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Improved production of human hemoglobin in yeast by engineering hemoglobin degradation

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ABSTRACT

With the increasing demand for blood transfusions, the production of human hemoglobin (Hb) from sustainable sources is increasingly studied. Microbial production is an attractive option, as it may provide a cheap, safe, and reliable source of this protein. To increase the production of human hemoglobin by the yeast *Saccharomyces cerevisiae*, the degradation of Hb was reduced through several approaches. The deletion of the genes *HMX1* (encoding heme oxygenase), *VPS10* (encoding receptor for vacuolar proteases), *PEP4* (encoding vacuolar proteinase A), *ROX1* (encoding heme-dependent repressor of hypoxic genes) and the overexpression of the *HEM3* (encoding porphobilinogen deaminase) and the *AHSP* (encoding human alpha-hemoglobin-stabilizing protein) genes — these changes reduced heme and Hb degradation and improved heme and Hb production. The reduced hemoglobin degradation was validated by a bilirubin biosensor. During glucose fermentation, the engineered strains produced 18% of intracellular Hb relative to the total yeast protein, which is the highest production of human hemoglobin reported in yeast. This increased hemoglobin production was accompanied with an increased oxygen consumption rate and an increased glycerol yield, which (we speculate) is the yeast's response to rebalance its NADH levels under conditions of oxygen limitation and increased protein-production.

1. Introduction

Hemoglobin (Hb) is a major blood-protein, whose main function is oxygen transport. In humans, during erythropoiesis in the bone marrow, megakaryocyte/erythroid progenitor cells (MEP) produce Hb that is carried by differentiated erythrocytes (red blood cells, RBCs) in blood circulation (Xavier-Ferrucio and Krause, 2018). Hb is the cofactor-containing tetrameric protein that is composed of two α- and two β-globin subunits ($\alpha_2\beta_2$) in adults. Each subunit carries one heme *b* (protoporphyrin IX) group with a ferrous iron atom ligated by the four nitrogen atoms at the center of the porphyrin ring (Bruice, 2004). While the iron atom acts as an active site for oxygen binding, the organic component of Hb contributes to the regulation of the Hb activity. For example, the organic component ensures the reversibility of the oxygen binding to Hb (Nagai et al., 1985). Human Hb is needed for blood transfusions and for artificial blood, but in recent years donated blood has had shortages, because of decreasing rates of donation.

As an alternative to human donations of blood, recombinant production of Hb is a promising technology, following the success of other valuable chemicals. Published research on the recombinant production of hemoglobin has reported the use of production hosts from bacteria, yeast, plants, and animals (Behringer et al., 1989; Hoffman et al., 1990; Wagenbach et al., 1991; Swanson et al., 1992; Dieryck et al., 1997). The degradation of hemoglobin in heterologous hosts is a common problem, which occurs due to lower stability of α-globin subunit (Kihm et al., 2002; Feng et al., 2004). In *E. coli,* the expression of the human-hemoglobin genes in a single operon increased the stochiometric quantities of α- and β-globins, which are used in Hb folding (Hoffman et al., 1990). In *E. coli*, site-directed mutagenesis increased the solubility of Hb (Weickert et al., 1999). In *E. coli,* the co-expression of the erythroid

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human α-hemoglobin stabilizing protein (AHSP) increased the yield of Hb production (Vasseur-Godbillon et al., 2006). Production of heme-containing proteins, particularly hemoglobins, is dependent on heme availability (Fibach et al., 1995). Internal availability of heme and its precursor 5-aminolevulinic acid (5-ALA) in *E. coli* are limited. The addition of external heme, 5-ALA, and the overexpression of heme transporters were reported beneficial for improved hemoglobin production in *E. coli* (Verderber et al., 1997; Graves et al., 2008; Fiege et al., 2018). The budding yeast *Saccharomyces cerevisiae* is a traditional model microorganism, which has become a preferred cell-factory for many products, such as flavors, industrial enzymes, and pharmaceuticals (Hong and Nielsen, 2012; Kutyna and Borneman, 2018; Nielsen 2019). Unlike in bacteria, for hemoglobin production yeasts do not require the external addition of costly heme or heme precursor, 5-ALA. *S. cerevisiae* produces heme endogenously in a complex pathway involving two cell compartments, cytosol and mitochondria, and is strictly regulated by the carbon source, oxygen, and iron availability (Zhang and Hach 1999; Hoffman et al., 2003; Liu et al., 2014). The expression of recombinant hemoglobin in yeast has been significantly improved by the strategies on enhancing the endogenous heme biosynthesis, balancing α- and β-globin gene expression, and also by engineering oxygen sensing (Keng 1992; Liu et al., 2014; Martínez et al., 2015). The heme biosynthesis capacity was increased by the overexpression of rate-limiting enzymes of the pathway (Liu et al., 2014; Martínez et al., 2015). As an example, the overexpression of *HEM3* gene (encoding porphobilinogen deaminase) on multi-copy plasmid results in up to 4-fold increase in intracellular free heme and up to 4% of Hb (Liu et al., 2014). The deletion of *HAP1* gene, encoding a transcription factor involved in the regulation of cellular respiration and heme biosynthesis, was successful to improve the hemoglobin production further, up to 7% of the total cell soluble protein content (Martínez et al., 2015).

We used new approaches to improve human Hb production in yeast, which are based on engineering reduced hemoglobin degradation (Fig. 1A and B): i) overexpressed the *HEM3* gene (encoding porphobilinogen deaminase); ii) deleted the *ROX1* gene (encoding heme-dependent repressor of hypoxic genes) to eliminate inhibition of

