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Efficient Secretory Expression for Mammalian Hemoglobins in *Pichia pastoris*

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Abstract: Mammalian hemoglobins (HB) are a kind of heme-binding proteins that play crucial physiological roles in various organisms. The traditional techniques employed for the extraction of HB are expensive and time-consuming, while the yields of mammalian HB in previous reports were quite low. The industrial *Pichia pastoris* is a highly effective platform for the secretory expression of heterologous proteins. To achieve efficient secretory expression of HB in *P. pastoris*, multiple strategies were applied, including the selection of a suitable host, the screening of optimal endogenous signal peptides, the knockout of *VPS10*, *VTH1*, and *PEP5*, and the co-expression of Alpha-Hemoglobin Stabilizing Protein (AHSP). In addition, the conditions for producing HB were optimized at shaking-flask level (BMMY medium with 100 mg/L of hemin, 2% methanol, and 24 °C). Based on these conditions, the higher titers of bovine hemoglobin (bHB, 376.9 ± 13.3 mg/L), porcine hemoglobin (pHB, 119.2 ± 7.3 mg/L), and human hemoglobin (hHB, 101.1 ± 6.7 mg/L) were achieved at fermenter level. The engineered *P. pastoris* strain and comprehensive strategies can also be applied to facilitate the synthesis of other high-value-added hemoproteins or hemoenzymes.

Keywords: *Pichia pastoris*; endogenous signal peptide; mammalian hemoglobins; AHSP; secretory expression

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1. Introduction

Mammalian hemoglobin (HB), a tetramer composed of two α-chains and two β-chains [1,2], plays a pivotal role in oxygen transport in vertebrates and many invertebrates [3–5]. Additionally, hemoglobin is essential for regulating intracellular pH, maintaining redox balance, and the metabolism of nitric oxide [6,7]. Each subunit of hemoglobin contains one heme group, which includes a ferrous iron atom coordinated by four nitrogen atoms in the core of the porphyrin ring [8]. Currently, these hemoglobins are extensively utilized as acellular oxygen transporters [9], bioavailable iron suppliers [10], and artificial metalloenzymes [11], leveraging HB's biochemical properties. Furthermore, the peptides derived from hemoglobins can be used as promising therapeutic agents [12,13]. Currently, one of the biggest challenges for food manufacturers is the creation of plant-based meat analogs with similar flavors and textures [14,15]. Hemoglobins are essential components that contribute to the taste and color of animal meat [16]. Since the plant-derived leghemoglobin

(LegH) synthesized by *P. pastoris* has been applied by Impossible Foods Inc. to develop the popular artificial meat products "Impossible Burgers" [17], the addition of mammalian hemoglobins to meat analogs can greatly improve their color and flavor [18].

Traditional methods of preparing hemoglobin, including solid-phase extraction from blood or anion-exchange chromatography from animal whole blood, are not suitable for large-scale production due to low yield and high cost [19,20]. Nowadays, the biosynthesis of hemoglobin has been developed as a feasible option by the advancements in synthetic biology [21]. Although mammalian hemoglobins have been successfully synthesized in *Escherichia coli* [22,23] and *Saccharomyces cerevisiae* [24–26], the challenges of low expressional level and complex process of purification still limited the large-scale production. Engineering the degradation pathway of heterologous proteins in *S. cerevisiae* increased the production of human hemoglobin to about 18% of the total cellular protein. However, when fused with α-Factor signal peptide, only trace amounts of human hemoglobin were detected by Western blot in the 360-fold concentrated fermentation broth [25]. *P. pastoris*, recognized for its robust capabilities of protein expression and post-translational modification [27], has emerged as an efficient system for the synthesis of hemoglobin [28,29]. The use of signal peptides in *P. pastoris* enables the direction of exogenous proteins into the secretory pathway, simplifying the purification process of products [30]. Furthermore, the U.S. Food and Drug Administration (FDA) has designated *P. pastoris* as a Generally Recognized as Safe (GRAS) strain to commercially produce LegH as a color additive (GRN No. 737) [31]. To meet the growing demand for mammalian hemoglobins in the fields of food and medicine, it is crucial to develop *P. pastoris* for high cell-density fermentation to express various types of hemoglobins [32].

The signal peptides (SP) are short peptides located in the N-terminus of nascent polypeptides, which act as a recognized component in nascent polypeptides for targeting proteins to endoplasmic reticulum or cell membrane via secretory pathways [33]. It has wide applications in various fields, including the production of recombinant proteins, immunology, and disease diagnosis [33]. The efficiency of SP significantly affects the secretory yield of target proteins [34]. Although the α-Factor SP is effective in *P. pastoris*, many endogenous SPs can be mined and used as an alternative to secrete heterologous proteins [35–38]. Recent research has demonstrated that endogenous SPs from *S. cerevisiae* can more effectively mediate the secretion of heterologous proteins compared to the α-Factor SP [39]. However, there has been little research on the prediction and evaluation of endogenous SPs from *P. pastoris* to mediate the secretion of target proteins [40].

Post-translational processing of secreted proteins is complicated by the transport of unfolded and misfolded proteins to vesicles, where they are degraded by protein hydrolases [41,42]. Vps10p and Vth1p are type I transmembrane sorting receptors and are pivotal in sorting and delivering multiple vesicular hydrolases within Golgi compartments [43]. These receptors facilitate multiple sorting cycles between the late Golgi and pro-vesicular endosomal-like compartments, ensuring accurate intracellular sorting and delivery of soluble vesicular proteins [42]. Additionally, Pep5p, the essential part of the HOPS (homotypic fusion and protein sorting) complex which is required for vacuolar SNARE (Soluble *N*-ethylmaleimide-sensitive factor attachment protein receptors) complex assembly, plays a crucial role during the trafficking of hydrolase precursors to the vacuole [44–46].

In this study, the secretory expression of mammalian hemoglobins was successfully achieved in the recombinant *P. pastoris* for the first time. Utilizing the engineered strains P1 (X33-Δ*ku70-Mit1*-Δ*yps1*) and P1H9 (X33-Δ*ku70-Mit1*-Δ*yps1*-HEME-9) previously developed [47], the more efficient chassis cells were selected to express bovine, human, and porcine hemoglobins. In addition, the best endogenous SP was explored and assessed for the secretion of mammalian hemoglobins in *P. pastoris*. Moreover, the impact of knocking out *VPS10*, *VTH1*, and *PEP5* genes on the expression of hemoglobins was examined to reduce the activities of vacuole proteases. Furthermore, the co-expression of AHSP (human alpha-hemoglobin-stabilizing protein) was performed to improve the stability and yield of hemoglobins. Finally, the highest yields of bovine, human, and porcine hemoglobins

were obtained at the fermenter level using the optimized fermentation conditions and fed-batch strategies.

2. Materials and Methods

2.1. Reagents, Strains, and Culture Conditions

The recombinant *E. coli* DH5α strain was cultured in Luria-Bertani medium, which contains 25 μg/mL zeocin (5 g/L yeast extract, 10 g/L tryptone, 10 g/L NaCl, pH 7.0), at 37 °C for the preservation or extraction of plasmids. The recombinant *P. pastoris* strains were cultivated on the solid YPD medium (10 g/L yeast extract, 20 g/L tryptone, 2% glucose, 20 g/L agar) at 30 °C to isolate single colonies. For seed culture, the engineered strains were inoculated in 50 mL of BMGY medium (1% (*v*/*v*) glycerol, 10 g/L yeast extract, 20 g/L tryptone, 13.4 g/L YNB, 4×10^{-5}% biotin, 100 mM potassium phosphate, pH 6.0) and incubated at 30 °C and 220 rpm. During the expression of heterologous hemoglobins, the engineered strains were cultured in BMMY medium (1% (*v*/*v*) methanol, 10 g/L yeast extract, 20 g/L tryptone, 13.4 g/L YNB, 4×10^{-5}% (*w*/*v*) biotin, 100 mM potassium phosphate buffer, pH 6.0).

Chemicals were bought from Sinopharm Chemical Reagent (Shanghai, China). The GeneJET Gel Extraction Kit, 10% Bis-Tris protein gel, yeast extract, and tryptone were acquired from Thermo Fisher Scientific (Shanghai, China). Hemin was procured from Aladdin (Shanghai, China). The extraction of the plasmid was performed using the SanPrep Column Plasmid Mini-Preps Kit (Sangon Biotech, Shanghai, China). Quick-cut enzymes *Not* I, *Xho* I, *Sac* I, and *Avr* II, along with PCR PrimeSTAR MAX DNA Polymerase, were obtained from TaKaRa (Dalian, China).

