Bioprocess optimization of glutathione production by *Saccharomyces boulardii*: biochemical characterization of glutathione peroxidase

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Abstract

The well-known probiotic GRAS *Saccharomyces boulardii* (CNCM I-745) was used for the first time to produce glutathione (GSH). The culture conditions affecting GSH biosynthesis were screened using a Plackett–Burman design (PBD). Analyzing the regression coefficients for 12 tested variables, yeast extract, glucose, peptone, cysteine, temperature and agitation rate had a positive significant effect on GSH production with a maximum yield 192 mg/L. The impact of kinetics of adding cysteine was investigated in 19 experiments during the growth time course (0–36 h), and the maximum yield of glutathione (235 mg/L) was obtained by addition of cysteine after 8 h post-inoculation. The most significant variables were further explored at five levels using central composite rotatable design (CCRD), giving a maximum production of GSH (552 mg/L). Using baffled flasks, the yield of GSH was increased to 730 mg/L, i.e., 1.32-fold increment. The two rate-limiting genes of GSH biosynthesis “γ-glutamyl cysteine synthetase (*GSH1*) and GSH-synthetase (*GSH2*)” were amplified and sequenced to validate the GSH biosynthetic potency of *S. boulardii*. The sequences of genes showed 99% similarity with *GSH1* and *GSH2* genes of *S. cerevisiae*. Glutathione peroxidase was purified and characterized from *S. boulardii* with molecular mass and subunit structure of 80 kDa and 35 kDa as revealed from native and SDS-PAGE, ensuring its homodimeric identity. The activity of GPx was reduced by 2.5-fold upon demetallization confirming its metalloproteinic identity. The GPx was strongly inhibited by hydroxylamine and DTNB, ensuring the implication of surface lysine and cysteine residues on the enzyme active site domains.

Keywords Glutathione · Nutritional optimization · *Saccharomyces boulardii* · Glutathione peroxidase

Introduction

During the last decades, biomaterials, fine chemicals, nutraceuticals, nanomaterials, and pharmaceutically relevant compounds have become increasingly biosynthesized by different microorganisms (El-Baz et al. 2011a, b, 2016; 2018; Cui et al. 2019; Hassan et al. 2019). Glutathione (γ-glutamyl-L-cysteinyl-glycine) is a non-protein thiol containing tripeptide, which is widely distributed in living organisms, and mainly in eukaryotic cells (Meister and Anderson, 1983; Lienkamp et al. 2020). Glutathione (GSH) is an essential molecule for cells in the protection mechanisms against UV, xenobiotics, and heavy metals (Lienkamp et al. 2020). GSH is an essential bioactive tripeptide widely used in food, medicine, and other industries (Cui et al. 2019; Dan et al. 2020). In humans, GSH deficiency has been linked to numerous disorders, such as cardiovascular diseases, pulmonary diseases, liver cirrhosis, diabetes, gastrointestinal, and pancreatic inflammations, immune, aging, and neurodegenerative
diseases (Ballatori et al. 2009). Another major use of GSH is its use as a supplementation in GSH-deficient disorders observed in liver diseases, cystic fibrosis, Alzheimer’s disease, Parkinson’s disease, age-related disorders, and HIV infection (Ballatori et al. 2009). Other uses of GSH are in various acceptable formulations such as emulsifiers, anti-aging and moisturizers for skin care, and anti-sun products. To date, the organisms of choice for efficient production of GSH are the yeasts, especially *S. cerevisiae* and *Candida utilis* (Liang et al. 2009). Yeasts provide advantage such as their ability for fast growing to high cell densities in low-price media (Bachhawat et al. 2009). Genetically modified bacteria, e.g., *Escherichia coli*, have been successfully used for GSH production (Wang et al. 2015). Recently, Cui et al. (2019) reported that the engineered strain *E. coli* JM109 produced 24 mM GSH, using fed-batch fermentation in 5-L bioreactor, and the yield of GSH was improved by 17.3% as compared to the control strain. However, consumers usually do not accept the use of GMOs, especially in food. GSH is produced industrially by *S. cerevisiae* in high concentration, which is generally recognized as safe (GRAS) (Li et al. 2004; Hong et al. 2019). However, it is worthwhile to search for a novel GSH producer GRAS microorganism. *Saccharomyces boulardii* is a non-pathogenic yeast strain widely prescribed for adults and children as a biotherapeutic agent (McFarland and Bernasconi 1993). *S. boulardii* is an asporogenous yeast that grows well at 37 °C and pH 2.0, and thus has better heat/acid tolerance (Du et al. 2012). In addition, *S. boulardii* was used as a “probiotic” in animal feeding, and has been effective for reducing pathogens and their corresponding toxins concentration, strengthening the microbial balance, in addition to stimulating the immune system. *S. boulardii* is genetically very close to *S. cerevisiae*; however, it shows a very different behavior, particularly, in relation to growth yield and resistance to stresses, such as temperature and acidic conditions, which are important features for any microorganism used in probiotic applications (Fietto et al. 2004). Lately, Hong et al. (2019) stated that the probiotic *S. cerevisiae* BOF (*S. boulardii*-based product) can produce (5.1 mg/g dry basis) of GSH. Glutathione is synthesized in two consecutive ATP-dependent reactions (Meister and Anderson 1983). Firstly, the dipeptide γ-glutamyl cysteine (γ-GC) is synthesized from L-cysteine and L-glutamic acid by γ-glutamylcysteine synthetase (EC 6.3.2.2). In the second step, glycine is added to the C-terminal site of γ-GC by GSH synthetase (EC 6.3.2.3) to form GSH. Both enzymes were purified from several biological sources and their corresponding genes cloned from yeast (Mutoh et al. 1995; Shi et al. 2020) bacteria (Cui et al. 2019), and animal tissues (Huang et al. 1995). The optimization and control of the production process for GSH were investigated, showing that the temperature, agitation rate, and the pH are the most significant factors affecting its production. Furthermore, the addition of amino acids such as cysteine, glycine, glutamate, and serine increased the yield of GSH by 2.67-fold as compared with the control medium free from these amino acids. Furthermore, Cha et al. (2004) reported that by the optimization of concentrations of glucose, yeast extract, K₂HPO₄, and cysteine in the medium, the productivity of GSH was increased 2.27-fold as compared with the basal medium.

