Balanced globin protein expression and heme biosynthesis improve production of human hemoglobin in Saccharomyces cerevisiae

Balanced globin protein expression and heme biosynthesis improve production of human hemoglobin in *Saccharomyces cerevisiae*

Lifang Liu [a], José L. Martínez [a], Zihe Liu [a], Dina Petranovic [a], Jens Nielsen [a,b,*]

a Novo Nordisk Foundation Center for Biosustainability,
Department of Chemical and Biological Engineering,
Chalmers University of Technology, SE-41296 Göteborg, Sweden
b Novo Nordisk Foundation Center for Biosustainability,
Technical University of Denmark, Fremtidsvej 3, DK-2970 Hørsholm

*Corresponding author:
Department of Chemical and Biological Engineering
Chalmers University of Technology
Kemivägen 10
SE-412 96 Göteborg, Sweden

Telephone: +46 31 772 3804
Fax: +46 31 772 3801
Email: nielsenj@chalmers.se

Abstract

Due to limitations associated with whole blood for transfusions (antigen compatibility, transmission of infections, supply and storage), the use of cell-free hemoglobin as an oxygen carrier substitute has been in the center of research interest for decades. Human hemoglobin has previously been synthesized in yeast, however the challenge is to balance the expression of the two different globin subunits, as well as the supply of the prosthetic heme required for obtaining the active hemoglobin ($\alpha_2\beta_2$). In this work we evaluated the expression of different combinations of α and β peptides and combined this with metabolic engineering of the heme biosynthetic pathway. Through evaluation of several different strategies we showed that engineering the biosynthesis pathway can substantially increase the heme level in yeast cells, and this resulted in a significant enhancement of human hemoglobin production. Besides demonstration of improved hemoglobin production our work demonstrates a novel strategy for improving the production of complex proteins, especially multimers with a prosthetic group.

Key words

Human hemoglobin, heme biosynthesis, prosthetic group, protein production, *Saccharomyces cerevisiae*

1. Introduction

Hemoglobin is the major oxygen transport protein in blood and is found in erythrocytes. The protein is a tetrameric metalloprotein consisting of four globin subunits ($\alpha_2\beta_2$) with each subunit containing a prosthetic heme group. One heme molecule is comprised of a protoporphyrin IX and a ferrous iron which has high affinity for binding oxygen (Belcher et al., 2010; Benesch and Benesch, 1963). Due to the concerns with whole blood for transfusion, e.g. limited resources, cross-typing requirements and transfusion transmitted disease, substantial efforts have been put into the development of safe and effective human blood substitutes (Looker et al., 1992; Wagenbach et al., 1991). Aside from synthetic perfluorocarbon (PFC) polymers (Biro, 1993) and erythrocyte products (which are purified hemoglobin from a variety of mammalian sources), cell-free hemoglobin-based oxygen carrier (HBOC) has been extensively studied for its high efficiency in oxygen transfer and its contribution to the maintenance of osmotic pressure (Lu et al., 2011). The main limitation with this product is protein instability due to tetramer dissociation into dimers, resulting in short retention time and renal toxicity, and high oxygen affinity in the body caused by absence of a co-factor 2, 3-bis (phospho) glycerate (2,3-BPG) (Kroeger and Kundrot, 1997; Looker et al., 1992; Wagenbach et al., 1991). A series of advances have been achieved including chemical modifications of natural hemoglobin to extend its half-life in circulation (Harris and Palmer, 2008) which have greatly stimulated further studies on HBOC (Lu et al., 2011).

With the development of recombinant DNA technology, it became possible to engineer hemoglobin with genetic engineering, which enabled designed modification to improve its biological properties as well as to enhance its production. A variety of strategies have been applied in different organisms since the late 80s ranging from bacteria (Hoffman et al., 1990) to higher organisms, including plants (Dieryck et al., 1997) and animals (Behringer et al., 1989; Martinez et al., 2012). The main advances include (i) expression of α and β globin chains simultaneously and refold together, with addition of exogenous heme *in vitro* (Hoffman et al., 1990) (ii) Co-expression of the methionine amino-peptidase (Met-AP) gene with the globin genes to reduce the N-terminal methionine in modified globins

produced in *E. coli* (Shen et al., 1993) (iii) Site-directed mutations in β globin chain in order to reduce the extreme oxygen affinity due to the lack of 2,3-BPG allosteric regulation (Hernan et al., 1992) (iv) Tandem fusion of two α globins to prevent tetramer disassociation into αβ dimers (Looker et al., 1992) (v) Co-expression of bacterial heme membrane transporter systems to increase heme uptake and further enhancement of hemoglobin production (Graves et al., 2008). In addition to this, several recent studies showed that α-hemoglobin stabilizing protein (AHSP) could prevent α globin precipitation and hereby have a positive effect on hemoglobin production (Feng et al., 2004; Feng et al., 2005; Vasseur-Godbillon et al., 2006).

The yeast *Saccharomyces cerevisiae* is a well-known cell factory for production of fuels (Lee et al., 2008), chemicals (Chemler et al., 2006; Nevoigt, 2008) and heterologous proteins, especially industrial enzymes and pharmaceuticals (Hou et al., 2012; Martinez et al., 2012). There are several reasons why yeast can potentially be a highly competitive expression host for production of human hemoglobin: it is general regarded as safe (GRAS organism) which avoids the risk of pathogen contaminations from human or animal resources, it is able to perform post-translational modifications, including correct processing the globin termini, folding and subunit assembling (Porro et al., 2005), being the prerequisite for proper reconstitution of a soluble heme-containing tetramer *in vivo*. Furthermore, being a long-term eukaryal model organism, numerous studies have been carried out on *S .cerevisiae* from which extensive genome and physiological information have been generated (Petranovic and Nielsen, 2008). For these reasons several recombinant proteins such as human insulin (Novo Nordisk), human bovine serum albumin (Novozymes) and hepatitis vaccines (GlaxoSmithKline, Merck) among others, have been industrially produced and approved for medical applications in *S. cerevisiae* (Martinez et al., 2012; Walsh, 2010).

Hemoglobin synthesis is highly conserved through evolution and the non-globin part, the prosthetic group protoporphyrin IX, is common among divergent species such as human and yeast , so one can utilize the endogenous yeast heme pathway for the production of human recombinant hemoglobin (Kumar, 1995). Studies suggest that recombinant hemoglobin produced in yeast remains fully

functional and shows identical rate of dissociation as the native human hemoglobin (Coghlan et al., 1992). However, with improved globin production it is questionable whether endogenous heme production is sufficient. Thus, it has been found that supplementation with hemin can accelerate hemoglobin synthesis in immature cultured erythroid cells, which suggests that heme availability is a rate-controlling step for hemoglobin production (Fibach et al., 1995). Additionally, heme association to globins was suggested to occur co-translationally and could therefore facilitate the formation of the proper tertiary structure on the ribosome (Komar et al., 1997). A coordinated optimization of endogenous heme level is therefore expected to improve heterologous hemoglobin production in yeast cells.

