed into six Eppendorf microcentrifuge tubes and centrifuged at 14,000 rpm for 2 min to wash and recover the biomass. The biomass was washed once with ice-cold RNase-free [dimethyl pyrocarbonate-treated] water. Total processing of the samples did not take more than 5 min. The washed biomass samples and the culture supernatant were frozen in liquid nitrogen and stored at −80°C until required. All measurements were performed in duplicate.

Biomass concentration was determined by weighing the cell pellets after drying in the oven at 105°C for 24 h. Intracellular thiols were extracted using 0.1 N formic acid (70°C, 1 h). Cell debris was removed by centrifugation, and the supernatant was derivatized with 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB). An ACQUITY UPLC system (Waters, USA), equipped with a C_{18} column (BEH C18, 100×2.1 mm, 1.7 μm, Waters, USA) and a photo diode array (PDA) detector ACQUITY PDA 2996 was used for the chromatographic determination of GSH, γGCS, and cysteine. A two-gradient mobile phase (A: water+0.1% formic acid and B: acetonitrile+0.1% formic acid) and a flow rate of 0.3 mL min^{-1} was used. Concentration of cysteine in the cultivation medium was expressed as the sum of the concentration of reduced and oxidized cysteine, determined simultaneously by the two UPLCs using derivatization with DTNB and an AccQFluor kit (Waters, USA), respectively. The standards (cysteine, γGCS, and GSH) used for external calibration were obtained from Sigma (USA). Concentration of hydrogen sulfide (H_2S) in the culture medium was determined as described by Moest (1975).

Expression of nine genes (*GSH1*, *GSH2*, *ECM38*, *SUL1*, *MET17*, *CYS3*, *CYS4*, *STR2*, and *STR3*; Fig. 1) was monitored by a solution hybridization method termed "Transcript Analysis with the aid of Affinity Capture" (TRAC) using a pool of oligonucleotide probes designed for the respective target genes, separable and quantifiable by capillary electrophoresis (Rautio et al. 2006, 2007). Yeast samples were prepared for the TRAC analysis by adding 25 to 50 mg (dwt) of harvested yeast to 500 μL of lysis buffer (PlexPress, Finland) to give a final concentration of 120–240 μg μL^{-1}. The yeast cells were disrupted twice with 500 μL acid-washed glass beads (Sigma) in a FastPrep cell homogenizer (ThermoSavant, Germany), using 6 m/s for 45 s. Lysed yeast samples (120–240 μg dwt/reaction) were added to hybridization buffer (PlexPress,

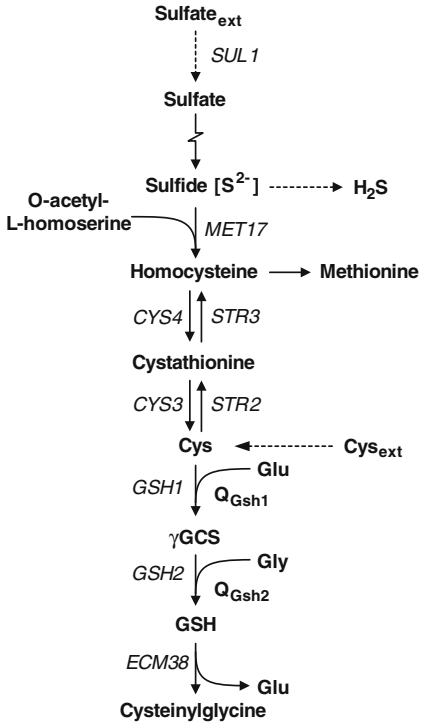

Fig. 1 Schematic representation of the main biosynthetic pathways involved in the metabolism of cysteine and related compounds. Q_{Gsh1}, Q_{Gsh2}, specific rates of γGCS and GSH biosynthetic reactions, respectively (micromole per gram per hour); *SUL1* high-affinity sulfate permease, *MET17* homocysteine synthase, *CYS4* cystathionine beta-synthase, *CYS3* cystathionine gamma-lyase, *STR3* cystathionine beta-lyase, *STR2* cystathionine gamma-synthase, *GSH1* gamma-glutamylcysteine synthetase, *GSH2* glutathione synthetase, *ECM38* gamma-glutamyltranspeptidase

Finland) containing target-specific probes and control ssDNA (PlexPress sequence ID 418) strand as an internal hybridization control. Affinity capture of hybrids, washing, and elution were carried out in 96-well plates using a magnetic bead particle processor KingFisher Flex (Thermo Electron, Finland) as follows: (1) affinity capture of hybridized RNA targets to 50 μg of streptavidin-coated TRACPACK® Beads (PlexPress, Finland) for 30 min at room temperature; (2) washing of the beads five times for 1 min in 150 μL of wash buffer (PlexPress, Finland) at room temperature; (3) elution of probes with 10 μL formamide (Applied Biosystems, USA) for 20 min at 37°C. The eluates were analyzed by capillary electrophoresis using an ABI PRISM 3100 Genetic Analyser (Applied Biosystems, USA).

To calibrate the separation of the detection probes by size, GeneScan-120LIZ size standard (Applied Biosystems, USA) was added to each sample. The identity of the probes was determined by the migration speed and the quantity by the peak area. To minimize non-biological variation in the TRAC assay, the signal intensities measured for the target genes were normalized between samples, using the signal measured for the internal hybridization control. The resulting signal intensities were either normalized to the biomass content in the hybridization mixture or related to the average signal of *ACT1* (YFL039C) and *SHR3* (YDL212W) signal level in the respective samples.

System equations

The specific biomass growth rate (μ, per hour) and biomass yield based on the substrate (sucrose) consumption (Y_{XS}, gram dry weight per gram) were calculated as follows:

$$\mu = \frac{d(X \times V)}{(X \times V)dt} \tag{1}$$

$$Y_{XS} = \frac{d(X \times V)}{dt\left[F_{in} \times S_S - \frac{d(S \times V)}{dt}\right]} \tag{2}$$

where V is the current fermenter volume (liters); X is biomass concentration in the fermenter (gram dry weight per liter); F_{in} is the addition rate of the feeding medium (liters per hour); S_S is the sucrose concentration in the feeding medium (grams per liter) and S is the sucrose concentration in the culture medium (grams per liter).

The specific rates of Gsh1p and Gsh2p reactions (Q_{Gsh1} and Q_{Gsh2}, micromole per gram per hour) were expressed as follows:

$$Q_{Gsh2} = \frac{d(GSH_X \times X \times V)}{dt(X \times V)} \tag{3}$$

$$Q_{Gsh1} = Q_{Gsh2} + \frac{d(\gamma GCS_X \cdot X \cdot V)}{dt(X \cdot V)} \tag{4}$$

where GSH_X and γGCS_X are concentrations of glutathione and γ-glutamylcysteine in the biomass (micromole per gram). Thus, the specific GSH accumulation rate in the biomass was equal to Q_{Gsh2} and that of γGCS to $Q_{Gsh1}-Q_{Gsh2}$.

The GSH molar yield based on the consumption of cysteine fed into the fermenter (mole per mole) was calculated as follows:

$$Y_{GSH/cys} = \frac{d(GSH_X \times X \times V)}{dt\left[F_{in} \times CYS_S - \frac{d(CYS \times V)}{dt}\right]} \tag{5}$$

where CYS_S is the concentration of cysteine in the feeding medium (micromolars); CYS is the concentration of cysteine in the culture medium (micromolars).

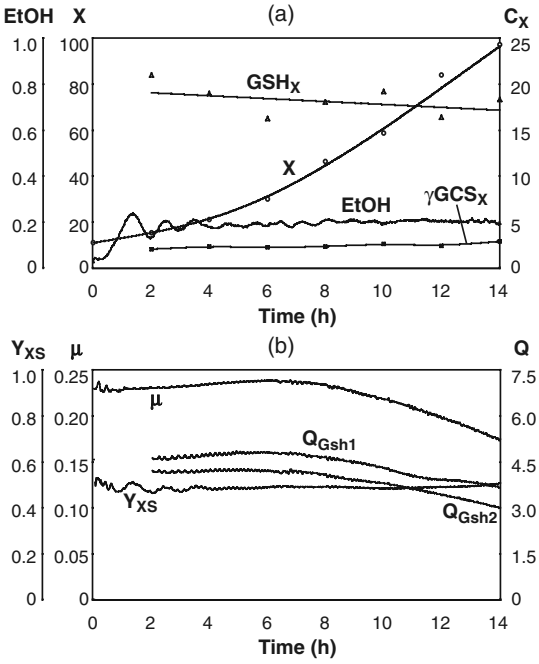

Fig. 2 Ethanol-stat fed-batch cultivation of *S. cerevisiae* LYCC7048 without cysteine and glycine addition. *EtOH* ethanol concentration in the cultivation medium (milligrams per milliliter); *X* biomass concentration in the cultivation medium (grams per liter); γGCS_X, GSH_X concentration of γGCS and GSH in the cells, respectively (micromoles per gram), Y_{XS} biomass yield based on substrate (sucrose) consumption (grams per gram), μ specific growth rate (per hour), Q_{Gsh1}, Q_{Gsh2} specific γGCS and GSH synthesis rates, respectively (micromoles per gram per hour)

Results

GSH biosynthesis in high-density fed-batch culture without cysteine addition

The specific GSH and γGCS synthesis rates, as well as the mRNA expression levels of the relevant genes were first determined under conditions of fed-batch culture using the basic feeding medium (i.e., without added cysteine and glycine). The ethanol-stat cultivation strategy ($EtOH_{\text{set-point}}$= 0.2 g L^{-1}) was used to grow the cells at near-maximum respiratory capacity (i.e., $\mu \approx \mu_{crit}$). Sucrose concentration corresponding to the given ethanol set-point in the culture medium was consistently very low (data not shown). A constant specific growth rate ($\mu \approx 0.24$ h^{-1}) was observed up to a biomass concentration of ~50 g L^{-1}. At higher biomass concentrations, a slow and consistent decrease in the specific growth rate was observed (Fig. 2b). As no decrease in the biomass yield occurred with this decreasing growth rate, the phenomenon is suggested to be related to growth rate inhibition rather than to nutrient limitation and decoupling of energy metabolism. With a few exceptions (notably *GSH1* and *GSH2*), the inhibitory effect of higher biomass concentrations was also observed on the mRNA concentrations of most of the studied target genes (Fig. 3a). The expression of *GSH1* and *GSH2* relative to the expression of housekeeping genes *ACT1* and *SHR3* increased with decreasing growth rate (Fig. 3b).

