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RESEARCH ARTICLE

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

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Abstract

The dipeptide γ-glutamylcysteine (γ-GC), the first intermediate of glutathione (GSH) synthesis, is considered as a promising drug to reduce or prevent plethora of age-related disorders such as Alzheimer and Parkinson diseases. The unusual γ-linkage between the two constitutive amino acids, namely cysteine and glutamate, renders its chemical synthesis particularly challenging. Herein, we report on the metabolic engineering of the non-conventional yeast *Yarrowia lipolytica* for efficient γ-GC synthesis. The yeast was first converted into a γ-GC producer by disruption of gene *GSH2* encoding GSH synthase and by constitutive expression of *GSH1* encoding glutamyl-cysteine ligase. Subsequently genes involved in cysteine and glutamate anabolism, namely *MET4*, *CYSE*, *CYSF*, and *GDH1* were overexpressed with the aim to increase their intracellular availability. With such a strategy, a γ-GC titer of 464 nmol mg^{-1} protein (93 mg $gDCW^{-1}$) was obtained within 24 h of cell growth.

KEYWORDS
antioxidant, γ-glutamylcysteine, sulfur metabolism, *Yarrowia lipolytica*

1 | INTRODUCTION

γ-Glutamylcysteine (γ-GC) is synthesized from glutamate and cysteine by glutamylcysteine ligase (GCL) through the formation of an unusual γ-glutamyl bond (Figure S1).[1] It is also the substrate with glycine of glutathione synthase (GS) for the formation of glutathione (γ-L-glutamyl-L-cysteinyl-glycine [GSH]), the most abundant intracellular non-protein thiol compound.[1] The intracellular concentration of γ-GC remains low as it is rapidly converted into GSH by GS whose activity is significantly higher than that of GCL.[2,3] Moreover, the γ-GC synthesis rate is constrained by the availability of cysteine, whose intracellular concentration is significantly lower than that of glutamate.[4,5]

GSH plays a pivotal role in numerous intracellular processes, including the maintenance of redox balance, the storage of cysteine, the detoxification of toxic compounds, and the neutralization of reactive oxygen species and other free radicals through its oxidation into glutathione disulfide (GSSG).[1,4,6] Glutathione reductase (GR) can hydrolyze GSSG in the presence of nicotinamide adenine dinucleotide phosphate (NADPH) to release GSH while it is not active on the disulfide form of γ-GC (*bis*-γ-glutamylcystine [bγ-GC]). By contrast, γ-glutamyltranspeptidase (γ-GT) that normally hydrolyze GSH into

Abbreviations: ATP, adenosine triphosphsate; BSA, bovine serum albumin; bγ-GC, *bis*-γ glutamylcystine; CDW, cell dry weight; ESI-Q-TOF, electrospray-ionization quadrupole time-of-flight; GCL, glutamylcysteine ligase; GPX1, glutathione peroxidase 1; GR, glutathione reductase; GS, glutathione synthase; GSH, γ-L-glutamyl-L-cysteinyl-glycine; GSSG, glutathione disulfide; NAC, *N*-acetylcysteine; NADPH, nicotinamide adenine dinucleotide phosphate; NMR, nuclear magnetic resonance; NsPCS, phytochelatin synthase-like; TOCSY, total correlation spectroscopy; γ-GC, γ-glutamylcysteine; γ-GT, γ-glutamyltranspeptidase.

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glutamate and cysteine-glycine dipeptide is also active on both γ-GC and bγ-GC with the release of glutamate and cysteine, and glycine and cystine (cyst-cyst), respectively.[7]

In mammals, the onset of ageing is associated with a significant decrease in both GCL activity and the availability of cysteine, resulting in a decrease in intracellular GSH content.[8] This reduced de novo GSH synthesis has been associated with the development of age-related disorders such as Alzheimer's (AD)[9] and Parkinson's diseases (PD).[10] Given that cysteine availability is a limiting factor for GSH synthesis, several cysteine prodrugs, including *N*-acetylcysteine (NAC) and NAC ethyl ester, have been employed in clinical practices.[8,11] However, they have not proven fully successful due to the low level of GCL activity, limiting de novo synthesis. Exogenous GSH supply has also proven ineffective in human therapy. GSH has a short half-life of 1.6 min in the plasma due to its rapid hydrolysis by membrane-bound γ-GT[8,11,12] and its passive transport from plasma to cells is not thermodynamically favorable.[13] γ-GC has emerged as an alternative drug to elevate intracellular GSH levels.[14,15] Recent studies have demonstrated that its oral administration increases GSH levels in the lymphocytes of healthy humans.[16] In mice with AD, it improved spatial memory while reducing neuroapoptosis and oxidative stress.[17] γ-GC was also found to reduce oxidative stress damage and neuroinflammation induced by soluble amyloid β-oligomers in human astrocytes[18] and human endothelial cells.[19] In vivo trials in mice showed that γ-GC reduced sepsis lethality and systemic inflammatory response, and also inhibited lipopolysaccharide-stimulated production of tumor necrosis factor α, as well as interleukin-1β.[20] γ-GC also alleviates insulin resistance and hepatic steatosis.[21] Furthermore, it was shown to replace GSH as a cofactor for glutathione peroxidase 1 (GPX1), a ubiquitous enzyme in many human tissues that protects cells from oxidative stress.[22] These examples underscore γ-GC potential for preventing or partially curing chronic diseases and age-related disorders.