For the aforementioned reasons, the main objective of the present work was to optimize the production of GSH using the GRAS *S. boulardii* for the first time, where 12 environmental and nutritional factors were screened using PBD, in conventional Erlenmeyer flasks. After analyzing the design and determining the significant factors, CCRD was applied to establish the response surface, fitting the model, and forecasting the optimum conditions for the biosynthesis of GSH. To maximize the production of GSH, a comparison between conventional Erlenmeyer and baffled flasks was investigated, with all the optimum conditions detected in CCRD. The two responsible genes for the biosynthesis of GSH (*GSH1* and *GSH2*) were sequenced to validate the potency of GSH biosynthesis, as well as glutathione peroxidase (GPx) was purified and biochemically characterized.

### Materials and methods

#### Chemicals

Reduced L-GSH and alloxan were obtained from Sigma-Aldrich Inc. (Sigma-Aldrich, St. Louis, MO, USA). Dibasic sodium phosphate and glucose were obtained from ADWIC (CAIRO, Egypt). Yeast extract, agar–agar and peptone were obtained from SAS chemical Co., (MUMBAI, India). Malt extract, glycine, L-cysteine and L-glutamic acid were bought from LOBA Chemie (MUMBAI, India). All solutions were prepared using deionized distilled water.

#### Strains, media, and plasmids

The strain *S. boulardii* was isolated from “Florastor®”, under aseptic conditions, in a laminar airflow (Heraeus, Germany), the contents of one capsule was dispensed into 500 mL Erlenmeyer flask containing 100 mL of sterilized yeast malt peptone (YMP) broth medium with the following components (g/L): yeast extract 3.0, malt extract 3.0, peptone 5.0 and dextrose 10.0, pH was adjusted to 6.5 using 1 N NaOH/1 M HCl, then mixed well and incubated in shaking incubator (New Brunswick™, Innova® 43) at 170 rpm and 30 °C for 24 h. *S. boulardii* was spread on YMP agar plates and incubated for 24 h, and the developed pure colonies were examined, transferred, maintained on YMP agar slants at 4 °C, and renewed every 30 days. *Escherichia coli* JM109 was used as the recipient strain for the recombinant plasmid.
and it was grown in Luria–Bertani medium supplemented with 100 μg/mL ampicillin. pGEM-T easy vector (Promega) was used as a cloning vector for various PCR products generated during this study.

**DNA extraction and PCR amplification of GSH1 and GSH2 genes**

Genomic DNA was extracted from 24 h pure *S. boulardii* culture using the Yeast DNA Extraction Kit (Thermo Scientific™ Cat.No. 78870) according to the manufacturer’s instructions. The DNA quality was checked by 1% agarose gel as well as spectrophotometrically (at λ<sub>260</sub> and λ<sub>280</sub> nm). The genes encoding γ-glutamyl cysteine (γ-GC) synthetase (GCS, EC 6.3.2.2) (*GSH1*) and glutathione synthetase (EC 6.3.2.3) (*GSH2*) were amplified by PCR. The forward and reverse primers for amplification of *GSH1* were 5′-GACT AGTATGGGACTCTTAGTTGGGC-3′ and 5′-TCAGAG TCTTTAACAT TTGCTTTCTATGGCTATTTT-3′, and for *GSH2* amplification were 5′-ACGGCTACG ATG GCCACATCACCCTTC-3′ and 5′-GCAGATCCTCA GTAAAGAATAACTGTCCAAACATGGG-3′, respectively (Yoshida et al. 2011). PCR was performed in a 50 μL reaction volume containing 1X PCR buffer (10 mM Tris–HCl pH 8.3; 50 mM KCl; 2 mM MgCl<sub>2</sub> and 0.01% (w/v) gelatin), 250 μM dNTPs, 2.5 units of *Taq* DNA polymerases (amaR OnePCR™, Cat.No. SM213-0250), 100 pmol of each primer and the DNA template. PCR reaction conditions were 94 °C for 4 min; 35 cycles of denaturation at 94 °C; annealing at 60 °C; and extension at 72 °C for 50, 60, 120 s, respectively, and finally followed by 7 min extension at 72 °C. The amplified PCR product was visualized by electrophoresis on 1.5% (w/v) agarose gel for confirmation of amplification. Purification of the PCR product was done using high pure PCR purification kit (Qiagen).

**Cloning, sequencing and in silico analyses of GSH1 and GSH2**

The PCR products were purified using high pure PCR purification kit (Qiagen) and ligated into pGEM-T vector with T4 DNA ligase overnight at 4 °C (Kebeish et al. 2016). The recombinant clones were transformed into *E. coli* JM109 competent cells according to the manufacturer’s instructions. The amplicons of PCR were purified and sequenced using ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystem, USA) and the DNA sequencer ABI 310 Genetic Analyzer. The obtained sequence data were further analyzed using the BLAST tool of NCBI.

The sequence data were translated using ExPASy online translation tools (www.expasy.com). The six frames translation analysis was used for predicting the open reading frames (ORF). The open reading frame without stop codons for γ-glutamyl cysteine synthetase (*GSH1*) and GSH synthetase (*GSH2*) were selected for further bioinformatic analysis by protein BLAST. The diversity of the sequence among the selected isolate was analyzed by multiple sequence alignment and comparative sequence analysis along with the *GSH1* and *GSH2* sequences of protein database of NCBI using CLUSTALW program. The obtained sequences of *GSH1* (Gsp1p) and *GSH2* (Gsp2p) genes were BLAST searched non redundantly on the NCBI database, and aligned with ClustalW muscle algorithm, the phylogenetic trees was constructed by MEGA5 neighbor-joining method with 1000 bootstrap replication (Tamura et al. 2011).

**Experimental designs**

**Inoculum and shake-flask culture conditions**

Seed culture was prepared by inoculating 24 h old *S. boulardii* slant into 250 mL YMP broth medium. The flask was incubated in shaking incubator at 30 °C and 170 rpm for 24 h. All shake-flask experiments were performed in 250 mL Erlenmeyer flasks (baffled or unbaffled), each containing 50 mL of culture medium and inoculated with 10% (v/v) of the seed culture.