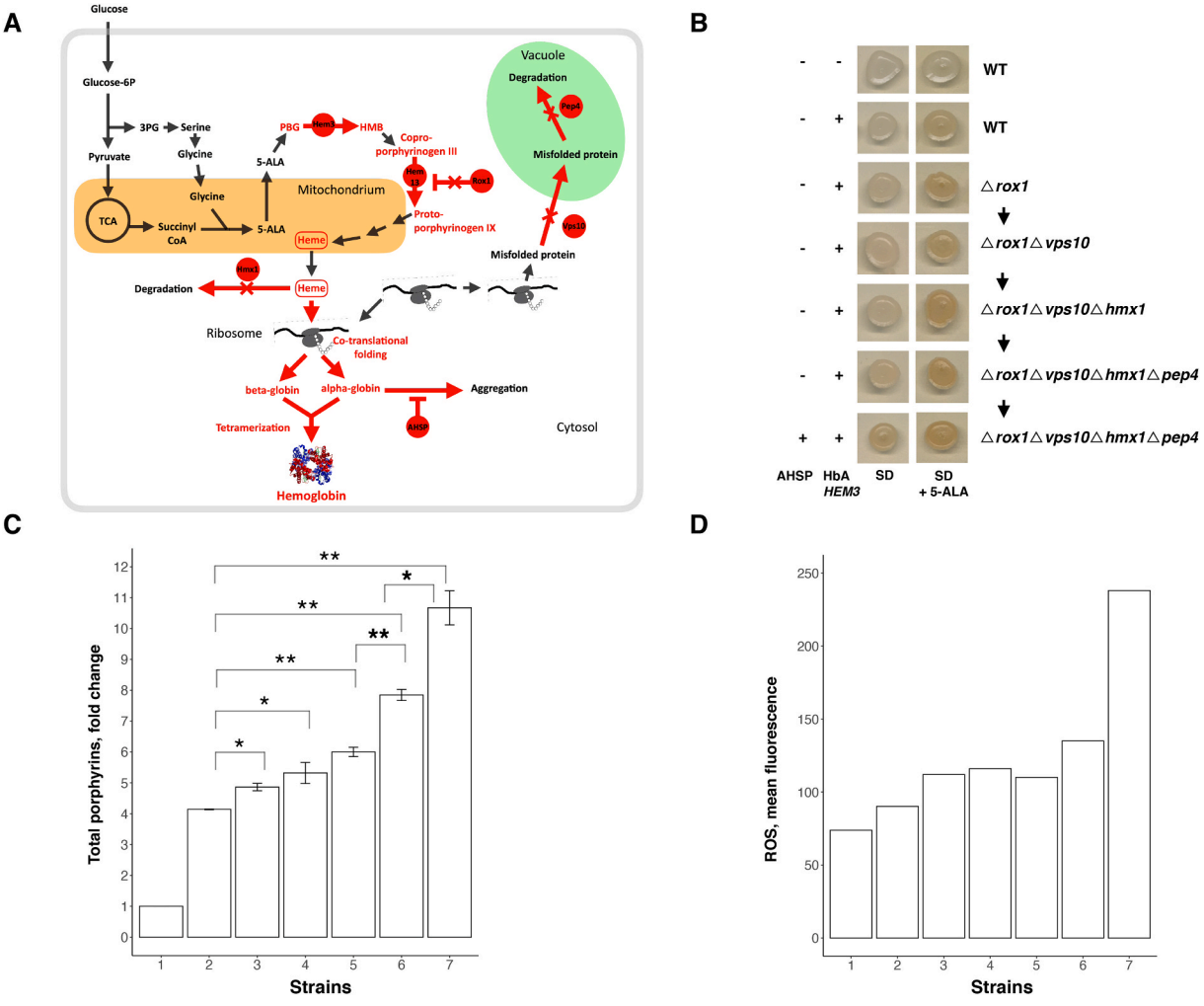

Fig. 1. Construction of yeast strains with reduced degradation of heme and hemoglobin. A) The overview of strains construction. The human adult form of hemoglobin (HbA) and human alpha-hemoglobin stabilizing protein (the *AHSP* gene) were expressed in *S. cerevisiae Δrox1* strain with modification of heme degradation pathway (carrying the *HMX1* gene deletion), and reduced targeting of misfolded proteins to vacuole (carrying the *VPS10* and *PEP4* deletions). B) The deletions of *VPS10, HMX1, PEP4* genes were introduced sequentially into *Δrox1* mutant strain background and their porphyrins production was compared to the wild-type strain in SD medium with and without 5-ALA. Each strain was transformed with HbA expression plasmids pIYC04 + HEM3 (HEM3) and pSP-GM1+ αaβ (HbA) or pIYC04 + HEM3+AHSP (AHSP) and pSP-GM1+ αaβ (HbA). C) Total porphyrins were assayed using 3 biological replicates, error bars represent standard deviation. Strain #1 was used to normalize the data. Statistical analysis was performed using one-way ANOVA (*$p \leq 0.03$, **$p \leq 0.003$). D) ROS accumulation of constructed strains at 24 h of glucose fermentation detected by dihydrorhodamine 123 staining. Mean fluorescence values of 5000 cells obtained by flow cytometry were compared. Strains: 1- WT/empty vectors, control; 2- WT/HEM3/αaβ; 3- *Δrox1*/HEM3/αaβ, 4 - *Δrox1Δvps10*/HEM3/αaβ, 5 - *Δrox1Δvps10Δhmx1*/HEM3/αaβ, 6 - *Δrox1Δvps10Δhmx1Δpep4*/HEM3/αaβ, 7 - *Δrox1Δvps10Δhmx1Δpep4*/HEM3+AHSP/αaβ.

HEM13 gene (encoding coproporphyrinogen III oxidase) by Rox1; iii) deleted the *HMX1* gene (encoding heme oxygenase) responsible for specific heme cleavage; iv) overexpressed human *AHSP* gene (encoding α-hemoglobin stabilizing protein) for increased stability of the α-globin; v) deleted the *VPS10* (encoding sorting receptor of vacuolar hydrolases) and *PEP4* (encoding vacuolar proteinase A) genes for reducing targeting of hemoglobin to the vacuoles for protein degradation. Among these 6 mutations we introduced, the modification of expression the genes *ROX1, HMX1, VPS10* and *AHSP* were not used before for hemoglobin production in *S. cerevisiae*. By combining the mutations in one genome, we showed their beneficial effect and engineered a novel *S. cerevisiae* strain with substantially reduced hemoglobin degradation and improved hemoglobin production, and which produces ~18% of human hemoglobin of its total protein content on glucose.

2. Results

2.1. Improving heme biosynthesis and reducing heme and hemoglobin degradation increased the yield of human hemoglobin

The transcriptional factor Rox1, which is an activator of hypoxic genes, is also a repressor of heme biosynthesis. The Rox1 inhibits the transcription of the *HEM13* gene (Keng 1992). In *S. cerevisiae,* the deletion of the *ROX1* gene or the reduction of its expression increase the production of heterologous proteins, such as α-amylase and insulin precursor (Liu et al., 2015). The deletion of the *ROX1* gene increases intracellular levels of heme (Zhang et al., 2017). When transformed with the plasmids for recombinant hemoglobin expression (pIYC04 + HEM3 and pSP-GM1+ ααβ, Table S1), the *Δrox1* strain accumulated 1.2 times more total porphyrins than the wild-type (WT) background strain (Fig. 1C). This was visible on the plates supplemented with the heme precursor 5-ALA (5-aminolevulinic acid): the *Δrox1* strain with hemoglobin plasmids had a red pigmentation, a consequence of the greater porphyrins-accumulation than the WT (Fig. 1B). Consequently, the *Δrox1* strain was selected for our engineering.