The heme biosynthesis pathway in yeast (Figure 1) contains eight steps starting from 5-aminolevulinic acid (ALA) synthesis from succinyl-CoA and glycine (catalyzed by Hem1p) in mitochondria. After being transported to the cytosol to form porphobilinogen (PBG) (catalyzed by Hem2p), the intermediate is further converted to hydroxymethylbilane (catalyzed by Hem3p) and then uroporphyrinogen III (by Hem4p) and finally into coproporphyrinogen III (catalyzed by Hem12p). The coproporphyrin III is transported back to the mitochondria and converted to protoporphyrinogen IX by Hem13p following synthesis of protoporphyrin IX (by Hem14p). The last step is the incorporation of an iron atom into protoporphyrin IX to form the heme molecule catalyzed by Hem15p.

In this study, we evaluated the effect of metabolic engineering the heme biosynthetic pathway on heterologous hemoglobin production (Figure 1): we overexpressed, individually or in different combinations (with promoters of different strengths), three genes coding for enzymes that represent confirmed (*HEM2* and *HEM3*) or suspicious (*HEM12*) rate-limiting steps in the heme biosynthetic pathway (Hoffman et al., 2003), and additionally co-expressed α and β globin genes in different ratios. Using this approach we could evaluate a large number of combinations with engineering the heme biosynthesis and globin subunit co-expression and hereby identify the optimal combination for high level human hemoglobin production.

2. Materials and Methods

2.1. Plasmids and strains

S. cerevisiae CEN.PK 113-11C (*MATa his3Δ1 ura 3-52 MAL2-8c SUC2*)(Entian and Kötter, 1998) was used as the host strain for all the experiments performed in this study. Plasmid constructions were performed following standard molecular biology techniques using *Escherichia coli* DH5α (Bethesda Research Laboratories). All yeast transformations were performed following standard lithium acetate method (Daniel Gietz and Woods, 2002) and selected on synthetic dextrose media SD-His (heme overexpression strains), SD-Ura (globin overexpression strains), and SD-His-Ura (heme and globin double overexpression strains) respectively. For cultivation, yeast strains were grown in liquid SD-His, SD-Ura and SD-His-Ura accordingly.

All the heme overexpression plasmids were constructed using a bidirectional expression plasmid pIYC04 (Chen et al., 2013). The heme genes were amplified from the chromosomal DNA of CEN.PK 113-11C (Table S1). Plasmids H2, H3 and H12 were constructed by inserting the corresponding genes into the expression cassette of pIYC04 under the *TEF1* promoter. Plasmids H2H3 and H2H12 were constructed by inserting *HEM3* and *HEM12* respectively into H2 under the *PGK1* promoter. H3H2H12 was built by inserting a *HEM3* expression cassette amplified from H3 into H2H12. The globin overexpression plasmids were constructed from a different bidirectional expression plasmid pSP-GM1 (Chen et al., 2012). The DNA sequences (with restriction sites at both ends), namely *HBA* (coding α), *HBB* (coding β) and *HBAA* (coding di-α) were synthesized by GeneScript, Inc., and codon optimized to facilitate their expression in *S. cerevisiae*. The *HBAA* sequence coding di-α was made by linking two *HBA* gene sequences (first one without stop codon) with one glycine linker. The *HBB* fragment was digested with *Sal*I and *Kpn*I and ligated under the *PGK1* promoter of pSP-GM1 first. Then the HBA and HBAA fragments were digested by *Not*I and *Sac*I and ligated under the *TEF1* promoter of pSP-GM1 to make B/A and B/AA respectively. Plasmid B/A/A was made by inserting the *HBA* expression cassette amplified from B/A into plasmid B/A between *Pst*I and *Mfe*I. Plasmid B/AA/B was constructed by inserting the HBB expression cassette amplified from B/A into the *Mfe*I

site of plasmid B/AA. All the primers and the synthesized globin gene sequences are listed in the supplementary file (Table S1, S2).

CEN.PK 113-11C was transformed with the heme and globin overexpression plasmids, resulting in different recombinant host strains (Figure 1B, Table 1).

2.2. Coproporphyrin assay

Coproporphyrin levels were measured by HPLC using the HPLC Porphyrin Kit from ALPCO Diagnostics Inc. Strains transformed with the heme overexpression plasmids were cultured in SD-His (containing 6.7 g/L of yeast nitrogen base w/o amino acids (Difco Laboratories, Sparks, MD), 0.77 g/L of complete supplement mixture (CSM, w/o histone) (MP Biomedicals, Solon, OH) and 2% glucose) medium at 30^{o}C with constant shaking at 180 rpm during 96 hours before being harvested. Cell pellets collected from 35ml of culture were resuspended into 1mL of 0.6% HCl after being washed twice with ddH_2O. The suspended cell pellets were kept in dark at 4 ^{o}C before being loaded onto HPLC (PU-2089 Plus pump, AS-2057 Plus auto sampler, and FP-920 fluorescence detector, JASCO). The eluents, standard, and reversed phased C_{18}-column were included in the kit and the running conditions were referred to the kit.

2.3. Heme/porphyrin determination assay

The intracellular heme concentration was measured using a protocol derived from (Sassa, 1976) and slightly modified from (Michener et al., 2012). Cells were grown to a desired density and harvested at OD_{600}*mL =8 (e.g. if OD_{600}=2, 4mL of culture is harvested). The cell pellets were resuspended in 500µL of 20mM oxalic acid and then kept in dark at 4 ^{o}C overnight. 500µL of preheated 2M oxalic acid was added and half of the mixture was taken into an amber tube and heated up to 95-98 ^{o}C for 30 minutes during which the other half mixture was kept at room temperature. 200µL of clear supernatant was transferred into a black 96 plate and fluorescence with excitation at λ= 400nm and emission at λ= 600nm was monitored. The total porphyrin level (bound heme and free porphyrins) corresponds to the heated sample, and the free porphyrins level corresponds to the non-heated sample.

2.4. SDS-PAGE assay

For protein extraction, yeast cell samples were taken at the indicated times and cells were harvested by centrifugation. Pellets were resuspended in 2ml of NaOH containing 7% 2-mercaptoethanol plus 2ml of trichloroacetic acid (TCA) and incubated 2 min at room temperature. After incubation, cells were washed with 1M Tris-HCl (pH 7.5) and centrifuged. The cell pellet was resuspended in loading buffer (SDS 3%, Glycerol 10%, 2-mercaptoethanol 5%, Tris pH 6.8 0.0625 M, 0.01 mg bromophenol blue) and heated up to 95°C during 5 min. Cells were discarded by centrifugation and the remaining protein suspension was used for loading into gradient (4-12%) polyacrylamide bis-tris gels (Novex, Invitrogen) at the selected protein concentrations. The marker ladder used was Novex Sharp Pre-stained Protein Standard from Invitrogen. Software GelPro Analyzer (Media Cybernetics) was used for quantification of band intensities.