While γ-GC can be chemically synthesized, large-scale production presents a complex process requiring several steps involving the protection, coupling, and deprotection of the reactive sulfhydryl group of cysteine. Coupling the constitutive amino acids through the unconventional γ-glutamyl bond occurs at a low yield (less than 10%) and necessitates the use of highly toxic solvents, raising environmental and safety concerns.[23] Although fermentation and enzymatic production processes have been patented,[24,25] the product has not yet reached the market, largely due to high process costs.[26] Recently, an enzymatic process based on the hydrolysis of GSH using a phytochelatin synthase-like (NsPCS) enzyme was developed at the lab scale.[24] While the reaction achieved a conversion yield of 0.8, it requires a pure GSH solution, as well as the production, purification, and immobilization of recombinant NsPCS, which can be cumbersome and time-consuming. We previously reported on the engineering of the yeast *Yarrowia lipolytica* for efficient GSH production,[27,28] achieving GSH titers and productivity rates among the highest reported in the literature. Therefore, we employed this yeast as a chassis strain for γ-GC production.

2 | MATERIALS AND METHODS

2.1 | Strains, media, and culture conditions

The strains used are listed in Tables 1 and S1. *Escherichia coli* was cultivated at 37°C in Luria-Bertani medium, supplemented with ampicillin (100 mg L^{-1}) or kanamycin sulfate (50 mg L^{-1}) as needed. Yeasts were cultured at 28°C in YPD medium (containing 20 g L^{-1} Difco bacto peptone, 10 g L^{-1} Difco yeast extract, and 20 g L^{-1} glucose) or in YNBD medium (comprising 1.7 g L^{-1} Difco yeast nitrogen base medium without amino acids and ammonium sulfate, 20 g L^{-1} glucose, 2 g L^{-1} Difco casamino acids, 1 g L^{-1} ammonium chloride, and 0.05 M phosphate buffer at pH 6.8), with the addition of leucine (0.2 g L^{-1}) or uracil (0.1 g L^{-1}) to fulfill the requirements of auxotrophic strains. YNBDplus medium was prepared by enriching YNB medium with glutamate (0.1 g L^{-1}) and/or cysteine (0.1 g L^{-1}). YPDox was supplemented with H_2O_2 at concentrations ranging from 0 to 24 mM. For solid media, agar (15 g L^{-1}) was added. All shake flask cultures were performed at least in biological triplicate. They were seeded at an initial optical density at 600 nm (OD600) of 0.2 from cells grown for 24 h in YPD medium and washed twice with phosphate buffer (50 mM, pH 6.8).

2.2 | General molecular techniques, vectors, and strains construction

Standard molecular genetic techniques were used.[29] Restriction enzymes and T4 DNA ligase were from New England BioLabs (MA, USA). PCR amplifications were conducted using GoTaq DNA polymerase (Promega, WI, USA) or Q5 High-Fidelity DNA polymerase (New England BioLabs) with primers listed in Table S2. PCR fragments were purified with the QiAgen Purification kit (Qiagen, Hilden, Germany) while DNA fragments were purified from the agarose gels using the Monarch DNA Gel Extraction kit (BioLabs, New England, USA). GeneJET plasmid Miniprep Kit (Thermo Scientific, Waltham, USA) was used for plasmid extraction and purification. Yeast genomic DNA was prepared, and yeast cell transformation was carried out as previously described.[30,31] qPCRs were performed following procedures outlined elsewhere, with the specific primers listed in Table S2.[27]

For the *GSH2* gene disruption, a 1 kb fragment (Pro_GSH2) upstream of the start codon and a 1.2 kb fragment (Ter_GSH2) downstream of the stop codon of gene *YALI0C17831g* were each PCR-amplified using the primer pairs GSH2pro_for/GSH2pro_rev and GSH2ter_for/GSH2ter_rev, respectively, and with *Y. lipolytica* RIY129 genomic DNA serving as the template. The URA3 selection marker was amplified using the primer pair Auxo_fo/Auxo_rev, utilizing plasmid RIP110 as the template. The GSH2 disruption cassette (Pro_GSH2-URA3-Ter_GSH2) was obtained by conducting an overlapping PCR with amplicons Pro_GSH2, Ter_GSH2, and the marker amplicon as templates, along with GSH2pro_for/GSH2ter_rev as the primer pair. The resulting 3.5 kb fragment was cloned into the pGEMT-Easy vector to

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TABLE 1 *Y. lipolytica* strains used in this study.

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

generate plasmid RIP295 (Table S1). It was subsequently utilized to transform strain RIY176. Transformants were selected on YNBG, and the correctness of the genotype was confirmed through analytical PCR using the primer pair GSH2_verif/Auxo_rev.