2.2. The Construction of Recombinant Plasmids

The plasmids and genes used in this study were summarized in Table S1. Tables S3 and S4, respectively provide the information of fragments for CRISPR/Cas9 knock-in and knock-out procedures and employed sgRNA sequences. The primer list for plasmid construction is available in Table S5, while Table S6 describes the elements of endogenous SPs. The *P. pastoris* GS115 genome from NCBI database (https://www.ncbi.nlm.nih.gov/) facilitated the creation of homologous fragments.

To establish an effective expression system, GenScript (Nanjing, China) synthesized all the codon-optimized genes for hemoglobins. Specifically, *LegH* and *bHBα* genes were amplified using primers LegH-F1, common-XhoI-F2/LegH-R, and bHBα-F1, common-XhoI-F2/bHBα-R, and the *bHBβ*, *hHBα*, *hHBβ*, *pHBα*, *pHBβ* genes were amplified by the same method. The pPICZαA plasmid was double-digested using *Not* I and *Xho* I restriction endonucleases, and the plasmid skeleton of pPICZαA was purified using a DNA gel extraction kit. The genes encoding hemoglobins with 6 × His tag and the skeleton fragment of the pPICZαA plasmid after double enzyme digestion were assembled into plasmids through Gibson assembly. For the substitution of LegH SP, primers were designed to span the entire sequence of target endogenous SPs, incorporating a segment of overlapping sequence at the 5′ end for the amplification with pPICZαA-αF-LegH as a template (Figure 1A). The assembled purified DNA fragments yielded the desired recombinant plasmid via Gibson assembly. To construct the expression plasmids for AHSP from different animal sources, using bovine AHSP as a model, the *bAHSP* gene was amplified by bAHSP-F/bAHSP-R primers, and the pGAPZαA backbone by pGAP-F/pGAP-R primers. These fragments were then purified and assembled using Gibson assembly.

To construct the knock-in cassettes for the genes encoding single-subunit of mammalian hemoglobins, using bHBα as an example, the plasmids pMD18T-Fg1 and pPICZαA-αF-bHBα were employed as templates (Figure 1B). Primers Fg1pMD18-F/Fg1pMD18-R and Fg1-HBα-F/Fg1-HBα-R were used to amplify the pMD18T-Fg1 backbone, which includes 1 kb upstream and downstream homology arms of P_{FLD1}UP-gRNA1 genome locus, and the expression cassette of P_{AOX1}-(*αF-bHBα*)-*AOX1TT*, respectively. Subsequently, the recombinant plasmid pMD18T-Fg1-αF-bHBα was constructed through Gibson assembly.

The amplification with primers Donor-Fg1-F/Donor-Fg1-R yielded the UpFg1-P$_{AOX1}$-(αF-bHBα)-*AOX1TT*-DoFg1 donor fragment, prepared for the operation of knock-in.

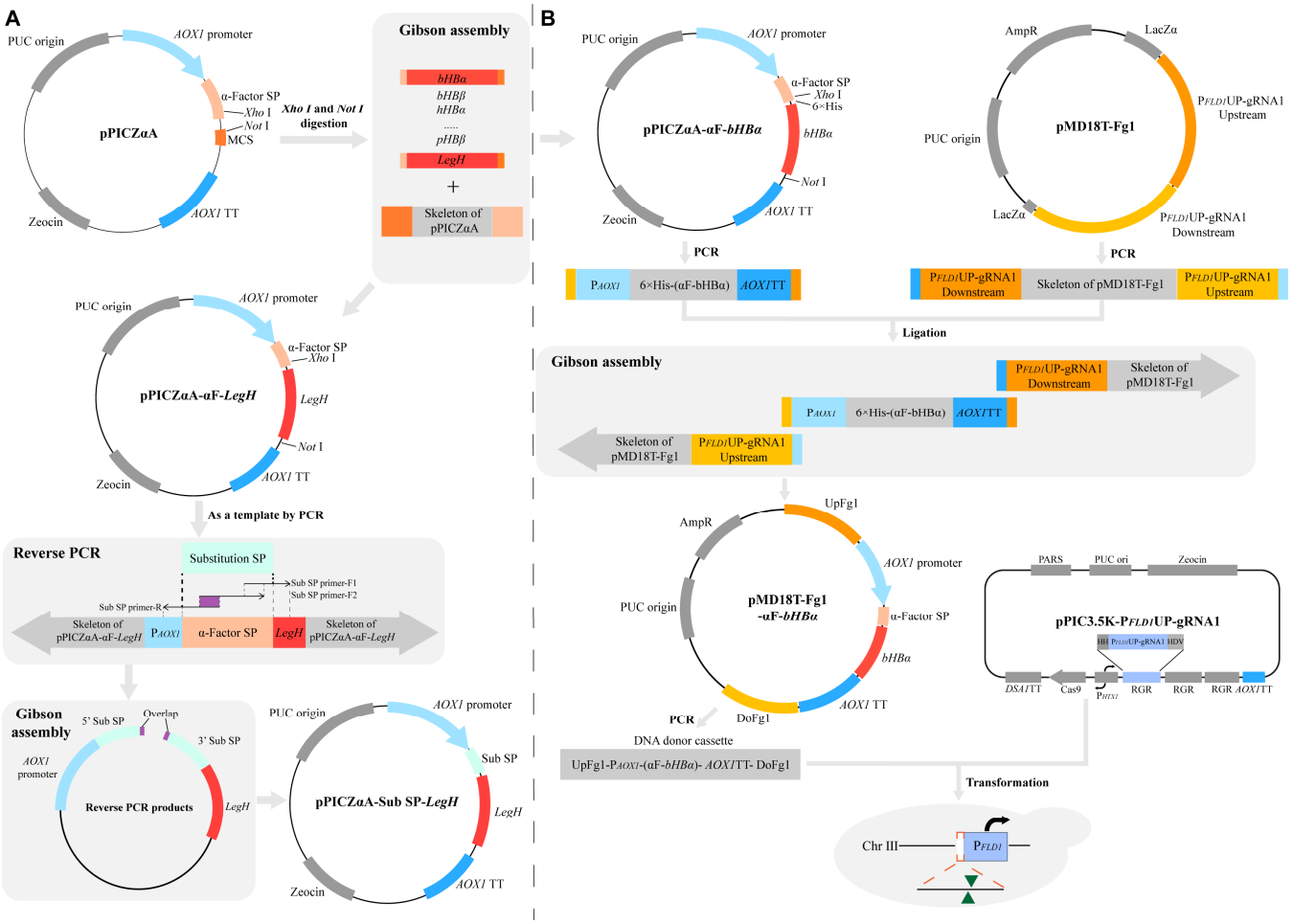

Figure 1. Schematic of constructing recombinant plasmids and engineered *P. pastoris* strains. (**A**) The method to construct recombinant plasmids for replacing α-Factor SP with endogenous SP. (**B**) Using the donor DNA cassette [UpFg1-P$_{AOX1}$-(aF-bHBa)-*AOX1*TT-DoFg1] for gene knock-in at the P$_{FLD1}$UP-gRNA1 genome locus as an example, the schematic shows the method of constructing the gene knock-in cassettes and the method of constructing engineered *P. pastoris* strains with CRISPR/Cas9-mediated genomic integration.

To construct the knock-out cassette for the *VPS10* gene, 1 kb upstream and downstream homology arms of the *VPS10* gene were amplified from X33 genomic DNA, using primers UpVPS-F/UpVPS-R and DoVPS-F/DoVPS-R. These arms were fused via overlap extension PCR to create the UpVPS10-DoVPS10 cassette. The pMD18T-*VPS10* recombinant plasmid was subsequently constructed through Gibson assembly. For amplification of the *VPS10* knockout cassette, the pMD18T-*VPS10* as templates utilized primers Up-Do-VPS-F/Up-Do-VPS-R. The sgRNA sequences for target genes were designed using the CHOPCHOP tool (http://chopchop.cbu.uib.no/) [48]. By substituting the sgRNA sequence in the pPIC3.5K-*ku70*-gRNA1 plasmid with primers VPS10g1-F1/Cas9-backbone-R1 and Cas9-backbone-F2/VPS10g1-R2, the pPIC3.5K-*VPS10*-gRNA plasmid was constructed. The similar method was used for the construction of *VTH1* and *PEP5* knockout cassettes and their corresponding CRISPR/Cas9 plasmids.

