

## Expression of *Vitreoscilla* Hemoglobin Enhances Glutathione Accumulation in *Saccharomyces Cerevisiae*

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**Abstract:** Glutathione (GSH) is an important antioxidant and free radical scavenger in the human body. Maintaining oxygen balance is a major problem in GSH production through fermentation. The study was used to the heterologous expression of *vgb*, encoding *Vitreoscilla* hemoglobin (VHb) involved in oxidative metabolic activity, to regulate redox potential in *Saccharomyces cerevisiae*. Plasmids *pYES2-vgb*, *pYES2-Su9-vgb*, and *pYES2-vgb-2A-Su9-vgb*, which subcellularly localize the expression of VHb in the cytoplasm, mitochondria, and cytoplasm and mitochondria, respectively, were constructed. And GSH production improved 24%, 83%, and 122%, respectively, compared to the parent strain. In this paper, the expression of VHb in the cytoplasm and mitochondria provides a novel approach for the industrial production of GSH by microbial fermentation and a solution for the problem of compromised cell growth and the accumulation of reducing agents during industrial fermentation.

**Keywords:** Glutathione, *Saccharomyces cerevisiae*, *Vitreoscilla* Hemoglobin, Location expression

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### I. Introduction

Glutathione (GSH),  $\gamma$ -L-glutamyl-L-cysteinylglycine, is the most abundant nonprotein thiol found in many organisms (Masip et al., 2006). GSH also plays key roles in redox signalling, the detoxification of xenobiotics and/or their metabolites, the modulation of cell-cycle progression and apoptosis, the storage of cysteine, and the regulation of immune function and fibrogenesis (Kunio et al., 2011; Shelly and Lu, 2013; Muller and Merrett, 2015). Moreover, it participates in numerous metabolic and cell signalling processes, including protein synthesis and amino-acid transport, DNA repair and the control of cell division, and cell-suicide programmes (García-Giménez, 2013). GSH is a valuable tripeptide that is widely used in the pharmaceutical, food, and cosmetic industries (Tian et al., 2010; Kritzing et al., 2013; Lassila et al., 2015; Sonthalia et al., 2016). At present, the methods of producing GSH include solvent extraction, chemical synthesis, microbial fermentation, etc., among which microbial fermentation is the most widely used. At present, the main microorganisms that can produce GSH are *S. cerevisiae*, *Pichia pastoris* and *Escherichia coli*. It is more convenient, faster, and more efficient to improve intracellular GSH levels by modifying the target strains through genetic engineering. Two problems are often encountered during the industrial production of GSH through *S. cerevisiae* fermentation. First, when the oxygen content in the environment is relatively high, GSH is oxidized to GSSG, which leads to a decrease in GSH production. Second, when in an oxygen-depleted environment, the cell-growth rate slows. Therefore, how to maintain the redox potential balance has become an key problem.

*Vitreoscilla* hemoglobin (VHb) is an oxygen-binding protein involved in oxygen-related metabolic activities, which increases metabolism by transferring oxygen to the respiratory chain, alters metabolism under hypoxic conditions, and affects expression of the target gene. The gene *vgb* encoding VHb has been successfully cloned and transformed into the cytoplasm of bacteria (Pablos et al. 2011; Deng et al., 2018), yeast (Chen et al., 2019; Mirończuk et al., 2019) and plant (Mu et al., 2017; Wang et al., 2019) to improve its growth and productivity. It also optimizes cell density, protein synthesis and oxidative metabolism, especially under anoxic conditions (Stark et al., 2015).

Su9 is a signal sequence that localizes proteins to the mitochondria. Westermann and Neupert (2000) described the Su9-directed localization of fluorescent proteins to the mitochondria. In constructing a polycistronic vector to achieve the co-expression of multiple genes, internal ribosome entry sites (IRES) and 2A elements are most commonly used. The 2A peptide sequences disrupt peptide-bond formation during translation, leading to two independent proteins, a process known as "ribosomal-skipping" (Subramanian et al., 2017).