**Plackett–Burman experimental design and data analysis**

Plackett–Burman design (PBD) is an efficient tool to screen important variables among many factors with the least number of experiments. In PBD, 12 variables including the glucose, peptone, malt extract, cysteine, glutamic, glycine, yeast extract; and temperature, initial pH, agitation rate, medium volume and incubation period were set in two levels; −1 and +1 for low and high levels, respectively. 4 of these variables (cysteine, glutamic, glycine, yeast extract) were investigated regarding their GSH inducing properties. A GSH inducer was defined as a substance that can be biologically transformed to GSH. The arrangement of each experiment was generated by MINITAB®17 (Minitab Inc., PA, USA). All experiments were conducted in duplicate and their mean values of GSH %, GSH content (g/L) and cell dry weight (CDW) were taken as a response (Table S1). A total of 44 experiments were conducted as the design was replicated. The analysis of variance (ANOVA) for the experimental data and the model coefficients were computed with MINITAB® 17 statistical analysis tool kit (Eq. 1).

\[ Y = \beta_0 + \sum \beta_i x_i, \]

where Y is the predicted response; \( \beta_0 \) is the model constant; \( \beta_i \) is the linear coefficient and \( x_i \) is the independent variable.
The effect of addition time of cysteine

To investigate the impact of addition time of cysteine (5 mM), a total of 19 experiments were carried out at 0–36 h with increments of 2 of incubation time, using PBD design (Experiment no. 5) to determine the best addition time for the biosynthesis of GSH.

Central composite rotatable design (CCRD)

The significant factors affecting GSH production by *S. boulardii* were identified using PBD as yeast extract, glucose, peptone, and cysteine concentrations as major independent variables. Based on these results, the experiment was further expanded to a central composite rotatable design (CCRD) to get the optimum levels of the tested variables leading to the maximum GSH production with fixing the temperature value and agitation rate at 31 °C and 225 rpm (Myers et al. 2016). Since the addition time of cysteine has an influential effect on both GSH and CDW, so the CCRD design was carried out twice: in the first one, cysteine was added at zero time; while in the second, it was added after 8 h of inoculation. The design matrix, variables, and their levels in coded units for both designs are listed in Table S1. All the experimental data analysis, 100 times of 16 factorial, 8 axial and 7 central points. Both designs were carried out concomitantly to alleviate and minimize all sources of errors originating from handling and measurement systems. To predict the optimal point, a second-order polynomial function was fitted to correlate the relationship between the GSH content as a response and all the independent variables (Myers et al. 2016) (Eq. 2).

\[
Y = \beta_0 + \beta_1X_1 + \beta_2X_2 + \beta_3X_3 + \beta_4X_4 + \beta_{12}X_1X_2 + \beta_{13}X_1X_3 + \beta_{14}X_1X_4 + \beta_{23}X_2X_3 + \beta_{24}X_2X_4 + \beta_{34}X_3X_4 + \beta_{11}X_1^2 + \beta_{22}X_2^2 + \beta_{33}X_3^2 + \beta_{44}X_4^2
\]

(2)

where *Y* is the predicted response; *β*₀ is the model constant; *X₁, X₂, X₃* and *X₄* are the independent variables; *β₁, β₂, β₃* and *β₄* are the linear coefficients; *β₁₂, β₁₃, β₂₃, β₂₄* and *β₃₄* are the cross product coefficients; and *β₁₁, β₂₂, β₃₃* and *β₄₄* are the quadratic coefficients.

Cell dry weight (CDW) and optical density (OD)

Yeast cultures were sampled periodically, and the cell biomass concentration was determined by the cell dry weight. Five milliliters of the growth culture was centrifuged at 5000 rpm for 10 min and the pellet was washed twice with 5 mL saline solution (0.9% NaCl) and dried at 105 °C until constant weight. For OD measurement, samples were withdrawn every 2 h and diluted appropriately with saline solution, then growth OD was assessed at λ₆₀₀ nm spectrophotometrically. The glucose concentration was assayed by a glucose meter (Accu-Chek® active glucose meter system).

Intracellular GSH assay

*Saccharomyces boulardii* was incubated at 95 °C for 20 min to release the GSH from the cells into the aqueous layer. Cells were then removed by centrifugation at 5000 rpm for 20 min and then the GSH content in the supernatant was assayed using alloxan method as described by Wen et al. (2005). GSH reacts with alloxan under the specified conditions of the experiment and its concentration was determined spectrophotometrically at 305 nm. Briefly, 1 g/L alloxan was freshly prepared in 0.1 M HCl solution and stored in amber glass bottle, glycine (0.1 M) was prepared using deionized water. Phosphate buffer (pH 7) was prepared by mixing equimolar solutions (0.24 M) of Na₂HPO₄ and NaH₂PO₄ in deionized water. Standard reduced GSH calibration curve (0–200 mM) was prepared using GSH serially diluted with deionized water. The intracellular GSH content (mg/L) was calculated from the calibration curve (Linear Regression). Each of the GSH standard concentrations was mixed in a cuvette containing 3.5 mL of 0.24 M Na₂HPO₄ and NaH₂PO₄ buffer and 0.5 mL of glycine (0.1 M). One mL of alloxan solution was added to initiate the reaction, left for 20 min, and then the absorbance was measured at 305 nm. For sample analysis, 1 mL of tested sample was used instead the standard GSH. The reduced GSH content was calculated based on the following formula:

\[
\text{Intracellular reduced GSH} (\%, W/W) = \frac{\text{GSH (g/L)}}{\text{CDW (g/L)}} \times 100
\]

Glutathione peroxidase activity (GPx)

The crude enzyme was extracted by pulverizing *S. boulardii* (5 g fresh weight) in liquid nitrogen, dispensing in sterile extraction buffer 50 mL Tris–HCl (10 mM, pH 7.0) containing 1 mM PMSF, 1 mM EDTA and 1 mM 2-mercaptoethanol (ElMekawy et al. 2013; El-Sayed et al. 2016, 2019a, b). The homogenate was thoroughly vortexed for 5 min, centrifuged at 8000 rpm for 10 min at 4 °C, and the
supernatant was used as the source of crude GPx. The activity of GPx was determined by 5,5’-dithiobis-2-nitrobenzoic acid (DTNB) (Ellman’s reagent). Briefly, the reaction mixture 10 mM GSH, 1 mM EDTA, 390 µM H2O2, dissolved in Tris–HCl (40 mM, pH 7.0) and 100 µL enzyme preparation in 1 mL total volume. Blanks of enzyme and substrate were prepared. After incubation at 37 °C for 15 min, the reaction was stopped by freezing at −20 °C for 5 min, amended with 100 µL DTNB reagent and incubated for 10 min. The residual concentration of GSH (GSH-TNB adducts) was measured spectrophotometrically at 412 nm (Tietze 1969). One unit of glutathione peroxidase was expressed by the amount of enzyme releasing 1 µmole of oxidized glutathione (GSSG) from reduced glutathione (GSH) as substrate per min per mg protein under standard assay. The protein content of the enzyme was determined by Bradford’s reagent using bovine serum albumin as standard (Bradford 1976).