2.3. The Construction of Engineered Pichia pastoris Strains

Three methods were employed to modify *P. pastoris* and the engineered strains were described in Table S2. For the recombinant plasmids derived from pPICZαA, it was digested by *Sac* I and integrated into the *P. pastoris* 5′*AOX1* site. Similarly, the recombinant plasmids from pGAPZαA were digested by *Avr* II and integrated into the 5′*GAP* site of *P. pastoris*. In the case of CRISPR/Cas9-mediated genome integration [49], the purified knock-in or knock-out cassette as fusion DNA donors of the target gene, along with the CRISPR/Cas9 plasmid containing the sgRNA sequence, was introduced into the specific sites in *P. pastoris* using 250 to 650 ng of CRISPR/Cas9 plasmid and 4 μg of donor DNA. The electrotransformation was carried out according to the instructions of the Invitrogen manual. Transformants were incubated on YPD plates with 100 μg/mL Zeocin to perform colony PCR at 30 °C. Positive clones were cultured in YPD medium without Zeocin to loss the CRISPR/Cas9 plasmid.

2.4. Cultural Conditions for Hemoglobin Expression at Shaking-Flask Level

To express hemoglobins, a single colony of engineered *P. pastoris* strain was inoculated into 5 mL of YPD medium in a 50 mL sterile centrifuge tube and cultured to log phase (OD_{600} = 2–6) at 30 °C and 220 rpm. The primary seed cultures were inoculated at 1% inoculum into 50 mL of BMGY medium in 250 mL shaking flasks and incubated at 30 °C and 220 rpm for 24 h. In the following, the medium was centrifuged at 3000× *g* for 10 min, and the supernatant was discarded. The cell pellets were washed twice with saline to remove residual glycerol. Finally, the cells were transferred to 250 mL shaking flasks containing 50 mL of BMMY medium and cultured at 30 °C and 220 rpm. Methanol was added every 24 h to maintain a final concentration of 1%.

2.5. Cultural Conditions for Hemoglobin Expression at 5-L Fermenter Level

The engineered strain was cultured on YPD plates at 30 °C for 2 days. A single colony was selected and inoculated into 5 mL of YPD medium and incubated with shaking at 220 rpm for 24 h. Subsequently, 1% inoculum of the primary seed culture was transferred into two 250 mL shaking flasks containing 50 mL YPD medium, and incubated at 30 °C and 220 rpm for 20 h. The secondary seed culture (100 mL) was then inoculated into a 5 L bioreactor (T&J Bioengineering, Shanghai, China) with 2.5 L of BSM medium (composition for 1 L: 26.7 mL of 85% H_3PO_4, 0.93 g $CaSO_4$, 18.2 g K_2SO_4, 14.9 g $MgSO_4{\cdot}7H_2O$, 4.13 g KOH, 40 g glycerol) along with 4.35 mL/L filter-sterilized PTM1 salt solution ($CuSO_4{\cdot}5H_2O$ 6.0 g, NaI 0.08 g, $MnSO_4{\cdot}H_2O$ 3.0 g, $Na_2MoO_4{\cdot}2H_2O$ 0.2 g, H_3BO_3 0.02 g, $CoCl_2$ 0.5 g, $ZnCl_2$ 20.0 g, $FeSO_4{\cdot}7H_2O$ 65 g, Biotin 0.2 g, and H_2SO_4 5.0 mL), and 2 mL/L of 10% antifoam 304 (Thermo Fisher Scientific, Shanghai, China). For DO-stat fed-batch culture, the temperature was controlled at 30 °C and 24 °C in two distinct stages, while the pH was maintained at 5.5 using 50% NH_4OH. To keep the dissolved oxygen (DO) level above 20% of air saturation, the agitation speed was adjusted from 700 to 1000 rpm, and the airflow rate was set at 3 L/min.

2.6. Quantification and Purification for Hemoglobins

The cell growth was monitored by measuring the optical density at 600 nm with a UV-1280 Spectrophotometer (Shimadzu, Kyoto, Japan). To determine wet cell weight (WCW), an aliquot of 5.0 mL of fermentation broth was sampled and centrifuged at 12,000× *g* for 20 min. After the supernatant was removed, the cell pellet was weighed.

For secreted mammalian hemoglobins (bHB, hHB, and pHB), the purification was performed using BeaverBeads™ His-tag Protein Purification (70501, Beaver Biomedical Engineering, Suzhou, China), processing 20 mL of supernatants with 500 μL of magnetic beads. The washing and elution buffers contained 50 mM and 500 mM imidazole in 20 mM PBS buffer (pH 7.4), respectively. Finally, 400 μL elution buffer was added to collect the protein, and the purified target proteins (concentrated 50-fold) were obtained. The process

of protein purification was carried out using the Four-dimensional Rotating Mixer (BE-1100, Kylin-Bell, Haimen, China).

Secreted hemoglobins were identified using 10% (*w*/*v*) polyacrylamide SDS-PAGE. Protein samples were prepared using NuPAGE™ LDS Sample 4X Buffer (Thermo Fisher Scientific, Shanghai, China) and heated up to 99 °C for 10 min. The 10uL protein sample added to each lane involves mixing 7.5 µL of fermentation broth supernatant or purified protein, with 2.5 µL of LDS loading buffer. The SDS-PAGE was performed using XCell SureLock Mini-Cell (Thermo Fisher Scientific, Shanghai, China), and SDS-gel staining was accomplished using the Common Stain Buffer (C516024-0500, Sangon Biotech, Shanghai, China).

The fermentation supernatant was used as a crude protein solution for Bradford assay to determine the secreted LegH at the shaking-flask level and the synthesis of mammalian hemoglobins at the fermenter level. Mammalian hemoglobins synthesized at shaking-flask level were purified and assayed by the Bradford method to test the effect of different strategies. The concentrations of hemoglobins were determined through a combination of Bradford protein concentration assay kit (P0006, Beyotime Biotech, Shanghai, China) and quantitative analysis with QuantityOne 4.6 (Bio-Rad, Hercules, CA, USA) analysis software. In the quantification of mammalian hemoglobins via the QuantityOne system, only the bands of α and β subunits, as well as their dimer were counted.

2.7. Determination of Hemoglobin Functional Activity and Protein Sequence

For functional activity assessment, 0.02 mM of purified hemoglobin and hemoglobin-ref (CAS Number: 9008-02-0, M = 64,500 Da) were incubated in PBS buffer for 10 min. The absorption spectroscopy from 300–700 nm was conducted using a Cytation Microplate Reader (Synergy H1, BioTek Instruments, Winooski, VT, USA). The standard hemoglobin from bovine (S12021), human (H7379), and porcine (XYPR9070) sources were acquired from Yuanye Biotechnology (Shanghai, China), Sigma-Aldrich (St. Louis, MO, USA), and Xinyu Biotechnology (Shanghai, China), respectively.

The specific peroxidase activity of hemoglobin was measured using 3,3′,5,5′-tetramethy lbenzidine (TMB) chromogen solution as the substrate (P0209, Beyotime Biotech, Shanghai, China). A total of 10 µL of purified hemoglobin, diluted 50-fold, were incubated for 3 min after adding 100 µL of TMB. Subsequently, the absorbance change was measured at 620 nm over a period of 6 min, and 1 unit was defined as increase of 0.01 per minute. Cpr stands for concentration of protein. The peroxidase activity pod (U/mg) was calculated as follows:

$$\mathrm{POD\ (U/mg)} = \frac{\Delta \mathrm{OD}_{620} \times \mathrm{V_{total}}}{0.01 \times \Delta \mathrm{T} \times \left(\mathrm{Cpr} \times \mathrm{V_{sample}}\right)}$$

To identify the synthesized mammalian hemoglobins, MALDI-TOF mass spectrometry (ultrafleXtreme, Bruker Daltonics, Billerica, MA, USA) was employed for the identification and analysis of protein sequence.