Concentration of γGCS in the cells was almost constant (~2 μmol g^{-1}) during the entire cultivation while a slight decrease of GSH concentration (from ~18 to 16 μmol g^{-1}) was observed (Fig. 2a). The initial rate of γGCS biosynthesis (Q_{Gsh1}) in the biomass was ~4.7 μmol $g^{-1}h^{-1}$ and that of GSH (Q_{Gsh2}) was ~4.5 μmol g^{-1} h^{-1} (Fig. 2b). Along with the decreasing growth rate at higher biomass concentrations, the specific rates of Gsh1p and Gsh2p reactions decreased in almost direct proportion.

Fed-batch cultivations with the shift to continuous addition of cysteine and glycine

Continuous addition of cysteine and glycine in ethanol-stat ($EtOH_{\text{set-point}}$=0.2 g L^{-1}) fed-batch culture was initiated by changing the basic feeding medium to one containing both cysteine and glycine at different concentrations of 56 μmol g^{-1} sucrose (exp. 1), 112 μmol g^{-1} sucrose (exp. 2), or 225 μmol g^{-1} sucrose (exp. 3). This change in media composition resulted in a significant accumulation of intracellular cysteine and a decrease in the specific growth rate in exp. 2 and exp. 3 (Fig. 4e, f). However, in exp. 1, a decrease in the specific growth rate was only observed at cell densities higher than 60 g L^{-1} (Fig. 4d).

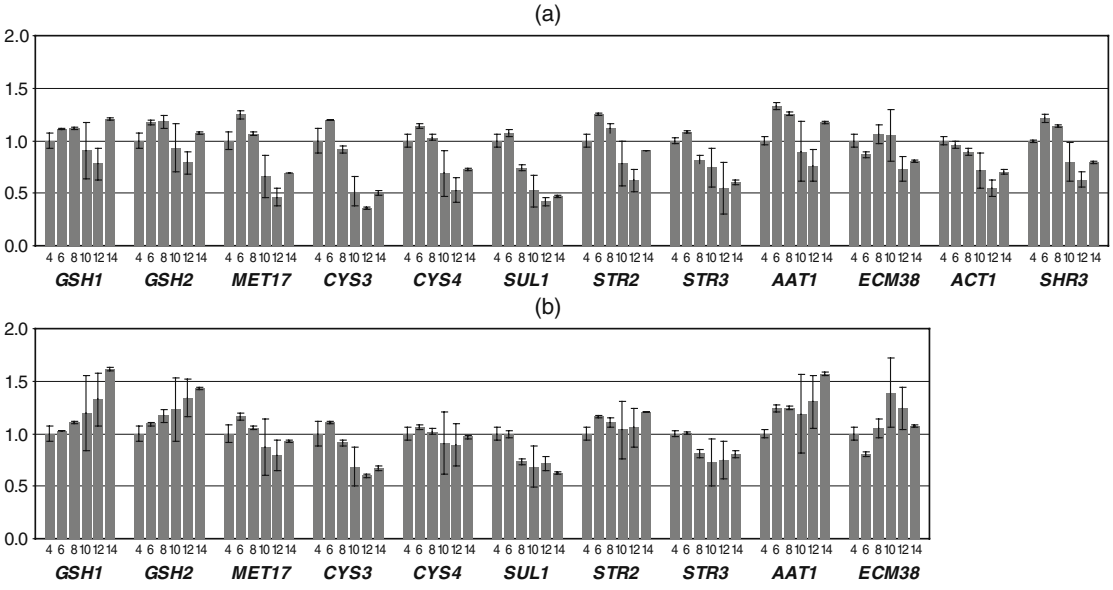

Fig. 3 Fold change of the expression of the target and reference genes (*ACT1* and *SHR3*) compared with the value at 4 h in the ethanol-stat fed-batch cultivation of *S. cerevisiae* LYCC7048 without cysteine and glycine addition: **a** relative to internal hybridization control and biomass content in the hybridization mixture and **b** relative to internal hybridization control and the expression of reference genes. *Error bars* represent the average absolute deviation of samples from their mean, the *numbers under the columns* represent the cultivation time in hours of sampling. For gene designations, see Fig. 1

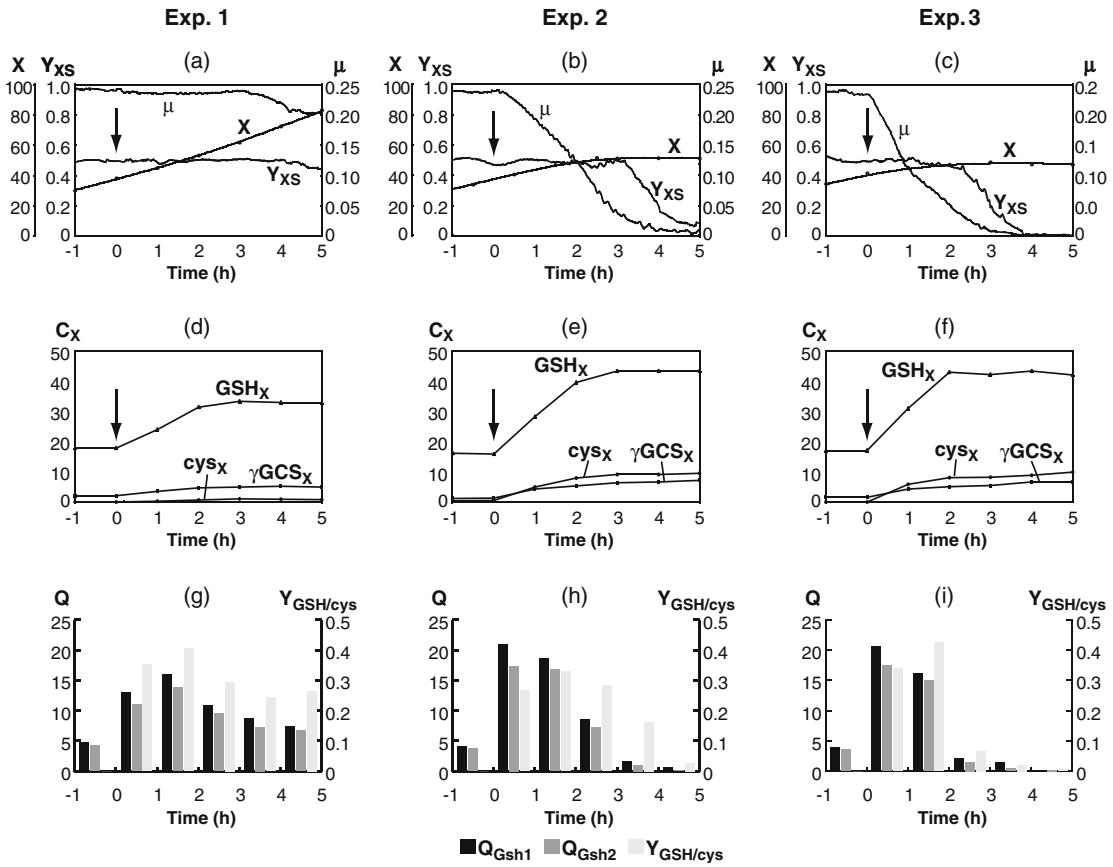

Fig. 4 Ethanol-stat fed-batch cultivation of *S. cerevisiae* LYCC7048 with a shift to a feeding medium containing cysteine and glycine: 56 μmol g^{-1} sucrose in exp. 1; 112 μmol g^{-1} sucrose in exp. 2; 225 μmol g^{-1} sucrose in exp. 3. *X* biomass concentration in the cultivation medium (grams per liter); Y_{XS} biomass yield based on substrate (sucrose) consumption (grams per gram); μ specific growth rate (per hour); cys_X, γGCS_X, GSH_X concentration of cysteine, γGCS, and GSH in the cells, respectively (micromoles per gram); Q_{Gsh1}, Q_{Gsh2} hourly averages of specific γGCS and GSH synthesis rates, respectively (micromoles per gram per hour); $Y_{GSH/cys}$ hourly average of cysteine accumulation yield into intracellular GSH (micromoles per micromole *cys*). The *arrows* denote the switch to the cultivation media containing cysteine and glycine

In each experiment, the shift to medium enriched with cysteine and glycine caused an immediate increase in the concentration of GSH in yeast. This effect was more pronounced at higher cysteine to sucrose ratios in the feed (exp. 2 and exp. 3; Fig. 4e, f). The average specific rates of γGCS and GSH biosynthesis (Q_{Gsh1} and Q_{Gsh2}, respectively) and molar yields per consumed cysteine ($Y_{GSH/cys}$) after the shifts of the feeding media were calculated at intervals of 1 h (Fig. 4g–i). The highest specific accumulation rate of GSH in all experiments was observed during the first 2 h following the media shift. The maximum accumulation rate in exp. 1 (14 μmol g^{-1} h^{-1}) was somewhat lower than that in exp. 2 and exp. 3 (17 μmol g^{-1} h^{-1}) where two and four times higher cysteine concentrations were used. This can be explained by the lower concentration of intracellular cysteine than was required for the saturation of Gsh1p enzymatic activity in exp. 1. Due to the accumulation of γGCS in cells, a higher reaction rate of Gsh1p compared with that of Gsh2p was calculated for all three experiments (Fig. 4g–i). The maximum GSH yields (0.35–0.4 mol mol^{-1}) based on cysteine consumption were similar in all experiments (Fig. 4g–i). Notably, both the GSH yield as well as the specific reaction rates Q_{Gsh1} and Q_{Gsh2} started to decrease when GSH reached its maximum value.

