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Metabolic Engineering of *Saccharomyces cerevisiae* for Fermentative Production of Heme

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ABSTRACT

Heme is a key ingredient required to mimic the color and flavor of meat in plant-based alternatives. This study aimed to develop a yeast-based microbial cell factory for efficient and sustainable production of heme. To this end, first, Hem12p (uroporphyrinogen decarboxylase) was identified as the rate-limiting enzyme in the heme biosynthetic pathway present in *Saccharomyces cerevisiae* D452-2. Next, we investigated the effects of disruption of the genes involved in the competition for heme biosynthesis precursors, transcriptional repression, and heme degradation (*HMX1*) on heme production efficiency. Of the knock-out strains constructed in this study, only the *HMX1*-deficient strain produced heme at a higher concentration than the background strain without gene disruption. In addition, overexpression of *PUG1* encoding a plasma membrane transporter involved in protoporphyrin IX (the precursor to heme biosynthesis) uptake led to a significant increase in intracellular heme concentration. As a result, among the various engineered strains constructed in this study, the $\Delta HMX1/H3\&12 + PUG1$ strain, the *HMX1*-deficient strain overexpressing *HEM3*, *HEM12*, and *PUG1*, produced the highest concentration of heme (4.6 mg/L) in batch fermentation, which was 3.9-fold higher than that produced by the wild-type D452-2 strain. In a glucose-limited fed-batch fermentation, the $\Delta HMX1/H3\&12 + PUG1$ strain produced 28 mg/L heme in 66 h.

1 | Introduction

Heme is an iron-containing porphyrin complex that is essential for the survival of almost all living organisms [1] because it is an important coenzyme for various enzymes involved in oxygen transport [2], energy production [3], and iron metabolism [4]. In particular, heme has recently received substantial attention as a key ingredient responsible for the flavor and color of meat as the market for plant-based meat alternatives expands [5]. The global market for plant-based meat alternatives is expected to reach USD 33.99 billion by 2027 [6].

Because of the important role of yeast extracts as flavoring enhancers in plant-based meat alternatives, *Saccharomyces cerevisiae* with high heme content could be directly used in the plant-based meat industry without heme isolation and purification processes [7]. For this reason, we sought to enhance the intracellular heme content of *S. cerevisiae* through a systematic metabolic engineering approach. In a recent study, the highest heme concentration (53.7 mg/L) produced in a yeast system was achieved through the metabolic engineering of *S. cerevisiae*, in which target genes were selected based on genome-scale modeling [8]. However, the heme production concentration was

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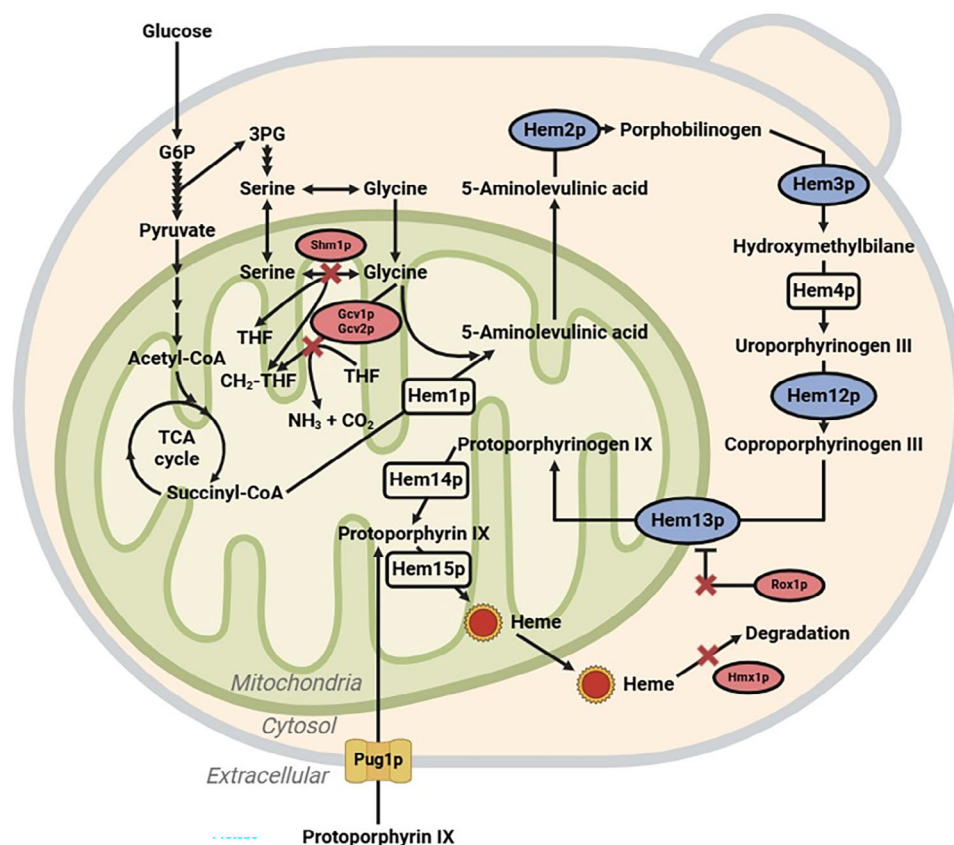