2.8. Statistical Analysis

All experimental measurements were conducted in triplicate with unique biological samples. Results were presented as mean ± standard deviation (SD). One-way analysis of variance (ANOVA) followed by Tukey's test and Two-tailed-Student's *t*-test was used to reveal the statistical significance using GraphPad Prism 8.0. Significance was indicated by NS (non-significance), * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$.

3. Results

3.1. Selection of an Efficient System to Express Globin Components in HB

To solve the challenges in heterologous expression of hemoglobins in *P. pastoris*, including the lower yield of globin and the significant degradation of globin, a modified P1 strain (X33-Δ*ku70-Mit1*-Δ*yps1*) was constructed in our previous research. The gene encoding

heterologous hemoglobin is driven by the P_{AOX1} promoter and activated by Mit1p [47]. In addition, the heme supply was enhanced in another P1H9 strain (X33-Δ*ku70-Mit1*-Δ*yps1-HEME-9*) by streamlining the heme synthetic pathway [47]. Based on these two engineered *P. pastoris* hosts, the genes encoding α- and β-subunits of three hemoglobins (bHB, hHB, and pHB) were controlled by the P_{AOX1} promoter and integrated at the P_{FLD1}UP-g1 and P_{TEF1}UP-g1 loci in P1 strain, and at the P_{FLD1}UP-g1 and P_{GAP}Up-g2 loci in P1H9 strain.

The results showed that all globins were successfully expressed in *P. pastoris* at shaking-flask level under the control of 1% methanol induction and 40 mg/L heme supplementation, including bHB (59.5 ± 1.5 mg/L), hHB (27.8 ± 3.0 mg/L), and pHB (47.5 ± 2.1 mg/L) in P1 strain, and bHB (54.2 ± 1.6 mg/L), hHB (22.0 ± 3.4 mg/L), and pHB (42.1 ± 1.5 mg/L) in P1H9 strain (Figure 2A). The titers of three different globins and the status of cell growth revealed that the P1 strain is more suitable for the synthesis of mammalian hemoglobins than the P1H9 strain. The possible reason is the synthesis of heme hinders cell growth by depleting cellular resources and increasing metabolic load in the P1H9 strain, thereby decreasing the expression of mammalian hemoglobins [23]. Thus, the P1 strain was selected to express mammalian hemoglobins in the following experiments.

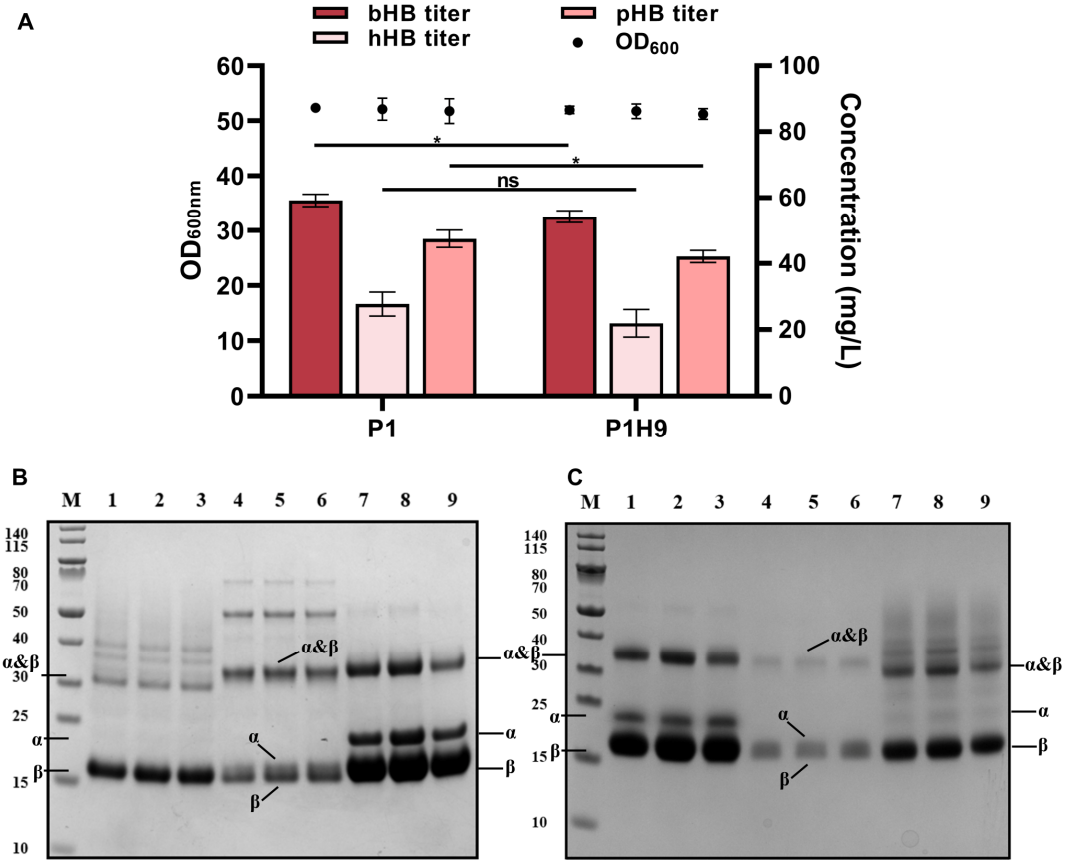

Figure 2. Optimization of the expression platform for mammalian hemoglobins. (**A**) Selection of a suitable host for bHB, hHB, and pHB globins. (**B**) SDS-PAGE analysis of the mammalian hemoglobins expressed in P1 strain. Lanes 1–3: porcine hemoglobin, Lanes 4–6: human hemoglobin, Lanes 7–9: bovine hemoglobin. (**C**) SDS-PAGE analysis of the mammalian hemoglobins expressed in P1H9 strain. Lanes 1–3: bovine hemoglobin, Lanes 4–6: human hemoglobin, Lanes 7–9: porcine hemoglobin. M indicates the protein ladder. Three independent strains were evaluated for the expression of mammalian hemoglobins. Statistical evaluation (*p*-value) was conducted by a two-tailed *t*-test. "*" $p < 0.05$, and "ns" stands for "not significant" ($p \geq 0.05$).

3.2. Optimization of Fermentation Conditions at Shaking-Flask Level

As the mammalian hemoglobins have never been synthesized in *P. pastoris*, the appropriate fermentation conditions are crucial to obtain the higher titer. Since plant hemoglobin and myoglobin have been expressed in *P. pastoris* by BMGY medium [50], this medium was chosen as the basic composition for the expression of mammalian hemoglobins at first. In addition, due to the critical role of heme in the synthesis of hemoproteins, it is necessary to optimize the additive amount of hemin (20.0, 60.0, 100.0, and 140.0 mg/L) for the high activities. The results showed that the expression of hemoglobin was influenced by the different concentrations of hemin and the effect of 140 mg/L hemin addition was not significantly better than that of 100 mg/L hemin addition (Figure 3A). Therefore, for the cost-effectiveness in large-scale production, the additive amount of hemin was maintained at 100 mg/L. Furthermore, the concentration of methanol (0.5%, 1%, 2%, and 4%) was optimized during induction and it found that the optimal expression of mammalian hemoglobin occurred at 2% methanol (Figure 3B).