Purification and molecular subunit structure of *S. boulardii* GPx

*S. boulardii* was grown on the nutritionally optimized media for GSH production as described above, incubated at standard conditions. After growth, the yeasts pellets (20 g) were collected, washed with sterile saline and pulverized in liquid nitrogen, dispensed in 50 mL Tris–HCl (10 mM, pH 7.0) with 1 mM PMSF, 1 mM EDTA and 1 mM 2-mercaptoethanol (Abdel-Monem et al. 2012; El-Sayed et al. 2016, 2019a; b). The homogenate was thoroughly vortexed for 5 min, centrifuged at 8000 rpm for 10 min, and the supernatant was used as the source of crude enzyme. The supernatant was fractionally concentrated with 20 kDa cut-off dialysis was used as the source of crude enzyme. The supernatant was collected, washed with sterile saline and pulverized in liquid nitrogen, dispensed in 50 mL Tris–HCl buffer and 1 mM EDTA and 1 mM 2-mercaptoethanol (Abdel-Monem et al. 2012; El-Sayed et al. 2016, 2019a; b). The homogenate was thoroughly vortexed for 5 min, centrifuged at 8000 rpm for 10 min, and the supernatant was used as the source of crude enzyme. The supernatant was fractionally concentrated with 20 kDa cut-off dialysis membrane (Cat. # 546-00051) against polyethylene glycol 6000. The enzyme preparations were purified by ion-exchange chromatography with DEAE-Sepharose column (1.5 x 40 cm). The pre-equilibrated column was load with the crude protein; the enzyme fractions were eluted with Tris–HCl buffer of NaCl gradients (100–300 mM) at 1 mL/min flow rate. The activity and concentration of GPx fractions were determined as described above. The most active homogenous fractions were collected and purified by gel-filtration chromatography using Sephadex G100 column (El-Sayed et al. 2016).

The molecular homogeneity and subunit structure of the purified GPx was assessed by denaturing-PAGE (Laemmli 1970). The entire molecular mass of the purified GPx was determined by Native-PAGE with slight modification. The native sample buffer containing 500 µg of purified GPx were vigorously shaken for 2 min, then loaded to the native-PAGE (10%) (El-Sayed et al. 2016). The gel was running for 2 h at 4 °C, washed with Tris–HCl buffer and soaked in 50 mL of ddH2O containing 1 mM cumene hydroxide and 1 mM GSH, incubated for 2 h and 80 rpm using shaker incubator. Subsequently, the gel was rinsed twice with distilled water and stained with 1% ferric chloride and 1% potassium ferrocyanide solution; the developed blue-black band indicates the activity of GPx (Li et al. 2000).

Active site mapping and catalytic identity of purified GPx

The catalytic identity of purified *S. boulardii* GPx was determined using the different cationic inhibitors and amino acids suicide analogs (El-Sayed et al. 2016). The enzyme preparation was demetallized by dialysis (Cat # 546-00051, Wako Chem., USA) against 50 mM Tris–HCl buffer (pH 7.5) containing 1 mM EDTA for 2 h. The apo-enzyme was amended with different cations, such as Ba2+, Fe3+, Ca2+, Hg2+, Fe3+, Al3+, Zn2+, Na+, Cu2+, and Na+ at 1 mM final concentration for 2 h at 4 °C, then the substrate was added and the enzymatic activity was measured by the standard assay. The influence of suicide amino acid reactive analogs, namely hydroxylamine, guanidine thiocyanate, DTNB, MBTH, H2O2 and PMSF (1 mM final conc.) on the activity of purified GPx was assessed. The enzyme was amended with each compound for 2 h, then the substrate was added, and the residual enzymatic activity was measured by the standard assay.

Statistical analysis

The experiments were conducted in biological triplicates and the results were expressed by means ± SD. The significance and F-test were calculated using one-way ANOVA with Fisher’s least significant difference of post hoc test.

Results and discussion

Screening of the significant culturing factors affecting GSH production using PBD

Genetically modified strains of *E. coli* were used for maximum GSH production that reached 5870 and 15.210 mg/L (Wang et al. 2016; Zhang et al. 2016). However, GMOs are not usually accepted especially in food products, as well as the problems concerning consumer acceptance. The GSH biosynthesis was mainly controlled by γ-glutamyl cysteine synthetase (*GSH1*) and glutathione synthetase (*GSH2*) (Johnston and Bloch 1951). *Saccharomyces boulardii* differs from *S. cerevisiae* in its taxonomic, metabolic and genetic properties (Lei et al. 2016); therefore, it is interesting to study the culture conditions for GSH production by *S. boulardii*. YMP medium components such as, glucose, yeast extract, peptone and malt extract were investigated. Glucose is the carbon and energy source, while the yeast
extract supplies vitamin B complex, other growth factors, which is needed to generate enough biomass. Peptone serves as a source of nitrogen, carbon, and essential nutrients, while malt extract represents an additional source of carbon. Since GSH is a tripeptide, its production has close relationship with certain intracellular amino acids, especially glutamic acid, cysteine and glycine. Therefore, the three amino acids (γ-glutamic acid, l-cysteine and glycine) could be important factors affecting its biosynthesis. Wang et al. (2016) reported that cysteine and glycine play a significant role in the synthesis of GSH. Likewise, the PBD analysis represented by Pareto chart (Fig. 1) shows that cysteine is the key amino acid in the GSH production by S. boulardii. However, both glutamic acid and glycine have minor effects on GSH production by S. boulardii as shown from their high p values and these results (Table S1) are in accordance with that obtained by Alfafara et al. (1992). Similar results correlated the production of GSH by yeasts with both cysteine and glycine significantly affected the GSH accumulation, while, glutamic acid did not (Wen et al. 2005). In our study, only cysteine significantly affected GSH production (Fig. 1).