In this study, we propose that Vhb might balance the growth of thalli and the accumulation of GSH through oxygen regulation. Using expression vectors that localized Vhb in the cytoplasm, mitochondria and both the cytoplasm and mitochondria, respectively, we explored the relationship between Vhb and GSH yield.

## II. Materials And Methods

### Strains and growth conditions

*E. coli DH 5α* was grown in Luria–Bertani (LB) broth (Sambrook and Russell, 2002). The *S. cerevisiae* 2.558 were routinely maintained on yeast extract peptone dextrose (YPD) medium (Sambrook and Russell, 2002). Putative transformants were purified in Synthetic Dropout Medium-Uracil (SD-ura) medium (Sambrook and Russell, 2002).

### Plasmid construction

The vector pYES2 (Invitrogen, P<sub>GAL1</sub>, CYC1 TT) was used as the backbone vector. The *vgb* gene, with flanking *EcoR* I and *BamH* I restriction sites, was amplified using *Vitreoscilla* genomic DNA as the template with PvgbF/PvgbR as primers. The recombinant plasmid, pMD19-T-*vgb*, was obtained by inserting the *vgb* gene into the pMD19-T vector. The pMD19-T-*vgb* and pYES2 plasmids were digested with *EcoR* I and *BamH* I to obtain the recombinant plasmid, pYES2-*vgb*, which can express Vhb in cytoplasm.

Pxlt1F/Pxlt1R were used as primers and pUC57-2A-Su9 as the template to obtain the Su9 sequence for recombination with pMD19-T. The recombinant plasmid, pYES2-Su9-*vgb*, which expressed GSH in the mitochondria was obtained by linking the plasmid, pMD19-T-Su9, with pYES2-*vgb* after cleavage with *EcoR* I.

A 2A-Su9 sequence with an *EcoR* I restriction site was synthesized and recombined with pYES2-*vgb* after *EcoR* I digestion, yielding pYES2-*vgb*-2A-Su9. Using the PxltvgbF/PxltvgbR primers and pYES2-*vgb* as the template, the *vgb* gene with *Sph* I restriction sites was amplified. The recombinant plasmid, pYES2-*vgb*-2A-Su9-*vgb*, which expressed Vhb in both the cytoplasm and the mitochondria, was obtained by the recombination of pYES2-*vgb*-2A-Su9 and *vgb* with *Sph* I restriction sites.

### Transformation and screening of transformants

The preparation of *S. cerevisiae* competent cells and the transformation with recombinant plasmids are based on the PEG/LiAc chemical conversion method of Gietz and Schiestl (2007).

Single colonies from the SD-ura plates were placed in 20 μL of lysis solution, incubated for 10 min in an 80°C water bath, and centrifuged at 1 5000 g for 5 min. The resulting supernatant was tested for the presence of recombinant plasmids. The strain 0 and strain 1 containing pYES2 and pYES2-*vgb* was detected by amplification with PYES2F/PYES2R primers; the strain 2 containing pYES2-Su9-*vgb* was verified using primers PvgbCDSF/PvgbCDSR, and the strain 3 containing pYES2-*vgb*-2A-Su9-*vgb* was verified using primers PY1F/PY1R.

### Fermentation and GSH detection

The parent strain and the transformant strains 0 — 3 were inoculated into YPD medium, cultured at 28°C, and shaken at 1 5000 g. After 16 h, the cultures were inoculated at 1:50 into the fermentation medium (glucose 20 g/L, peptone 7 g/L, (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> 7 g/L, KH<sub>2</sub>PO<sub>4</sub> 6 g/L, K<sub>2</sub>SO<sub>4</sub> 3.6 g/L, MgSO<sub>4</sub> 1.5 g/L) and incubated for 72 h on a rotary shaker (1 5000 g) at 30 °C. Cells were collected in weighed sterile 50-mL centrifuge tubes, centrifuged at 1 5000 g for 3 min, and the residual medium was blotted with filter paper and dried the *S. cerevisiae* before weighing and calculating the cell mass. The amount of GSH was determined by HPLC as follows: 10 mL of the cell lysate, adjusted to pH 3 using concentrated sulfuric acid, was boiled for 1 min in a beaker, absorbed 1 mL mixture, and immediately placed on the ice.