FIGURE 1 | Metabolic engineering strategies for efficiently producing heme in *Saccharomyces cerevisiae*. Fluxes in the heme biosynthetic pathway were amplified by overexpressing the rate-limiting enzymes (Hem2p, Hem3p, Hem12p, and Hem13p). The *ROX1* and *HMX1* genes were disrupted to derepress *HEM13* expression and prevent heme degradation, respectively. In addition, the *SHM1*, *GCV1*, and *GCV2* genes were disrupted to increase 5-aminolevulinic acid availability, and the *PUG1* gene was overexpressed to increase protoporphyrin IX availability. CH₂-THF indicates 5,10-methylenetetrahydrofolate; Gcv1p, glycine cleavage complex 1; Gcv2p, glycine cleavage complex 2; G6P, glucose 6-phosphate; Hem1p, 5-aminolevulinic acid synthase; Hem2p, aminolevulinic acid dehydratase; Hem3p, porphobilinogen deaminase; Hem4p, uroporphyrinogen III synthase; Hem12p, uroporphyrinogen decarboxylase; Hem13p, coproporphyrinogen III oxidase; Hem14p, protoporphyrinogen oxidase; Hem15p, protoporphyrin ferrochelatase; Hmx1p, heme oxygenase 1; 3PG, glycerate 3-phosphate; Pug1p, protoporphyrin uptake protein; Rox1p, repressor of the hypoxic functions; Shm1p, mitochondrial serine hydroxymethyltransferase; THF, tetrahydrofolate.

still low compared to engineered bacterial strains, which can produce up to 1.03 g/L of heme [9].

In *S. cerevisiae*, heme is produced through the C4 pathway and protoporphyrin-dependent pathway [10]. Heme biosynthesis begins in the mitochondrial fraction with 5-aminolevulinic acid synthase (Hem1p), which catalyzes the production of 5-aminolevulinic acid (5-ALA) from succinyl-CoA and glycine. In the cytoplasm, aminolevulinic acid dehydratase (Hem2p), porphobilinogen deaminase (Hem3p), uroporphyrinogen III synthase (Hem4p), uroporphyrinogen decarboxylase (Hem12p), and coproporphyrinogen III oxidase (Hem13p) synthesize protoporphyrinogen IX from 5-ALA (Figure 1). After protoporphyrinogen IX is transported back into the mitochondria, it is converted to heme in two consecutive reactions catalyzed by protoporphyrinogen oxidase (Hem14p) and protoporphyrin ferrochelatase (Hem15p).

To improve the intracellular heme content in *S. cerevisiae*, three bottlenecks must be resolved. The first bottleneck is the low expressional level of rate-limiting enzymes involved in the heme biosynthetic pathway. Previous studies reported that the rate-

limiting enzymes in heme biosynthesis are Hem2p, Hem3p, Hem12p, and Hem13p [11–14]. In particular, a repressor of the hypoxic functions (ROX1) represses the expression of various hypoxic genes, including *HEM13*, when intracellular heme content is high [15, 16]. Accordingly, we investigated the effects of the following on heme biosynthesis efficiency: (1) Overexpression of *HEM2*, *HEM3*, *HEM12*, and *HEM13* under the transcriptional control of strong promoters and (2) derepression of *HEM13* expression by disrupting the *ROX1* gene (Figure 1).

The second bottleneck is the limited availability of heme biosynthesis precursors. Because the initial precursor to heme production (5-ALA) is synthesized from succinyl-CoA and glycine, the concentration of succinyl-CoA and glycine in the mitochondria is important for the synthesis of 5-ALA and heme. Mitochondrial serine hydroxymethyltransferase (SHM1), glycine cleavage complex 1 (GCV1), and glycine cleavage complex 2 (GCV2) use glycine competitively with Hem1p, diverting carbon flux away from 5-ALA [17]. Therefore, we disrupted the *SHM1*, *GCV1*, and *GCV2* genes to reduce byproduct formation and direct more carbon flux into heme production (Figure 1). As mentioned above, the last step of heme biosynthesis is catalyzed by Hem15p, which

converts protoporphyrin IX (PPIX) to heme. A previous study reported that protoporphyrin uptake protein 1 (PUG1) has the dual activity of PPIX uptake and heme excretion [18]. Therefore, if the heme excretion activity is strong, *PUG1* disruption will be beneficial to increase the intracellular heme concentration, and conversely, if the PPIX uptake activity is strong, *PUG1* overexpression will be beneficial. To this end, we investigated the effects of disruption and overexpression of *PUG1* on the intracellular heme concentration in *S. cerevisiae* (Figure 1).