For marker rescue, the resulting strain RIY441 was further transformed with the replicative vector RIE132, as previously described.[32] The correctness of the resulting RIY448 was verified by PCR, employing the primer pair URA-Fo/URA-Rev. Strain RIY441 was transformed with plasmid RIP111 to confer prototrophy in the resulting RIY454 strain.

For constitutive overexpression of *GSH1* (*YALI0E30129g*), vectors RIP210 (LEU2ex) and RIP211 (URA3ex) were subjected to *Not*1 digestion, and the expression cassettes were subsequently gel purified. Vector RIP210 was utilized to transform strain RIY441, while both vectors were employed to transform strain RIY448. Transformants were selected on YNBD, and the correctness of the genotype was assessed through analytical PCR, using primer pair pTEF_forw/GSH1_rev. The resulting strains were denoted as RIY455 and RIY445, respectively (Table 1). The auxotrophic derivative of RIY445, named RIY476, was obtained via transformation with vector RIP132, as previously described.[32] Marker excision was confirmed using analytical PCR, utilizing primer pair LoxP-Fo/LoxR-Rev.

Genes *MET4* (*YALI0D04466g*), *CYSTE* (*YALI0E08536g*), and *CYSTF* (*YALI0F14047g*) were PCR-amplified using primer pairs Met4-Fo/Met4-Rev, CystE-Fo/CystE-Rev, CystF-Fo/CystE-Rev, respectively, and subsequently cloned into the pGEMT-easy vector to generate vectors RIP316, RIP317, and RIP318, respectively. The amplified sequences were confirmed for accuracy through DNA sequencing, employing the primer pair M13fo/M13rev. The *MET4* coding sequence was excised from RIP316 by *BamH*I/*Avr*II digestion and integrated into the corresponding sites of vectors RIP136 and RIP137 to generate vectors RI320 and RIP319, respectively. Similarly, the *CYSTE* and *CYSTF* coding sequences were released from RIP117 and RIP318 vectors by *BamH*I/*Avr*II digestion, and they were inserted at the corresponding sites of vector RIP136 to yield vectors RIE322 and RIE324, respectively. Prior to yeast transformation, pTEF-based vectors underwent *Not*1 digestion, and the corresponding expression cassettes were subsequently purified from agarose gel. Strain RIY476 was transformed with expression cassettes released from vectors RIP310, RIP320, RIP322, and RIP324 to yield strains RIY500 to RIY503, respectively (Table 1). Strains RIY500, RIY502, and RIY503 were further transformed with the purified expression cassette released from plasmid RIP319 to generate strains RIY504 to RIY506, respectively. Finally, strains RIY500 to RIY503 were transformed with vector RIP110 to generate prototrophic strains RIY507 to RIY510. The correctness of the genotype was verified through analytical PCR, using primer pTEF-Fo and a primer annealing in the coding sequence of the corresponding gene, namely Gdh_verif, Met4_verif, CystE_verif, and CystF_verif. Auxotrophy was confirmed by PCR using the primer pair Auxo-fo/Auxo_rev. In the constructed strains, the expression of genes *GSH1*, *MET4*, *GDH1*, *CYSE*, and *CYSF* was quantified through qPCR, using the primers listed in Table S1.

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2.3 | Analytical methods

The cell dry weight (CDW, gCDW L^{-1}) was used for biomass quantification. For cell extract preparation, cells from 1 mL of culture broth were collected by centrifugation at 5000 × *g* at room temperature for 10 min. The cell pellet was then washed twice with phosphate-buffered saline (PBS, pH 6.8) and was subsequently re-suspended in 1 mL of the same buffer. Cell disruption was carried out utilizing a FastPrep-24 instrument (MP Biopmedicals, Eschwege, Germany) for four cycles of 2 min. each at a speed of 6.5 m s^{-1}, and with 0.3 g of glass beads per mL of cell suspension (acid-washed, Sigma). Subsequently, cellular debris were separated by centrifugation (10,000 × *g* for 10 min at 4°C). The total protein concentration in the cell extract was determined using the Coomassie protein assay reagent (Thermo Scientific). Protein concentration was calculated from a calibration curve obtained with standard solutions of bovine serum albumin (BSA) (Thermo Scientific). The γ-GC concentration in the cell extracts was determined using the Fluorometric Thiol Quantitation kit (Sigma–Aldrich). Preceding the measurement, cell extracts underwent pretreatment with a 5-sulfosalicylic acid solution (10%) to precipitate all soluble proteins in the samples. All measurements were conducted in triplicate for each biological replicate, and subsequently, the means and standard deviations were calculated. The γ-GC titer was calculated in nmol per mg of protein in the cell extract (nmol mg^{-1} prot) or in mg per gram of dry cell weight (mg $gDCW^{-1}$).