Results of the mRNA expression of the target genes relative to the expression of the housekeeping genes *ACT1* and *SHR3* for all three experiments are given on Fig. 5. Although an increase in intracellular GSH concentration

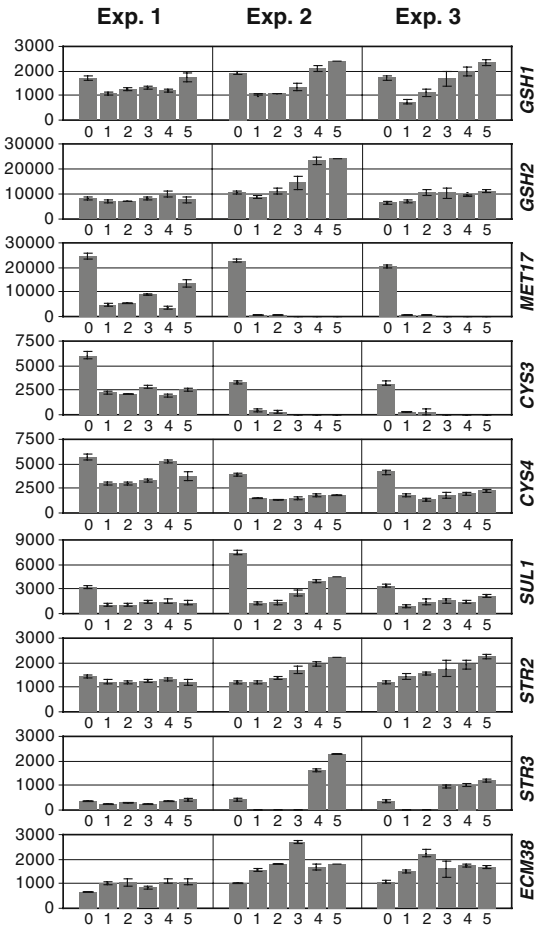

Fig. 5 Expression of the target genes (in fluorescence intensity units), relative to internal hybridization control and the expression of reference genes (*ACT1* and *SHR3*) in the ethanol-stat fed-batch cultivation of *S. cerevisiae* LYCC7048 with a shift to a feeding medium containing cysteine and glycine: 56 μmol g^{-1} sucrose in exp. 1; 112 μmol g^{-1} sucrose in exp. 2; 225 μmol g^{-1} sucrose in exp. 3. *Error bars* represent the average absolute deviation of samples from their mean values, the *numbers under* the *columns* represent the cultivation time (in hours from the switch of the feeding). For gene designations, see Fig. 1

was observed during the first 2 h following the shift to medium containing cysteine and glycine, up-regulation of the genes involved in the GSH biosynthetic pathway (*GSH1* and *GSH2*) was not detected. Instead, a 25–50% down-regulation in the expression of *GSH1* was observed after the shift. The extent of this down-regulation increased with the increasing cysteine/sucrose ratio in the feed. This suggests that the observed initial increase in the reaction rates Q_{Gsh1} and Q_{Gsh2} is most likely to be independent of control at the transcriptional level. The most remarkable down-regulation was observed in the expression of genes encoding the enzymes involved in homocysteine and cysteine biosynthesis, homocysteine synthase (*MET17*) and cystathionine gamma-lyase (*CYS3*). In exp. 2 and exp. 3 the expression of these genes ceased almost completely. Interestingly, an increased production of H_2S was observed under conditions where *MET17* expression was restricted (Fig. 6). Expression of cystathionine beta-synthase (*CYS4*) decreased more than 50% once cysteine addition was initiated; however, unlike *CYS3* and *MET17*, it was subsequently observed to maintain a stable relative expression. A similar behavior (down-regulation by at least 50%) was also observed for high-affinity sulfate permease (*SUL1*) under each set of conditions. Interestingly, the expression of *STR2* (cystathionine gamma-synthase) was observed to increase in conjunction with an increasing intracellular cysteine concentration during exp. 2 and exp. 3. A similar behavior would have been expected for cystathionine beta-lyase (*STR3*); however, the fluorescence signal intensity of *STR3* was observed to be close to the detection threshold (400 U) and consequently, the missing expression signal in some data points of exp. 2 and exp. 3 is believed to be a result of detection issues, rather than down-regulation of the gene. While no changes in the expression of gamma-glutamyltranspeptidase (*ECM38*), responsible for the degradation of GSH, were observed in exp. 1, the expression increased remarkably under the conditions encountered during exp. 2 and exp. 3. Although this suggests an increase in the concentration of Ecm38p, we were not able to determine any traces of cysteinylglycine, the intermediate of GSH catabolism in the cells.

Discussion

In the present work, we have studied the accumulation of GSH and the expression of related genes in ethanol-stat fed-batch fermentations, which allowed starting the experiments with a well-defined high-density culture. The gradual addition of cysteine prevented its excessive accumulation, which has been reported to result in prompt inhibition of growth when introduced using a shot-wise addition protocol (Alfafara et al. 1992a, b; Wang et al. 2007).

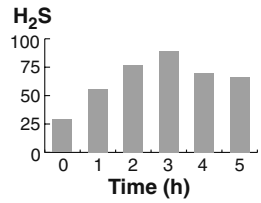

Fig. 6 Concentration of H_2S in the culture medium (micrograms per liter) in ethanol-stat fed-batch cultivation of *S. cerevisiae* LYCC7048 (exp. 3) after the switch to the feeding medium containing cysteine and glycine (225 μmol g^{-1} sucrose)

Prior to switching the feeding medium, the culture characteristics were virtually identical in all experiments (X=39±1 g L^{-1}, μ=0.24 h^{-1}, GSH_X=17±1 μmol g^{-1} and Q_{Gsh1}=4.5±0.5 μmol g^{-1} h^{-1}). The switch to enriched medium resulted in a rapid increase in intracellular cysteine, γGCS, and GSH concentrations (Fig. 4d–f). Surprisingly, along with increasing γGCS and GSH synthesis rates, a decrease in *GSH1* expression, relative to that of the housekeeping genes *ACT1* and *SHR3*, was observed (Fig. 4g–i; Fig. 5). However, this decrease was only temporary and was followed by a full recovery at higher GSH concentrations in conditions of decreasing GSH synthesis rate. Thus, the extent of repression of *GSH1* expression is not directly related to the concentration of intracellular GSH, but may depend on the rate at which the GSH, γGCS, or cysteine concentration changes[1]. Based on the results of Menant et al. (2006), the rapidly increasing cysteine concentration in the cells could be the cause of the temporary down-regulation of *GSH1*. While the exact mechanisms causing this down-regulation remain to be elucidated, the results show that, under the studied conditions, the transcriptional regulation has a relatively minor role in GSH accumulation. The increase of γGCS and GSH synthesis rates and concentrations is most likely to be caused by the rapid increase of substrate concentrations for Gsh1p and Gsh2p reactions. The decrease in GSH synthesis rate at higher GSH concentrations (Fig. 4) suggests that the feedback control of Gsh1p activity by GSH was significant in determining its maximum concentration in the cells. Notably, similar GSH accumulation and gene expression patterns were also observed in preliminary studies with a different baker's yeast strain under the conditions identical to those of exp.1 and exp. 2 (data not illustrated).

In each experiment, the cellular uptake of the total added cysteine was higher than 85%. However, only ~50% of the consumed cysteine was identified in the composition of γGCS, GSH, and as free intracellular cysteine. The up-regulation of *STR2* and *STR3* and the down-regulation of *SUL1, MET17*, *CYS4*, and *CYS3* in the two experiments with the higher cysteine content in the feed (Fig. 5) indicate that cells are switching from sulfate to cysteine as a preferred sulfur source. Considering the average methionine and cysteine content in yeast protein fractions (Halasz and Lásztity 1991), our mass balance calculations showed that the total cysteine utilization for GSH and protein synthesis was close to 100% (data not illustrated) in case of complete halt of de novo synthesis of cysteine.

[1] We have recently shown that the magnitude of the expression of stress response protein Hsp12p in *S. cerevisiae* is similarly dependent on the rate of change of the concentration of stressor compounds in the cultivation medium (Nisamedtinov et al. 2008).

A high cysteine concentration in the feeding resulted in an accumulation of H_2S (Fig. 6), repression of respiration, and a decrease in specific growth rate (Fig. 4). H_2S as well as sulfite have been reported to inhibit cellular respiration (Schimz 1980; Marzluf 1997; Grieshaber and Völkel 1998; Sohn et al. 2000). If Met17p reaction is completely blocked, the incomplete inhibition of Sul1p, Met3p (ATP sulfurylase), Met14p (APS kinase), Met16p (PAPS reductase), and/or Met10p (sulfite reductase) reactions may cause an accumulation of sulfite and sulfide in the cells. On the other hand, operation of this pathway under conditions of excess cysteine would result in a significant waste of metabolic energy, and it is likely to be down-regulated through the degradation of Met4p transcription factor (Menant et al. 2006). Therefore, catabolization of cysteine can also be considered a possible source of sulfite and thus H_2S. Unfortunately, no comprehensive data is available on cysteine catabolism in *S. cerevisiae*. Thus, further studies are required to elucidate the pathways of sulfur metabolism in the presence of cysteine.