2.5. Assay for active hemoglobin

Washed cell pellets were resuspended into 500µl of 100mM of PBS buffer supplemented with 1x protease inhibitor (Halt Protease Inhibitor Cocktail, Thermo), pH 7.0. The cell suspension was loaded into a FastPrep™ Lysing tube with an equal volume of glass beads (425-600µm diameter, Sigma). Cells were broken by applying 8 pulses of 20 seconds with the speed of 6m/s using a FastPrep 24 instrument (MP, Biomedicals, LLC). The sample tubes were placed on ice for 3 minute between pulses. Clear supernatants were harvested by centrifugation at full speed for active hemoglobin assay and protein quantification. Lipid removal agent Cleanascite (X2555-100, BIOTECH SUPPORT GROUP) was added according to product description in case of unclear suspensions in the supernatant. Protein concentration was measured by BCA protein assay kit (Cat 23225, Pierce). The active hemoglobin was measured using the QuantiChromTM Hemoglobin Assay Kit (DIHEMOGLOBIN-250, Clonagen), based on an improved Trition/NaOH method, in which the hemoglobin is converted into a uniform colored end product that can be measured by spectrophotometer.

2.6. Relative gene transcription levels by qPCR

Yeast cells were cultured at 30°C with shaking at 180 rpm in shake flask containing 50 ml of SD-His. Total RNA was isolated from 10 ml of cells with OD_{600} around 1. The cells were disrupted by Fast-Prep 24 (MP-Biomedicals) using glass beads (Lysing matrix C, MP-Biomedicals). Extracted RNA was purified using the RNeasy kit (Qiagen). 200 ng of total RNA was used to synthesize cDNA from which 2 µl of the synthesized cDNA was used as the template for the qPCR reaction to a final reaction volume of 20 µl, using the Brilliant III Ultra Fast SYBRGreen QPCR Master mix (Stratagene) in Mx3005P QPCR System (Agilent Technologies). Cycle thresholds (Ct) were normalized to the Ct value of *S. cerevisiae ACT1*.

2.7. Batch fermentations

Fresh colonies were picked to inoculate into 50 mL of SD- His-Ura media (containing 6.7 g/L of yeast nitrogen base w/o amino acids (Difco Laboratories, Sparks, MD), 0.75 g/L of complete supplement mixture (CSM, w/o histone and uracil) (MP Biomedicals, Solon, OH) and 2% glucose) to make seed cultures. After growing for 24 hours at 30°C at 180 rpm, the seed cultures were inoculated into the bioreactors with an initial OD_{600} of 0.1. Fermentations were performed in DasGip 1.0-liter stirrer-pro vessels (DASGIP GmbH, Germany) with a working volume of 600 mL in SD -His-Ura media. The temperature was controlled at 30°C with agitation at 600 rpm and aeration at 1 vvm. Dissolved oxygen was controlled above 30% throughout the experiment. The impeller speed was automatically adjusted in response to the sensor. The pH was maintained at 6.0 using 2M KOH in response to the pH sensor (Mettler Toledo, Switzerland). All fermentations were performed in biological duplicates. Dry cell weight was measured by filtering 5mL of cell culture through a 0.45µm filter membrane and calculating the increased weight of the dried filter. Supernatants filtered through 0.45µm membrane were loaded to HPX-87G column (BIORAD, USA) on a Dionex Ultimate 3000 HPLC (Dionex Softron GmbH, Germany) to measure the concentrations of glucose, ethanol, and glycerol in the cultures. The samples were running with a flow rate of 0.6mL/min at 65°C using 5mM H_2SO_4 as mobile phase.

Results and Discussion

To investigate the impact of increased endogenous heme synthesis on heterologous hemoglobin production in *S. cerevisiae*, a series of recombinant strains were constructed by transforming different expression plasmids in the yeast strain CEN.PK 113-11C (Fig 1, Table 1).

3.1. Increased endogenous heme level by overexpression of heme genes

By transforming the heme overexpression plasmids H2, H3, H12, H2H3, H2H12, and H3H2H12 into CEN.PK 113-11C, strains with the engineered heme biosynthesis pathway were constructed (Table 1). The strains were designated with the same names as the corresponding plasmids H2, H3, H12, H2H3, H2H12, and H3H2H12 (Table 1). The cytosolic intermediate of the heme biosynthetic pathway, coproporphyrin was measured in all the engineered strains and used as an indicator for heme and porphyrin levels in the cells (Fig 2A). All strains were compared with the strain transformed with pIYC04 (empty plasmid) and the fold values were calculated relative to it. Strain H3 showed a four times increase on coproporphyrin level compared to the control strain, pIYC04, followed by H12 and H3H2H12 whose coproporphyrin levels were 3.2 and 2.2 times to pIYC04 respectively (Fig 2A). In terms of bound heme level, H3 still stood out with almost 5 fold of pIYC04, followed by H3H2H12, 2.8 fold, and H12, 2.4 fold (Fig 2B).

HEM13 transcription is known to be negatively regulated by oxygen and heme (Zagorec and Labbe-Bois, 1986) through *HAP1* and *ROX1* (Keng, 1992). Overexpression of the genes in the heme biosynthesis pathway (strains H2, H3, H12, H2H3, H2H12 and H3H2H12) resulted in certain improvements on heme levels (Fig 2B) which might induce feedback inhibition on the synthesis of Hem13p which converts coproporphyrinogen III into protoporphyrinogen IX, causing subsequent accumulation of coproporphyrins (Michener et al., 2012). The coproporphyrin levels (Fig 2A) may therefore represent reliable indicators of the heme levels in the strains, and Hem13p might be another potential bottleneck in addition to the reported rate-limiting steps catalyzed by Hem2p and Hem3p.

Both driven by *TEF1* promoter, *HEM3* overexpression (H3) showed a better effect than *HEM12* overexpression (H12) on coproporphyrin accumulation suggesting a greater rate-limiting effect of Hem3p than Hem12p during heme biosynthesis. Overexpression of *HEM3* released the bottleneck in this step providing more intermediate for further (catalyzed by Hem4p and Hem12p) conversion to coproporphyrin. On the other hand, also driven by the *TEF1* promoter, *HEM2* overexpression (H2) exhibited a very limited improvement on the coproporphyrin level in the cells although the Hem2p has been confirmed as a rate-controlling enzyme under aerobic conditions (Hoffman et al., 2003; Zhang and Hach, 1999). This is very likely due to the enzymes blocking the subsequent steps, especially Hem3p and Hem12p, were not overexpressed in H2. The point could be supported by the higher coproporphyrin levels presented in H2H3 and H2H12 than in H2. Since *HEM3* in H2H3 and *HEM12* in H2H12 are both under the *PGK1* promoter which has a reported weaker strength than *TEF1* promoter (Valérie Nacken, 1996) driving *HEM2* in these strains, the steps catalyzed by Hem3p and Hem12p may still being blocked to certain extends which could partly explain the not so obvious improvements on the coproporphyrin levels in these strains. Additional reason could be that with more gene cassettes compulsory for expression, the resource for expressing one gene has to be distributed to more genes and therefore each gene gets lesser/weaker expression. The expressions of *HEM2*, *HEM3* and *HEM12* in H2H3 and H2H12 might have been reduced to certain levels resulting in the minor improvement of coproporphyrin accumulation than H2. Similarly, the expression burden was even heavier for the cells in the H3H2H12 strain and consequently the proteins especially Hem3p which has been indicated to be the most effective enzyme compared to Hem2p and Hem12p to block heme synthesis, were less expressed, which could be the reason that H3H2H12 did not show higher or even comparable level of coproporphyrin than H3 and H12.