To increase the production of Hb, we planned to reduce the intracellular degradation of both hemoglobin and heme (Fig. 1A). We deleted the *VPS10*, *PEP4*, and *HMX1* genes in the *S. cerevisiae* genome and overexpressed the human α-hemoglobin-stabilizing protein, which is encoded by the *AHSP* gene (Fig. 1A). The final gene combination was the result of sequential experimentation. We experimentally verified the impact of each deletion. If the deletion was successful, this new strain was further engineered with a new mutation; otherwise, when the mutation was not successful, we continued with alternative genes until we reached an apparent local maximum that substantially improved on previous results. We chose the *VPS10* and *PEP4* genes as they affect the targeting of misfolded proteins to the vacuole for their vacuolar degradation, and the *HMX1* gene is part of the heme-degradation pathway (Hong et al., 1996; Protchenko and Philpott, 2003; Marques et al., 2006). The three gene-deletions (*Δvps10*, *Δhmx1,* and *Δpep4*, Fig. 1B) were sequentially introduced into the *Δrox1* strain background by the Cre-lox system with *kanMX* as a selective marker. After each deletion, the *kanMX* marker was removed by the induction of Cre-recombinase expression (Materials and methods). The human *AHSP* gene was cloned and expressed on the pIYC04 + HEM3 plasmid as a synthetic peptide with its codons optimized for expression in *S. cerevisiae*. The four deletion strains were transformed with adult form of hemoglobin (HbA) expression plasmids (pIYC04 + HEM3 and pSP-GM1+ααβ or pIYC04 + HEM3+AHSP and pSP-GM1+ααβ [Table S1]).

The production of porphyrins and hemoglobin was increased with each step (Fig. 1B and C, Fig.S1). In erythrocytes and in *E. coli*, human AHSP prevents the degradation and oxidation of α-globin by forming a stable complex with a free α-globin subunit (Kihm et al., 2002; Feng et al., 2004; Vasseur-Godbillon et al., 2006; Mollan et al., 2010). In our yeast model, the overexpression of *AHSP* gene increased hemoglobin production by 58% (Fig. 2A and B). After 24 h, the total porphyrins level in the *Δrox1Δvps10Δhmx1Δpep4*/HEM3+AHSP/ααβ strain was 2.6 times higher than in the WT/HEM3/ααβ strain (Fig. 1C). At 24 h we observed that the increased hemoglobin production positively correlated with increased ROS production (Fig. 1C and D), as it occurs in other protein production strains (Tyo et al., 2012). On the other hand, the AHSP strain had slightly reduced level of ROS at the early stage of glucose fermentation, at 6 h (Fig.S2); reduced ROS is associated with antioxidant AHSP activity (Kihm et al., 2002; Feng et al., 2004; Mollan et al., 2010). As hemoglobin production increased, the growth rate was reduced (Fig.S3), likely due to higher protein production in the cell or toxicity of hemoglobin to yeast or both. After the *PEP4* deletion, the α-globin band of hemoglobin was detected both by Western blotting and by protein SDS gel, which indicated the high production of hemoglobin (Fig. 2, Fig.S1).

The hemoglobin activity in yeast was detected by the absorption

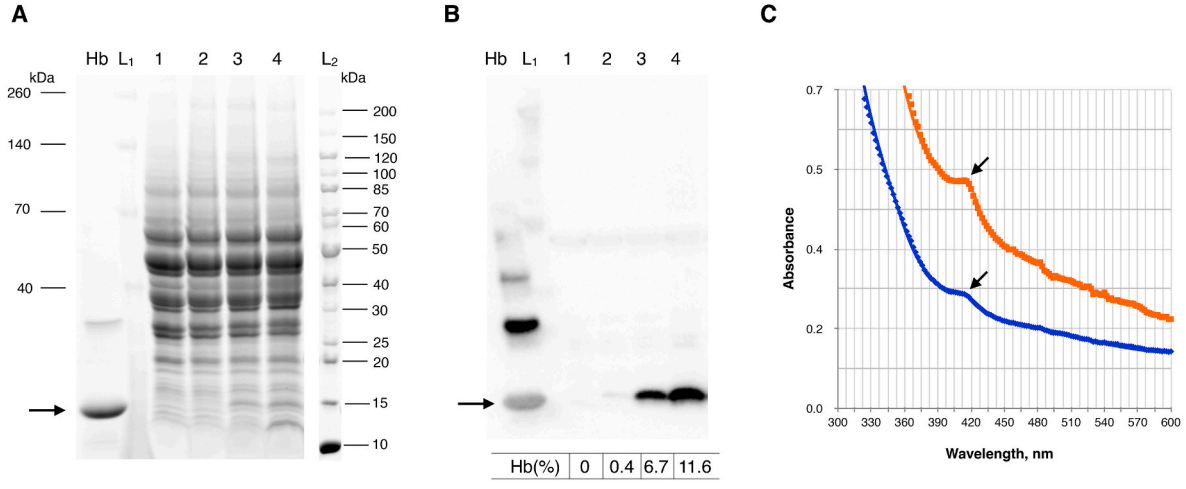

Fig. 2. Hemoglobin expressed in yeast and binds carbon monoxide. A) SDS PAGE gel of TCA protein extracts of yeast strains expressing hemoglobin in flasks. The arrow indicates the hemoglobin band. B) Western blotting using anti-alpha globin antibodies. Hb-A commercial hemoglobin (control), L1 – Spectra™ Multicolor Broad Range Protein Ladder, L2 - PageRuler™ Unstained Protein Ladder. The arrow indicates the hemoglobin band. Hemoglobin production, %, per total protein was calculated from hemoglobin band intensity for each strain: #1 - 0%; #2–0.4%; #3–6.7%, and #4–11.6%. C) Absorption spectra of supernatant of yeast crude extracts of strain #2 (blue) and strain #4 (orange) treated with CO-releasing compound CORM-3. The arrows indicate the peak of carboxyhemoglobin. Strains: 1- WT/empty vectors, control; 2- WT/HEM3/ααβ; 3- *Δrox1Δvps10Δhmx1Δpep4*/HEM3/ααβ, 4- *Δrox1Δvps10Δhmx1Δpep4*/HEM3+AHSP/ααβ. . (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

spectra of carboxyhemoglobin (Hb-CO) formed after the treatment of cellular extracts with carbon monoxide-generating compound (CORM-3) (Fig. 2C). The strain *Δrox1Δvps10Δhmx1Δpep4*/HEM3+AHSP/ααβ had a CORM-3 treated cell-free extract with an absorption peak at 419 nm (corresponding to the Hb-CO), which was 3.1-times greater than the peak of the CORM-3 treated extract from the WT/HEM3/ααβ strain (Fig. 2C).