PBD was used to screen the factors affecting GSH production by S. boulardii. Results in Table S1 show that there was a variation in GSH production, which indicates the importance of studying the medium composition to obtain higher GSH productivity. Maximum GSH% of 2.274 was obtained in the 8th trial run, whereas a minimum GSH% of 0.578 was obtained in the 22nd trial run. This improvement was achieved on using glucose (70 g/L), yeast extract and peptone (12 g/L), malt extract (0 g/L), pH (3), temperature (37 °C), cysteine and glycine (10 mM), glutamic acid (0 mM), agitation rate (300 rpm), culture volume (25 mL) and cultivation period (24 h). Also, the main effects of the examined variables on GSH production were calculated and graphed as a Pareto chart (Fig. 1), which displayed the magnitude of each estimate and showed the ranking of the variable estimates. The effects of these variables and their significance were estimated by their p values (Table S1). P value < 0.05 indicated that the influence of the variable was significant. Experimental data (Fig. 1) show that six variables, including the time for cysteine addition, glucose, peptone, and yeast extract conc, temperature and agitation rate, were identified as the significant variables. The R² and the adjusted R² values of the model are 95.56%, and 93.63%, respectively, indicating a high correlation. The best obtained results of both the yeast biomass and the GSH content revealed that at the fermentation conditions, maximum cell growth did not exactly correspond to the best one found for high GSH production. The obtained results revealed that the optimum pH for high GSH production was 5.5, where S. boulardii could tolerate gastric acidity and could survive in a wide pH range. S. boulardii produced better biomass and high GSH content at 31 °C; and these results are consistent with that of Du et al. (2012). Adequate oxygen can initiate

![Fig. 1 Pareto chart rationalizing the effect of each variable on the production of GSH by S. boulardii using PBD. The vertical line defines the 95% confidence interval](image-url)

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the strain growth (Du et al. 2012) and consequently GSH production. The high \( p \) values of 0.967 and 0.077 for the medium volume and incubation period, respectively, suggest their insignificant effect on GSH production, whereas the agitation rate (Table S1 and Fig. 1) has a low \( p \) value (0.019) indicating its significant effect.

**The effect of addition time of cysteine**

To modulate the GSH metabolism in the fermentation process, the appropriate time for addition of cysteine was studied. Therefore, 19 experiments were carried out for cysteine addition at 0, 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34 and 36 h of growth using PBD design (Experiment no. 5). Results obtained (Fig. 2) show that cysteine addition after 8–14 h of incubation had significant effect on GSH content with a maximum yield 235 mg/L after 8 h. It could be concluded that cysteine worked well on the rapidly growing cell, which was in accordance with the results obtained by Alfaara et al. (1992) and Liang et al. (2009), who reported that the optimal time for GSH addition is during the stationary phase of *S. cerevisiae*, and that cysteine was the only amino acid that can enhance GSH production. Cysteine is the substrate of gamma-glutamyl cysteine synthetase, which is the first committed enzyme in GSH biosynthesis; however, at the same time, cysteine can act as a growth inhibitor (Li et al. 2004). Consistently, addition of the precursor amino acids "cysteine, glycine and glutamate" has been extensively investigated to increase the intracellular GSH content (Alfaara et al. 1992). Coincidentally, cysteine represents the key amino acid for GSH production and is the most common inducer for GSH overproduction.

**Central composite rotatable design (CCRD), model fitting and ANOVA**

The intracellular concentration of glutathione by *S. boulardii* was maximized upon nutritional optimization by response surface methodology using Plackett-Burman design (PBD). Response surface methodology (RSM) use quantitative data to build an empirical model that describes the relationship between each factor investigated and the response. This model considers the effects of each factor, interactions between factors, and curvature (Myers et al. 2016). CCRD is the most popular class of second-order designs and widely used in RSM. Based on the PBD and the cysteine addition experiments, a CCRD experimental design was carried out to provide a proper model for the optimization of GSH production by *S. boulardii*, with four factors (glucose, yeast extract, peptone and cysteine concentrations) at five levels with cysteine addition after 8 h post-inoculation or at zero time. Results in Table S1 show that GSH was increased from 218 to 559 mg/L by the addition of cysteine 8 h post-inoculation as compared to its addition at zero time (Exp. No. 20), indicating that the addition time is a crucial factor affecting GSH production by *S. boulardii*. Udeh and Achremowicz (1997) reported that total GSH concentration obtained at the optimal conditions was 160 mg/L, which was nearly two-fold that of the control without cysteine addition. Similarly, Lorenz et al. (2015) reported that the duration of cysteine addition was 6 h, and he developed a repeated fed-batch procedure using cysteine (0.40 mol/mol), to obtain a high
biomass of 84.2 g/L and GSH content of 1.3047 g/L using S. cerevisiae Sa-07346. An analysis of variance (ANOVA) was used to determine whether the constructed model was adequate to describe the observed data (Table S1). The coefficient of determination ($R^2$) indicates the percentage of the variability of the optimization parameters that is elucidated by the model. The response equation in which the parameters were fitted and interactions between them were determined as a quadratic equation (Eq. 3) was as follows:

$$
\text{Total GSH (Y) } = -0.424 + 0.00525X_1 + 0.0373X_2 + 0.0448X_3 + 0.1732X_4 - 0.000041X_1^2 \\
- 0.001231X_2^2 - 0.001314X_3^2 - 0.01377X_4^2 + 0.000107X_1 \times X_2 + 0.000022X_1 \times X_3 \\
- 0.000322X_1 \times X_4 - 0.000694X_2 \times X_3 - 0.001833X_2 \times X_4 + 0.000107X_1 \times X_3 \\
$$

This equation revealed that cysteine concentration was the most significant factor as it shows a regression coefficient of 0.1732, followed in order by peptone concentration (0.0448), yeast extract (0.0373) and glucose (0.00525), exerting a statistically significant overall effect ($p$ value < 0.05) on the response. In addition, a positive sign represents a synergistic effect, whereas a negative one indicates an antagonistic effect.