In conclusion, under conditions of continuous cysteine addition, an initial increase in GSH synthesis rate is caused by the increased substrate (cysteine) availability for Gsh1p reaction. Our results indicate that the subsequent feedback inhibition of Gsh1p activity resulting from elevated intracellular GSH concentrations is the main factor in determining the maximum attainable GSH concentration in *S. cerevisiae*.

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PUBLICATION II

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Metabolic changes underlying the higher accumulation of glutathione in *Saccharomyces cerevisiae* mutants

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BIOTECHNOLOGICAL PRODUCTS AND PROCESS ENGINEERING

Metabolic changes underlying the higher accumulation of glutathione in *Saccharomyces cerevisiae* mutants

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Abstract Molecular mechanisms leading to glutathione (GSH) over-accumulation in a *Saccharomyces cerevisiae* strain produced by UV irradiation-induced random mutagenesis were studied. The mutant accumulated GSH but also cysteine and γ-glutamylcysteine in concentrations that were several fold higher than in its wild-type parent strain under all studied cultivation conditions (chemostat, fed-batch, and turbidostat). Transcript analyses along with shotgun proteome quantification indicated a difference in the expression of a number of genes and proteins, the most pronounced of which were several fold higher expression of *CYS3*, but also that of *GSH1* and its transcriptional activator *YAP1*. This together with the higher intracellular cysteine concentration is most likely the primary factor underlying GSH over-accumulation in the mutant. Comparative sequencing of *GSH1* and the fed-batch experiments with continuous cysteine addition demonstrated that the feedback inhibition of Gsh1p by GSH was still operational in the mutant.

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Introduction

Glutathione (GSH) is the most abundant non-protein thiol in eukaryotic cells. Due to a wide range of cellular physiological functions, in particular antioxidative functions, it has become an important compound for the treatment of a host of diseases and malfunctions, including cancer and neurodegenerative diseases (Martin and Teismann 2009; Galant et al. 2009). Due to the increasing number of practical applications for GSH, the efficiency of production technologies are increasingly important. Even though a number of alternative technologies exist for the industrial production of GSH (e.g., chemical and enzymatic synthesis), microbial synthesis in yeast, in particular *Saccharomyces cerevisiae*, has remained the mechanism of choice (Bachhawat et al. 2009). However, despite the simplicity of the fermentation process, this technique has several shortcomings. One of the major drawbacks in using yeast as a cell factory for industrial GSH production is that the synthesis of GSH is rigorously controlled by means of different and complex mechanisms. Thus, considering that the efficiency of the production of GSH relies on maximizing the concentration of this molecule inside cells, the main challenge lies in overcoming the regulatory mechanisms which restrict the high accumulation of GSH in yeast.

The biosynthesis of GSH occurs over two consecutive ATP-dependent reactions, catalyzed by γ-glutamylcysteine synthetase (Gsh1p) and GSH synthetase (Gsh2p). Generally, the first reaction has been considered to be the rate-limiting step in GSH biosynthesis as it is controlled via the feedback inhibition of GSH at both the transcriptional and post-

translational levels (Pócsi et al. 2004). Transcriptional regulation of the *GSH1* gene to the availability of GSH is a complex process and mediated by the coupling of two transcriptional activators – redox sensitive Yap1p and Met4p, a principal factor mediating regulation of sulfur assimilatory pathways (Wheeler et al. 2003). Under conditions of increased GSH levels Yap1p is deactivated via thioredoxin-mediated reduction of an intramolecular disulfide bond and its following export from the nucleus to cystoplasm (Delaunay et al. 2000; Trotter and Grant 2003). Post-translational regulation is a non-allosteric feedback inhibition of Gsh1p activity (Soltaninassab et al. 2000). The structural basis of this feedback inhibition of Gsh1p activity in *S. cerevisiae* was recently published (Biterova and Barycki 2010). In addition to these two control mechanisms, cysteine has been shown to be the major limiting precursor for GSH accumulation (Alfafara et al. 1992a) and thus the regulation of sulfur assimilatory pathways is also important from the point of view of cysteine biosynthesis.

Different strategies have been proposed to increase GSH accumulation in yeast. To overcome the substrate limitation, modulation of precursor amino acids, in particular cysteine, in the cultivation media has been used (Alfafara et al. 1992b; Wen et al. 2006; Wang et al. 2007; Liang et al. 2008). Grant et al. (1997) over-expressed the two GSH biosynthetic enzymes Gsh1p and Gsh2p in *S. cerevisiae* and observed nearly 70% increase in intracellular GSH levels when cells were grown in a medium supplemented with appropriate amino acids. Overproduction of a few key enzymes in sulfur assimilatory pathways leading to cysteine biosynthesis have also been suggested (Bachhawat et al. 2009), however, to the best of our knowledge, the effect of such genetic modifications has not been demonstrated until now.

In addition to genetically engineered strains, several mutation strategies have also been described in the literature for the isolation of GSH over-accumulating variants. The physical or chemical mutagenesis methods used included UV, X-radiation, γ-radiation, and *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine treatment, while resistance to highly toxic compounds such as ethionine, 1,2,4-triazole and sodium cyanide was used to screen GSH overproducers (Li et al. 2004). Thus, Lai et al. (2008) were able to select a mutant which accumulated GSH in fourfold higher concentrations than the respective wild-type strain. Although GSH over-accumulation has been related to mutations in the *GSH1* gene and the resulting disruption or release of the feedback inhibition of GSH on Gsh1p, there have been no reports confirming this hypothesis to date.

Although random mutagenesis can cause a wide range of mutations which may result in severe growth defects, analysis of mutants prepared in this manner, and the resulting changes in the metabolism which underlie the higher GSH accumulation could assist in the generation of improved GSH accumulating yeast strains by means of genetic engineering. The aim of the present work was to study the molecular mechanisms responsible for GSH over-accumulation in a *S. cerevisiae* strain selected via random mutagenesis.

Materials and methods

Yeast strains and cultivation media composition

The mutant (LYCC6267-56267) used in this study was selected for resistance to 1,2,4-triazole and azide after UV mutagenesis as described by Hamada et al. (1986).

For chemostat and turbidostat culture experiments, sucrose containing mineral medium was used: sucrose 40 g L^{-1} (100 g L^{-1} in turbidostat), NaCl 1 g L^{-1}, KH_2PO_4 6 g L^1, NH_4Cl 0.7 g L^{-1} (6 g L^{-1} in turbidostat), $MgSO_4{\cdot}7H_2O$ 1 g L^{-1}, $CaCl_2{\cdot}2H_2O$ 33 mg L^{-1}, $FeSO_4{\cdot}7H_2O$ 5 mg L^{-1}, $MnSO_4{\cdot}5H_2O$ 1.6 mg L^{-1}, $ZnSO_4{\cdot}7H_2O$ 1.6 mg L^{-1}, $CoCl_2{\cdot}5H_2O$ 0.3 mg L^{-1}, $CuCl_2{\cdot}5H_2O$ 0.3 mg L^{-1}, $Na_2MoO_4{\cdot}H_2O$ 0.3 mg L^{-1} and vitamins (mg L^{-1}): myo-inositol 20; thiamine 4.4; pyridoxine 1.2; Ca-pantothenate 0.5; D-biotin 0.03. The pH of the feeding was adjusted to pH 4.6 with H_2SO_4.

The initial and feeding media composition used during fed-batch experiments was identical to that described previously (Nisamedtinov et al. 2010). Feeding media containing the precursor amino acids cysteine and glycine in different concentrations (56 and 112 μmol of each per gram of sucrose in Exp.1 and Exp. 2, respectively) were prepared by dissolving the amino acids in basic feeding medium, previously deoxygenated by flushing with N_2 for at least 10 min. Notably, glycine was included in equimolar concentrations with cysteine to prevent its possible limitation and any effects of this deficiency on the results.

Cultivation system and cultivation process routines

Chemostat and turbidostat culture experiments were carried out in a 1 L Biobundle fermenter (Applikon, The Netherlands) equipped with an ADI 1030 bio-controller (Applikon) using the cultivation control software BIOXPERT (Applikon). The fermenter was equipped with pO_2, pH, temperature, and OD sensors. The fermenter and the feeding media vessels were weighed (Sartorius, Germany) to precisely control the dilution rate. Two variable speed pumps (Cole Parmer, USA) were used to supply the dilution medium and keep the culture volume constant (V_{SET}=400 mL). The pH of the culture (pH_{SET}=4.6) was controlled by titration with 1 M NH_4OH. Both cultivations were started by inoculating

20 mL of overnight (48 h in the case of mutant) YPD-grown culture into the fermenter containing 380 mL of dilution medium and grown in batch mode for 12–24 h before starting the continuous cultivation. In chemostat, the dilution rate D=0.1 h^{-1} was used. In turbidostat the simple control algorithm allowing two-level control of the feeding rate (F) was used based on the culture optical density (OD) value, such as described in Kasemets et al. (2007): $F = V \times D_{LOW}$ or $V \times D_{HIGH}$, where V is the culture volume and D is the dilution rate. The set-point of OD was 20 U (λ=600 nm), which corresponded to biomass concentration of 5 g L^{-1}. The D_{HIGH} and D_{LOW} were chosen equal to $1.5 \times \mu_{max}$ and to $0.3 \times \mu_{max}$, respectively. All cultivations were carried out at 30°C.