2.4 | Characterization of γ-GC by UPLC-QTOF/MS

Yeast cell extract was deproteinized with a 10% solution of 5-sulfosalicylic acid, as previously described for thiol quantitation. Standard solutions (2–50 μM) of γ-GC, GSH, and *bis*-γ-glutamylcystine (bγ-GC) were prepared in ultrapure water. Five μL of either cell extract, standard solutions, YNBD medium or culture supernatants were injected per analysis. The detection and quantification of the different molecules were conducted using an Agilent 1290 Infinity II HPLC system coupled to a mass detector (Jet Stream ESI-qTOF 6530, Agilent). The molecules were separated on a C18 Acquity UPLC BEH column (2.1 × 50 mm × 1.7 μm; Waters) using a mixture of 0.1% formic acid (solvent A) and acetonitrile acidified with 0.1% formic acid (solvent B) as the mobile phase. The flow rate was set at 0.3 mL min^{-1}, and the column temperature was maintained at 40°C. For elution, the mobile phase was initially held at 100% of solvent A for 1.5 min before transitioning to 100% of solvent B in 0.2 min. Following 5 min under these conditions, the mobile phase composition was returned to its initial state in 0.1 min. Mass spectra were recorded in positive mode with the following mass detector parameters: capillary voltage: 3.5 kV; nebulizer pressure: 35 psi; drying gas: 8 L min^{-1}; drying gas temperature: 300°C; flow rate of sheath gas: 11 L min^{-1}; sheath gas temperature: 350°C; fragmentor voltage: 175 V; skimmer voltage: 65 V; octopole RF: 750 V. Data analysis was performed using MassHunter Qualitative Analysis software (Agilent). The identification of the eluted molecules was based on their retention time and accurate masses compared to those of commercial standards (Sigma–Aldrich).

2.5 | Characterization of γ-GC by nuclear magnetic resonance

The deproteinization of yeast cell extract was carried out using the same procedure as previously described for thiol quantitation. Standard solutions of γ-GC, GSH, and GSSG at concentrations ranging from 2 to 50 μM in a 50 mM phosphate buffer at pH 6.8 were used as reference standards. Cell extracts obtained from both the wild-type strain RIY129 and the γ-GC producing strain (i.e., RIY445) were analyzed by nuclear magnetic resonance (NMR) utilizing a 700 MHz Bruker Avance III HD instrument equipped with a helium-cooled triple nuclei (HCN) probe. NMR spectra were recorded at 298 K, with the sample composed of 570 μL of cell lysate and 30 μL of D_2O for the internal frequency lock signal. For 1D 1H spectra, water suppression was achieved through the utilization of the Bruker pulse sequence "ZGESGPPE," and 256 transients were accumulated before Fourier transformation. The relaxation delay between each transient was set to 1 s. In the case of the 2D HH total correlation spectroscopy (TOCSY), the Bruker pulse sequence "MLEVESGPPH" was applied, with a 256-time increment comprising 64 transients each. The spin-lock time and relaxation time were set at 80 ms and 1 s, respectively.

2.6 | Statistical analysis

Statistical signification of results was assessed by unpaired *t*-test. Alpha value was set at 0.05. All statistical tests were performed using GraphPad Prism 9 software.

3 | RESULTS AND DISCUSSION

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

In *Y. lipolytica*, genes *YALI0E30129g* (*GSH1*) and *YALI0C17831g* (*GSH2*) encode GCL and GS, respectively.[27] With the goal of constructing a γ-GC producer strain, gene *GSH2* was disrupted in strain RIY176, resulting in the generation of strain RIY441 (Table 1). Subsequently, in the disrupted strain, gene *GSH1* was overexpressed under the control of the strong constitutive promoter pTEF, both in one and two copies, leading to the strains RIY455 and RIY445, respectively. To demonstrate the synthesis of γ-GC instead of GSH, mass spectrometric analysis was conducted on cell extracts from the engineered strains, and the results were compared to those of the parental strain RIY129. Electrospray-ionization quadrupole time-of-flight (ESI-Q-TOF) spectra of RIY129 displayed a molecular ion $[M + H]^+$ at *m/z* 308.0917, corresponding to GSH. Conversely, such a specific molecular ion was not detected

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TABLE 2 Detected ions (*m/z*) for γ-glutamylcysteine, γ-glutamylcystine (γ-glutamyl cysteine oxidized form), glutathione and glutathione disulfide (glutathione oxidized form) in cell extract of *Y. lipolytica* strain RIY129, RIY455 (*gsh2Δ*, *GSH1*), and RIY445 (*gsh2Δ*, *GSH1*, *GSH1*).

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

Data are representative of measurements performed on two biological replicates. n.d.: no ion detected. Corresponding extracted ion chromatogram (EIC) and mass spectra are presented in Figure S1.

TABLE 3 γ-GC peptide proton chemical shift (ppm) observed in the 2D TOCSY for cell lysate of strain RIY445 (*gsh2Δ*, *GSH1*, *GSH1*).