From an industrial perspective, total GSH (g/L) was selected in this study, so media components were optimized with respect to total GSH as a response. CCRD results (Table S1) showed that the fermentation components formula that maximized cell growth (CDW) did not support the GSH accumulation. Run 23 showed the maximum cellular growth represented by CDW of 12.74 g/L, accompanied by low GSH production (0.324 g/L) as compared with other runs in the design. On the other hand, run 29 showed the highest GSH production (0.552 g/L) accompanied by CDW of 10.66 g/L. The GSH content was almost doubled from 0.212 to 0.552 g/L, and its percentage (2.448–5.178) by cysteine addition 8 h post-inoculation, as compared to cysteine addition at zero time. The GSH concentration decreased when L-cysteine concentration was decreased, as indicated by the positive sign in the equation. These results are consistent with those of Wen et al. (2005). It was reported that cysteine addition during the fermentation course promotes the increase of the GSH concentration. The increased activity of GSH synthetase upon cysteine addition may account for the increased conversion of γ-glutamyl cysteine to GSH (Wang et al. 2016). On the contrary, Fig. 3 shows that when cysteine was increased from 1 to 3.75 mM,
the GSH increased markedly to reach its maximum concentration of 0.559 g/L, and then decreased gradually when cysteine concentration was increased above 3.75 mM. The result of Alfafar et al. (1992) indicated that the positive effect on the specific GSH production rate was obtained when the cysteine concentration was above 3 mM. Results in Fig. 3 show that when cysteine concentration was 5.12 mM, the GSH was decreased to 0.523 g/L. This can be explained by the inhibitory effect of cysteine on the yeast growth which was reflected by a decrease in its CDW. This was also confirmed by the reduction in yeast biomass when cysteine was added at zero time (Table S1).

The effect of glucose, cysteine, yeast extract and peptone concentrations and their interactive effects on GSH production are shown in Fig. 3 and Table S1. The produced GSH remained relatively constant (0.549 and 0.559 g/L) when the yeast extract was increased from 9.5 to 12.25 g/L, respectively, before its slight reduction to 0.549 g/L when the yeast extract was increased to 15 g/L (Fig. 3; Table S1). In contrast, Cha et al. (2004) reported that 0.204 g/L of GSH was produced, when 30 g/L of yeast extract was used in the fermentation medium. However, Zhang et al. (2007) optimized GSH production by S. cerevisiae using uniform experimental design and 0.740 g/L of GSH was produced with 3 g/L yeast extract, in shake-flask culture. Similarly, changing the peptone concentration exerts the same effect, where GSH remained around 0.550 g/L when the peptone concentration increased from 8.3 to 11.3 g/L (Fig. 3). Overall, the optimization plot of CCRD model predicted that GSH was to be 0.559 g/L when using the optimal growth medium of 69.32 g/L glucose, 12.21 g/L yeast extract, 11.12 g/L peptone and 3.75 mM cysteine (Eq. 3). The experimental result gave a yield of 0.544 g/L GSH, which was very close to the predicted one using CCRD analysis and close to the best trial (Run no. 20) in Table S1. The comparison of the predicted and experimental values showed good correlation between them, implying that the empirical model derived from CCRD can be used adequately to describe the relationship between all tested reaction parameters and the biosynthesis of GSH by S. bouardii. Hence, this model could then be used to predict GSH production, within the experimental range.

The effect of using baffled flasks on GSH production

Shake-flask design is particularly important as it determines the performance of microbial culture in terms of produced GSH by establishing oxygen transfer conditions and hydrodynamics (Li et al. 2013). PBD and CCRD experiments were carried out in unbaffled conventional Erlenmeyer flasks. Several modifications to the conventional Erlenmeyer flasks have been proposed to improve mass transfer; nevertheless, the simplest and more effective one has been the baffling of the flasks. The comparison between unbaffled and baffled flasks emphasizes the advantages of efficient mass transfer, increasing oxygen availability, and distinctive shear property in baffled flasks (Li et al. 2013). Shaking flask culture of microorganisms confirm the aerobic conditions, providing sufficient stirring and oxygen to the culture, enabling microorganisms to be batch cultured in parallel at low cost (Ge and Rao 2012). The experiment was carried out using all the optimized conditions obtained from the CCRD analysis which are: glucose 69 g/L, yeast extract 12 g/L, peptone 11 g/L, cysteine 3.75 mM added after 8 h of incubation, temperature was set at 31 °C, agitation rate at 225 rpm, initial pH of 5.5 and incubation period of 32 h.

Fig. 4 Effect of using baffled flasks on cell dry weight (CDW) of S. bouardii and its production of GSH in yeast malt peptone medium (YM), optimized medium in normal flasks (opti-normal) and optimized medium in baffled flasks (opti-baffled)
experiment (Fig. 4) was repeated using the optimized conditions in baffled flasks, the GSH was increased to 730 mg/L, which was 4.5-fold that obtained in non-optimized YMP medium and 1.31-fold that obtained using un-baffled flasks; indicating that good aeration is an important factor in the biotechnological production process of GSH by S. boulardii. Similar results were reported for C. utilis, when the cysteine metabolism was combined with controlling the dissolved oxygen, thus promoted efficient GSH production (Liang et al. 2009). Consistently, the cellular growth and metabolic productivities of E. coli K12 baffled cylindrical flask was increased by about 1.3- to 1.5-fold than in the Erlenmeyer and Sakaguchi flasks (Takahashi and Aoyagi 2020).

**Amplification, sequencing, phylogenetic, in silico analyses of GSH1 and GSH2 genes**

To confirm the capability of S. boulardii to synthesize GSH, GSH1 and GSH2 genes were amplified, cloned, and sequenced. Based on the reported sequence in the GenBank database, the genomic DNA that encodes the two genes were amplified with two synthetic primers that contained the SpeI and BglI sites for the first gene (GSH1) and SalI and BglII sites for the second gene (GSH2). Two size products (2.2 and 1.5 kb) for GSH1 and GSH2 genes, respectively, were detected and confirmed using agarose gel electrophoresis (Fig. S1). These products were cloned into the plasmid pGEM-T and expressed in E. coli JM109, the transformants were selected, and the genes were amplified and sequenced by automatic sequencing in Bionex Inc., Korea. The nucleotide sequences of GSH1 and GSH2 genes were submitted to the GenBank under the accession numbers, MT583477 and MT583478.