Fed-batch experiments with continuous cysteine addition were carried out as described in Nisamedtinov et al. (2010). In short, 25 mL of overnight (48 h in the case of mutant) grown inoculum in YPD medium was inoculated into 2 L of starting medium and grown in a batch culture until ethanol was almost completely consumed. Subsequently, the fed-batch was started in ethanol-stat mode (ethanol set-point, $EtOH_S$=0.2 g L^{-1}), allowing cultivation at a near-critical growth rate ($\mu \approx \mu_{crit}$). At the biomass concentration of ~40 g dwt L^{-1} a shift from the basic feeding medium to one containing cysteine and glycine (56 and 112 μmol (g sucrose)$^{-1}$ in Exp. 1 and Exp. 2, respectively) was carried out and the cultivation was continued as described above.

Analytical methods

In the fed-batch experiments with continuous addition of cysteine the GSH accumulation kinetics and expression of genes of sulfur assimilatory pathway and GSH metabolism were determined in the mutant and its parent strain. Culture samples (6–7 mL) were collected rapidly into 15-mL tubes on ice; 1-mL aliquots were distributed into six Eppendorf microcentrifuge tubes and centrifuged at 14,000 rpm for 2 min to wash and recover the biomass. The biomass was washed once with ice-cold RNasefree (dimethyl pyrocarbonate-treated) water. Total processing of the samples did not take more than 5 min. The washed biomass samples and the culture supernatant were frozen in liquid nitrogen and stored at −80°C until required.

Quantification of cysteine (cys), γ-glutamylcysteine (γGCS) and GSH, calculation of the specific reaction rates of Gsh1p and Gsh2p (Q_{Gsh1p} and Q_{Gsh2p}, respectively), and expression of the genes (*GSH1, GSH2, ECM38, SUL1, MET17, CYS3, CYS4, STR2, STR3, YAP1,* and *MET4*) in ethanol-stat fed-batch experiments were carried out as described in Nisamedtinov et al. (2010). All analyses were carried out in duplicates. The specific reaction rates of Gsh1p and Gsh2p in chemostat and turbidostat cultures were determined as follows:

$$Q_{Gsh2p} = \frac{\frac{dGSH_X}{dt} + pmp_{out} \cdot X \cdot GSH_X}{V \cdot X} \quad (1)$$

$$Q_{Gsh1p} = \frac{\frac{d\gamma GCS_X}{dt} + pmp_{out} \cdot X \cdot \gamma GCS_X}{V \cdot X} + Q_{Gsh2p} \quad (2)$$

where Q_{Gsh1p} and Q_{Gsh2p}—specific rates of Gsh1p and Gsh2p reactions, respectively (μmol g^{-1} h^{-1}); GSH_X and γGCS_X—concentration of glutathione and γ-glutamylcysteine in the biomass (μmol g^{-1}); pmp_{out}—culture outflow from the fermenter (L h^{-1}); V—current fermenter volume (L); X—biomass concentration in the fermenter (g dwt L^{-1}).

The comparative analysis of expression of proteins in the mutant and its parent strain was carried out with four samples (two for each strain) collected from the chemostat experiments after steady state conditions were achieved (i.e., after passing 4–5 culture volumes of dilution media). Samples were washed once with ice-cold water, snap-frozen in liquid nitrogen and stored at −20°C. Proteins were extracted in yeast extract buffer (50 mM 2-morpholinoethanesulfonic acid, 10 mM EDTA, and 10 mM $MgCl_2$ at pH 4.5) using glass beads (212–300 μm) coupled with vigorous vortexing at 4°C for 15 min. Protein concentration was determined by BCA protein assay kit (Thermo Scientific, Rockford, IL) and the amount of 100 μg of each protein sample was precipitated with ice-cold acetone. Aliquots of 100 μg acetone precipitated proteins from each sample were processed for labeling with iTRAQ 4plex reagents (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. The combined peptide mixtures were separated into ten fractions using a cation-exchange cartridge system (Applied Biosystems, Foster City, CA) and cleaned by StageTips (Rappsilber et al. 2007). All fractions were analyzed twice by LC-MS/MS using an Agilent 1200 series nanoflow system (Agilent Technologies, Santa Clara CA) connected to a Thermo Scientific LTQ Orbitrap mass-spectrometer (Thermo Electron, San Jose, CA) equipped with a nanoelectrospray ion source (Proxeon, Odense, Denmark). The mass spectrometry method employed combined HCD and CID spectrums according to Köcher et al. (2009). The data generated was compared against *S. cerevisiae* sequence data (downloaded from KEGG Genes Database 20042010) by MassMatrix search tool (Xu and Freitas 2009). The reversed decoy database was used for the detection of false positives. Only proteins with confidence interval of more than 95% were accepted for further data analysis.

For sequencing, the *GSH1* gene was amplified by PCR in two fragments (approximately 1,200 bp) using genomic DNA as a template. The amplified fragments were cloned

into a pJET1.2 vector as described by the manufacturer (Fermentas, Lithuania). Four clones *per* fragment were sequenced using plasmid specific primers flanking the cloning site. The sequencing reactions were set up using a BigDye kit as described by the manufacturer (Applied Biosystems, USA). The genomic sequences obtained were analyzed using BLAST.

Results

GSH accumulation in the mutant and wild-type strain

The intracellular concentrations of GSH and its precursors in the mutant and its wild-type parent strain were compared under three different culture conditions: (1) chemostat cultivation (fully respiratory growth, $\mu<\mu_{crit}$); (2) fed-batch cultivation in ethanol-stat mode (near-critical growth rate, $\mu\approx\mu_{crit}$); and (3) turbidostat cultivation (fermentative growth, $\mu=\mu_{max}$) (Table 1). Under all culture conditions the concentration of GSH in the mutant was more than twofold higher than in the wild-type strain; close to ~50 μmol g^{-1} in both chemostat and turbidostat cultures and somewhat lower (~42 μmol g^{-1}) in ethanol-stat fed-batch culture. The respective concentrations for the wild-type strain were ~13 μmol g^{-1} in ethanol-stat and turbidostat cultures and ~23 μmol g^{-1} in the chemostat culture. Notably, the concentrations of cysteine and γGCS, the precursors of GSH were also markedly higher in the mutant (Table 1). The corresponding calculated Gsh1p and Gsh2p reaction rates were two to four times greater than those observed in the wild-type strain. The presence of extracellular GSH could not be determined in case of either strain.

Higher thiol accumulation in the mutant was accompanied by a lower maximum respiratory growth rate (μ_{crit}) in ethanol-stat fed-batch culture and maximum growth rate (μ_{max}) in turbidostat culture (Table 1). Biomass yield based on sucrose consumption (Y_{XS}) was also found to be somewhat lower in the mutant. These changes in the growth characteristics may be caused by higher thiol levels and/or other metabolic changes induced by random mutagenesis.

Effect of cysteine addition

Supplementation of cysteine to a yeast culture is a common means of promoting GSH accumulation. To determine how the addition of cysteine affects the concentration of GSH in the studied strains, ethanol-stat fed-batch cultivations with a switch to a feeding medium supplemented with cysteine were carried out.

The shift of the feeding medium increased the intracellular concentration of cysteine in both strains but an obvious increase in GSH concentration and the specific rates of Gsh1p (Q_{Gsh1p}; Fig. 1) and Gsh2p (not illustrated) reactions were only observed in the wild-type culture. This rapid increase in GSH concentration and Q_{Gsh1p} suggested that the Gsh1p reaction step was cysteine limited in this strain.

Regardless of the cysteine concentration in the feeding medium, the maximum calculated values of Q_{Gsh1p} in the two experiments with the wild-type strain (15 and 17 μmol g^{-1} h^{-1} in Exp. 1 and Exp.2, respectively) were approximately twofold higher than in the experiments with the mutant (7 and 9 μmol g^{-1} h^{-1} in Exp.1 and Exp. 2, respectively) (Fig. 1). Nevertheless, the maximum attained GSH level in the wild-type strain (~25 μmol g^{-1}) remained lower than in the mutant (~45 μmol g^{-1}). Moreover, a rapid decrease of Q_{Gsh1p} in the wild-type strain was observed shortly after reaching its maximum value, which can be explained by the feedback inhibition of Gsh1p reaction by

Table 1 Concentration of intracellular cysteine (cys), γ-glutamylcysteine (γGCS) and glutathione (GSH), the specific reaction rates of Gsh1p (Q_{Gsh1p}) and Gsh2p (Q_{Gsh2p}), and the specific growth rate (μ) and biomass yield based on sucrose consumption (Y_{XS}), determined for mutant and wild-type strain under three different growth conditions