The last bottleneck is heme oxygenase 1 (HMX1)-mediated degradation of heme. HMX1, whose expression is regulated by the major iron-dependent transcription factor (AFT1), promotes the ability of cells to use heme as a source of nutritional iron [19]. Therefore, the *HMX1* gene was disrupted to enhance intracellular heme concentration by preventing heme degradation (Figure 1). Herein, efficient heme production was achieved in *S. cerevisiae* by optimizing the expression levels of the rate-limiting enzymes, enhancing the pool of heme biosynthesis precursors, and blocking heme degradation.

2 | Experimental Section

2.1 | Strains and Plasmids

Escherichia coli TOP10 (Invitrogen, Carlsbad, CA, USA) was used as a host strain for plasmid construction. *S. cerevisiae* D452-2 (*MAT α* , *leu2*, *his3*, *ura3*, and *can1*) [20] was used for the production of heme. All plasmids and strains used in this study are listed in Table 1.

2.2 | Genetic Manipulation

All gene cloning experiments were carried out using NEBuilder HiFi DNA Assembly Master Mix (New England Biolabs, Ipswich, MA, USA), as specified by the manufacturer. The primer sets used for the amplification of the *HEM2*, *HEM3*, *HEM12*, *HEM13*, and *PUG1* genes, yeast integrative plasmids (YIps), and guide RNA (gRNA) plasmids are listed in Table S1. The resulting PCR products were combined using NEBuilder HiFi DNA Assembly Master Mix (New England Biolabs) to construct YIps for heme production and gRNA plasmids for CRISPR/Cas9-based genome editing system.

To construct *S. cerevisiae* knock-out strains, the genome editing system based on CRISPR/Cas9 was used, as previously described [21]. Briefly, repair DNA fragments were amplified with primers listed in Table S1. The resulting repair DNA fragments and gRNA plasmids were co-transformed into *S. cerevisiae* strains harboring pCas9_AUR to disrupt genes of interest.

2.3 | Media and Culture Conditions

E. coli strains were grown in Luria–Bertani (LB) medium (10 g/L of tryptone, 5 g/L of yeast extract, and 10 g/L of NaCl) containing 50 μ g/mL of ampicillin. *S. cerevisiae* strains were pre-cultured at 25°C and 250 rpm for 48 h in yeast synthetic complete (YSC) medium (6.7 g/L of yeast nitrogen base) containing 20 g/L of

glucose with appropriate nucleotides, amino acids, and antibiotic. Cells at the stationary phase of the preculture were harvested and inoculated into main cultures with an initial optical density at 600 nm (OD₆₀₀) of 1.0. Batch fermentation experiments were performed at 25°C and 250 rpm for 48 h in a baffled flask containing 100 mL of yeast peptone (YP) medium (10 g/L of yeast extract and 20 g/L of Bacto™ Peptone) containing 50 g/L of glucose.

Fed-batch fermentation was performed in a 2.5 L bioreactor (Kobitech Co., Incheon, Republic of Korea) containing 1 L of 40YP medium (40 g/L of yeast extract and 20 g/L of Bacto™ Peptone) with 20 g/L of glucose and an initial OD₆₀₀ of 1.0. After depletion of the initially added glucose, a feeding solution consisting of 280 g/L of yeast extract, 140 g/L of Bacto™ Peptone, and 350 g/L of glucose was fed at a rate from 6.4 to 20.8 mL/h. The agitation speed, air supply, temperature, and medium pH were maintained at 1000 rpm, 3.0 vvm, 25°C, and pH 4.5, respectively.

2.4 | Analytical Methods

Cell concentration at OD₆₀₀ was monitored using a spectrophotometer (OPTIZEN POP, Mecasys, Daejeon, Republic of Korea). Analysis of extracellular metabolites, including glucose, glycerol, acetic acid, and ethanol, was performed using a high-performance liquid chromatography (HPLC) system (Thermo Fisher UltiMate 3000, Thermo Fisher Scientific, Waltham, MA, USA) equipped with a reflective index detector and a Rezex ROA-Organic Acid H⁺ column (Phenomenex, Torrance, CA, USA). Metabolites were separated at isocratic temperature (70°C) and a flow rate of 0.6 mL/min in 5 mM H₂SO₄.