The obtained sequence data (Fig. S2) were further subjected to in silico translation to obtain the uninterrupted ORF. Amino acid sequences were subjected to BLAST analysis, which clearly revealed its identity to the amino acid sequences of GSH1 and GSH2 of other organisms. *Saccharomyces boulardii* GSH1 and GSH2 exhibited a 99% and 96% similarity with *S. cerevisiae* YJM1447 GSH1 with accession AJR71399.1 and with *S. cerevisiae* AWR1631 GSH2 with accession EDZ69442.1, respectively. From the phylogenetic tree (Fig. S2), the ORF sequence of *S. boulardii* GSH1 displayed a 99% similarity with GSH1 from *S. cerevisiae*.

### Table 1 Overall purification profile of glutathione peroxidase from S. boulardii

| Purification step       | Total protein (mg) | Total activity (U) | Specific activity (μmol/mg/min) | Purification fold | Yield  |
|-------------------------|--------------------|--------------------|-------------------------------|-------------------|--------|
| Crude                   | 10                 | 585                | 58.5                          | 1                 | 100    |
| Dialysis by 20 kDa cutoff | 4.5               | 449                | 99.7                          | 1.71              | 76.8   |
| DEAE-Sepharose           | 1.04               | 172                | 165.4                         | 2.84              | 29.5   |
| Sephadex G100            | 0.20               | 89                 | 445                           | 7.7               | 15.4   |

**Purification and molecular subunit structure of S. boulardii GPx**

GPx was purified from *S. boulardii* by fractional concentration using dialysis membrane (20 kDa cutoff), gel-filtration and ion-exchange chromatography (El-Sayed et al. 2019a, b). After purification, GPx activity was increased by about 7.7-fold with 15.4 yield (Table 1). Overall, the specific activity of purified *S. boulardii* GPx was 445 μmol/min, with protein concentration 0.2 mg/L. The entire molecular mass and subunit structure of the purified GPx was about 80 kDa and 35 kDa as revealed from the native-PAGE and denaturing-PAGE, respectively (Fig. 5), ensuring the homodimeric identity of purified enzyme. Similar purification protocols for GPx from *S. cerevisiae* have been reported (Liu et al. 2008). Consistently, GPx with molecular subunit structures 35–39 kDa has been reported from yeast and humans (Brigeli-Flohé and Maiorino 2013).

**Biochemical properties of purified GPx**

The catalytic and structural identities of the purified GPx were assessed in response to various inhibitors and amino acids suicide analogs (El Baz et al. 2017; El-Sayed and Ali 2020). The enzyme preparation was demetallized by dialysis against 20 mM Tris–HCl containing 1 mM EDTA. The activity of holo-GPx was reduced from 445 μmol/min to 180 μmol/min upon demetallization, (2.5-fold), ensuring the metalloproteinic identity of the enzyme (Table 2). The enzyme activity was completely restored upon addition of Se+ cations, followed by Na+ suggesting the possibility of...
Fig. 5 Purification and molecular subunit structure of glutathione peroxidase (GPx) from *S. boulardii*. The enzyme was extracted and purified from the yeast pellets by ion-exchange, gel-filtration chromatography and its homogeneity was checked by denaturing and non-denaturing PAGE. A Activity of GPx fractions from ion exchange at 200 mM NaCl. B Activity of *S. boulardii* GPx fractions purified by gel-filtration chromatography. The most active fractions from the last chromatographic column was concentrated, and the enzyme subunit structure and entire molecular mass was determined from the SDS-PAGE (C) and Native-PAGE (D). M protein marker (Cat. # PG-PMT2962, 315–10 kDa), S refers to sample.

Table 2 Relative activity of purified *S. boulardii* glutathione peroxidase in response to various inhibitors and amino acid suicide analogues

|                | Conc. (mM) | Specific activity (μmol/mg/min) | Relative activity |
|----------------|------------|---------------------------------|-------------------|
| Holo-GPx       | 0          | 445 ± 45                         | 100               |
| Demetallized GPx* | 0          | 180 ± 25                         | 40.4              |
| KCl            | 10         | 202 ± 30                         | 45.3              |
| BaCl₂          | 10         | 230 ± 34                         | 51.6              |
| HgCl₂          | 10         | 270 ± 35                         | 60.6              |
| FeCl₃          | 10         | 301 ± 41                         | 67.6              |
| CaCl₂          | 10         | 359 ± 45                         | 80.6              |
| ZnCl₂          | 10         | 389 ± 42                         | 87.4              |
| CuSO₄          | 10         | 469 ± 47                         | 105.3             |
| AlCl₃          | 10         | 296 ± 35                         | 66.5              |
| SeCl₂          | 10         | 458 ± 49                         | 102.9             |
| NaCl           | 10         | 398 ± 30                         | 89.4              |
| MgSO₄          | 10         | 260 ± 26                         | 58.4              |
| Hydroxylamine  | 20         | 110 ± 22                         | 24.7              |
| Iodoacetate    | 20         | 254 ± 19                         | 57.2              |
| 6-Diazo-5-oxo-L-norleucine | 20 | 268 ± 29                     | 60.3              |
| DTNB           | 20         | 203 ± 32                         | 45.7              |
| MBTH           | 20         | 260 ± 35                         | 58.5              |
| PMSF           | 5          | 260 ± 37                         | 58.5              |
their implantation as enzyme cofactor. No obvious positive effect on the activity of Apo-GPx was noticed upon addition of divalent cations such as Cu$^{2+}$, Ba$^{2+}$, Mg$^{2+}$, Hg$^{2+}$, Ca$^{2+}$, Zn$^{2+}$ and trivalent cations Fe$^{3+}$ and Al$^{3+}$. Similar results confirm the dependency of GPx from different organisms on the monovalent cations Se$^{+}$ as cofactor (Brigelius-Flohé and Maiorino 2013). The highest restoring activity of GPx in response to Na$^+$ might be due the molecular proximity of Na$^+$ with Se$^{+}$.