The transcriptional levels of *HEM3* and *HEM12* were examined and as shown in Fig 3, the mRNA levels of the heme genes *HEM3* and *HEM12* were much higher in the strains transformed with the corresponding vectors than with the empty vector. The transcriptional level of *HEM3* in strain H3 is extremely high (131.6 folds) corelating to the highest coproporphyrin accumulation in the H3 strain.

The acidic-heat method can discriminate between heme bound to proteins and the free porphyrins in cells. As shown in Fig 2B, the heme level in H3 was the most abundant followed by H3H2H12 and H12, showing the same trend as that for coproporphyrin. The higher heme level in H3 than in H12 might indicate a bigger flux of coproporphyrins converted to protoporphyrin IX than in H12, even though the coproporphyrins accumulated in H3 were also more than in H12. The free porphyrins include more than coproporphyrins which might explain the higher free porphyrin levels in H12 compared to H3. Both assays indicate that *HEM3* overexpression alone generates the highest heme levels in cells compared to the other strains.

3.2. Co-overexpression of heme and globin constructs increased heterologous hemoglobin production

The top three heme overexpression plasmids H3, H3H2H12 and H12 were selected to be co-transformed together with a range of globin overexpression plasmids B/A, B/AA, B/AA/B, B/A/A and the empty vector pSP-GM1 (Fig 1B). The combination of α and β globin genes on the globin vectors were designed based on i) α globin is more unstable than β globin (Vasseur-Godbillon et al., 2006) and ii) αβ dissociation can be prevented by linking two α globin genes with a glycine linker (Looker et al., 1992). The expression levels were examined by SDS-PAGE (Fig 4). By co-overexpression with H3, the globin construct B/A/A showed the highest protein expression, however, there was nearly no band of the right size (around 16 KDa) when H3 was expressed in combination with other globin constructs (Fig 4A). Very weak signal of α and β globins were detected by ELISA (data not shown) and based on this result, co-overexpressions of B/A/A with the other two heme overexpression plasmids H12 and H3H2H12 were also checked by SDS-PAGE: with heme overexpressed, the globin synthesis was also improved (Fig 4B). Expression of B/A/A with the empty plasmid pIYC04 did not show clear expression, thus further supporting the hypothesis that endogenous heme levels are insufficient to achieve high heterologous globin production (Smith et al., 2011).

3.3. Effect of B/A/A co-overexpression on heme/porphyrin level

In order to understand if an improved hemoglobin expression is correlated with an increased heme level, the heme/porphyrin level in the strains co-overexpressed with B/A/A was examined.

The relative fold change was calculated relative to the heme level in pIYC04+pSP-GM1. As shown in Fig 5, both the bound heme level and the free porphyrins level in H3+B/A/A are higher than in H3+pSP-GM1, and in the H3H2H12+B/A/A strain compared with H3H2H12+pSP-GM1. With B/A/A co-overexpressed, we found an increase of 87% with H3 co-overexpression. In erythroid cells, heme export from the mitochondria requires a corresponding synthesis of heme-binding proteins (Lynch et al., 2009). One hypothesis could be that with higher levels of globin expressed, an initial decrease in the pool of free cytosolic heme occurs which induces an increase in the production rate of porphyrins resulting in a global increase of both free porphyrins and consequently the hemoproteins. The bound heme levels that we measured would be thus higher.

3.4. Physiological characterization and active hemoglobin production in batch fermentations

The physiological properties of the recombinant hemoglobin strains were characterized in batch fermentations, as shown in Table 2. The specific growth rates did not vary significantly which were 0.25, 0.28, 0.28 and 0.27 respectively for H3+B/A/A, H3H2H12+B/A/A, pIYC04+B/A/A and pIYC04+pSP-GM1. At the end of cultivation, the percentages of the active hemoglobin in the whole extracted soluble proteins were assayed and calculated. The result showed that the strain H3+B/A/A had the most active hemoglobin accounting for 4.09% of the whole cell soluble protein followed by H3H2H12+B/A/A with 3.82%. There was activity accounted for 2.8% of the whole cell protein in the pIYC04+pSP-GM1 strain, most probably contributed by the endogenous flavohemoglobin (Zhao et al., 1996).

The specific growth rate of strain H3+B/A/A was slightly lower when compared with other strains. It also had the most soluble hemoglobin production and less biomass yield. Declines on specific growth rate due to plasmid-encoded recombinant gene expression have been extensively noticed in bacteria, yeast and filamentous fungi (Hoffmann and Rinas, 2004; Pakula et al., 2005; Tyo et al., 2012) all

pointing to a higher stress and impairment to cellular process. In the strain H3+B/A/A, a coordinated over production of heme and globin units have been achieved which further assembled to the correct tetramer. This process might result in a flux switching from normal cellular processes to recombinant hemoglobin production, and consequently affect cell performance. The heme and globins production is less effective in the other two recombinant strains thus the normal growth have not been impaired significantly.

The cell growth reached the end of the glucose phase and the beginning of the ethanol phase at around 20 and 36 hours after inoculation respectively (Fig 6A). Interestingly, we found that the heme level in H3+B/A/A significantly decreased before the ethanol phase started (Fig 6B) indicating a negative correlation between heme/porphyrin synthesis and respiration. In contrast, in H3H2H12+B/A/A and pIYC04+pSP-GM1, the heme level kept improving after the glucose phase was finished with a decrease in the free porphyrin level suggesting that porphryin synthesized in the glucose phase were gradually converted into heme. The correlation between the heme levels and the hemoglobin production was also measured by SDS-PAGE (Fig 6C). Clearly, the level of hemoglobin produced in the glucose phase is higher than in the beginning of the ethanol phase (about 1.3 fold) for H3+B/A/A, confirming that efficient heterologous hemoglobin production requires a low rate of respiration, as it occurs during the glucose consumption phase in the fermentation process.

Conclusion

Previous studies have reported successful expression of recombinant human hemoglobin in the yeast *S. cerevisiae* (Adachi et al., 1992; Coghlan et al., 1992; Martin de Llano et al., 1993; Ogden et al., 1994; Wagenbach et al., 1991). In this study, yeast heme synthesis pathway was engineered for the first time to facilitate the synthesis of recombinant hemoglobin, resulting in an increase of 1.3 times (H3+B/A/A against pIYC04+B/A/A) in hemoglobin production. This work has generated a plasmid platform for further improving heterologous hemoglobin production in yeast. Further improvement might be achieved by engineering the heme membrane transport and/or the ion trans-membrane pathway (e.g. ferric reductase and permease-oxidase complex required for iron uptake in *S. cerevisiae*) (Dancis et al., 1990; Ginzburg et al., 2009; Graves et al., 2008; Stearman et al., 1996; Villarreal et al., 2008). In addition to hemoglobin, a similar strategy can be applied to enhance the production of other hemoproteins, e.g. cytochromes, catalases and P450 enzymes which are powerful catalysts for synthesis of drug metabolites (Jung et al., 2011). This work is further demonstrating the possibility for efficient production of complex proteins (e.g. multimer with a prosthetic group), as we have demonstrated that the production of a prosthetic group can be increased through metabolic engineering and hence eliminate the need for exogenous supplementation with expensive precursors.