Calculation method of GSH content in unit cell:

$$\text{GSH content of per gram yeast wet cell (mg/g)} = \frac{\text{Sample peak area} \times 0.01 \text{ mg/ml} \times 10 \text{ ml}}{\text{Peak area of standard product} \times \text{Sample weight}}$$

GSH standard (0.01 mg/L) was prepared as follows: 0.01 g GSH, dissolved in 100 mL of ultra-pure water with an appropriate amount of vitamin B to prevent oxidation, was quickly placed in a 1.5-mL centrifuge tube and then in a 4°C refrigerator. The GSH content was determined by HPLC using a C18 reverse column (250 mm×4.6 mm (i.d), 5 μm); the mobile phase consisted of ammonium dihydrogen phosphate (6.8 g/L), heptane sulfonic acid (1.1 g/L), and methanol (40 mL/L). The flow rate was 1 mL/min, the column temperature was 25°C, the detection wavelength was 210 nm, and the sample injection volume was 10 μL.

### III. Results

#### The expression of Vhb in the cytoplasm and HPLC analysis of GSH

The recombinant plasmid, pYES2-*vgb*, was amplified using the PvgbCDSF/PvgbCDSR primers, and the CDS region of target gene *vgb* was 441bp. The recombinant plasmid pYES2-*vgb* was shown to be successfully constructed by electrophoresis (Figure 1). The plasmid pYES2-*vgb* was successfully transformed into the *S. cerevisiae* 2.558 to obtain strain 1 and the *S. cerevisiae* 2.558 successfully transformed with plasmid pYES2 was Strain 0 as blank control. The results showed the dry weights of strain 0 and strain 1 were 0.049g and 0.067g respectively (Table 3). The GSH yield per g of strain 0 was 8.17 mg and the GSH yield per g of strain 1 was 10.17 mg, the increase in GSH production per g dry weight of strain 1 compared to the strain 0 was 24 % (Table 3).

#### The expression of Vhb in mitochondria and HPLC analysis of GSH

Using primers PY1F/ PY1R the predicted size of the recombinant gene is approximately 1,020 bp. The recombinant plasmid, pYES2-Su9-*vgb*, was shown to be successfully constructed by electrophoresis (Figure 2). The plasmid pYES2-Su9-*vgb* was successfully transformed into the *S. cerevisiae* 2.558 to obtain strain 2. The result showed the dry weight of strain 2 was 0.081g, the GSH yield per g of strain 2 was 14.91 mg. The increase in GSH production per g dry weight of strain 2 compared to the strain 0 was 83 % (Table 3).

#### The expression of Vhb in both the cytoplasm and mitochondria and HPLC analysis of GSH

The recombinant plasmid, pYES2-*vgb*2ASu9*vgb*, was amplified using primers PY1F/ PY1R, and the predicted size of the target fragment is approximately 1,400 bp. Electrophoresis showed that the recombinant plasmid, pYES2-*vgb*-2A-Su-9*vgb*, was successfully constructed (Figure 3). The plasmid pYES2-*vgb*-2A-Su9-*vgb* was successfully transformed into the *S. cerevisiae* 2.558 to obtain strain 3.

The result showed the dry weight of strain 2 was 0.086g, the GSH yield per g of strain 3 was 18.71 mg. GSH production per g dry weight of strain 3 was increased 122% compared to strain 0 (Table 3).