2.2. The hemoglobin and heme degradation product bilirubin was diminished

Our engineered strains were analyzed for the accumulation of the hemoglobin degradation product, bilirubin, using a bilirubin-binding biosensor the UnaG protein from an eel muscle (Kumagai et al., 2013). The mCherry-UnaG fusion from the plasmid mCherry-FDD (Navarro et al., 2016) was cloned under the yeast promoter *PGK1* on the vector pIYC04 + HEM3 (Table S1). The constructed plasmid pIYC04 + HEM3+mCherry-UnaG (Table S1) was transformed into the four deletion-strains and the wild-type strain to estimate the effect of the introduced mutations on bilirubin formation (Fig. 3A). Upon 100 μM Fe^{3+} supply, the WT strain had the highest fluorescence yield at 12 h of growth, which provided a standard for the successive deletion-strains. First, the *ROX1* deletion had the lower fluorescence during fist 10 h of growth but after 15 h the fluorescence was higher than in WT strain (Fig. 3B). The *rox1* mutation increases the expression of anaerobic genes (Ter Linde and Steensma, 2002). Second, the additional *VPS10* deletion reduced the fluorescence by ~40% (Fig. 3B). Third, the additional deletion of the *HMX1* gene in the *rox1vps10* background reduced the fluorescence further by ~40% (Fig. 3B); *HMX1* encodes the heme oxygenase that catalyzes the formation of bilirubin's precursor biliverdin. In contrast, the deletion of the *PEP4* gene in the *rox1vps10* background strain did not significantly affect the bilirubin formation (Fig. 3B). The fusion hemoglobin, which is a more stable hemoglobin form (Chakane, 2017; Chakane et al., 2017), had less fluorescence of the bilirubin biosensor and thus less bilirubin than HbA form (Fig.S4).

2.3. Strains with increased hemoglobin-production had increased cell-size and cell-density

The AHSP strain (*Δrox1Δvps10Δhmx1Δpep4*/HEM3+AHSP/ααβ) with increased hemoglobin production increased its average cell-size (Fig. 4). Larger cells contain more proteins (Marguerat and Bähler, 2012.). Forcing the high production of protein hinders ribosomal activity, so the yeast decreases the growth rate and increases the cell size (Kafri et al., 2016). In our study, the cell length was 30% greater in the AHSP strain than in the control strain WT/HEM3/ααβ (Fig. 4A). Flow cytometry analysis by both side-scattered and forward-scattered light showed two populations, the AHSP strain and control strain WT/HEM3/ααβ (Fig. 4B), suggesting that they have different cell size or cell density. This suggestion was confirmed by the results of the cell sorter, which showed that the AHSP strain had cell diameter and cell volume 1.2- and 1.8-times (respectively) greater than the control strain WT/HEM3/ααβ (Fig.4C, Fig.S5). The AHSP strain had the greatest cell-volume (Fig. 4, Fig.S5). However, the intermediate strain *Δrox1Δvps10Δhmx1Δpep4*/HEM3/ααβ, which produced 58% lower Hb than AHSP (Fig. 2B), had a cell-size from 10% to 36% higher than control strain, WT/HEM3/ααβ (Fig.S5).

2.4. Iron supplementation increased recombinant hemoglobin production and reduced bilirubin formation

The final step of heme biosynthesis in *S. cerevisiae* is the incorporation of iron into the porphyrin ring by a ferrochelatase encoded by the *HEM15* gene (Labbe-Bois, 1990). Doubling the iron concentration in the medium increased porphyrin synthesis 25% and the hemoglobin protein production 1.5-fold in AHSP strain (Fig.S6). Finally, increasing 2-fold the concentration of iron (Fe^{3+}) decreased the formation of bilirubin by ~12% (Fig.S6).

2.5. Hemoglobin production increased in bioreactors

The hemoglobin production in the constructed strains was studied in batch bioreactors with constant aeration and pH (Materials and

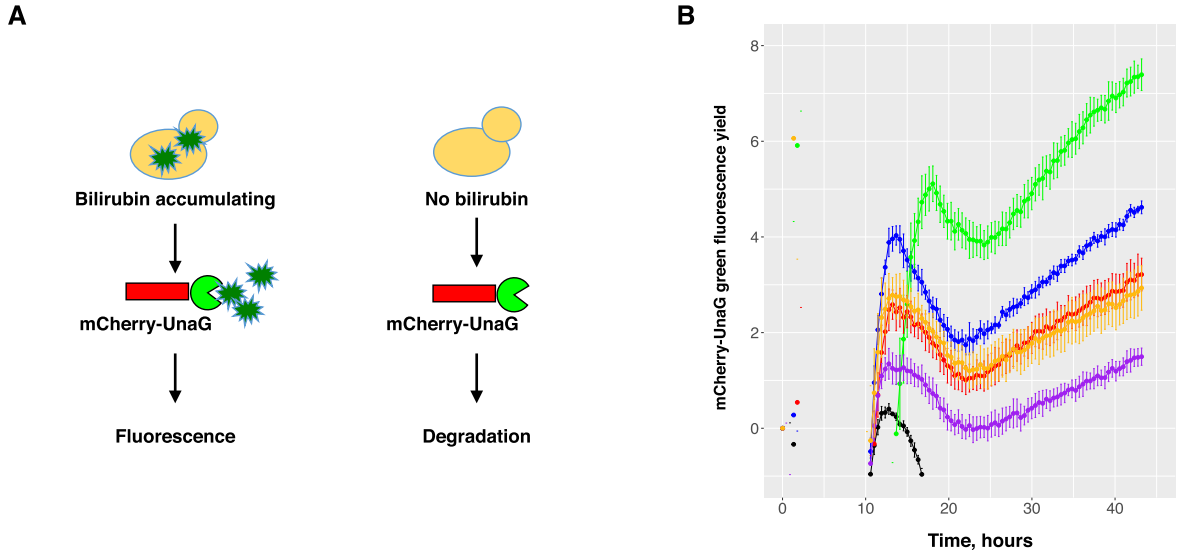

Fig. 3. Bilirubin biosensor expressed in yeast. A) The mCherry-UnaG construct was expressed in yeast under promoter *PGK1* to study the accumulation of heme/hemoglobin degradation product bilirubin. The construct is fluorescent when bilirubin is present but is degraded when no bilirubin is formed. B) mCherry-UnaG green fluorescence yield (fluorescence normalized by biomass) in different strains expressing HbA: wild-type (Strain 2, blue); *Δrox1* (Strain 3, green); *Δrox1Δvps10* (Strain 4, red); *Δrox1Δvps10Δhmx1* (Strain 5, purple); *Δrox1Δvps10Δpep4* (Strain 6, gold). Wild-type strain expressing empty vector was used as a control (Strain 1, black). Each strain was grown in SD his-ura- medium with 2% glucose and 100 μM Fe^{3+} and was analyzed in BioLector® bioreactor. Error bars represent standard deviation of 4 biological replicates. . (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