	Wild-type	Mutant
Chemostat cultivation ($\mu=D=0.1$ h^{-1})		
Y_{XS}, g g^{-1}	0.485±0.005	0.426±0.009
cys, μmol g^{-1}	0.40±0.02	1.91±0.18
γGCS, μmol g^{-1}	3.98±0.64	28.2±0.6
GSH, μmol g^{-1}	23.3±1.6	50.1±1.3
Q_{Gsh1p}, μmol g^{-1} h^{-1}	2.5±0.3	7.5±0.2
Q_{Gsh2p}, μmol g^{-1} h^{-1}	2.3±0.1	4.8±0.1
Ethanol-stat fed-batch cultivation ($\mu\approx\mu_{crit}$)		
μ_{crit}, h^{-1}	0.190±0.005	0.125±0.005
Y_{XS}, g g^{-1}	0.490±0.010	0.425±0.005
cys, μmol g^{-1}	0.22±0.11	0.87±0.13
γGCS, μmol g^{-1}	2.8±0.1	13.4±0.5
GSH, μmol g^{-1}	12.7±1.8	41.6±2.9
Q_{Gsh1p}, μmol g^{-1} h^{-1}	2.9±0.2	8.2±0.7
Q_{Gsh2p}, μmol g^{-1} h^{-1}	2.3±0.2	6.4±0.3
Turbidostat cultivation ($\mu\approx\mu_{max}$)		
μ_{max}, h^{-1}	0.293±0.018	0.230±0.007
Y_{XS}, g g^{-1}	0.137±0.004	0.113±0.004
cys, μmol g^{-1}	N/A	N/A
γGCS, μmol g^{-1}	0.8±0.2	4.5±0.7
GSH, μmol g^{-1}	12.8±1.0	50.1±1.8
Q_{Gsh1p}, μmol g^{-1} h^{-1}	3.7±0.3	14.1±2.7
Q_{Gsh2p}, μmol g^{-1} h^{-1}	3.5±0.3	13.0±2.4

The means of independent experiments ± average absolute deviations from the means are shown. The means of independent experiment include the number of individual experiments carried out with both strains: fed-batch (2), chemostat (3), and turbidostat (3)

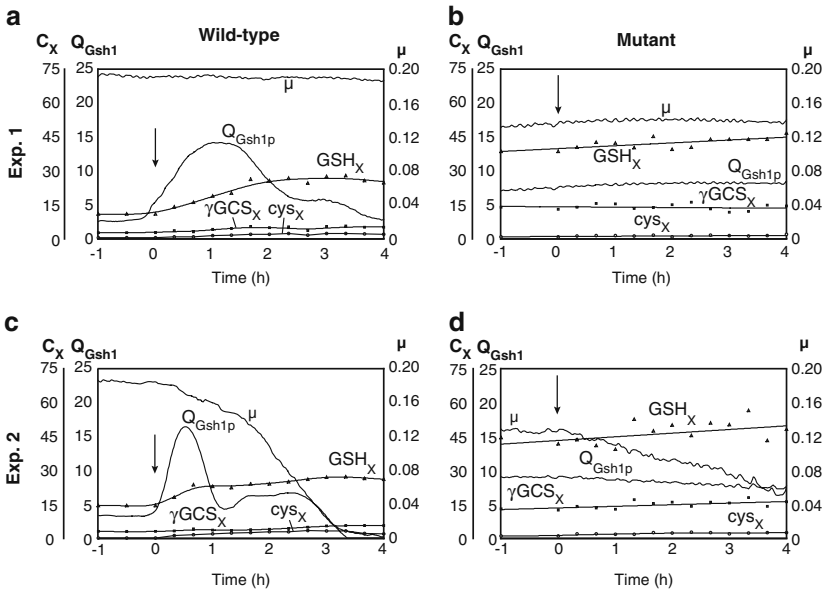

Fig. 1 Ethanol-stat fed-batch cultivation of the wild-type strain and mutant with switch to feeding containing cysteine: 56 μmol g^{-1} sucrose in Exp. 1 and 112 μmol g^{-1} sucrose in Exp. 2. C_X concentration of cysteine (cys_X), γ-glutamylcysteine (γGCS_X), and glutathione (GSH_X) in the biomass (μmol g^{-1}), Q_{Gsh1p} specific rate of Gsh1p reaction (μmol^{-1} g^{-1} h^{-1}), μ specific growth rate (h^{-1})

increased levels of GSH. In contrast, the concentration of intracellular GSH remained largely constant in the mutant and no significant change in the Q_{Gsh1p} values was observed when cysteine was fed to the culture.

Comparative sequence analysis of GSH1

In order to determine whether the higher level of GSH in the mutant was caused by mutation in the *GSH1* gene, a comparative *GSH1* sequence analysis was carried out. Comparison of the *GSH1* sequence in the mutant with that of the wild-type strain revealed that one allele in the mutant was harboring a deletion (T at the position 582) in addition to several substitutions further downstream. Another allele was identified to contain a mis-sense mutation, with a replacement of C with T at the position 933 resulting in a replacement of cysteine with arginine. However, it should be noted that the same replacement was detected when comparing the *GSH1* gene sequence in the wild-type strain with that to both the *S. cerevisiae* S288c sequence (GenBank: BK006943.1) and the EC1118 sequence (GenBank:FN393075.2). These results suggest that it is most likely that there were no mutations leading to a disruption or release of the feedback inhibition of GSH on Gsh1p in the mutant.

Transcript analysis of genes involved in the metabolic pathways related to GSH

A total of 11 genes involved in the sulfate assimilation pathway and in cysteine and GSH metabolism (Fig. 2) were selected for analysis. Each gene was analyzed to investigate the possible causes of the higher intracellular thiol accumulation in the mutant strain, and differences in GSH accumulation kinetics between the mutant and wild-type strain in the fed-batch experiments with cysteine addition.

Comparison of the initial mRNA levels of the respective genes, relative to the house-keeping genes *ACT1* and *SHR3* before cysteine addition revealed a threefold higher transcription of *CYS3* in the mutant (Fig. 3), suggesting a higher Cys3p (cystathionine γ-lyase) reaction capacity. A

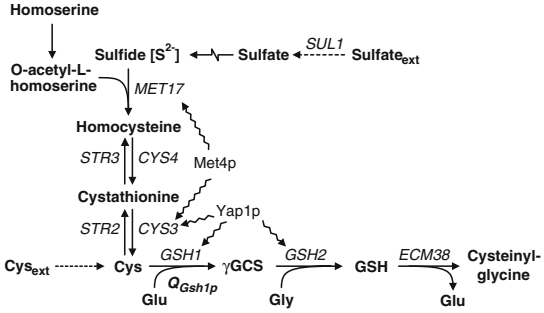

Fig. 2 Schematic representation of the main biosynthetic pathways involved in the metabolism of cysteine and GSH. Q_{Gsh1p} specific rate of Gsh1p reaction (μmol g^{-1} h^{-1}), *SUL1* high affinity sulfate permease, *MET17* homocysteine synthase, *CYS4* cystathionine β-synthase, *CYS3* cystathionine γ-lyase, *STR3* cystathionine β-lyase, *STR2* cystathionine γ-synthase, *GSH1* γ-glutamylcysteine synthetase, *GSH2* glutathione synthetase, *ECM38* γ-glutamyltranspeptidase, Met4p leucine zipper transcriptional activator, Yap1p redox sensitive transcriptional activator

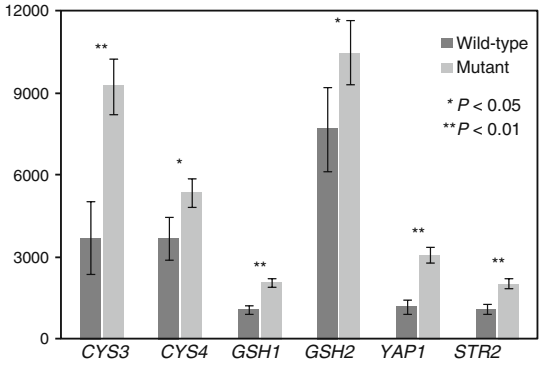

Fig. 3 The initial expression levels of the genes of cysteine and GSH biosynthesis relative to internal hybridization control and the expression of reference genes (*ACT1* and *SHR3*) in wild-type and mutant strains. The averages of two independent experiments are shown ±SD. The means were compared using two-sample Student's *t* test

higher relative transcription was also observed for *CYS4* (cystathionine β-synthase) and *STR2* which encodes cystathionine γ-synthase, catalyzing a reverse reaction to that of Cys3p. In addition to the genes involved in cysteine metabolism, the mRNA levels of genes within the GSH biosynthesis pathway, *GSH1* (γ-glutamylcysteine synthetase) and *GSH2* (glutathione synthetase) and their transcriptional activator *YAP1* were higher in the mutant. It is suggested that the higher relative transcription of the genes involved in the cysteine and GSH biosynthetic pathways could result in a greater capacity of their respective enzymatic reactions and might provide a rationale for the higher accumulation of GSH in the mutant. No statistically significant differences in the mRNA levels of the other studied genes could be demonstrated.

The dynamic response of genes to the increasing intracellular cysteine concentration was very similar in both strains. Up-regulation of *GSH1* mRNA levels was not observed in either strain. Rather, a small and short-term down-regulation occurred immediately after cysteine addition was started, followed by a complete recovery of transcription level with further cysteine addition and at higher intracellular GSH levels (Fig. 4). Thus, accumulation of GSH in either strain was most likely not controlled at transcript level under these conditions.

A down-regulation by at least twofold of the genes involved in homocysteine and cysteine biosynthesis; *MET17* (homocysteine synthase), *CYS4* and *CYS3,* was observed for both strains immediately after the start of cysteine addition, indicating a decrease in the de novo synthesis of this molecule (Fig. 4). This behavior was similar for *SUL1* (high affinity sulfate permease), suggesting that cells switched from sulfate to cysteine as a preferred sulfur source.

Proteome quantification

To determine whether differences in the transcription of the key genes of cysteine and GSH biosynthesis coincide with those in the respective protein concentrations, a quantitative shotgun proteomic analysis was carried out in the cellular extracts from chemostat cultivations. A total of 660 proteins, including Gsh2p, Cys4p, and Cys3p were examined in both strains. The expression of proteins of the main metabolic pathways in the mutant, relative to that in the wild-type strain is shown in Fig. 5. The majority of the proteins analyzed, including Gsh2p and Cys4p, did not show a statistically significant difference in their level of expression (i.e., less than 1.5-fold difference between the expression levels). However, Cys3p was observed to exhibit an expression level of more than two times higher in the mutant strain (data not illustrated), correlating with the results obtained during transcription analysis. Approximately twofold higher expression was also determined for Hom6p (homoserine dehydrogenase), involved in sulfur amino acid biosynthesis, and also for Sod1p (superoxide dismutase), an enzyme which is involved in oxidative stress defense.