Acknowledgement

The authors would like to thank J. K. Michener for assistance with heme measurement experiment. We thank the financial support from the European Research Council ERC project INSYSBIO (Grant no. 247013), the Novo Nordisk Foundation, the Chalmers Foundation, and the Knut and Alice Wallenberg Foundation

Reference

Adachi, K., Konitzer, P., Lai, C. H., Kim, J., Surrey, S., 1992. Oxygen Binding and Other Physical Properties of Human Hemoglobin Made in Yeast. Protein Eng. 5**,** 807-810.

Behringer, R. R., Ryan, T. M., Reilly, M. P., Asakura, T., Palmiter, R. D., Brinster, R. L., Townes, T. M., 1989. Synthesis of functional human hemoglobin in transgenic mice. Science. 245**,** 971.

Belcher, J. D., Beckman, J. D., Balla, G., Balla, J., Vercellotti, G., 2010. Heme degradation and vascular injury. Antioxid Redox Signal. 12**,** 233-48.

Benesch, R., Benesch, R. E., 1963. Some Relations between Structure and Function in Hemoglobin. J Mol Biol. 6**,** 498-505.

Biro, G. P., 1993. Perfluorocarbon-based red blood cell substitutes. Transfus Med Rev. 7**,** 84-95.

Chemler, J. A., Yan, Y., Koffas, M. A., 2006. Biosynthesis of isoprenoids, polyunsaturated fatty acids and flavonoids in *Saccharomyces cerevisiae*. Microb Cell Fact. 5**,** 20.

Chen, Y., Daviet, L., Schalk, M., Siewers, V., Nielsen, J., 2013. Establishing a platform cell factory through engineering of yeast acetyl-CoA metabolism. Metabolic Engineering. 15**,** 48-54.

Chen, Y., Partow, S., Scalcinati, G., Siewers, V., Nielsen, J., 2012. Enhancing the copy number of episomal plasmids in *Saccharomyces cerevisiae* for improved protein production. FEMS Yeast Res. 12**,** 598-607.

Coghlan, D., Jones, G., Denton, K. A., Wilson, M. T., Chan, B., Harris, R., Woodrow, J. R., Ogden, J. E., 1992. Structural and functional characterisation of recombinant human haemoglobin A expressed in *Saccharomyces cerevisiae*. Eur J Biochem. 207**,** 931-6.

Dancis, A., Klausner, R., Hinnebusch, A., Barriocanal, J., 1990. Genetic evidence that ferric reductase is required for iron uptake in *Saccharomyces cerevisiae*. Molecular and cellular biology. 10**,** 2294-2301.

Daniel Gietz, R., Woods, R. A., 2002. Transformation of yeast by lithium acetate/single-stranded carrier DNA/polyethylene glycol method. Meth. Enzymol. 350**,** 87-96.

Dieryck, W., Pagnier, J., Poyart, C., Marden, M. C., Gruber, V., Bournat, P., Baudino, S., Mérot, B., 1997. Human haemoglobin from transgenic tobacco. Nature. 386**,** 29.

Entian, K. D., Kötter, P., 1998. 23 Yeast Mutant and Plasmid Collections. Method Microbiol. 26**,** 431-449.

Feng, L., Gell, D. A., Zhou, S., Gu, L., Kong, Y., Li, J., Hu, M., Yan, N., Lee, C., Rich, A. M., 2004. Molecular mechanism of AHSP-mediated stabilization of α-hemoglobin. Cell. 119**,** 629-640.

Feng, L., Zhou, S., Gu, L., Gell, D. A., Mackay, J. P., Weiss, M. J., Gow, A. J., Shi, Y., 2005. Structure of oxidized α-haemoglobin bound to AHSP reveals a protective mechanism for haem. Nature. 435**,** 697-701.

Fibach, E., Kollia, P., Schechter, A., Noguchi, C., Rodgers, G., 1995. Hemin-induced acceleration of hemoglobin production in immature cultured erythroid cells: preferential enhancement of fetal hemoglobin. Blood. 85**,** 2967.

Ginzburg, Y. Z., Rybicki, A. C., Suzuka, S. M., Hall, C. B., Breuer, W., Cabantchik, Z. I., Bouhassira, E. E., Fabry, M. E., Nagel, R. L., 2009. Exogenous iron increases hemoglobin in beta-thalassemic mice. Exp Hematol. 37**,** 172-83.

Graves, P. E., Henderson, D. P., Horstman, M. J., Solomon, B. J., Olson, J. S., 2008. Enhancing stability and expression of recombinant human hemoglobin in *E. coli*: Progress in the development of a recombinant HBOC source. Biochim Biophys Acta. 1784**,** 1471-9.

Harris, D. R., Palmer, A. F., 2008. Modern cross-linking strategies for synthesizing acellular hemoglobin-based oxygen carriers. Biotechnol Prog. 24**,** 1215-25.

Hernan, R., Hui, H., Andracki, M., Noble, R., Sligar, S., Walder, J., Walder, R., 1992. Human hemoglobin expression in *Escherichia coli*: importance of optimal codon usage. Biochemistry. 31**,** 8619-8628.

Hoffman, M., Gora, M., Rytka, J., 2003. Identification of rate-limiting steps in yeast heme biosynthesis. Biochem Biophys Res Commun. 310**,** 1247-53.

Hoffman, S. J., Looker, D. L., Roehrich, J. M., Cozart, P. E., Durfee, S. L., Tedesco, J. L., Stetler, G. L., 1990. Expression of fully functional tetrameric human hemoglobin in *Escherichia coli*. Proc Natl Acad Sci U S A. 87**,** 8521-5.

Hoffmann, F., Rinas, U., 2004. Stress induced by recombinant protein production in *Escherichia coli*. Physiological Stress Responses in Bioprocesses. 73-92.

Hou, J., Tyo, K., Liu, Z., Petranovic, D., Nielsen, J., 2012. Engineering of vesicle trafficking improves heterologous protein secretion in *Saccharomyces cerevisiae*. Metab Eng. 14**,** 120-7.

Jung, S. T., Lauchli, R., Arnold, F. H., 2011. Cytochrome P450: taming a wild type enzyme. Curr Opin Biotechnol. 22**,** 809-17.

Keng, T., 1992. *HAP1* and *ROX1* form a regulatory pathway in the repression of HEM13 transcription in *Saccharomyces cerevisiae*. Molecular and cellular biology. 12**,** 2616.

Komar, A. A., Kommer, A., Krasheninnikov, I. A., Spirin, A. S., 1997. Cotranslational folding of globin. J Biol Chem. 272**,** 10646-51.

Kroeger, K. S., Kundrot, C. E., 1997. Structures of a hemoglobin-based blood substitute: Insights into the function of allosteric proteins. Structure. 5**,** 227-237.

Kumar, R., 1995. Recombinant Hemoglobins as Blood Substitutes - a Biotechnology Perspective. P Soc Exp Biol Med. 208**,** 150-158.