To prepare the sample for heme concentration measurements, cells were harvested at OD₆₀₀ × culture (mL) = 60 (i.e., if the OD₆₀₀ was 150, then 0.4 mL of culture was harvested). The harvested cells were resuspended in 0.4 mL of Y-PER™ (Thermo Fisher Scientific) and lysed, as specified by the manufacturer. Sample preparation for the determination of heme concentration was carried out according to a previous study [22]. Briefly, acetonitrile was used to extract the heme present in the yeast lysate, and the acetonitrile layer was filtered (0.2 μ m) before HPLC analysis. Heme concentration was measured using an HPLC system (Thermo Fisher UltiMate 3000, Thermo Fisher Scientific) equipped with a YMC-Pack ODS-A column (YMC, Kyoto, Japan). Heme was separated at constant temperature (50°C) with mobile phase A and mobile phase B in a gradient mode (0 min, A:B = 80:20; 10–11.1 min, A:B = 0:100; 12–25 min, A:B = 80:20; v/v) at a flow rate of 0.4 mL/min and then passed through a UV detector (400 nm). Mobile phase A was a mixture of acetonitrile and water (5:95, v/v) containing 1.2 g/L of formic acid, and mobile phase B was a mixture of acetonitrile and water (95:5, v/v) containing 1.2 g/L of formic acid.

2.5 | Statistical Analyses

Statistical analyses were carried out using IBM SPSS version 28 (IBM, Armonk, NY, USA). All data are presented as the means ± standard deviations. One-way analysis of variance was

TABLE 1 | List of strains and plasmids used in this study.

Name	Description	Reference
Strains		
<i>Escherichia coli</i> TOP10	F [−] <i>mcrA</i> Δ(<i>mrr</i> - <i>hsdRMS</i> - <i>mcrBC</i>) ϕ80 <i>lacZ</i> Δ <i>M15</i> Δ <i>lacX74</i> <i>recA1</i> <i>araD139</i> Δ(<i>ara-leu</i>)7697 <i>galU</i> <i>galK</i> <i>rpsL</i> (Str ^R) <i>endA1</i> <i>nupG</i>	Invitrogen (Carlsbad, CA, USA)
<i>Saccharomyces cerevisiae</i> D452-2	<i>MAT</i> α, <i>leu2</i> , <i>his3</i> , <i>ura3</i> , and <i>can1</i>	[20]
GPD-H2	D452-2 <i>HIS3::GPDp-HEM2-CYC1t</i>	In this study
GPD-H3	D452-2 <i>LEU2::GPDp-HEM3-CYC1t</i>	In this study
GPD-H12	D452-2 <i>URA3::GPDp-HEM12-CYC1t</i>	In this study
GPD-H13	D452-2 <i>CS6::GPDp-HEM13-CYC1t</i>	In this study
H2	D452-2 <i>HIS3::tHXT7p-HEM2-CYC1t</i>	In this study
H3	D452-2 <i>LEU2::tHXT7p-HEM3-CYC1t</i>	In this study
H12	D452-2 <i>URA3::tHXT7p-HEM12-CYC1t</i>	In this study
H13	D452-2 <i>CS6::tHXT7p-HEM13-CYC1t</i>	In this study
H2&3	D452-2 <i>HIS3::tHXT7p-HEM2-CYC1t</i> <i>LEU2::tHXT7p-HEM3-CYC1t</i>	In this study
H3&12	D452-2 <i>LEU2::tHXT7p-HEM3-CYC1t</i> <i>URA3::tHXT7p-HEM12-CYC1t</i>	In this study
H2&12	D452-2 <i>HIS3::tHXT7p-HEM2-CYC1t</i> <i>URA3::tHXT7p-HEM12-CYC1t</i>	In this study
H2,3&12	D452-2 <i>HIS3::tHXT7p-HEM2-CYC1t</i> <i>LEU2::tHXT7p-HEM3-CYC1t</i> <i>URA3::tHXT7p-HEM12-CYC1t</i>	In this study
HEM2,3,12&13	D452-2 <i>HIS3::tHXT7p-HEM2-CYC1t</i> <i>LEU2::tHXT7p-HEM3-CYC1t</i> <i>URA3::tHXT7p-HEM12-CYC1t</i> <i>CS6::tHXT7p-HEM13-CYC1t</i>	In this study
ΔHMX1/H3&12	D452-2 ΔHMX1 <i>LEU2::tHXT7p-HEM3-CYC1t</i> <i>URA3::tHXT7p-HEM12-CYC1t</i>	In this study
ΔROX1/H3&12	D452-2 ΔROX1 <i>LEU2::tHXT7p-HEM3-CYC1t</i> <i>URA3::tHXT7p-HEM12-CYC1t</i>	In this study
ΔSHM1/H3&12	D452-2 ΔSHM1 <i>LEU2::tHXT7p-HEM3-CYC1t</i> <i>URA3::tHXT7p-HEM12-CYC1t</i>	In this study
ΔGCV1/H3&12	D452-2 ΔGCV1 <i>LEU2::tHXT7p-HEM3-CYC1t</i> <i>URA3::tHXT7p-HEM12-CYC1t</i>	In this study
ΔGCV2/H3&12	D452-2 ΔGCV2 <i>LEU2::tHXT7p-HEM3-CYC1t</i> <i>URA3::tHXT7p-HEM12-CYC1t</i>	In this study
ΔPUG1/H3&12	D452-2 ΔPUG1 <i>LEU2::tHXT7p-HEM3-CYC1t</i> <i>URA3::tHXT7p-HEM12-CYC1t</i>	In this study
H3&12+PUG1	D452-2 <i>LEU2::tHXT7p-HEM3-CYC1t</i> <i>URA3::tHXT7p-HEM12-CYC1t</i> <i>HIS3::tHXT7p-PUG1-CYC1t</i>	In this study
ΔHMX1/H3&12+PUG1	D452-2 ΔHMX1 <i>LEU2::tHXT7p-HEM3-CYC1t</i> <i>URA3::tHXT7p-HEM12-CYC1t</i> <i>HIS3::tHXT7p-PUG1-CYC1t</i>	In this study
Plasmids		
pRS403tHXT7p	<i>HIS3</i> , tHXT7 promoter, <i>CYC1</i> terminator, an integrative plasmid, Amp ^R	[29]