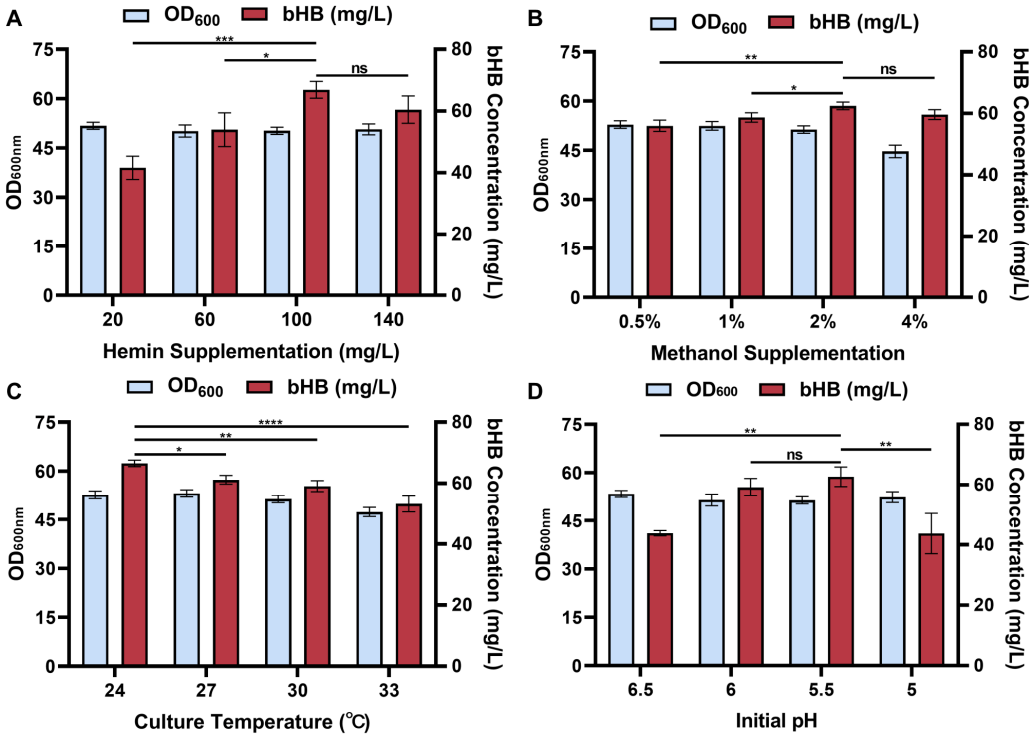

Figure 3. Optimization of bHB Expression at shaking-flask level. (**A**) Impact of hemin supplementation on bHB expression. (**B**) Influence of methanol concentration on bHB expression. (**C**) Effect of culture temperature on bHB expression. (**D**) Effect of initial pH in the medium on bHB expression. "*" $p < 0.05$ vs. compared group, "**" $p < 0.01$ vs. compared group, "***" $p < 0.001$ vs. compared group, and "****" $p < 0.0001$ vs. compared group; "ns" stands for "not significant".

In the following, the fermentation temperature was optimized at 24 °C, 27 °C, 30 °C, and 33 °C. It observed a significant decrease in hemoglobin expression at 33 °C, suggesting that the higher temperatures are not appropriate for the stable expression of multi-subunit hemoglobin. In addition, compared with 30 °C, the notably higher content of dimer hemoglobin presented at 24 °C (Figure 3C). Furthermore, concerning the extracellularly expressed hemoglobins, the moderately acidic environment can diminish the activity of protease and inhibit the degradation of target proteins [51]. Through the tests at various initial pH values (6.5, 6.0, 5.5, 5.0), the optimal initial pH was identified at 5.5 for the expression of mammalian hemoglobins (Figure 3D). The SDS-PAGE analysis for optimization of bHB fermentation conditions was presented in Supporting Information (Figure S1).

3.3. Screening Efficient Endogenous Signal Peptides in Pichia pastoris

Previously, α-Factor SP was used for effective secretion through directed evolution [52,53]. Further exploration of the potential of endogenous SPs in yeast for directing protein secretion is essential based on the recent research in *S. cerevisiae* [39]. Therefore, it is necessary to identify the efficient endogenous SPs for the secretory expression of mammalian hemoglobins in *P. pastoris*. Dependent on the complete genome sequence from NCBI (GenBank: GCA_001708105.1), which includes 5051 open reading frames (ORFs), SignalP 6.0 was utilized to identify candidate SP sequences, resulting in 248 distinct SPs. The analysis showed that the proportion of genes that possess SPs ranges from 3.0% to 6.0% for the four chromosomes of *P. pastoris*: Chr I (5.62%), Chr II (4.76%), Chr III (5.01%), and Chr IV (3.58%).

Besides the bioinformatics analysis, the detection of native secreted proteins during the high-cell-density fermentation by secretome is another feasible way to obtain efficient SPs in *P. pastoris* [54]. Thus, thirty proteins with higher expressional intensity in the secretome were selected [55]. Most of them are involved in the biosynthesis of cell walls and associated functions [55]. In the following, the SPs of these proteins were obtained by the prediction using SignalP 6.0.

In summary, 68 endogenous SPs from *P. pastoris* were evaluated in this study, including the top 40 SPs predicted by SignalP 6.0 (D-score value > 0.98), and 30 SPs originated from the secreted proteins in the fermentation broth [55]. The BA75_01931T0 and Sun4p, BA75_01272T0, and Cpr5p proteins share the same secreted SP. As LegH has been successfully secreted in *P. pastoris* [56], it was used as the model protein to test the efficiency of candidate SPs (Table S6).

The results showed that except for signal peptides BA75_04506T0, Yps1-1p, 7435_chr4-0690, and Cda2p, 64 endogenous SPs could mediate the secretion of LegH. However, compared to the control group using α-Factor SP, only 13 natural SPs predicted by SignalP 6.0 could improve the secretion of LegH, including the SPs of PpPIR2, Bgl2p, 7435_Chr3-1225, Flo5-1p, Sun4p, Uth1p, Kar2p, 7435_Chr3-1213, and Dse4p (Figure 4A–D). Among these SPs, the SPs of Dse4p and Sun4p have the best effect on the secretion of LegH. It should be noted that the endogenous Dse4p SP has been reported to mediate the efficient secretion of EGFP in *P. pastoris* [35].

Based on the secreted efficiency of LegH, the SPs of Dse4p and Sun4p were selected to replace the original α-Factor SP to enhance the secreted efficiency of bovine, human, and porcine hemoglobins. The analysis of SDS-PAGE confirmed the successful secretion of bovine, human, and porcine hemoglobins by the SPs of Dse4p and Sun4p (Figure 5B). In addition, the secretion of porcine hemoglobin increased by 8.79% using the Dse4p SP compared to the α-Factor SP (Figure 5A). However, the results showed the secretion of bovine and human hemoglobins decreased. Thus, the α-Factor SP remains the optimal choice for the secretion of bovine and human hemoglobins (Figure 5A). Subsequently, the P1-αF-bHB, P1-αF-hHB, and P1-DSE4-pHB strains were developed, using the α-Factor SP for bovine and human hemoglobin secretion and the Dse4p SP for porcine hemoglobin. These strains were designated as P1b, P1h, and P1p, respectively. These findings suggest that the mining of endogenous SPs is a useful way for the secretion of other heterologous proteins in *P. pastoris*.