Discussion

Several mutation strategies have been introduced for isolating GSH over-producing mutants of *S. cerevisiae* and other yeasts (Ikeno et al. 1977; Kono et al. 1977; Hamada et al. 1986) but the exact mechanisms underlying higher GSH accumulation in those mutants has remained rather obscure. In the present work we have studied the molecular mechanisms leading to GSH over-accumulation in UV-mutagenized *S. cerevisiae* strain.

The concentration of GSH in the mutant (40–50 μmol g^{-1}) was several-folds higher than in the wild-type parent strain under all of the cultivation conditions studied. This is comparable to what has been reported for *S. cerevisiae* strains proposed for biotechnological GSH production (Li et al. 2004). The results of our experiments suggest that the higher concentration of free cysteine is probably one of the most important factors underlying this higher GSH accumulation in the mutant. Increased cysteine concentration can be explained by the higher expression of *CYS3* and Cys3p. In addition to Cys3p, the proteome analysis results also indicated over twofold higher expression of Hom6p (homoserine dehydrogenase), an enzyme which catalyses the last step of homoserine biosynthesis, in the mutant. Being a precursor of homocysteine, the higher intracellular levels of homoserine may also contribute to the higher cysteine accumulation in the mutant.

Nevertheless, the higher concentration of free cysteine may not be the only factor underlying higher GSH

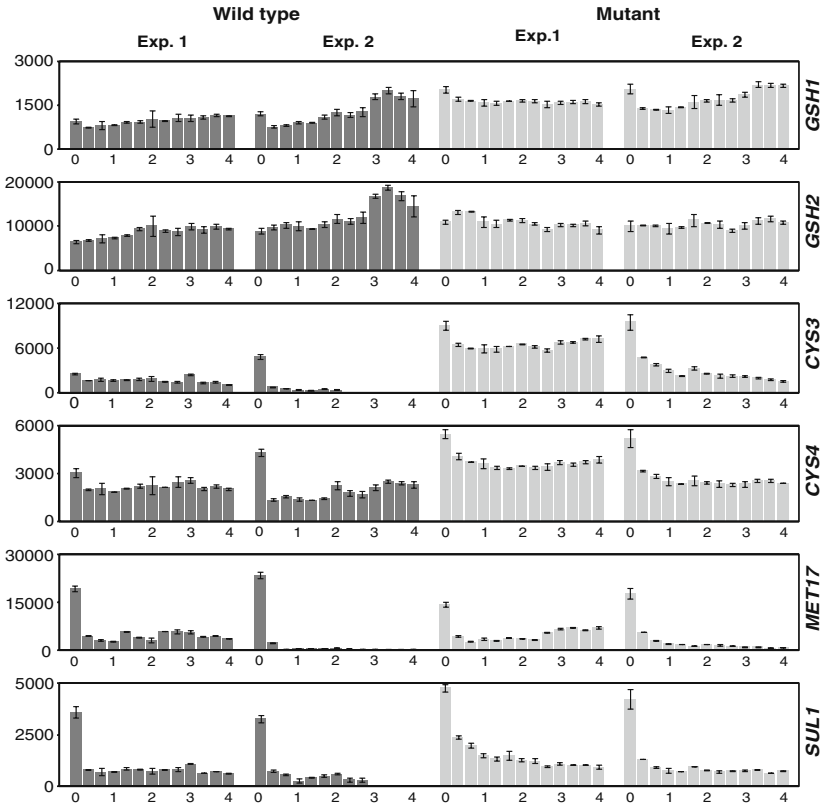

Fig. 4 Expression of the target genes, relative to internal hybridization control and the expression of reference genes (*ACT1* and *SHR3*) after the switch to cysteine supplemented feeding medium: 56 μmol cys g^{-1} sucrose in Exp. 1; 112 μmol cys g^{-1} sucrose in Exp. 2. The means of duplicates are shown ±average absolute deviation. The numbers under the columns represent the cultivation time (in hours from the switch of the feeding media)

accumulation in the mutant, as indicated by experiments with continuous cysteine addition. The switch to a medium supplemented with cysteine during fed-batch growth resulted in a considerable increase in GSH concentration in the wild-type strain, but its maximum levels still remained significantly lower than in the mutant. One way to explain this could be the lack of feedback inhibition of Gsh1p reaction by GSH in the mutant, which has been suggested to be the major mechanism for GSH over-accumulation in strains obtained by random mutagenesis (Li et al. 2004). This possibility, however, was ruled out by our comparative sequencing results of *GSH1* in the mutant and wild-type strains.

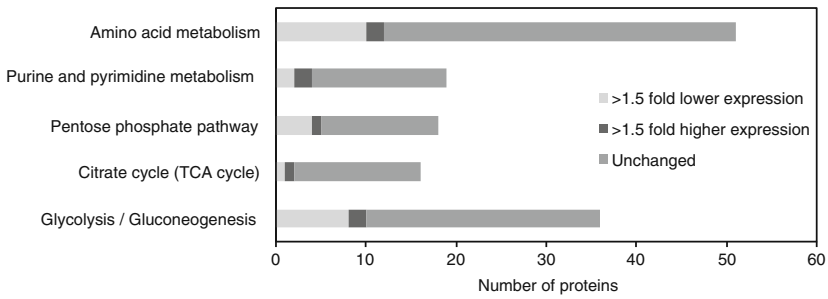

Fig. 5 The expression of proteins involved in the main metabolic pathways in the mutant, relative to that of the wild-type strain

Consequently, an alternative mechanism must be present to allow higher GSH accumulation in the mutant under conditions of cysteine abundance. When analyzing the mRNA levels of the respective genes, we noticed that in addition to *CYS3*, the transcription of *GSH1* was also significantly higher in the mutant, suggesting higher Gsh1p concentrations in the cells. This in turn could result in a higher capacity of the respective enzymatic reaction and thus, a shift in its saturation level. The initial intracellular cysteine concentration in the mutant was much higher than the respective K_m (0.17 mM) of the Gsh1p enzyme in *S. cerevisiae* (Biterova and Barycki 2010). This suggests that in the mutant strain it is likely that Gsh1p reaction had become saturated prior to cysteine addition and therefore, in contrast to the wild-type strain, the increase in intracellular cysteine concentration did not result in a significant increase in GSH concentration, or specific Gsh1p reaction rate.

Another possible explanation for the limited increase in GSH concentration in the mutant upon cysteine addition could have been the down-regulation of *GSH1* in response to increasing intracellular cysteine and/or GSH concentrations. However, no significant down-regulation of *GSH1* (or *GSH2*) was observed in either strain (Fig. 4), indicating that the transcriptional control of GSH accumulation was not the dominant factor in this instance.

Our results demonstrated that the higher accumulation of GSH in the mutant used in the present study is caused primarily by the higher transcription rate of *CYS3* and *GSH1*, resulting in an increased cysteine biosynthesis and a greater capacity of the Gsh1p reaction step, respectively. Nevertheless, the precise locations of the mutations causing the higher transcription of these genes remain to be elucidated. In general, genes in the sulfate assimilation and S-containing amino acid biosynthesis pathways are believed to be regulated coordinately and the transcriptional activator Met4p is suggested to be required for their expression (Thomas and Surdin-Kerjan 1997; Hiraishi et al. 2008). In yeast, Met4p activity is negatively regulated via ubiquitination in response to increased intracellular cysteine levels (Thomas and Surdin-Kerjan 1997; Kaiser et al. 2000; Rouillon et al. 2000; Menant et al. 2006). It could be possible that mutations occur in *MET4* and/or in the genes encoding enzymes which participate in the ubiquitination of *MET4*, resulting in the absence or only partial negative regulation of Met4p in the mutant. This, however, is unlikely as the initial expression levels of *MET17*, the transcription of which is under control of Met4p, were similar in both studied strains. Also, a rapid down-regulation of *MET17* and *CYS3* in response to cysteine addition was observed in both strains.

Our transcript analysis showed that the mRNA levels of *YAP1* were approximately twofold higher in the mutant. Yap1p controls the transcription of genes encoding proteins with antioxidative properties such as Sod1p (superoxide dismutase) but also Gsh1p and Gsh2p (Lee et al. 1999; Sugiyama et al. 2000). Triazole used for strain screening in the present study is known as an inducer of oxidative stress (Bagnyukova et al. 2005) which may result in higher expression of *YAP1*. This, in turn, could cause the higher expression of Sod1p and Gsh1p and resistance of the mutant to the highly oxidative selective pressure. Similarly to *GSH1* and *SOD1*, there is also a Yap1p response element (YRE motif; −235 to −229) in the 5′-upstream region of *CYS3* (Lee et al. 1999). The activity of Yap1p is regulated by its cellular localization between the nucleus and the cytoplasm which is determined by intracellular redox state and the respective conformational changes in the Yap1p structure (Kuge et al. 1997; Delaunay et al. 2000; Gulshan et al. 2005). The localization of Yap1p was not determined in this work; nevertheless, the twofold higher transcription of *YAP1* may result in an increased accumulation of Yap1p in the nucleus and explain the higher transcription of *GSH1*, *GSH2* and *CYS3* in the mutant.