(Continues)

TABLE 1 | (Continued)

Name	Description	Reference
pRS405tHXT7p	<i>LEU2</i> , tHXT7 promoter, <i>CYC1</i> terminator, an integrative plasmid, Amp ^R	[29]
pRS406tHXT7p	<i>URA3</i> , tHXT7 promoter, <i>CYC1</i> terminator, an integrative plasmid, Amp ^R	[29]
pKCK21	G418 ^R , <i>tHXT7p-GSH2-CYC1t</i> , an integrative plasmid, Amp ^R	[30]
pHLK40	pRS403tHXT7p harboring <i>HEM2</i>	In this study
pHLK41	pRS405tHXT7p harboring <i>HEM3</i>	In this study
pHLK42	pRS406tHXT7p harboring <i>HEM12</i>	In this study
pHLK43	pKCK21 harboring <i>tHXT7p-HEM13-CYC1t</i>	In this study
pHLK47	pRS403tHXT7p harboring <i>PUG1</i>	In this study
pCas9_AUR	Aur ^R , p414- <i>TEF1p-Cas9-CYC1t</i> , modified Cas9 expression plasmid, Amp ^R	[21]
pgRNA- <i>TRP1</i> -HYB	Hyg ^R , 2 μ origin, <i>SNR52p-gTRP1-SUP4t</i> , Amp ^R	[31]
pgRNA- <i>HMX1</i> -HYB	Hyg ^R , 2 μ origin, <i>SNR52p-gHMX1-SUP4t</i> , Amp ^R	In this study
pgRNA- <i>ROX1</i> -HYB	Hyg ^R , 2 μ origin, <i>SNR52p-gROX1-SUP4t</i> , Amp ^R	In this study
pgRNA- <i>SHM1</i> -HYB	Hyg ^R , 2 μ origin, <i>SNR52p-gSHM1-SUP4t</i> , Amp ^R	In this study
pgRNA- <i>GCV1</i> -HYB	Hyg ^R , 2 μ origin, <i>SNR52p-gGCV1-SUP4t</i> , Amp ^R	In this study
pgRNA- <i>GCV2</i> -HYB	Hyg ^R , 2 μ origin, <i>SNR52p-gGCV2-SUP4t</i> , Amp ^R	In this study
pgRNA- <i>PUG1</i> -HYB	Hyg ^R , 2 μ origin, <i>SNR52p-gPUG1-SUP4t</i> , Amp ^R	In this study

carried out, and statistical significance was assessed using Tukey HSD tests at a *p*-value of < 0.05.

3 | Results and Discussion

3.1 | Enhanced Heme Production in *S. cerevisiae* by Optimizing the Expression Levels of Rate-Limiting Enzymes in the Heme Biosynthetic Pathway

To overexpress genes coding for enzymes (Hem2p, Hem3p, Hem12p, and Hem13p) predicted to be rate-limiting in heme biosynthesis, we replaced the upstream ~100 bp of the target genes with a constitutive strong promoter, the GPD promoter (GPDp) or truncated HXT7 promoter (tHXT7p), using the CRISPR/Cas9-based genome editing system (Figure S1) according to a previous study [23]. In contrast to the previous study [23], which reported the successful overexpression of target genes, the same strategy could not enhance the transcript levels of the *HEM2*, *HEM3*, *HEM12*, and *HEM13* genes (data not shown); hence, all engineered strains with the promoter replacement produced similar or lower levels of heme than the control *S. cerevisiae* D452-2 strain (Figure S2). We speculated that regulatory components of promoters, including upstream activating or repressing sequences, present upstream of the *HEM2*, *HEM3*, *HEM12*, and *HEM13* genes might interfere with the interaction between RNA polymerase and GPDp/tHXT7p [24, 25].