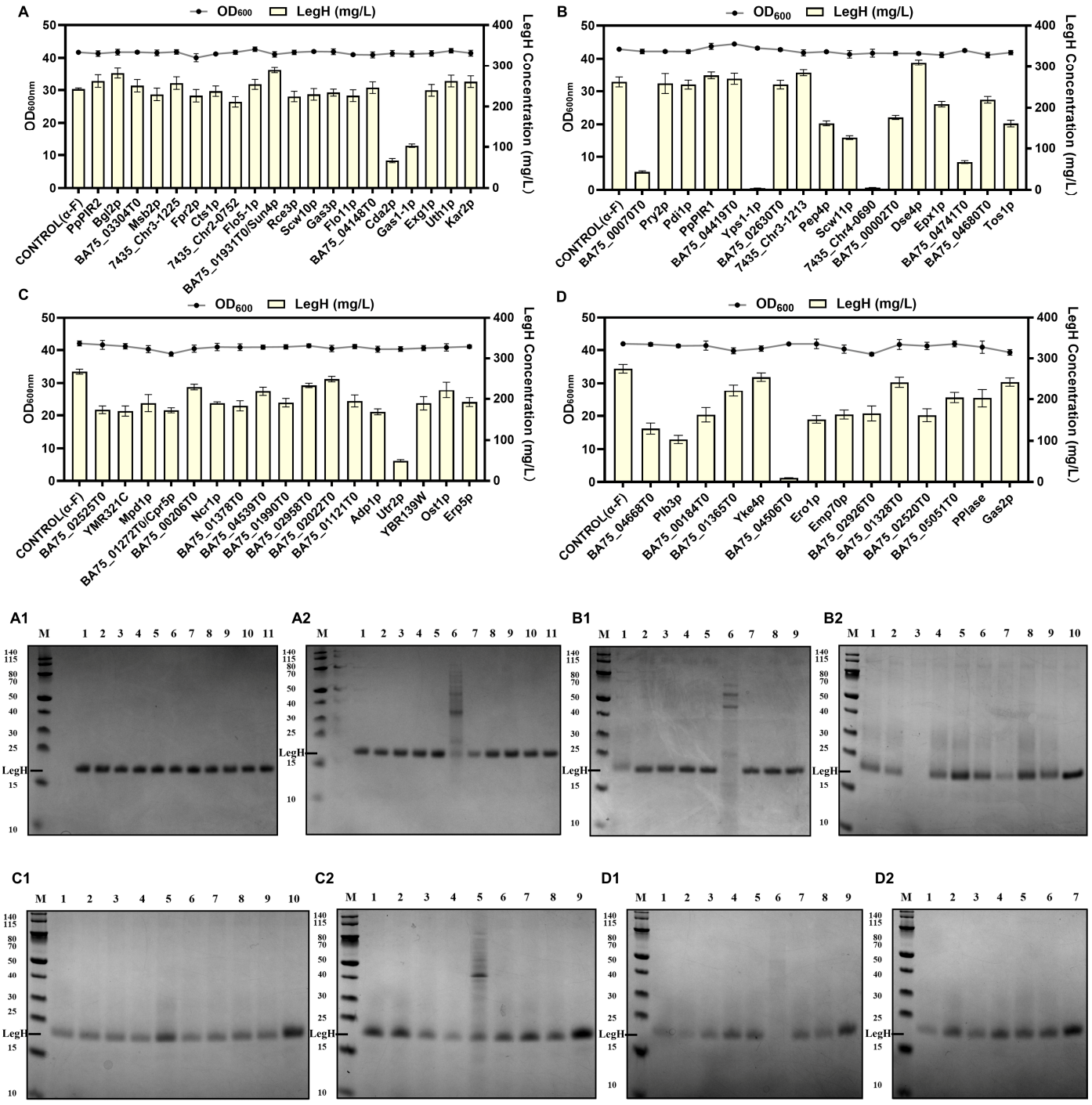

Figure 4. Secreted expression of LegH mediated by the endogenous secretion signals from *P. pastoris*. (**A**–**D**) The secreted expressional levels of LegH by the recombinant strains utilizing various endogenous signal peptides. (**A1**–**D2**) Analysis of recombinant LegH expression by SDS-PAGE. (**A1**) Lanes 1–10 correspond to PpPIR2 to Sun4p strains in (**A**); Lane 11: α-Factor control. (**A2**) Lanes 1–10 represent Rce3p to Kar2p strains in (**A**); Lane 11: α-Factor control. (**B1**) Lanes 1–8 represent BA75_00070T0 to 7435_Chr3-1213 strains in (**B**); Lane 9: α-Factor control. (**B2**) Lanes 1–10 correspond to Pep4p to Tos1p strains in (**C**); Lane 9: α-Factor control. (**C1**) Lanes 1–9 represent BA75_02525T0 to BA75_01990T0 strains in (**C**); Lane 10: α-Factor control. (**C2**) Lanes 1–8 represent BA75_02958T0 to Erp5p strains in (**C**); Lane 9: α-Factor control. (**D1**) Lanes 1–8 represent BA75_04668T0 to Emp70p strains in (**D**); Lane 9: α-Factor control. (**D2**) Lanes 1–6 correspond to BA75_02926T0 to Gas2p strains in (**D**); Lane 7: α-Factor control. M indicates the protein ladder.

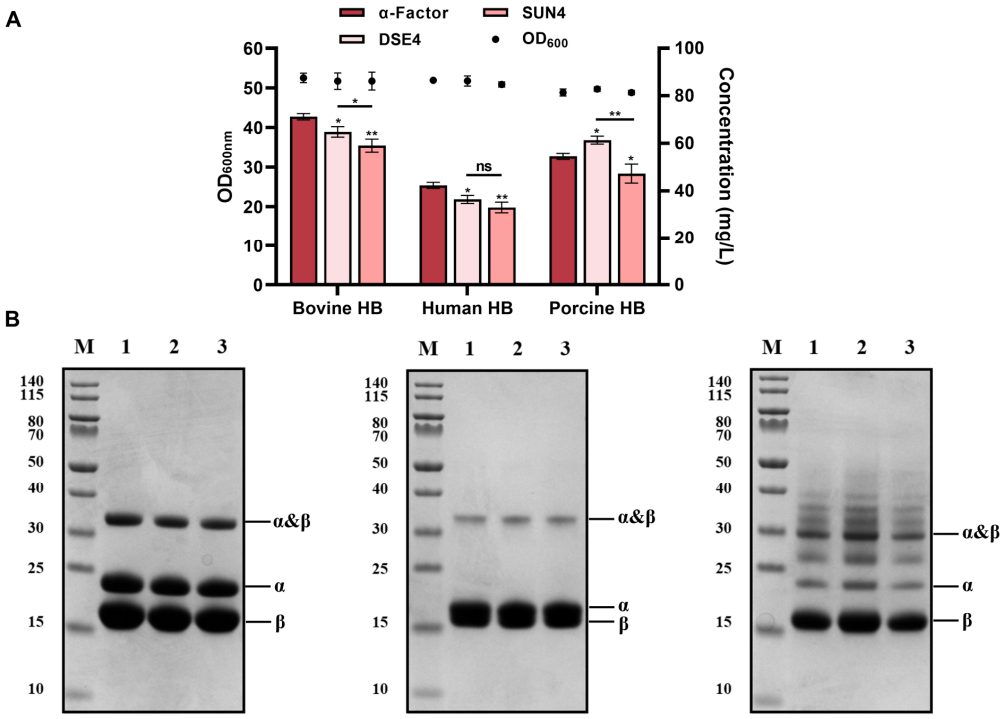

Figure 5. Secreted expression of mammalian hemoglobins mediated by the endogenous secretion signals from *P. pastoris*. (**A**) Secreted expressional levels of three hemoglobins (bHB, hHB, pHB) in the recombinant strains using various endogenous signal peptides. (**B**) The SDS-PAGE results of secreted bHB, hHB, and pHB are mediated by α-Factor, Dse4p, and Sun4p, respectively. "*" $p < 0.05$ vs. compared group, "**" $p < 0.01$ vs. compared group; "ns" stands for "not significant".

3.4. Enhancing the Synthesis of Mammalian Hemoglobins through Combinatorial Metabolic Engineering Strategies

AHSP is a small molecular chaperone that can stabilize α-globin by forming a complex and preventing its degradation and oxidation [57,58]. By integrating bovine, human, and porcine *AHSP* genes into the 5′*GAP* locus using the pGAPZαA plasmid, the significant increases in the titers of bHB (10.8%), hHB (14.6%), and pHB (9.3%) were achieved in the P1b/P1h/P1p strains (Figure 6A).

In *P. pastoris*, the higher expressional levels of vacuolar proteases Pep4p and Yps1-1p appeared during the phase of post-batch [55], which was attributed to secretory stress [59]. The degradation of target proteins by the proteases is a notable issue in the biosynthesis of hemoproteins, which can be confirmed by the reduced yield of porcine myoglobin and significant degradation of LegH in the fed-batch fermentations [47,56]. Our previous research found that the effect of knocking out the *YPS1-1* (GPI-anchored aspartyl protease) gene was better than that of knocking out the *PEP4* (vacuolar aspartyl protease) or *PRB1* (encoding vacuolar serine protease) gene on the control of globin degradation in *P. pastoris* [47]. To further eliminate the degradation of mammalian hemoglobins, the knock-out of vacuolar hydrolase-sorting proteins Vps10p and Vth1p, E3 ubiquitin-protein ligase Pep5p were investigated in this study.

To explore the influence of *VPS10*, *VTH1*, and *PEP5* on the secreted expression of mammalian hemoglobins in *P. pastoris*, CRISPR/Cas9 gene editing was employed. The recombinant plasmids were constructed, including pPIC3.5K-*VPS10*-gRNA, pPIC3.5K-*VTH1*-gRNA, pPIC3.5K-*PEP5*-gRNA, along with the corresponding donor DNA fragments UpVPS10-DoVPS10, UpVTH1-DoVTH1, and UpPEP5-DoPEP5. In the following, the P1b-Δ*Vth1*, P1b-Δ*Vps10*, and P1b-Δ*pep5* engineered strains were obtained by individually knocking out *VPS10*, *VTH1*, and *PEP5* genes, respectively.