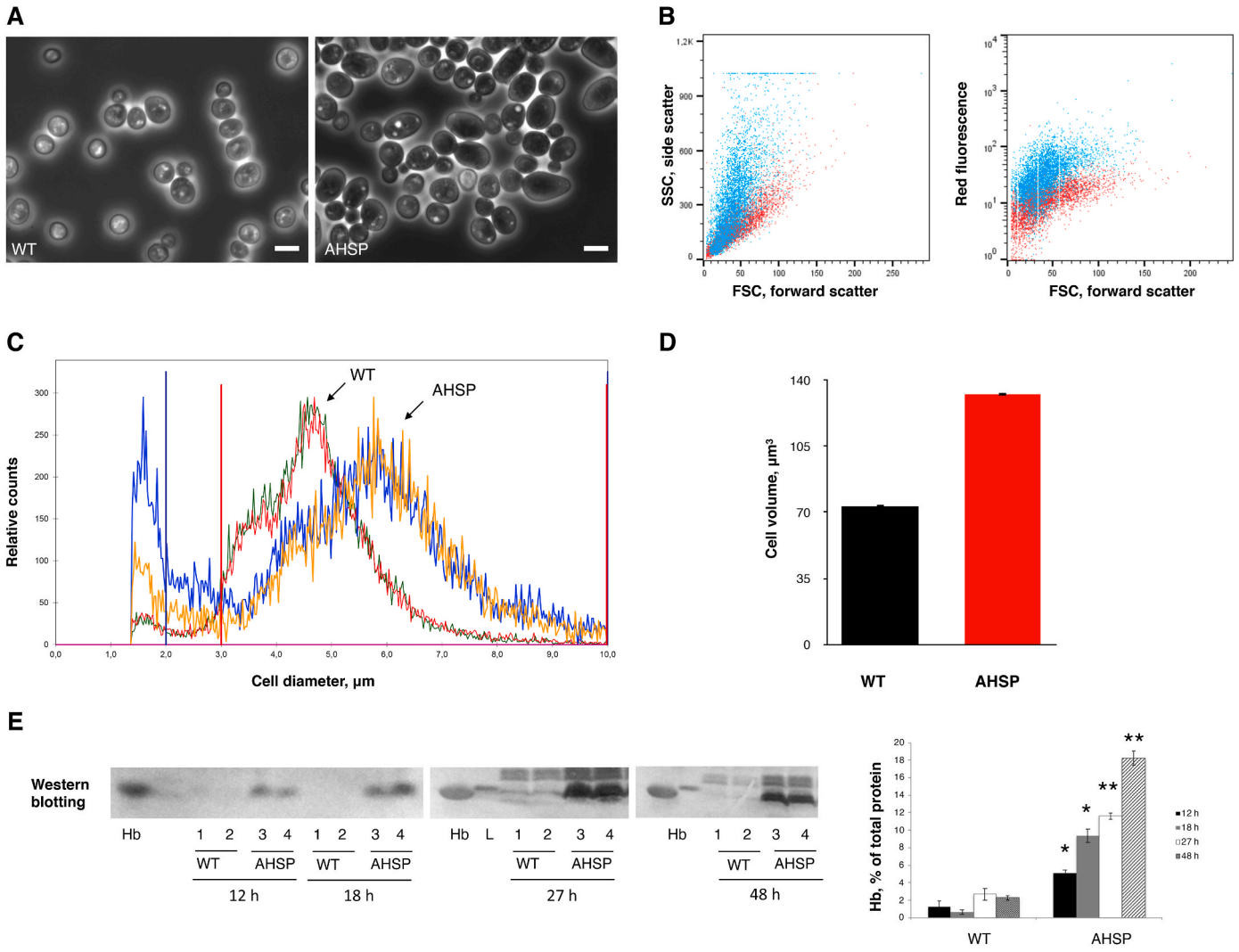

Fig. 4. AHSP strain had increased cell size and complexity. A) Phase contrast microscope images showing cell size of hemoglobin strains (WT and AHSP). Scale bar represents 10 µm. B) Flow cytometry analysis of 5000 cells shows two cell populations for WT (red) and AHSP (blue) strains. Cell diameter (C) and cell volume (D) estimated by CASY Model TT Cell Counter for WT and AHSP strains. E) Estimation of hemoglobin production during cultivation in bioreactors of WT and AHSP strains by Western blotting using anti-hemoglobin α antibodies. Hb, % was calculated by band intensity normalized by total protein loaded in each lane. The error bars represent standard deviation of two replicates. The AHSP was found to produce significantly greater Hb amount as estimated by one-way ANOVA (12 h: $*p \leq 0.03$; 18 h: $*p \leq 0.04$; 27 h: $**p \leq 0.007$; and 48 h: $**p \leq 0.002$). Strains: WT- WT/HEM3/ααβ; AHSP - *Δrox1Δvps10Δhmx1Δpep4*/HEM3+AHSP/ααβ. . (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Methods). Under these controlled conditions, the maximum specific growth rate of the AHSP strain was 20% less than that of the control strain WT/HEM3/ααβ (Table S2). The AHSP strain increased the yields of glycerol 100% and acetate 30%, while it increased the oxygen consumption rate 15% and decreased the CO_2 yield 6% (Table S2). In the AHSP strain, the increased glycerol, acetate-production, and oxygen-consumption-rate replicated the general finding that these changes indicate that the cell is coping with the redox imbalance (van Dijken and Scheffers, 1986). In the bioreactors, after 48 h of cultivation, the AHSP strain produced 18% of its total protein content in hemoglobin (Western blot). This Hb-production was 500% greater than in the control strain WT/HEM3/ααβ (Fig. 4E). The intermediate strain, *Δrox1Δvps10Δhmx1Δpep4* lacking AHSP protein, produced only ~8% of Hb of its total protein content (Fig.S7), while the control strain, WT/HEM3/ααβ, accumulated ~3–4% of intracellular hemoglobin (Liu et al., 2014), a result that we replicated (Fig. 4E). In the AHSP strain, after 24 h 12% of its total cellular protein was hemoglobin concurrently increasing the total cell protein content by 6%; after 48 h, 18% of its total cellular protein-content was hemoglobin, concurrently increasing the total protein content of the cell by 21% (Fig.4E, Fig.S8).