### IV. Discussion

To date, scholars have been devoted to increasing the production of GSH with great success, including the development of the commonly-used enzymatic synthesis of GSH using engineered *S. cerevisiae* (Chen et al., 2013). By optimizing the cell dosages of two engineered strains (S.TS013/GSH1, S.TS013/GSH2) and a two-stage reaction, GSH productivity increased by 84 % over that of the host strain. Another is an adaptive laboratory evolution strategy using acrolein resistance to obtain a strain with an enhanced GSH-accumulation phenotype (Patzschke et al., 2015 ). In addition, some scholars used the novel engineered *S. cerevisiae* GSH degradation protein and GSH uptake protein-deficient strains for extracellular GSH fermentation (Kiriya et al., 2012). However, the contradiction between GSH accumulation at lower redox potential and cell growth at higher redox potential cannot be solved.

Now, Our study provides an idea that introducing Vhb into cell. When the oxygen content in the environment is higher, Vhb may bind oxygen, thereby preventing GSH oxidation; when the oxygen content in the environment is lower, Vhb may release bound oxygen, thereby facilitating cell growth. In this study, recombinant plasmids containing the Vhb expressing gene, *vgb*, were transformed into *S. cerevisiae* such that Vhb was expressed in the cytoplasm, mitochondria, and both the cytoplasm and mitochondria. The results showed that the GSH production was improved 24%, 83%, and 122%, compared with the parent strain, respectively, indicating that Vhb increases the accumulation of GSH, especially when expressed in the cytoplasm and mitochondria simultaneously. In this paper, the expression of Vhb in the cytoplasm and mitochondria provides a novel approach for the industrial production of GSH by microbial fermentation and a solution for the problem of compromised cell growth and the accumulation of reducing agents during industrial fermentation.

We hypothesize that the expression of Vhb increases the efficiency of dissolved-oxygen utilization in cells, and increases cell metabolism and biomass. In the case of insufficient oxygen supply, the metabolism of *E. coli* and the metabolism of tricarboxylic acid are not balanced (Wei and Chen, 2018). Vhb can accelerate the tricarboxylic acid cycle and increase the cellular consumption rate of acetyl-CoA. Further, on the one hand, it is possible to promote an increase in cell biomass, and on the other hand, to suppress the formation of metabolic by-products such as acetic acid. Heterologous expression of Vhb in a variety of hosts has been shown to improve microaerobic cell growth and enhance oxygen-dependent product formation (Wei et al., 2018). The action of Vhb is likely linked to respiration and electron transfer, changing the carbon flux toward ethanol production in *S. cerevisiae* (Chen et al., 1994). Vhb improved the efficiency of oxidative metabolism by participating in an oxygen-limited respiration chain, which offered an extra advantage in hypoxic growth (Yang et al., 2001).

GSH is particularly important in the mitochondrial defence against both physiologically- and pathologically-generated oxidative stresses (Fernández-Checa et al., 1997; Garcia-Ruiz and Fernández-Checa, 2006). Severe oxidative stress depletes cellular GSH (Lu, 2009) severe mitochondrial GSH depletion leads to increased levels of ROS and RNS, mitochondrial dysfunction, and ATP depletion, converting apoptotic to necrotic cell death (Garcia-Ruiz and Fernández-Checa, 2007). This may explain why the effect of VHB is more pronounced when expressed in the mitochondria than in the cytoplasm.

### Compliance with Ethical Standards

No conflict of interest exists in the submission of this manuscript, and manuscript is approved by all authors for publication. I would like to declare on behalf of my co-authors that the work described was original research that has not been published previously, and not under consideration for publication elsewhere, in whole or in part. All the authors listed have approved the manuscript that is enclosed. The manuscript does not contain experiments using animals and human studies.

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### Conflict of Interest

The authors declare that they have no conflict of interest.

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### Figure legends

Table 1. Primers used for this study

Table 2. System for plasmid transformation *Saccharomyces cerevisiae*

Table 3. The GSH determination and comparison

Figure 1. The electrophoresis of plasmid pYES2-*vgb* detection

Figure 2. The electrophoresis of plasmid pYES2-Su9-*vgb* detection

Figure 3. The electrophoresis of plasmid pYES2-*vgb*-2A-su9-*vgb* detection

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