In conclusion, GSH over-accumulation in the studied mutant is caused by the combined effect of higher cysteine synthesis and increased flux through the Gsh1p reaction, determined by higher transcription rate of *CYS3* and *GSH1* whereas the feedback control of Gsh1p by GSH is still functional. The exact mechanisms causing higher *CYS3* and *GSH1* transcription rate in the mutant remain to be elucidated.

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PUBLICATION III

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**

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Rapid Communication

YAP1 over-expression in *Saccharomyces cerevisiae* enhances glutathione accumulation at its biosynthesis and substrate availability levels

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Microbiological production of glutathione using genetically engineered yeast strains has a potential to satisfy the increasing industrial demand of this tripeptide. In the present work accumulation of glutathione in response to *YAP1* over-expression in *Saccharomyces cerevisiae* was studied. The over-expression resulted in intracellular glutathione level over two times higher than in the parent strain. Transcript analyses revealed that, in addition to the genes encoding enzymes in the glutathione biosynthesis pathway (*GSH1* and *GSH2*), the expression levels of the genes in the cysteine biosynthesis pathway (*CYS3* and *CYS4*) were also significantly higher in the *YAP1* over-expressed strain. This suggests that *YAP1* over-expression affects glutathione accumulation at both its biosynthesis and substrate availability levels.

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1 Introduction

Glutathione (GSH) is the most abundant non-protein thiol in eukaryotic cells. Due to its antioxidant characteristics there is an increasing demand for application of this tripeptide in several industrial areas, including cosmetics, pharmaceutical products, and foods. For example, GSH is used in a formulation of various pharmaceuticals as a detoxicant and as a diet integrator for the treatment of diabetes, neurodegenerative diseases, cancer, liver cirrhosis, and human immunodeficiency virus (HIV) [1]. In recent years an interest to use GSH for reducing the oxidation related defects (e.g., aroma defects and browning) in white wines has been expressed by oenologists [2]. In addition, GSH-enriched yeast extract has increasingly drawn attention as a food additive due to a characteristic kokumi flavor [3]. Presently the estimated global annual production of pure crystalline GSH in the world exceeds 200 tons (Lallemand, Inc., personal communication).

Currently the most common method for the industrial production of GSH is the microbiological synthesis in yeasts *Saccharomyces cerevisiae* and *Candida utilis* [4]. As with other physiologically important compounds in the cell, the accumulation of GSH in yeast is tightly controlled at different levels. GSH is synthesized from three precursor amino acids glutamate, cysteine and glycine over two consecutive ATP-dependent reactions, catalyzed by γ-glutamylcysteine synthetase (Gsh1p) and GSH synthetase (Gsh2p). In general, the first reaction (Gsh1p) is considered to be the rate-limiting step and is controlled at both transcriptional and post-translational levels [5]. The accumulation of GSH is also regulated via the supply of precursor amino acids, in particular cysteine, for Gsh1p reaction [6]. The two reactions in the transsulfuration pathway that yield cysteine from homocysteine, catalyzed by cystathionine beta-synthase (Cys4p), and cystathionine gamma-lyase (Cys3p) are known to be

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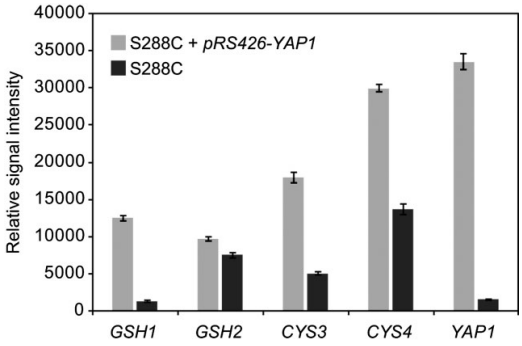

Figure 1. The expression levels of the key genes of cysteine and GSH biosynthesis relative to internal hybridization control and the expression of reference genes *ACT1* and *SHR3* (determined using TRAC [13]) in wild-type and *YAP1* over-expressed strains. The average levels from two independent glucose-limited chemostat experiments are shown ±SD. The means were compared using two-sample Student's *t*-test and $p < 0.01$ in all cases.

controlled by the transcriptional activator Met4p. The latter governs the *MET* genes network responsible for the assimilation of extracellular sulfate into methionine and cysteine [7].

The expression of *GSH1* and *GSH2* is known to be regulated by Yap1p, a transcriptional activator and a key determinant in oxidative stress resistance in yeast [8–10]. Notably, a Yap1p response element (TTA(C/G)TAA) has also been found in the 5′-upstream region of cystathionine gamma-lyase (*CYS3*) [8]. The activity of Yap1p is determined by its cellular localization between the nucleus and the cytoplasm in response to the intracellular redox state and the respective conformational changes in Yap1p structure [11].

In our previous studies with GSH over-accumulating mutant of *S. cerevisiae* we observed that higher mRNA levels of *GSH1* and *GSH2*, but also *CYS3* and *CYS4* were accompanied with higher *YAP1* expression, but not that of *MET4* [12]. This suggests that Yap1p may control GSH accumulation at both its biosynthesis and substrate availability levels. To verify this, we studied the accumulation of cysteine and GSH as well as the expression of the genes in the biosynthesis pathways of both compounds in chemostat cultures of *S. cerevisiae* S288C and its *YAP1* over-expressed mutant.

2 Materials and methods

Wild-type *S. cerevisiae* strain (S288C) and a respective *YAP1* over-expressing mutant (S288C + *pRS426-YAP1*) were grown in glucose limited chemostat cultures (dilution rate $D = 0.07\ h^{-1}$, cultivation temperature $T = 30°C$). Expression levels of the selected genes (*GSH1*, *GSH2*, *CYS3*, *CYS4*, and *YAP1*) were monitored by transcript analysis with the aid of affinity capture (TRAC) [13]. The cultivation system and dilution media composition as well as the methods for cysteine and GSH quantification were identical to those described elsewhere [12]. Details about the DNA manipulations used for *YAP1* over-expression are provided in Supporting Information (Appendix I).

3 Results and discussion

Upregulation of *YAP1* expression by at least an order of magnitude was achieved in S288C + *pRS426-YAP1* (Fig. 1). The resulting GSH concentration was over twofold higher than that in the parent strain S288C (32.5 ± 0.3 and 14.5 ± 0.1 μmol/g, respectively). Notably, the concentration of cysteine in S288C + *pRS426-YAP1* was also significantly higher than in S288C (17.7 ± 2.6 and 12.4 ± 1.0 μmol/g, respectively). The concentration of γ-glutamylcysteine was 1.24 ± 0.02 and 0.67 ± 0.05 μmol/g in S288C and S288C + *pRS426-YAP1*, respectively. Over-expression of *YAP1* resulted in significantly higher mRNA levels of *GSH1*, *GSH2*, *CYS3*, and *CYS4* in S288C + *pRS426-YAP1* (Fig. 1). This confirms our previous hypothesis about the combined effect of *YAP1* over-expression on GSH accumulation via transcriptional activation of the genes in the biosynthesis pathways of both GSH and its precursor cysteine.

The exact mechanisms leading to the higher expression levels of *GSH1*, *GSH2*, *CYS3*, and *CYS4* in response to *YAP1* over-expression remain to be elucidated. Yap1p-related transcriptional activation is dependent on its accumulation in the nucleus under conditions of oxidative stress and the resulting loss of the complex with nuclear export protein Crm1p which is responsible for the transport of Yap1p from nucleus to cytoplasm [11]. Since our chemostat experiments with both strains were carried out using identical environmental conditions, the over-expression of *YAP1* must have resulted in an increased accumulation of Yap1p in the nucleus regardless of the intracellular redox state. This may result from the different ratios of Crm1p to Yap1p concentrations in the studied strains and the inability of Crm1p to export all Yap1p from the nucleus in case of the mutant. To confirm this, a comparative determination of Yap1p localization between nucleus and cytoplasm in both strains should be carried out.

4 Concluding remarks

Various strategies have been proposed to increase GSH accumulation in yeast, including cysteine supplementation as well as application of special producer strains [4]. The use of genetically engineered strains allows for significantly higher GSH production yields compared to strains of wild-type origin. In the present work we have provided evidence that the over-expression of a single gene *YAP1* allows to satisfy the two important premises for GSH accumulation in yeast – increase both the biosynthesis of cysteine as well as the throughput of the two enzymatic reactions leading to GSH accumulation. Although there have been earlier reports on the possible dependence of the transcription of *CYS3* on Yap1p [8], to the best of our knowledge this is the first time to demonstrate this experimentally. The over-expression of *YAP1*, either alone or in combination with other genetic modifications could be applied for the improvement of GSH producer strains.

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The authors declare no conflict of interest.

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Appendix I

Standard techniques were used for DNA manipulations [14]. The *YAP1* gene with its 5′ upstream (361 bp) and 3′ downstream (195 bp) regions was amplified by PCR using *Pfu* DNA polymerase (Fermentas) and primers YAP1N (5′-GCGACTCGAGGTAACCAGCCCTAGCTGTTTGG) and YAP1C (5′-CATTGAAGGTGATACAGAGAATTC). The restriction sites, *Xho*I and *Eco*RI, used for the cloning are underlined. For the construction of pRS426-YAP1, the obtained PCR fragment was digested with *Xho*I and *Eco*RI and ligated into pRS426 [15] cut with the same enzymes. The inserted sequence was verified by sequencing.
The S288C-derived strain TYSC99 (*MATa/MATalpha ura3-52/ura3-5 trp1Δ63/+ leu2Δ1/+*) was used for transformation. Rich (yeast extract peptone dextrose, YPD) and selective (synthetic complete, SC) media were prepared as described [16]. Yeast transformation was performed by the lithium acetate method [17]. For plasmid selection, transformed cells were plated out on the SC plates lacking uracil.

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