Lee, S. K., Chou, H., Ham, T. S., Lee, T. S., Keasling, J. D., 2008. Metabolic engineering of microorganisms for biofuels production: from bugs to synthetic biology to fuels. Curr Opin Biotechnol. 19**,** 556-63.

Looker, D., Abbott-Brown, D., Cozart, P., Durfee, S., Hoffman, S., Mathews, A. J., Miller-Roehrkh, J., Shoemaker, S., Trimble, S., Fermi, G., 1992. A human recombinant haemoglobin designed for use as a blood substitute. Nature. 356**,** 258-260.

Lu, X., Ma, G., Su, Z., 2011. Hemoglobin Based Blood Substitutes-Preparation Technologies and Challenges. In: Editor-in-Chief: Murray, M.-Y., (Ed.), Comprehensive Biotechnology (Second Edition). Academic Press, Burlington, pp. 713-727.

Lynch, J., Fukuda, Y., Krishnamurthy, P., Du, G. Q., Schuetz, J. D., 2009. Cell Survival under Stress Is Enhanced by a Mitochondrial ATP-Binding Cassette Transporter That Regulates Hemoproteins. Cancer Res. 69**,** 5560-5567.

Martin de Llano, J., Schneewind, O., Stetler, G., Manning, J., 1993. Recombinant human sickle hemoglobin expressed in yeast. Proc Nebr Acad Sci. 90**,** 918.

Martinez, J. L., Liu, L., Petranovic, D., Nielsen, J., 2012. Pharmaceutical protein production by yeast: towards production of human blood proteins by microbial fermentation. Curr Opin Biotechnol.

Michener, J. K., Nielsen, J., Smolke, C. D., 2012. Identification and treatment of heme depletion attributed to overexpression of a lineage of evolved P450 monooxygenases. Proceedings of the National Academy of Sciences. 109**,** 19504-19509.

Nevoigt, E., 2008. Progress in metabolic engineering of *Saccharomyces cerevisiae*. Microbiol Mol Biol Rev. 72**,** 379-412.

Ogden, J. E., Harris, R., Wilson, M. T., 1994. Production of recombinant human hemoglobin A in *Saccharomyces cerevisiae*. Methods Enzymol. 231**,** 374-90.

Pakula, T. M., Salonen, K., Uusitalo, J., Penttilä, M., 2005. The effect of specific growth rate on protein synthesis and secretion in the filamentous fungus *Trichoderma reesei*. Microbiology. 151**,** 135-143.

Petranovic, D., Nielsen, J., 2008. Can yeast systems biology contribute to the understanding of human disease? Trends Biotechnol. 26**,** 584-590.

Porro, D., Sauer, M., Branduardi, P., Mattanovich, D., 2005. Recombinant protein production in yeasts. Mol Biotechnol. 31**,** 245-59.

Sassa, S., 1976. Sequential induction of heme pathway enzymes during erythroid differentiation of mouse Friend leukemia virus-infected cells. J Exp Med. 143**,** 305-15.

Shen, T. J., Ho, N. T., Simplaceanu, V., Zou, M., Green, B. N., Tam, M. F., Ho, C., 1993. Production of unmodified human adult hemoglobin in *Escherichia coli*. Proc Natl Acad Sci U S A. 90**,** 8108-12.

Smith, B. J., Gutierrez, P., Guerrero, E., Brewer, C. J., Henderson, D. P., 2011. Development of a method to produce hemoglobin in a bioreactor culture of *Escherichia coli* BL21(DE3) transformed with a plasmid containing Plesiomonas shigelloides heme transport genes and modified human hemoglobin genes. Appl Environ Microbiol. 77**,** 6703-5.

Stearman, R., Yuan, D. S., Yamaguchi-Iwai, Y., Klausner, R. D., Dancis, A., 1996. A permease-oxidase complex involved in high-affinity iron uptake in yeast. Science. 271**,** 1552-1557.

Tyo, K. E. J., Liu, Z., Petranovic, D., Nielsen, J., 2012. Imbalance of heterologous protein folding and disulfide bond formation rates yields runaway oxidative stress. BMC biology. 10**,** 16.

Valérie Nacken, T. A. a. E. D., 1996. Probing the limits of expression levels by varying promoter strength and plasmid copy number in *Saccharomyces cerevisiae*. Gene. Volume 175**,** 253-260

Vasseur-Godbillon, C., Hamdane, D., Marden, M. C., Baudin-Creuza, V., 2006. High-yield expression in *Escherichia coli* of soluble human alpha-hemoglobin complexed with its molecular chaperone. Protein Eng Des Sel. 19**,** 91-7.

Villarreal, D. M., Phillips, C. L., Kelley, A. M., Villarreal, S., Villaloboz, A., Hernandez, P., Olson, J. S., Henderson, D. P., 2008. Enhancement of recombinant hemoglobin production in *Escherichia coli* BL21(DE3) containing the Plesiomonas shigelloides heme transport system. Appl Environ Microbiol. 74**,** 5854-6.

Wagenbach, M., O'Rourke, K., Vitez, L., Wieczorek, A., Hoffman, S., Durfee, S., Tedesco, J., Stetler, G., 1991. Synthesis of wild type and mutant human hemoglobins in *Saccharomyces cerevisiae*. Nat Biotechnol. 9**,** 57-61.

Walsh, G., 2010. Biopharmaceutical benchmarks 2010. Nat Biotechnol. 28**,** 917-924.

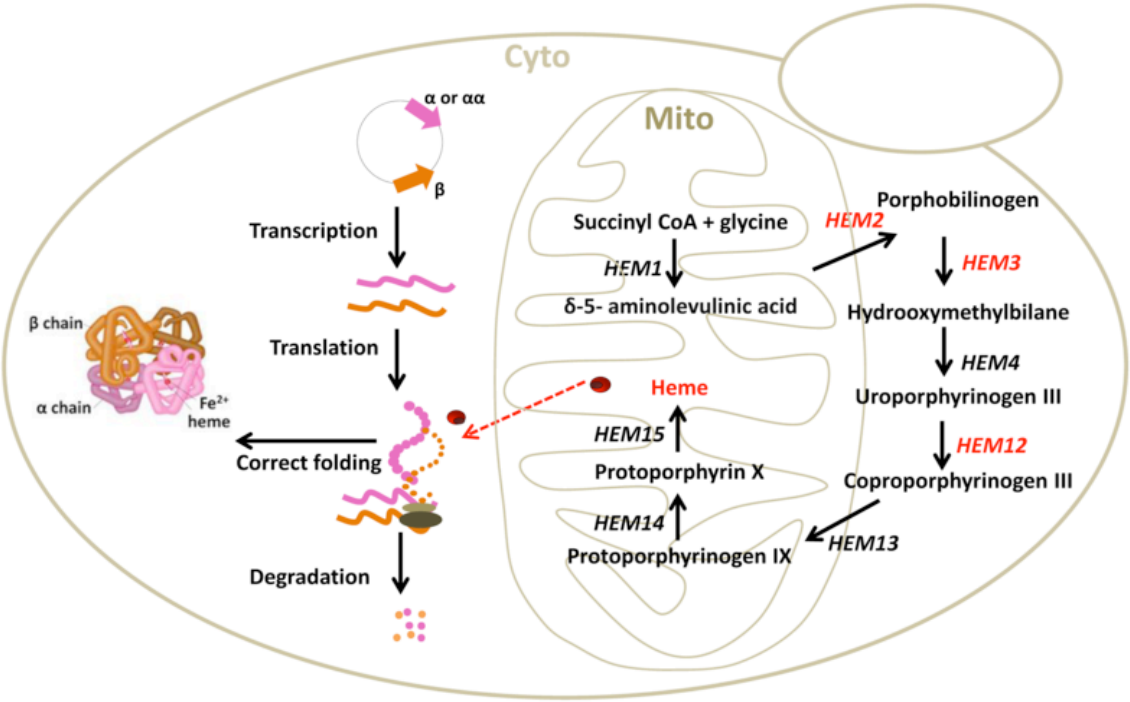
Zagorec, M., Labbe-Bois, R., 1986. Negative control of yeast coproporphyrinogen oxidase synthesis by heme and oxygen. Journal of Biological Chemistry. 261**,** 2506-2509.