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

γ-GC, γ-glutamylcysteine; GSH, glutathione; TOCSY, total correlation spectroscopy.

in the cell extracts of strains with *GSH2* disruption (RIY455, RIY445, Table 2, Figures S2–S4). In contrast, molecular ions $[M+H]^+$ characteristic of γ-GC and its oxidized form bγ-GC (*m/z* 251.0696 and 499.1163, respectively) were observed in the cell extracts of strains RIY455 and RIY454. These ions could not be detected in the cell extract from strain RIY129. Moreover, the cell extract of strain RIY445 was analyzed by 2D TOSCY-NMR to highlight the specific γ-GC proton chemical shifts, which were preliminarily identified using a pure γ-GC solution (Table 3, Figure S5). Furthermore, to confirm the presence of the dipeptide in the cell lysate of strain RIY445, an oxidation followed by the addition of freshly reduced γ-GC peptide was conducted. For this purpose, the cell extract was left at room temperature for 48 h to oxidize the cysteine moiety, that is, the conversion of γ-GC into bγ-GC. The 1D 1H NMR spectrum effectively demonstrated the disappearance of γ-GC characteristic signals at 8.11 and 2.52 ppm (Figure 1, red trace). The addition of reduced γ-GC to cell extracts (at a final concentration of 100 μM) restored the characteristic signals at 8.11 and 2.52 ppm (Figure 1, green trace). Based on these analyses, it can be concluded that the *gsh2Δ* strains (i.e., RIY455 and RIY445) produce γ-GC instead of GSH.

In the *gsh2Δ* strain RIY454, the free thiol content was slightly increased as compared to the parental strain (94 nmol γ-GC mg^{-1} protein and 71 nmol GSH mg^{-1} protein, respectively; Figure 2). In comparison to the wild-type strain RIY129, the expression level of *GSH1* was increased by 43- and 169-fold in strains RIY455 and RIY445, respectively (data not shown). As shown in Figure 2, the γ-GC titer was also significantly increased in the latter two strains (249 and 283 nmol mg^{-1} protein) when contrasted with the parental strain RIY454 (94 nmol mg^{-1} protein, *gsh2Δ*, Figure 2). These γ-GC titers fall within the range of the GSH titer obtained with strain RIY231, which overexpresses *GSH1* and *GSH2* (340 nmol GSH mg^{-1} protein).[27]

3.2 | γ-GC can substitute GSH in *Y. lipolytica*

In *Saccharomyces cerevisiae*, it was demonstrated that *GSH2*, encoding GS, is non-essential for growth, although the growth of a *gsh2Δ* mutant was affected in minimal media.[33,34] For a *S. cerevisiae* mutant disrupted for the GCL-encoding gene, preincubation of cells with γ-GC prior exposure to H_2O_2 was found to be as efficient for cell survival as preincubation with GSH.[35] To assess whether γ-GC can substitute for GSH in *Y. lipolytica* when subjected to oxidative stress, strains RIY129 (WT) and RIY445 (*gsh2Δ*, *GSH1*, *GSH1*) were cultivated in YPDox media at various concentrations of hydrogen peroxide (0–24 mM), known to trigger the formation of reactive oxygen species. As shown in Figure 3, the reduction in biomass under increasing oxidative stress was not significantly different ($p < 0.05$) between strains RIY129 and RIY445. This demonstrates that γ-GC can effectively substitute for GSH in the *gsh2Δ* mutant strain.

3.3 | γ-GC synthesis is limited by the availability of constitutive amino acid

Although γ-GC was significantly increased in strain RIY455 (*gsh2Δ*, GSH1, 249 nmol mg^{-1} protein) compared to strain RIY454 (*gsh2Δ*, 75 nmol mg^{-1} protein, Figure 2), no significant increase in γ-GC titer could be observed upon the overexpression of *GSH1* in one (RIY445, 249 nmol mg^{-1} protein) or two copies (RIY455, 277 nmol mg^{-1} protein) (Figure 2).

In *S. cerevisiae*, various strategies for constitutive amino acid supplementation have been developed with the aim of increasing GSH productivity.[6] To identify any intracellular limitations in γ-GC constitutive amino acids in *Y. lipolytica*, the two strains were cultured in YNBD minimal medium supplemented with glutamate and cysteine, alone or in combination. In the non-supplemented medium, the γ-GC titer was lower compared to the supplemented medium, especially for strain RIY445 (two copies of *GSH1*) grown in a medium supplemented with both glutamate and cysteine (a 2.8-fold increase) (Figure 4). This highlights that the intracellular availability of constitutive amino acids, especially cysteine, limits γ-GC synthesis.

3.4 | Increasing intracellular amino acid in γ-GC producing strain

Only a few reports exist regarding the characterization of cysteine and glutamate synthesis in *Y. lipolytica*. Using a combined approach of