2.6. Reducing hemoglobin degradation increases hemoglobin secretion

Adapting the hemoglobin fusion construct for the bacterium *E. coli* (Chakane et al., 2017) for use in yeast, we first codon-optimized it for yeast expression and then fused it to the yeast α-factor leader-sequence to target hemoglobin to the endoplasmic reticulum (ER) and the secretory pathway for extracellular secretion through the transport vesicles (Rexach et al., 1994). The α-factor leader-hemoglobin fusion-construct was expressed in three strains: INVSc1 (Invitrogen's diploid strain for protein expression), 184M (an amylase producer developed for secretion by mutagenesis [Huang et al., 2015]), and our *Δrox1Δvps10Δhmx1Δpep4* (mutant of CENPK113-11c with reduced hemoglobin degradation) [Fig. 5A]. No hemoglobin was detected before the concentration of the medium. However, after concentrating the strains' media using Amicon 10 kDa centrifugation filters (Merck Millipore), hemoglobin was detected by Western blotting in two strains, 184M and *Δrox1Δvps10Δhmx1Δpep4* (but not in INVSc1) Fig. 5B. In both these strains,

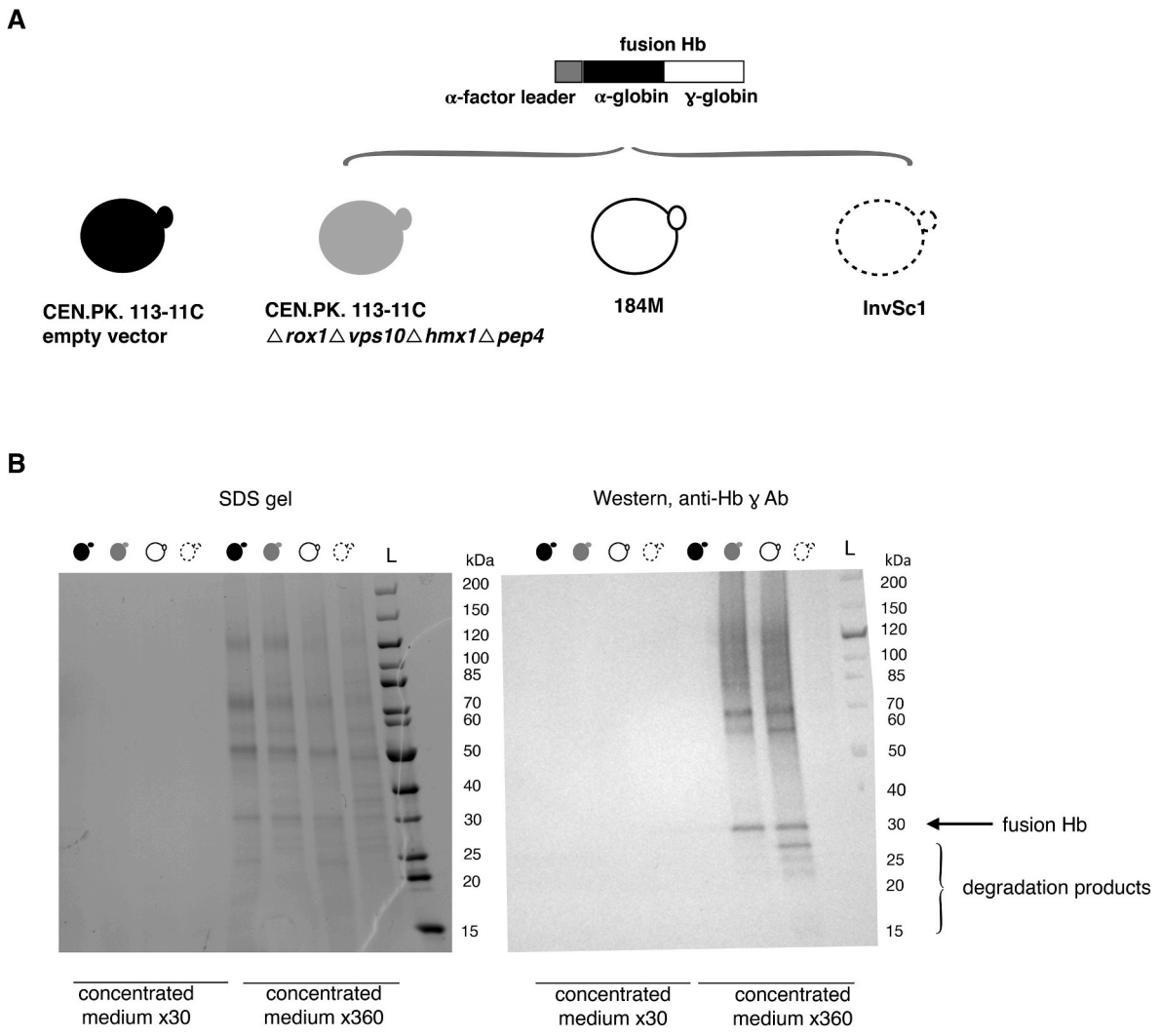

Fig. 5. Expression of hemoglobin fusion through secretory pathway in different yeast strains. A) Hemoglobin was expressed as a fusion peptide (αɣ-globin Hb fusion with *S. cerevisiae* native α-factor leader sequence) in CENPK113-11c *Δrox1Δvps10Δhmx1Δpep4*, 184M, and INVSc1 strains. CENPK113-11c carrying empty vector was used as a control. B) SDS protein gel and Western blotting with polyclonal antibodies against ɣ-globin chain were used to detect the fusion Hb (αɣ-globin, 33 kDa) construct after α-factor leader removal in secretory pathway. Media from the yeast cultures were concentrated using TCA and acetone either 30- or 360-fold.

the protein band of ~30 kDa corresponding to fusion hemoglobin was detected; however, in the 184M strain, 4 smaller bands were additionally observed (Fig. 5B), which may indicate the partial degradation of hemoglobin in the 184M strain, we speculate. The 184M strain has mutations that down-regulate the *ROX1* and *VPS10* introduced by mutagensis; its *PEP4* and *HMX1* gene-expressions were unchanged (Huang et al., 2017).

3. Discussion

Hb contains common prosthetic group (heme *b*), which is incorporated into the globin chains co-translationally and important for the polypeptide-folding (Komar et al., 1993). Heme availability is not only crucial for hemoglobin synthesis but for its major function, because a heme-less hemoglobin will be unable to bind oxygen.