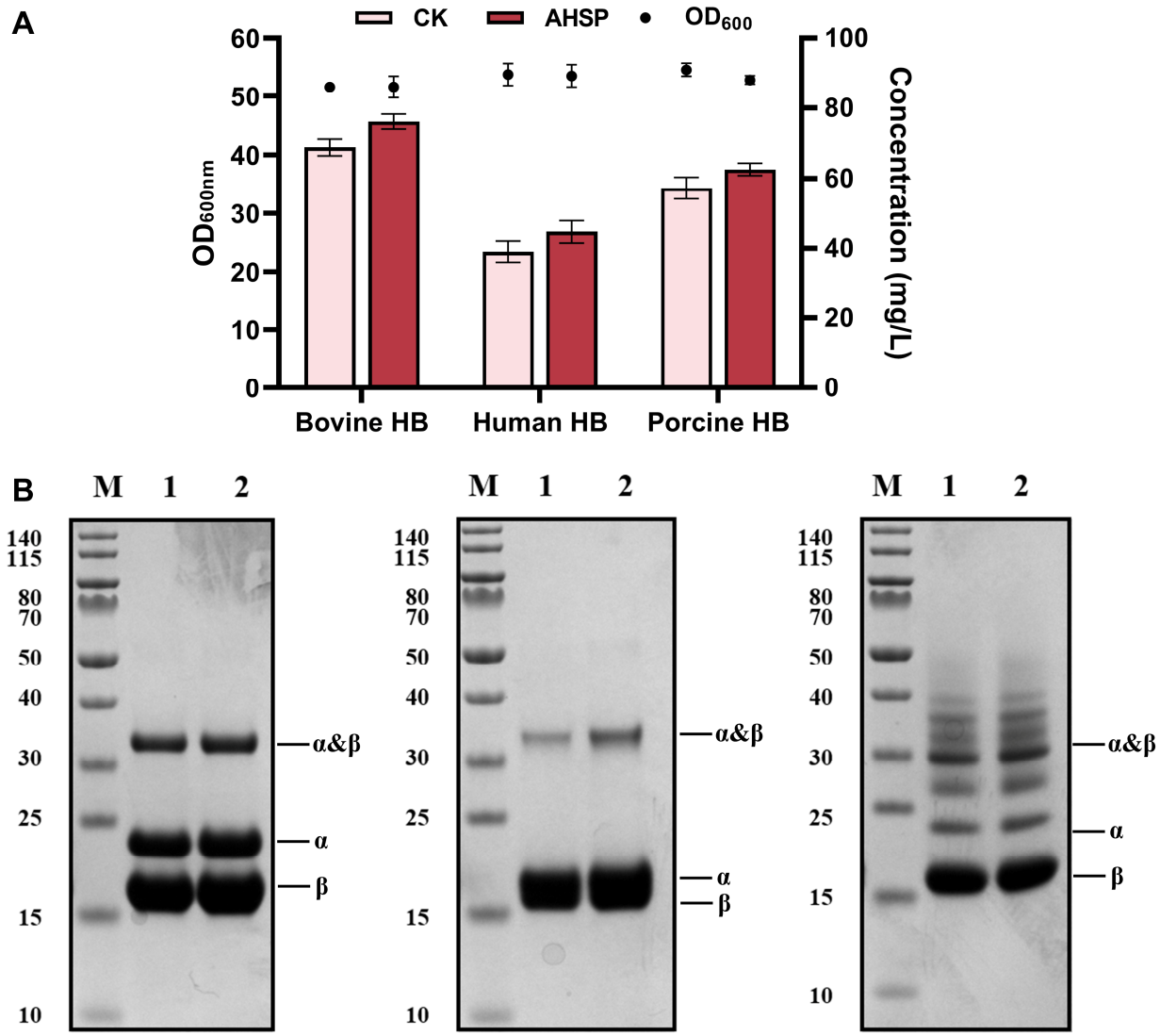

Figure 6. The effect of AHSP on the synthesis of mammalian hemoglobins in P1b, P1h, and P1p strains. (**A**) The change of hemoglobin titers (bHB, hHB, and pHB) after the co-expression of AHSP; the quantitative analysis was based on the bands in Figure 5B–D. "CK" denotes the strains without *AHSP* gene. (**B**) The SDS-PAGE analysis of bHB, hHB, and pHB in the engineered strains with *AHSP* gene.

The results showed that the secretion of mammalian hemoglobins was significantly enhanced in the P1b/P1h/P1p-Δ*Vth1* strains (bHB 93.1 mg/L, hHB 49.8 mg/L, pHB 67.3 mg/L), increasing by 1.21-fold, 1.18-fold, and 1.11-fold compared to the original P1b/P1h/P1p strains (Figure 7). Finally, the co-expression of AHSP was applied in the P1b/P1h/P1p-Δ*Vth1* strains, resulting in the final engineered P1b/P1h/P1p-Δ*Vth1-AHSP* strains (bHB 96.1 mg/L, hHB 55.7 mg/L, pHB 72.6 mg/L). It found that the co-expression of AHSP led to a 3.22% increase in the titer of bovine hemoglobin, an 11.84% increase in the titer of human hemoglobin, and a 7.88% increase in the titer of porcine hemoglobin, compared to the *VTH1* gene knockout strains (Figure 7). When normalized to cell density, the hemoglobin synthesized from engineered P1b/P1h/P1p-Δ*Vth1-AHSP* strains yielded 1.70 mg/L/OD_{600} for bovine, 0.98 mg/L/OD_{600} for human, and 1.27 mg/L/OD_{600} for porcine.

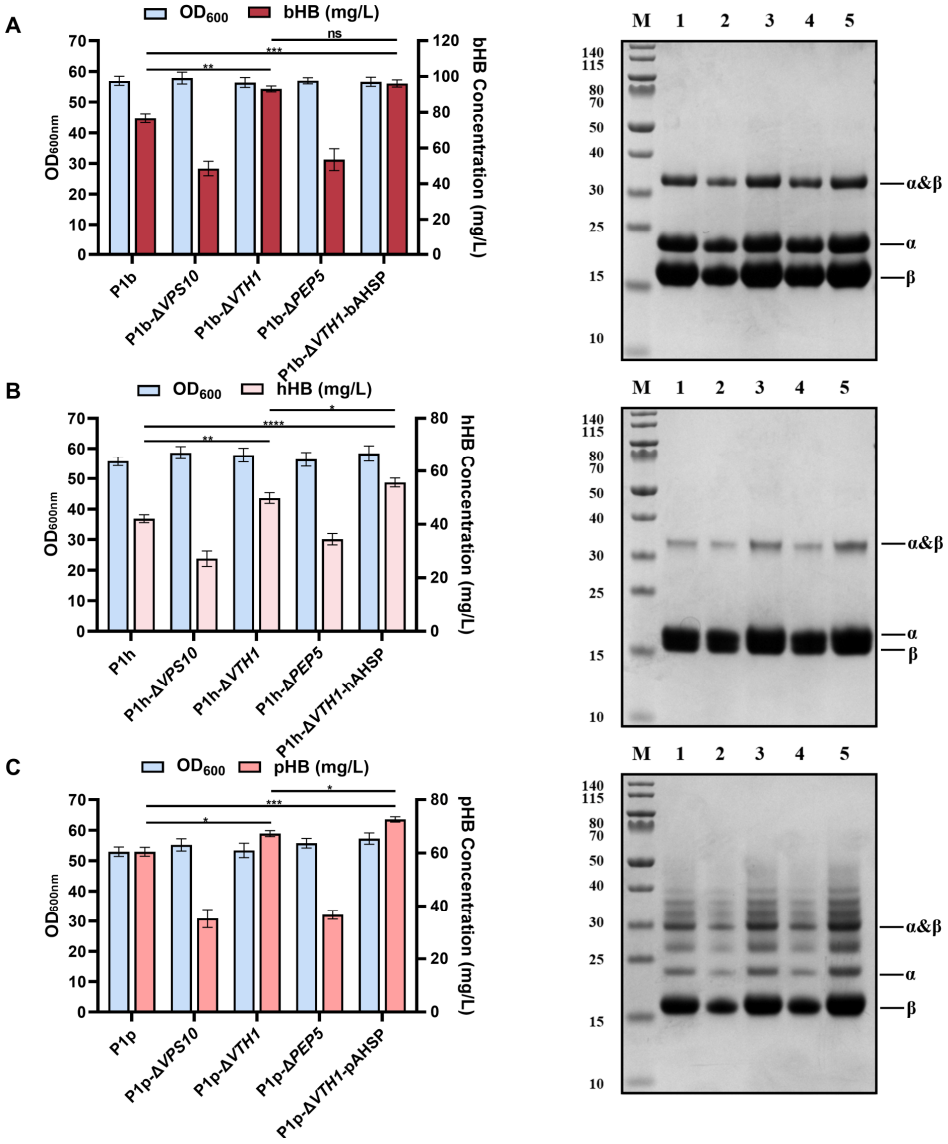

Figure 7. Effects of knocking out Heterologous Protein Degradation Gene and the co-expression of AHSP on the expression of mammalian hemoglobins. (**A**) The titer of bHB and the SDS-PAGE analysis of the engineered strains. (**B**) The titer of hHB and the SDS-PAGE analysis of the engineered strains. (**C**) The titer of pHB and the SDS-PAGE analysis of the engineered strains. "*" $p < 0.05$ vs. compared group, "**" $p < 0.01$ vs. compared group, "***" $p < 0.001$ vs. compared group, and "****" $p < 0.0001$ vs. compared group; "ns" stands for "not significant".