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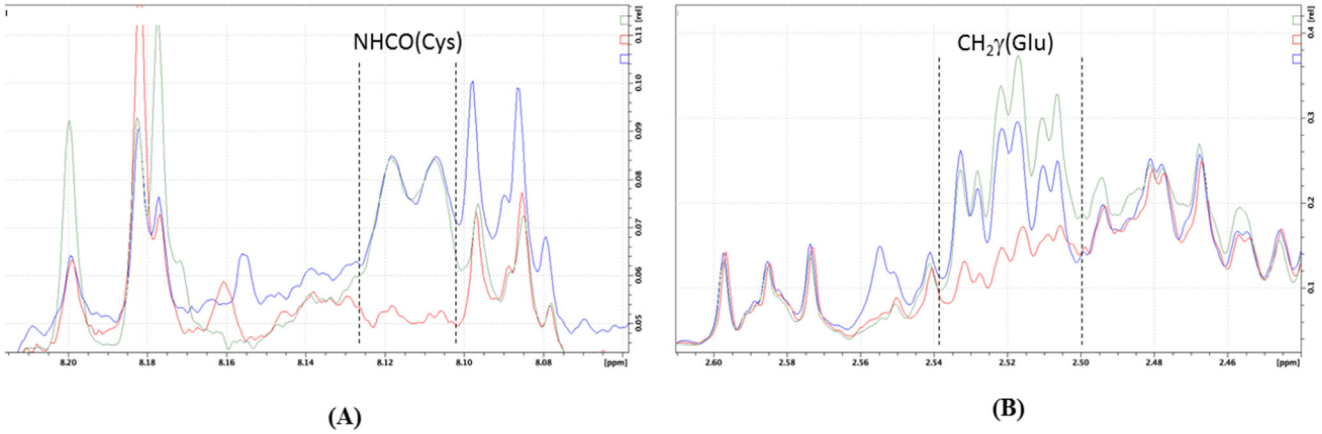


FIGURE 1 γ-Glutamylcysteine 1H 1D NMR spectra of cell extract of strain RIY445 (*gsh2Δ*, *GSH1*, *GSH1*). Color code: blue, fresh cell extract from 48 h culture; green, cell extract oxidized at RT for 48 h; green, oxidized cell extract spiked with 100 μM of γ-GC. A: NHCO signals, B: CH_2g signals. GSH, glutathione; NMR, nuclear magnetic resonance.

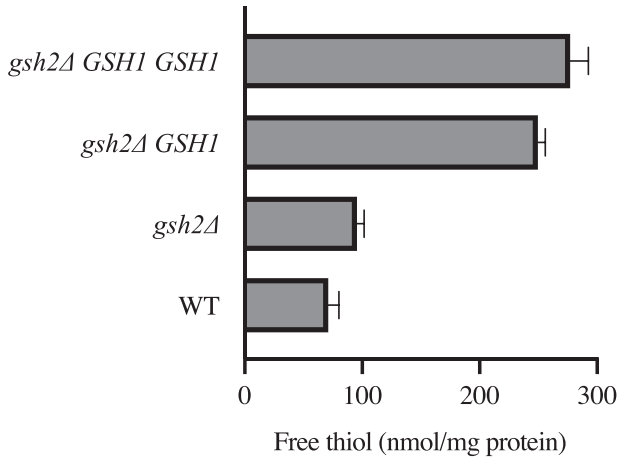


FIGURE 2 Free thiol (γ-GC or GSH) intracellular concentration for the RIY129 wild-type strain (WT, GSH), RIY454 (*gsh2Δ*, γ-GC), RIY455 (*gsh2Δ*, *GSH1*; γ-GC), and RIY445 (*gsh2Δ*, *GSH1*, *GSH1*; γ-GC) after 24 h of YNBD medium. Data are the mean and standard deviation of triplicate experiments. Free thiols concentration is given in nmol of thiol per mg of protein in the cell extract. γ-GC, γ-glutamylcysteine; GSH, glutathione.

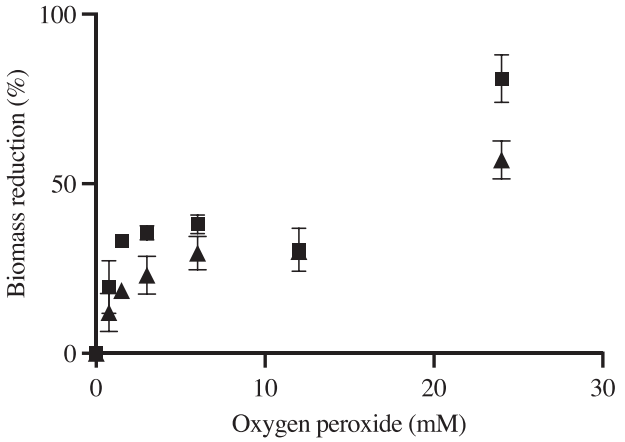


FIGURE 3 Biomass of strain RIY129 (WT, square) and RIY445 (*gsh2Δ*, *GSH1*, *GSH1*, triangle) after 24 h of growth in YNBD medium supplemented or not (w/o) hydrogen peroxide. Biomass from culture in non-supplemented medium was used as a reference to calculate the decrease, in percent, of biomass obtained in H_2O_2 supplemented medium. Data are the mean and standard deviation of triplicate experiments. GSH, glutathione.

transcriptomic and metabolite profiling, genes *YALI0E08536g* (*CYSE*) and *YALI0F14047g* (*CYSF*) were identified as two orthologues of *S. cerevisiae* gene YGR012w encoding cysteine synthase.[36] The corresponding enzyme converts *o*-acetyl-L-serine and hydrogen sulfide into cysteine and acetate. In the same study, a homolog of the *MET4* gene, encoding a transcriptional activator of the sulfur amino acid pathway, was identified as gene *YALI0D04466g*. We previously demonstrated that overexpression of the *MET4* gene in *Ogataea* (*Hansenula*) *polymorpha* led to a significant increase in GSH productivity.[37]