Therefore, we decided to use a conventional gene overexpression system based on Yips [26]. To this end, a set of the following

eight strains overexpressing one of the four genes (*HEM2*, *HEM3*, *HEM12*, and *HEM13*) under the transcriptional control of GPDp or tHXT7p was constructed (Table 1). Among the engineered *S. cerevisiae* strains, the GPD-H12 and H12 strains overexpressing *HEM12* under the transcriptional control of GPDp and tHXT7p exhibited significantly higher heme concentrations than the control D452-2 strain (Figure S2). As the magnitude of the increase (4.0-fold) in heme concentration in the H12 strain was higher than that (2.2-fold) in the GPD-H12 strain, tHXT7p was selected as the optimal promoter for further experiments.

Although the H12 strain produced heme at a maximum level at 48 h, the H2, H3, and H13 strains produced significantly lower heme at 48 h compared to the corresponding values at 72 h (Figure S2). This result indicated that *HEM12* overexpression improved heme production efficiency in *S. cerevisiae*, reducing the fermentation time required to reach the maximum heme concentration. When comparing intracellular heme concentrations at 48 h of cultivation, the H2, H3, and H13 strains produced heme at 6%–60% higher concentrations than the control D452-2 strain (Figure 2). Among the engineered strains, the H12 strain produced the highest concentration of heme (2.2 mg/L), which was 4.0-fold higher than the corresponding value for the D452-2 strain. Collectively, from these observations, we concluded that Hem12p is the major rate-limiting enzyme in heme biosynthesis. Previous studies have reported that Hem2p or Hem3p is the major rate-limiting enzyme [11, 13, 27]. However, we identified that Hem12p can act as a major rate-limiting enzyme, likely due to the difference in the genetic background of host strains.

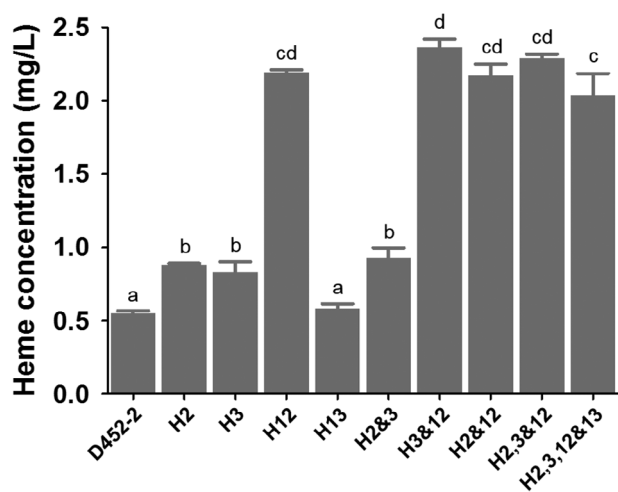


FIGURE 2 | Comparison of heme concentration in the control *Saccharomyces cerevisiae* D452-2 and the engineered strains overexpressing the rate-limiting enzymes after cultivation for 48 h. Results are the mean of two experiments, and error bars indicate standard deviation. Different letters represent significantly different means (Tukey HSD tests, $p < 0.05$).

We next investigated whether additional overexpression of the *HEM2*, *HEM3*, and *HEM13* genes in the H12 strain would enhance intracellular heme concentration. However, no synergistic effect was observed between Hem12p and the other three enzymes (Figure 2). We observed a slight increase in the heme production efficiency of the strain overexpressing both *HEM3* and *HEM12* (i.e., strain H3&12) compared to the H12 strain. As a result, the H3&12 strain produced 2.4 mg/L of heme. Therefore, the H3&12 strain was chosen as the host for further experiments on heme production. Because *HEM13* overexpression in the H3&12 background strains did not increase the intracellular heme concentration (Figure 2), the Δ ROX1/H3&12 strain (Table 1), which lacks *HEM13* repression by *ROX1* disruption, produced heme at a concentration similar to that produced by the H3&12 strain (Figure 3).

3.2 | Validation of Genetic Perturbations Capable of Increasing Heme Concentration in the H3&12 Strain

The production yield of target compounds often remains low due to the limited availability of precursors and/or degradation of target compounds. Therefore, we investigated the effects of the following genetic perturbations designed to reduce byproduct formation on the intracellular heme concentration in the H3&12 strain: (1) disruption of the *SHM1*, *GCV1*, and *GCV2* genes to increase 5-ALA availability and (2) overexpression of the *PUG1* gene to increase PPIX availability. While disruption of the *SHM1*, *GCV1*, and *GCV2* genes did not increase the intracellular heme concentration, the H3&12 + *PUG1* strain (the H3&12 strain overexpressing *PUG1*) (Table 1) produced 18% more heme than the background H3&12 strain (Figure 3). This result indicated that 5-ALA synthesis catalyzed by Hem1p is not a rate-limiting step in heme biosynthesis in the H3&12 strain. In contrast to previous reports that Hem1p is not the rate-limiting enzyme in heme production in wild-type yeast strains producing low