To improve heme availability, we overexpressed the heme biosynthesis gene *HEM3*, which limits the biosynthesis of heme. The gene *HEM3* increases both heme and hemoglobin production (Hoffman et al., 2003; Liu et al., 2014). Under the overexpression of the *HEM3* and human hemoglobin genes, the *Δrox1* strain produced more porphyrins.

The intracellular level of heme is tightly regulated in response to the oxygen availability by the transcription factor Hap1, which activates the Rox1. In turn, Rox1 represses the expression of the *HEM13* gene (Keng 1992; Ter Linde and Steensma, 2002). In *S. cerevisiae*, the deletion of the *ROX1* gene increases heme-production (Zhang et al., 2017), activates hypoxia-induced genes (Keng 1992), and increases the production of other heterologous proteins, such as insulin, invertase, and α-amylase (Liu et al., 2015).

Heterologous-protein synthesis is inhibited by the protein degradation mechanisms in the host, and consequently these processes' suppression increases protein-production. Outside the erythrocytes and without erythroid protective enzymes, the hemoglobin is degraded to globin and heme. Released heme is further degraded to biliverdin and bilirubin. Heme is naturally produced by yeast and is degraded when it is in access or upon iron limitations (Protchenko and Philpott, 2003). To suppress the protein-degradation processes, we engineered a hemoglobin-producing *S. cerevisiae* strain with reduced degradation of both the hemoglobin protein and its heme cofactor. Engineered with the *ROX1, VPS10*, *HMX1*, and *PEP4* gene-deletions, our yeast produced enough hemoglobin to be detected on the protein gel even without Western blotting while the degradation product of hemoglobin and heme bilirubin was reduced.

These four gene-deletions have already been found important in yeast biology. The heme oxygenase (encoded by the *HMX1* gene)

degrades heme upon iron starvation and oxidative stress (Protchenko and Philpott, 2003). Under conditions of high protein production, misfolded proteins are targeted to the vacuole degradation. The *VPS10* gene, encoding type I transmembrane sorting receptor for multiple vacuolar hydrolases, is involved in vacuolar targeting of unfolded proteins (Marcusson et al., 1994). The *VPS10* gene's deletion of the *VPS10* gene increases the production of other heterologous proteins (Hong et al., 1996), as does its suppression (Huang et al., 2017). The *PEP4* gene encodes a vacuolar aspartyl protease, which is important for the recycling of the proteins damaged by oxidative stress. The mutation of the *PEP4* gene increased the production of heterologous proteins in yeast (Marques et al., 2006; Wang et al., 2007). Introducing the deletions of *VPS10* and *HMX1* genes decreased the formation of the hemoglobin degradation-product bilirubin, as confirmed by the bilirubin biosensor.

In our strains, doubling the iron-concentration increased the hemoglobin-yield and reduced the bilirubin-formation. The heme-oxygenase expression is regulated by iron and oxidative stress (Protchenko and Philpott, 2003), and this expression is decreased in the medium with a doubled iron-concentration and in *Δrox1*.

Hemoglobin production was increased 58% by the overexpression of the human *AHSP* gene (α-hemoglobin stabilizing protein), which prevents its degradation and oxidation in erythrocytes (Kihm et al., 2002; Feng et al., 2004; Mollan et al., 2010). This *AHSP*-overexpression also reduced the ROS formation during the glucose-phase of fermentation.

Our engineered AHSP-strain produced the hemoglobin level 18% with respect to the total intracellular protein content. This was 2.6 times greater than the 7% previously reported (Martínez et al., 2015). This increased level of hemoglobin was associated with cells having an increased volume and an increased protein-content while having a 3-fold decrease in the growth rate. Such phenotypic changes occur in cells burdened with increased protein-synthesis (Kafri et al., 2016).

The engineered strain carried the mutation of *Δhmx1* (causing iron depletion) and had overexpressed hemoglobin-genes (causing iron depletion). Hemoglobin production was increased by doubling the iron concentration in the media. When cells are deprived of iron, their respiratory-chain malfunctions and the cells accumulate NADH; such stressed cells respond by increasing the expression of the *GPD2* gene, so producing glycerol (Ansell and Adler, 1999). Increased the intracellular production of hemoglobin changed the fermentation profile. The AHSP strain increased oxygen-consumption; it also increased the production of glycerol and acetate, which are produced to satisfy NADH/NADPH balance (Villadsen, et al., 2011). This cofactor imbalance could be addressed to improve the hemoglobin-production in the future.

In conclusion, we engineered a *Saccharomyces cerevisiae* strain that produced 18% (of total cell protein) as human hemoglobin. This strain could be used as a sustainable and safe source of hemoglobin for the development of hemoglobin-based oxygen carriers (HBOCs) or meat substitutes.

4. Materials and Methods

4.1. Media and strains growth conditions

Strains used in this study are listed in Table S3. The strains of *Saccharomyces cerevisiae* CEN.PK 113-11C (*MATa his3Δ1 ura 3–52 MAL2-8c SUC2*) (Entian and Kötter, 1998) and its *Δrox1* mutant (Liu et al., 2015) were used as hosts for human hemoglobin production. Yeast strains were maintained at 30 °C in a complete rich medium YPD (5 g/L yeast extract, 10 g/L peptone, 20 g/L glucose). Transformants with hemoglobin A expression plasmids pIYC04 + HEM3 and pSP-GM1+ααβ (Liu et al., 2014) were selected on synthetic complete medium SD without both uracil and histidine (6.9 g/L yeast nitrogen base with ammonium sulphate w/o amino acids (Formedium™), 0.75 g/L synthetic complete drop-out mixture w/o histidine and uracil (Formedium™), pH 6.0) containing 20 g/L glucose as carbon source. Additional iron was added to the SD medium (SD with Fe^{3+}) (100 μM of ferric citrate, Sigma-Aldrich). Deletion mutants were selected on YPD medium with G418 at the concentration of 0.2 g/L. For *kanMX* marker removal by Cre recombinase induction, transformants were grown on YPG medium (5 g/L yeast extract, 10 g/L peptone, 10 g/L galactose) overnight. For the evaluation of porphyrin production in *Δrox1* strain, 5-aminolevulinic acid (5-ALA) was added in SD media at the concentration of 1 mM.