Regarding glutamate, gene *YALI0F17820*, the homologue of *GDH1* and *GDH3* in *S. cerevisiae*, has been characterized as coding for a glutamate dehydrogenase that converts α-ketoglutarate into glutamate.[38] With the aim of increasing the intracellular concentrations of cysteine and glutamate and, consequently, further increasing the γ-GC titer, these genes were overexpressed alone or in combination under the control of the strong pTEF promoter in strain RIY476, an auxotroph derivative of strain RIY445 (*gsh2Δ*, *GSH1*, *GSH1*). Overexpression of the corresponding gene was first confirmed by qPCR (data not shown). Subsequently, cultures were conducted for the different constructed strains in YNBD medium, and the γ-GC titer was measured during the growth phase (i.e., 24 h) and stationary phase (i.e., 48 h). At 24 h, the highest γ-GC titer was observed for the strain that overexpressed the *MET4* gene, resulting in a 1.7-fold increase compared to the parental strain RIY445 (Figure 5; 277 and 464 nmol mg^{-1} protein, respectively). For strains that overexpressed the *CYSF* and *GDH1* genes, γ-GC titers increased in the same range (i.e., 1.3-fold, 366 and 351 nmol mg^{-1} protein, respectively). Surprisingly, the biomass obtained at 24 h for

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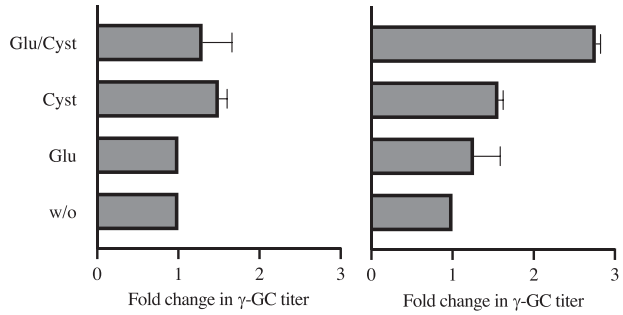


FIGURE 4 Fold change in γ-glutamyl-cysteine intracellular concentration for strains RIY455 (panel A, *gsh2Δ*, *GSH1*) and RIY445 (panel B, *gsh2Δ*, *GSH1*, *GSH1*) after 48 h of growth in YNBD medium supplemented or not (w/o) with glutamate (Glu) and/or cysteine (Cyst). Data are the mean and standard deviation of triplicate experiments. γ-GC concentrations were calculated in nmol of γ-GC per mg of protein in the cell extract and normalized to the value obtained for the non-supplemented medium (w/o). γ-GC, γ-glutamylcysteine; GSH, glutathione.

the strain overexpressing *GDH1* was 1.7-fold lower (i.e., 5.4 ± 0.3 gDCW L^{-1}) compared to the mean biomass value of all tested strains (9.5 ± 0.5 gDCW L^{-1}, data not shown). Overexpression of *CYSE* was detrimental for γ-GC production (i.e., 160 nmol mg^{-1} protein) but not for cell growth (i.e., 16.2 ± 0.3 gDCW L^{-1}, data not shown). Unexpectedly, the overexpression of *MET4* in combination with *CYSE*, *CYSF*, or *GDH* did not lead to any significant increase in γ-GC titer. At 48 h, the γ-GC titers were significantly lower, especially for the strain overexpressing *MET4* (7-fold reduction, 63 nmol mg^{-1} protein). γ-GC titer remained in the same range for both sampling times for strains overexpressing *MET4-CYSF* (RIY503, 267 vs. 218 nmol mg^{-1} protein) and *MET4-GDH* (RIY505, 302 vs. 236 nmol mg^{-1} protein).

Several hypotheses can be proposed to account for the observed phenomena at 48 h. Firstly, the culture supernatant of strains RIY445 (*gsh2Δ*, *GSH1-2c*) and RIY508 (*gsh2Δ*, *GSH1-2c*, *MET4*) was analyzed by ULPC-QTOF/MS to detect the presence of both γ-GC and bγ-GC. An increase in dipeptide content (i.e., γ-GC and bγ-GC) in the culture supernatant was noted after 48 h of growth compared to 24 h, with values of 39% and 71% for strains RIY445 and RIY508, respectively (Table S3). Therefore, the reduced titer observed after 48 h of growth can be attributed, at least in part, to a leakage of γ-GC from the cells. Secondly, mass spectrometry analysis was performed on the cell extract from strains RIY445 and RIY455, and the relative proportion of γ-GC and bγ-GC was calculated based on the peak area value on the extracted ion chromatogram (EIC). Between 24 and 48 h of culture, the relative proportion of bγ-GC exhibited a six-fold increase, confirming the accumulation of *bis*-γ-glutamylcystine over time inside the cell (Figure S6). This can be linked to the inability of the cell to reduce bγ-GC, as it has been demonstrated that it is not a substrate of GR.[39] Consequently, the intracellular pool of γ-GC gradually undergoes oxidation over time into bγ-GC, particularly during stationary phases when metabolic activity, and thus de novo γ-GC synthesis, are lower. The third hypothesis for explaining the lower γ-GC observed after 48 h of growth can be related to Met4 activity. Indeed, the expression of genes involved in synthesizing sulfur-containing amino acids, including cysteine, is regulated by the transcription factor Met4. It does not directly bind to DNA but interacts with DNA-binding proteins such as Cbf1, Met31, and Met32.[40] The inactivation of Met4 through its ubiquitination occurs when sulfur-containing amino acids are abundant in the cell.[41] It can be assumed that the demand for sulfur-containing amino acids decreases during the stationary phase when cell metabolism is reduced. Consequently, it can be postulated that during the stationary phase (i.e., 48 h of growth), when cell metabolism is lower, the demand for cysteine is reduced, leading to the ubiquitination of Met4. This, in