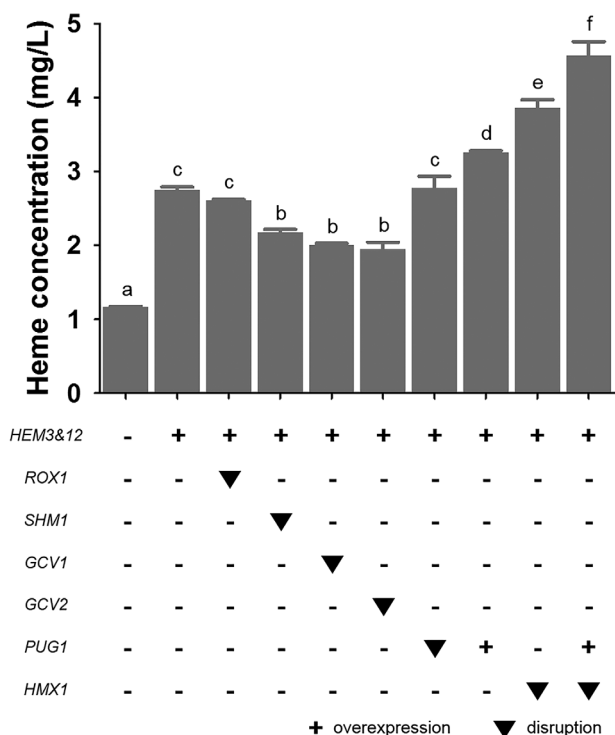


FIGURE 3 | Comparison of heme concentration in the control *Saccharomyces cerevisiae* D452-2 and various engineered strains after cultivation for 48 h. Results are the mean of $n \geq 2$ experiments, and error bars indicate standard deviation. Different letters represent significantly different means (Tukey HSD tests, $p < 0.05$).

levels of heme [11–14], a recent study reported that increasing 5-ALA availability in engineered yeast strains producing high levels of heme led to a significant increase in heme production [8]. The results of this study and earlier studies suggest that the effects of genetic perturbations for increasing 5-ALA availability on heme production are dependent on the intracellular heme concentrations of the background host strains. In other words, 5-ALA availability may only be limited if the heme biosynthetic pathway in *S. cerevisiae* is engineered to be highly efficient; thus, it was assumed that heme biosynthesis in the H3&12 strain is not yet optimized.

In light of a previous report that the *PUG1* transporter has heme excretion activity in addition to PPIX uptake activity [18], we also investigated the effects of *PUG1* disruption on the intracellular heme concentration of the H3&12 strain. However, *PUG1* disruption did not have a significant effect on heme production in the H3&12 strain (Figure 3), and heme was undetectable in the extracellular fractions of both the H3&12 and Δ PUG1/H3&12 strains (data not shown). These results are likely due to the very low concentration of free heme (i.e., protein-unbound heme) in *S. cerevisiae*. We note that heme produced by all engineered *S. cerevisiae* strains constructed in this study was bound to hemoproteins; hence, free heme was not detected (data not shown). This result is consistent with a previous study reporting very low intracellular heme concentrations in *S. cerevisiae* under physiologic conditions [28]. The results of this study and earlier studies suggested that the heme excretion activity of *PUG1* is

negligible under normal fermentation conditions because the intracellular free heme concentration is very low.

Next, we investigated the effects of disruption of the *HMX1* gene on heme production in the H3&12 strain. Heme oxygenase 1 (encoded by *HMX1*) is involved in heme degradation, and *HMX1* disruption in the H3&12 strain increased intracellular heme concentration by 40% (Figure 3). Having established the positive effect of *PUG1* overexpression and *HMX1* disruption on heme production, we combined these genetic perturbations into the H3&12 strain to construct the Δ HMX1/H3&12 + *PUG1* strain (Table 1). As a result, the Δ HMX1/H3&12 + *PUG1* strain produced 4.6 mg/L of heme with a yield of $91.4 \mu\text{g}_{\text{heme}}/\text{g}_{\text{glucose}}$, which was 18% higher than the corresponding values of the Δ HMX1/H3&12 strain without *PUG1* overexpression (Figure 3). We note that the intracellular heme concentration (4.6 mg/L) obtained from the Δ HMX1/H3&12 + *PUG1* strain was 3.9-fold higher than that produced by the wild-type D452-2 strain.