The active sites and catalytic identities of the purified S. boulardii GPx were evaluated from the amino acid suicide analogs such as hydroxylamine, iodoacetate, 6-diazo-5-oxo-L-norleucine, DTNB, MBTH, H$_2$O$_2$ and PMSF. The activity of purified GPx was reduced by fourfold upon the addition of hydroxylamine, by twofold in response to DTNB, and by 1.7-fold in response to iodoacetate, MBTH, PMSF and 6-diazo-5-oxo-L-norleucine, as compared to the holo-GPx activity (El-Sayed and Ali 2020). The strong reduction to the GPx activity in response to hydroxylamine and DTNB ensures the implication of surface lysine and cysteine residues on the active site domains of enzyme (El-Sayed et al. 2015, 2016). The mild negative effect on the GPX activity by MBTH ensures the inaccessibility of the asparagine, glutamine, and arginine residues for reactivity, which might be due their presence in the enzyme core, with lower frequency of these residues on the enzyme surface. Also, lower negative response of purified GPx in response to PMSF assures the limiting of accessiblity of surface aspartic acid residues (El-Sayed et al. 2019a). The catalytic efficiency affinity of the purified GPx from S. boulardii toward different substrates of proteinous amino acids, non-proteinous amino acids and tripeptides was assessed. The activity of GPx was measured as thiols peroxidase and deaminase activities (El-Sayed et al. 2016). The results obtained (Table 3) show that the highest affinity of GPx was measured toward reduced GSH (100%), followed by L-cysteine (95%) and homocysteine (75%) as substrates. The affinity of GPx toward these substrates could be due the presence of accessible reactive free thiols. However, the affinity of GPx toward oxidized GSH (GSSG), L-cystine, and homocystine as substrates was relatively low (15–23%), being reasonable due to the absence of free reactive thiols. The purified GPx displayed a very low deaminase activity toward the different tested substrates, especially oxidized GSH (22%), and the other tested amino acids. Furthermore, the kinetic properties of the purified GPx toward the reduced GSH, L-cysteine and homocysteine were determined based on thiol peroxidation. The highest affinity ($K_m$) of purified GPx was reported for reduced GSH ($K_m = 8$ mM), followed by L-cysteine ($K_m = 13$ mM), and oxidized GSH ($K_m = 39$ mM). As well as the highest turnover number ($K_{cat}$) and catalytic efficiency for GPx was recorded for reduced GSH ($K_{cat}$

### Table 3: Substrate specificity of the purified S. boulardii glutathione peroxidase

| Substrate                  | Con. (mM) | SH peroxidase relative activity (%) | Deaminase relative activity (%) |
|---------------------------|-----------|------------------------------------|--------------------------------|
| Reduced glutathione (GSH) | 10        | 100 ± 12                           | 12 ± 1.2                       |
| Oxidized glutathione (GSSG)| 10        | 15 ± 2                             | 22 ± 3.5                       |
| L-Cysteine                | 10        | 95 ± 5                             | 16 ± 2.6                       |
| L-Cystine                 | 10        | 23 ± 4                             | 10 ± 1.9                       |
| Homocysteine              | 10        | 75 ± 6                             | 9 ± 2.1                        |
| Homocystine               | 10        | 18                                 | 6 ± 1.7                        |
| L-Methionine              | 20        | 0                                  | 4 ± 0.9                        |
| L-Asparagine              | 20        | 0                                  | 3 ± 0.8                        |
| L-Glutamine               | 20        | 0                                  | 8 ± 0.7                        |
| L-Phenylalanine           | 20        | 0                                  | 0                              |
| L-Ornithine               | 20        | 0                                  | 4 ± 0.2                        |
| L-Arginine                | 20        | 0                                  | 5 ± 0.3                        |
| Bovine serum albumin      | 5         | 16 ± 2.4                           | 8 ± 0.4                        |

### Table 4: Kinetics parameters of the purified S. boulardii glutathione peroxidase

| Substrate                  | $K_m$ (mM) | $V_{max}$ (μmol/mg/min) | $K_{cat}$ (s$^{-1}$) | $K_{cat}/K_m$ (mM$^{-1}$ s$^{-1}$) |
|---------------------------|------------|-------------------------|----------------------|-----------------------------------|
| Reduced glutathione (GSH) | 8          | 450                     | 380                  | 208                               |
| Oxidized glutathione (GSSG)| 39         | 98                      | 114                  | 85                                |
| L-Cysteine                | 13         | 320                     | 218                  | 129                               |
Similar kinetic properties were reported for GPx from *S. cerevisiae* and humans (Liu et al. 2008).

**Conclusions**

The production of GSH-enriched yeasts for food applications might be the only way to achieve high intracellular GSH content as usually GMOs are not accepted. Molecular study was used to assure the genetic relatedness of *S. boulardii* isolate to the *Saccharomyces* spp. and GSH enzyme production. This study revealed that the application of PBD and CCRD statistical analyses were efficient strategies to optimize the cultural conditions for GSH production by *S. boulardii*. Under the optimized conditions, the GSH was increased from 192 to 552 mg/L in conventional Erlenmeyer flasks. However, using baffled flasks, the GSH was significantly increased to 730 mg/L.

Optimum culture conditions for maximum GSH production were: glucose 69 g/L, yeast extract 12 g/L, peptone 11 g/L, agitation rate of 225 rpm, initial pH of 7 and 3.75 mM cysteine added after 8 h of incubation at 31 °C. GPx was purified and characterized with 35–39 kDa subunits using SDS-PAGE. The activity of holo-GPx was reduced by 2.5-fold upon demetallization, ensuring the metalloproteinic identity of this enzyme. The strong reduction in the GPx activity in response to hydroxyamine and DTNB ensures the implication of surface lysine and cysteine residues on the enzyme active site domains. The process strategy applied in this study showed the potential of using the GRAS *S. boulardii* with the aim of maximizing GSH production and the probiotic features of this specific yeast.

**Supplementary Information** The online version contains supplementary material available at https://doi.org/10.1007/s00203-021-02584-0.

**Declarations**

Conflict of interest All authors declare that there are no financial/commercial conflicts of interest.

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