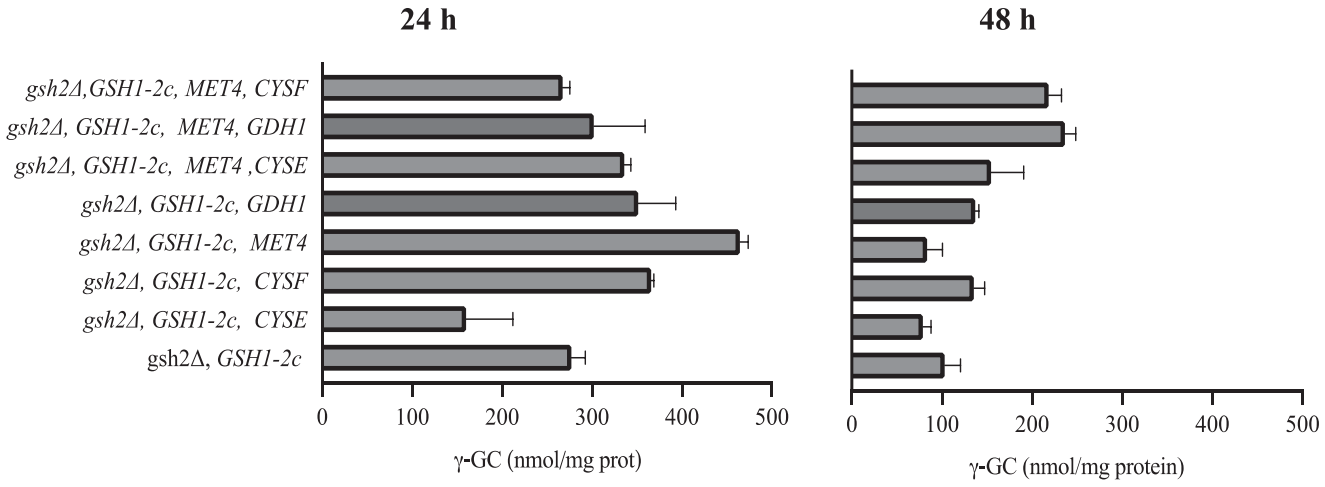


FIGURE 5 γ-glutamyl-cysteine intracellular concentration for strains RIY445 (gsh2D, GSH1-2c), RIY507 (gsh2Δ, GSH1-2c, CYSE), RIY510 (gsh2Δ, GSH1-2c, CYSF), RIY508 (gsh2Δ, GSH1-2c, MET4), RIY509 (gsh2 Δ, GSH1-2c, GDH1), RIY504 (gsh2 Δ, GSH1-2c, MET4, CYSE), RIY503 (gsh2 Δ, GSH1-2c, MET4, CYSF), and RIY505 (gsh2 Δ, GSH1-2c, MET4, GDH1) after 24 and 48 h of growth in YNBD medium. Data are the mean and standard deviation of triplicate experiments. γ-GC concentration is given in nmol of γ-GC per mg of protein in cell extract. GSH1-2c denote that mutant strains contain 2 copies of pTEF-GSH1 expression cassette. γ-GC, γ-glutamylcysteine; GSH, glutathione.

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turn, may result in lower synthesis of γ-GC due to reduced availability of intracellular cysteine.

4 | CONCLUSIONS

Interest in γ-GC as a therapeutic drug has been increasing over recent years. Surprisingly, reports on efficient producing strains or optimized production processes are scarce compared to those available for GSH. Only a few processes for chemical or enzymatic γ-GC synthesis have been proposed, but so far, these are still associated with low productivity, low yield, or high cost. In *S. cerevisiae*, the best mutants obtained by chemical mutagenesis produce a mixture of γ-GC and GSH, with a γ-GC titer of less than 5 mg $gDCW^{-1}$.[42,43] Herein, *Y. lipolytica* has been engineered to produce γ-GC, not GSH, with a titer of 464 nmol mg^{-1} protein, which corresponds to 93 mg $gDCW^{-1}$ within 24 h.

AUTHOR CONTRIBUTIONS

Diem Do, Mümine Guruk, Christian Damblon, and Anthony Arguelles-Arias: Investigation. Małgorzata Kus-Liśkiewicz: Writing-review & editing. Huseyin Erten: Funding acquisition, Writing-review & editing. Patrick Fickers: Conceptualization, Methodology, Supervision, Funding acquisition, Writing-original draft.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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