3.3 | Heme Production by the Δ HMX1/H3&12 + *PUG1* Strain in a Fed-Batch Fermentation

Considering that the difference in the intracellular heme concentrations between the Δ HMX1/H3&12 and Δ HMX1/H3&12 + *PUG1* strains was not very significant, we decided to select the final strain by comparing the heme production concentrations in fed-batch fermentation. After the depletion of initially added glucose, the feeding solution was fed at rates of 6.4–17.6 mL/h for the Δ HMX1/H3&12 strain and 6.4–20.8 mL/h for the Δ HMX1/H3&12 + *PUG1* strain to maximize both cell growth and heme production. Notably, the Δ HMX1/H3&12 + *PUG1* strain exhibited 33% and 50% higher maximum specific growth rate and maximum dry cell weight compared to the Δ HMX1/H3&12 strain (Figure 4). This result is consistent with a previous report that an increased heme synthesis resulted in increased ATP levels and increased transcription of genes required for the electron transport chain, citric acid cycle, and oxidative phosphorylation [16]. It is worth noting that the fed-batch culture of the Δ HMX1/H3&12 + *PUG1* strain resulted in the maximum heme production concentration of 28 mg/L, which was 25% higher than the corresponding value for the Δ HMX1/H3&12 strain. Therefore, the Δ HMX1/H3&12 + *PUG1* strain was selected as the final heme producer because it produced the highest amount of heme in both batch and fed-batch fermentations. It is noteworthy that while fed-batch fermentation often results in lower yields of target products compared to batch fermentation; in this study, the yields from the two fermentation methods were almost identical.

4 | Conclusion

Herein, the efficient synthesis of heme was achieved by reconstructing the heme biosynthetic pathway in *S. cerevisiae*. For this purpose, we identified Hem12p as the rate-limiting enzyme in heme synthesis. In addition, heme production efficiency was further improved by increasing the availability of the heme precursor PPIX and preventing heme degradation, mediated by *PUG1* overexpression and *HMX1* disruption, respectively. Finally, a glucose-

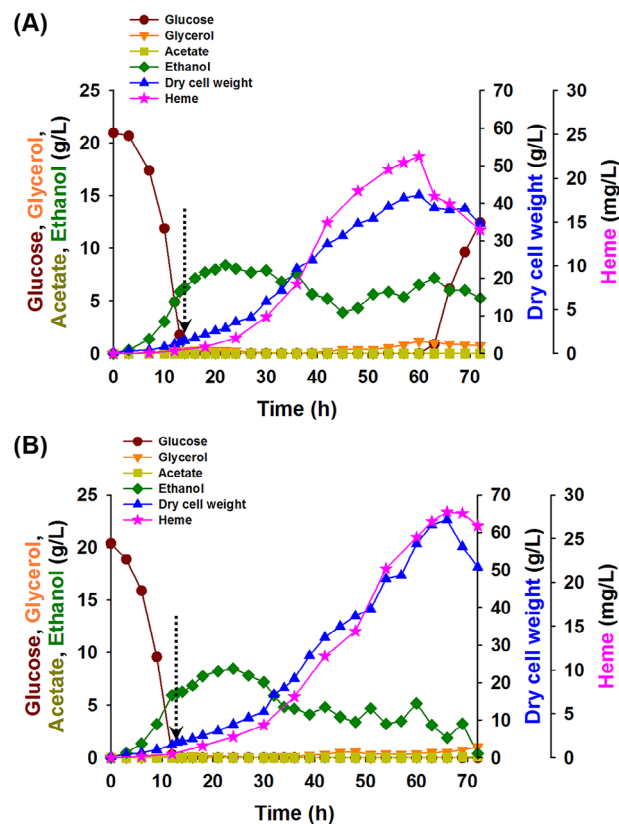


FIGURE 4 | Fed-batch production of heme in the Δ HMX1/H3&12 (A) and Δ HMX1/H3&12 + *PUG1* (B) strains. After the depletion of initially added glucose, the feeding solution consisting of 350 g/L of glucose, 280 g/L of yeast extract, and 140 g/L of Bacto™ Peptone was added into the bioreactor (black dot arrow). The agitation speed, air supply, temperature, and medium pH were maintained at 1000 rpm, 3.0 vvm, 25°C, and pH 4.5, respectively, throughout the cultivation. All values were measured in duplicate; the average values with less than 5% standard deviations are provided.

limited fed-batch fermentation using the Δ HMX1/H3&12 + *PUG1* strain, the *HMX1*-deficient strain overexpressing *HEM3*, *HEM12*, and *PUG1*, resulted in 28 mg/L of heme.

Author Contributions

Hyun-Jae Lee: investigation (lead), validation (equal), visualization (lead). **Dong Joo Shin:** methodology (lead), validation (equal). **Soo Bin Nho:** data curation (equal), visualization (supporting). **Ki Won Lee:** formal analysis (supporting), writing-review and editing (equal). **Sun-Ki Kim:** conceptualization (lead), formal analysis (equal), supervision (lead), writing-original draft (lead).

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Conflicts of Interest

The authors declare no conflicts 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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Supporting Information

Additional supporting information can be found online in the Supporting Information section.