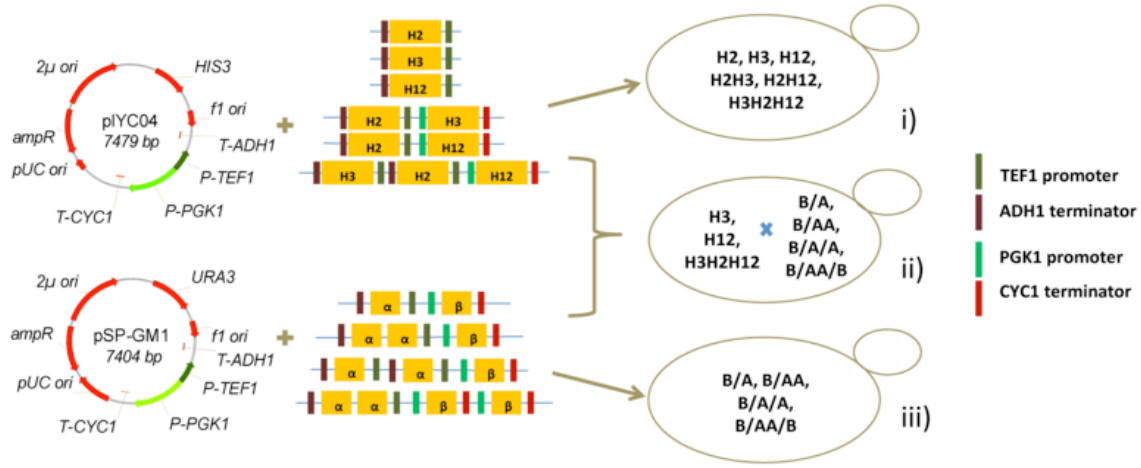
Zhang, L., Hach, A., 1999. Molecular mechanism of heme signaling in yeast: the transcriptional activator Hap1 serves as the key mediator. Cell. Mol. Life Sci. 56**,** 415-426.

Zhao, X. J., Raitt, D., P, V. B., Clewell, A. S., Kwast, K. E., Poyton, R. O., 1996. Function and expression of flavohemoglobin in *Saccharomyces cerevisiae*. Evidence for a role in the oxidative stress response. J Biol Chem. 271**,** 25131-8.

Figure legend

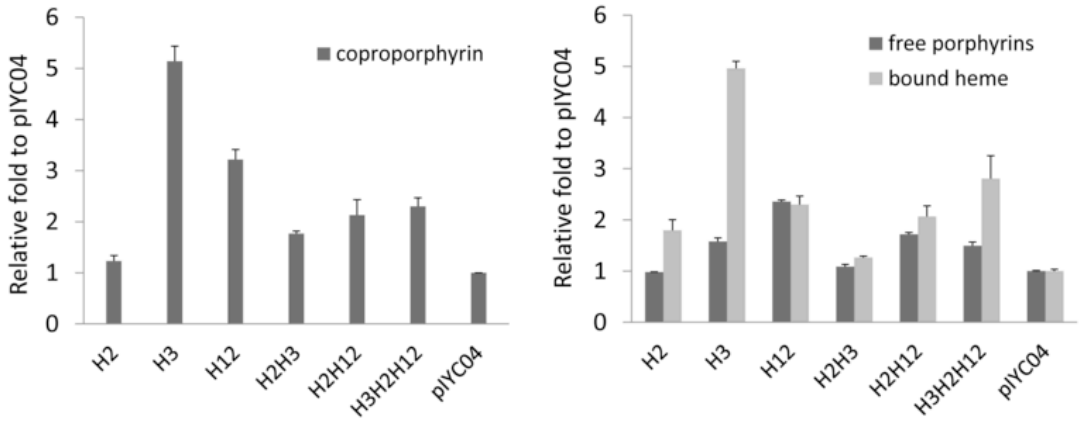
Fig. 1. Engineering strategy for optimizing the production of human hemoglobin. (A) Strategy overview: Yeast genes *HEM2*, *HEM3* and *HEM12* (highlighted in red) in heme biosynthesis pathway were overexpressed for an improved level of heme in cell cytosol. Human α and β globins were overproduced on plasmid in cytosol. Correct folding and assembling resulted in completed hemoglobin, failure in assembling resulted in degradation. (B) Strain construction: CEN.PK 113-11C as the expression host being transformed with i) heme overexpression plasmids: gene *HEM2*, *HEM3* and *HEM12* were inserted into the expression cassette *TEF1-ADH1*, either individually or in different combinations in the cassettes *TEF1-ADH1* and *PGK1-CYC1* on pIYC04. ii) globin overexpression plasmids: *HBA*, *HBB*, and *HBAA* were inserted into pSP-GM1 in the cassettes of *TEF1-ADH1* and *PGK1-CYC1* in different combinations. iii) heme and globin overexpression plasmids: The top three best heme/coproporphyrin producing plasmids H3, H12 and H3H2H12 were transformed in different combinations together with the globin overexpression plasmids B/A, B/AA, B/A/A and B/AA/B. Hemoglobin structure was modified from (Lu et al., 2011). Heme biosynthesis pathway was modified from (Hoffman et al., 2003)