4.2. Generation of gene knockout strains

Oligonucleotide primers and plasmids used in this study are listed in Table S1. The deletion cassettes with the dominant selection marker *kanMX* expressed under control of *Ashbya gossypii TEF1* (Steiner and Philippsen, 1994) were used for the gene knockouts. The subsequent marker removal was done by the Cre-lox system (Cre recombinase was expressed under promoter *GAL1* of *S. cerevisiae*). The deletion cassettes carried *kanMX* and Cre-recombinase flanked with LoxP and ~50 bp of nucleotide sequences homologous to *HMX1*, *VPS10*, and *PEP4* target genes of *S. cerevisiae*. Each deletion cassette was amplified in 2 fragments from template plasmids pDel1 and pDel2 (Table S1 [Wenning et al., 2017],), containing 335 bp overlapping region of *kanMX* gene to be repaired *in vivo* in yeast after the transformation (fragment 1): target gene 5′-sequence-loxP-half *kanMX* gene; fragment 2: *kanMX* gene (second half with overlap)-GAL-promoter-Cre recombinase-LoxP-target gene 3′ sequence, that were then co-transformed into *Δrox1* mutant. The *HMX1* gene deletion cassette was amplified by Del-HMX1-1 and Del1-rev primers pair (PCR fragment 1), Del2-for and Del2-HMX1-2 (PCR fragment 2). The *VPS10* gene deletion cassette was amplified by VPS10-1 and Del1-rev (PCR fragment 1), Del2-for and VPS10-2 (PCR fragment 2). The *PEP4* gene deletion cassette was amplified by PEP4-4 and Del1-rev (PCR fragment 1), Del2-for and PEP4-2 (PCR fragment 2) (Table S1). The transformants with deletion cassettes were selected on YPD medium with G418 at the concentration 0.2 g/L. The gene deletions were verified by PCR analysis and obtained mutants were selected for further studies. To induce the Cre recombinase expression, the transformants were grown overnight in rich medium with galactose (YPG) and then plated on YPD. The transformants, that lost the ability to grow on YPD with G418 after this treatment, were selected for further studies.

4.3. Glucose fermentations and metabolites analysis

Batch glucose fermentations were performed in flasks and under strictly controlled conditions in bioreactors. The shake flask fermentations were performed at 30 °C in 25 ml of liquid medium at 200 rpm, inoculated with an initial OD_{600} of 0.2 from the pre-cultures. The batch fermentations were performed in 1.0 L Biostat Qplus© bioreactors (Sartorius Stedim Biotech, Germany) with a working volume of 500 ml. The temperature was maintained at 30 °C and pH at 6.0. Bioreactors were inoculated with an initial OD_{600} of 0.1 from the pre-cultures. The amount of dissolved oxygen was measured by oxygen sensors and maintained above 30%. The volumetric flow (aeration) was set to 60 L/h (2 vvm) and constant agitation stirrer speed at 600 rpm. The dry weight was measured by collecting the biomass on membrane filters (0.45 μm, MontaMil® MCE, Frisenette, Denmark) with subsequent drying. The metabolites in the cultivation media were measured in the cultivation media by HPLC (Dionex Ultimate 3000 HPLC (Model 1100–1200 Series HPLC System, Agilent Technologies, Germany) with HPX-87H column (BIO-RAD, USA)). The off-gas from the bioreactors was passed through a foam-trap and analyzed by a mass spectrometer (Model Prima PRO Process MS, Thermo Fisher Scientific™, United Kingdom).

4.4. ROS detection

The ROS level was measured *in vivo* using the dihydrorhodamine 123 dye by the protocol described before (Johansson et al., 2016.). For this purpose, after 6 h of fermentation, the cells were harvested by

centrifugation and washed with 50 mM sodium citrate buffer. The cells were further incubated with 50 mM sodium citrate buffer supplemented with 50 μM dihydrorhodamine 123 for 30 min in the dark. After staining, the cells were spun down and washed with 50 mM sodium citrate buffer. The formation of rhodamine (oxidized form of dihydrorhodamine 123) was detected by fluorescence using the FLUOstar Omega microplate reader (with the excitation 485 nm and emission 520 nm filters) and Guava easyCyte™ 8HT flow cytometer (Millipore).

4.5. Porphyrins content analysis

Cellular heme and porphyrin content were determined as described before (Liu et al., 2014). Free cellular heme and total porphyrin content was determined after oxalic acid treatment by their fluorescence with excitation at $\lambda = 400$ nm and emission at $\lambda = 600$ nm on a FLUOstar Omega plate reader spectrophotometer.

4.6. Detection of carboxyhemoglobin by absorption spectra

The buffer to obtain cells crude extracts using glass beads contained 100 mM potassium phosphate with protease inhibitor cocktail (Fisher Scientific), CO-releasing compound CORM-3 (Sigma-Aldrich) at 0.6 mg/ml, 2 mM $MgCl_2$, 1 mM dithiothreitol and 1 mM EDTA. After cells debris removal, the carboxyhemoglobin amount was determined by spectra analysis of protein extracts of samples with the same protein concentration (13 mg/ml).

4.7. Determination of cell volume

The yeast cell volume was determined using CASY Model TT Cell Counter and Analyzer (Roche Diagnostics International Ltd). Cells were collected from bioreactors at 24, 48, 72 and 96 h of cultivation, re-suspended in CASY ton buffer and analyzed using capillary of 60 μm.

4.8. Protein extraction and Western blotting

Total protein was extracted by TCA treatment as described in (Baerends et al., 2000) and proteins separated by electrophoresis on precast SDS–polyacrylamide gels (4–20% gradient, Mini-PROTEAN® TGX Stain-Free™ Precast Gels, BIO-RAD), electro-transferred to PVDF membrane (Trans-Blot®Turbo Mini PVDF Transfer Packs, BIO-RAD) and hybridized with anti-hemoglobin antibodies (Hemoglobin α antibody (D-16): sc-31110, goat polyclonal, Santa Cruz Biotechnology). For hemoglobin signal detection, secondary antibodies were used conjugated with either alkaline phosphatase (Anti-goat IgG, Sigma-Aldrich) or horseradish peroxidase (Anti-goat IgG, Fisher-Scientific). The signal intensity was analyzed in Image Lab ™ (BIO-RAD).

Statistical analysis

The software packages R (Version 3.6.1 [R Development Core Team (2008). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria]) and Minitab^{R} 18.1 were used to analyze the data.

Consent for publication

Not applicable.

Ethics approval and consent to participate

Not applicable.

Author contributions

Olena P. Ishchuk and Dina Petranovic: Conceptualization. Olena P. Ishchuk, August T. Frost, Facundo Muñiz-Paredes, Saki Matsumoto, Nathalie Laforge, and Nélida Leiva Eriksson: Investigation. Olena P. Ishchuk, August T. Frost, Nélida Leiva Eriksson, José L. Martínez, and Dina Petranovic: Formal analysis. Olena P. Ishchuk: Writing-Original Draft. Olena P. Ishchuk: Visualization. Dina Petranovic: Supervision.

Declaration of competing interest

OPI and DP have filed a patent application related to the present work.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ymben.2021.05.002.

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