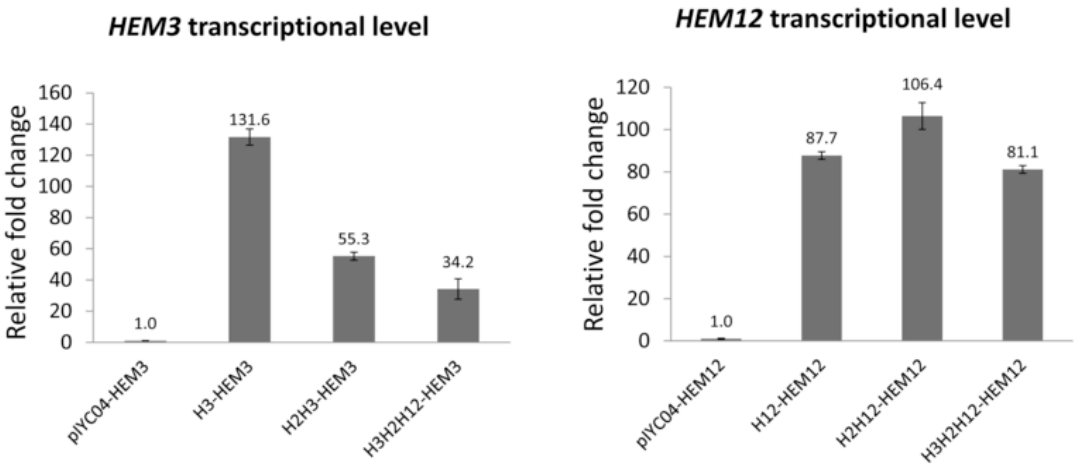
539

540 Fig. 2. Porphyrin/heme levels in heme over-expression strains. (A) Level of corproporphyrin. (B)
541 Level of free porphyrins and bound heme. The relative fold change is calculated based on the
542 fluorescence reads (excitation at λ= 400nm and emission at λ= 600nm) of the sample strains against
543 the control strain, pIYC04.



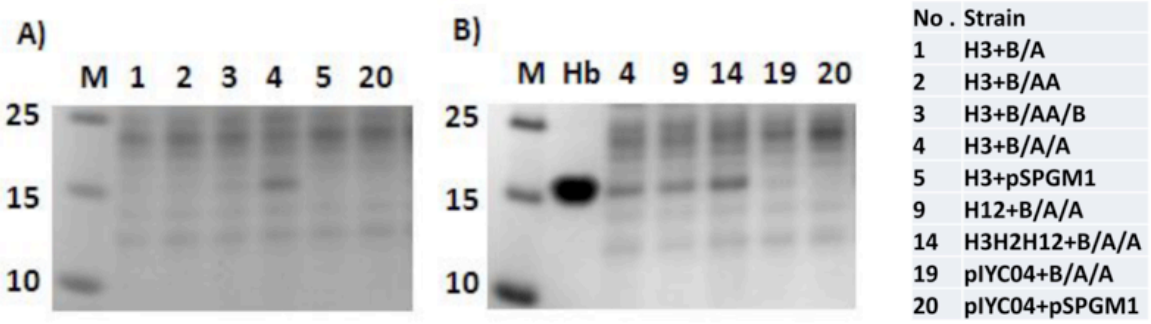
544

545 Fig. 3. Transcription level of (A) *HEM3* and (B) *HEM12* in the engineered strains transformed with
546 Heme plasmids. In both cases, the relative fold change is calculated based on the transcription levels of
547 the genes (relative to *ACT1*) in the sample strains against the control strain, pIYC04.



548

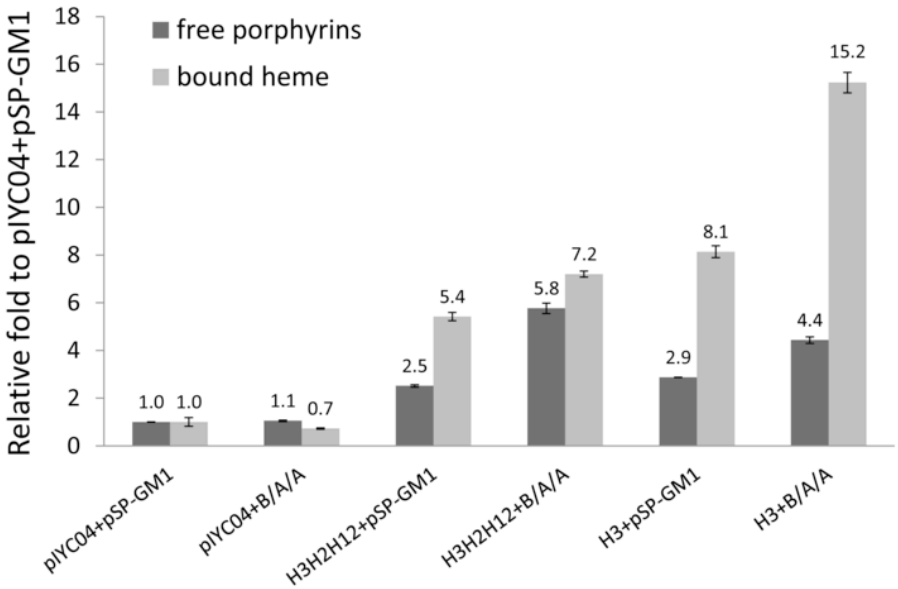
549 Fig. 4. SDS-PAGE analysis of the engineered strains with heme and globin co-overexpressed. (A) co-
550 overexpression of H3 with the globin constructs. (B) co-expression of B/A/A with the heme constructs.
551 M stands for the protein ladder, Hb stands for the standard hemoglobin (16 kDa) and the numbers
552 indicate the strains listed in the table on the side. 50µg of total protein was loaded per lane.



No .	Strain
1	H3+B/A
2	H3+B/AA
3	H3+B/AA/B
4	H3+B/A/A
5	H3+pSPGM1
9	H12+B/A/A
14	H3H2H12+B/A/A
19	pIYC04+B/A/A
20	pIYC04+pSPGM1

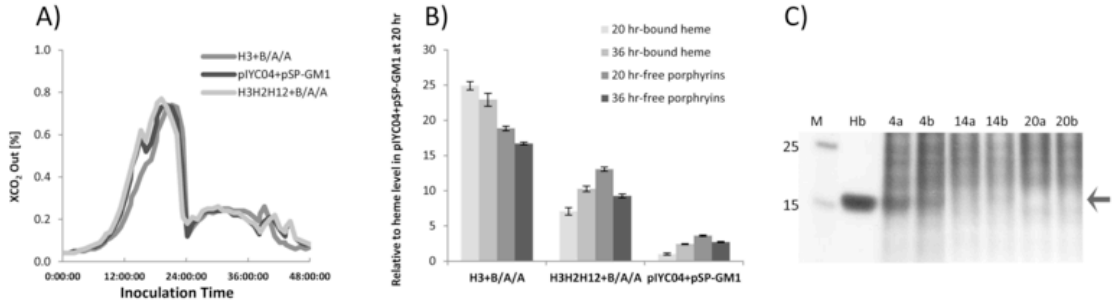
553

554 Fig. 5. Effects of B/A/A plasmid co-overexpression on heme/porphyrin level. The relative fold change
555 is calculated based on the fluorescence reads (excitation at λ= 400nm and emission at λ= 600nm) of
556 the sample strains to that of the control strain, pIYC04.



557

558 Fig. 6. Heme and hemoglobin production during batch fermentations. (A) Offgas profile: showing that
559 glucose phase finished at around 20 hrs and ethanol phase started at around 36 hrs. (B) Heme and
560 porphyrin levels in strains after inoculation for 20 and 36hrs respectively. (C) SDS-PAGE analysis of
561 the globin patterns produced at 20 and 36 hrs respectively. 4, 14, 20 represent strains H3+B/A/A,
562 H3H2H12+B/A/A and pIYC04+pSP-GM1 respectively. a and b represent 20 and 36 hrs after
563 inoculation. 25µg protein loaded.



564