3.5. Secretory Expression of Mammalian Hemoglobins at 5-L Fermenter Level

Based on the final engineered P1b/P1h/P1p-Δ*Vth1-AHSP* strains, the high-cell-density fermentations were performed using a two-stage feeding strategy. The optimal shake flask culture conditions during induction, including temperature, heme supplementation, and initial pH value, were subsequently applied to fermenter operations to facilitate a coherent scale-up strategy. Initially, after the first dissolved oxygen rebound, 50% *w*/*v* glycerol was fed at a flow rate of 35 mL per hour for 20 h. After glycerol depletion, the feeding of methanol started when the rebound of dissolved oxygen emerged again at approximately 48 h to induce the expression of mammalian hemoglobins. The induction strategy employs the traditional DO-stat control approach, as previously reported [60,61]. When the dissolved oxygen (DO) level rose above its upper limit of 20%, the methanol-feeding medium was initiated to activate the metabolic pathway. The feeding of methanol results in a decrease in DO tension to the set value, at which point the feeding is halted. Therefore, this method

prevented the accumulation of methanol and avoided the limitation of oxygen. The period of induction lasted over 100 h and hemin was added at a final concentration of 100 mg/L after 52 h. The concentration of mammalian hemoglobins were examined every 12 h. The results showed that the highest titer of bHB, hHB, and pHB reached 376.9 ± 13.3 mg/L, 101.1 ± 6.7 mg/L, and 119.2 ± 7.3 mg/L (Figure 8A,C).

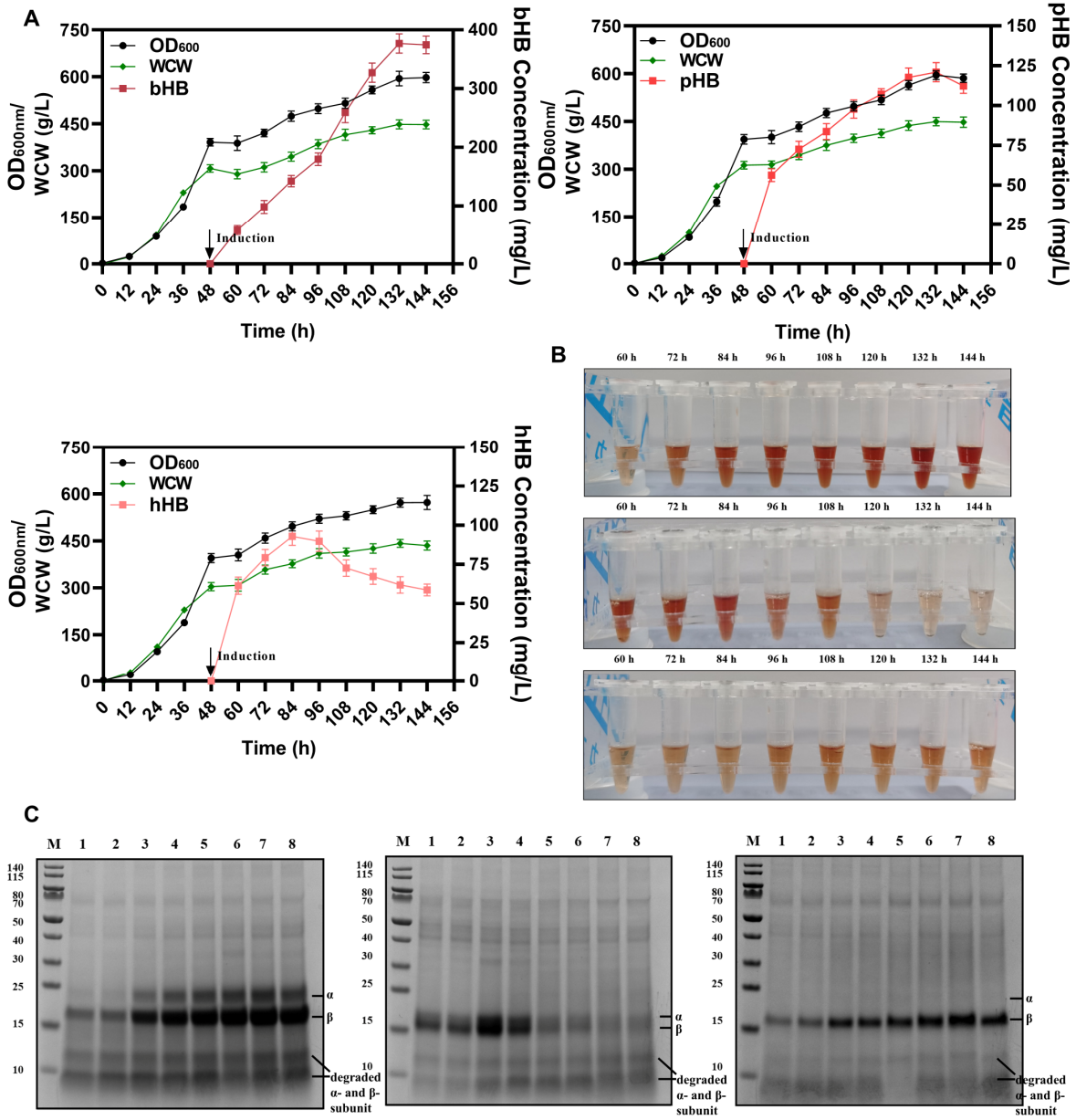

Figure 8. Synthesis of mammalian hemoglobins by fed-batch fermentations at 5-L fermenter scale. (**A**) The values of OD_{600} and wet cell weight (WCW) and the titers of bHB, hHB, and pHB during the fed-batch fermentations. (**B**) The purified samples of mammalian hemoglobins from 60 h to 144 h. The sequence of photos from top to bottom presented the bHB-purified samples, hHB-purified samples, and pHB-purified samples. (**C**) The SDS-PAGE results of the fermentation supernatant (bHB, hHB and pHB) for 60 h, 72 h, 84 h, 96 h, 108 h, 120 h, 132 h, and 144 h.

3.6. Biochemical Properties of Various Mammalian Hemoglobins Synthesized in Engineered Pichia pastoris Strains

To ensure the applications of synthesized mammalian hemoglobins in artificial meat alternatives, acellular oxygen carriers, and metalloenzymes, the biochemical properties of these hemoglobins were examined. The commercial native bovine (bHB-ref), human

(hHB-ref), and porcine (pHB-ref) hemoglobins were used as reference standards. The absorption spectra of were observed for these hemoglobins between 300–700 nm. The results showed that all synthesized mammalian hemoglobins displayed the characteristic spectra in the Soret band region, peaking at 410 nm. This spectral similarity is attributed to heme moiety binding in globin components, which was consistent with the previous research (Figure 9A) [26,62]. It demonstrated that the activities of synthesized HBs were close to the native HBs, suggesting they are suitable for applications in food processing and biocatalytic processes. In addition, hemoglobins synthesized from the cultured strains of the same batch were purified and used for peroxidase activity assay, and SDS-PAGE results of the samples are displayed in Figure 7A–C. The P1 strain without any hemoglobin gene was used as a blank control. 3,3′,5,5′-Tetramethylbenzidine (TMB) chromogen solution was used to assay peroxidase activity in purified bHB, hHB, and pHB samples (Figure 9B).

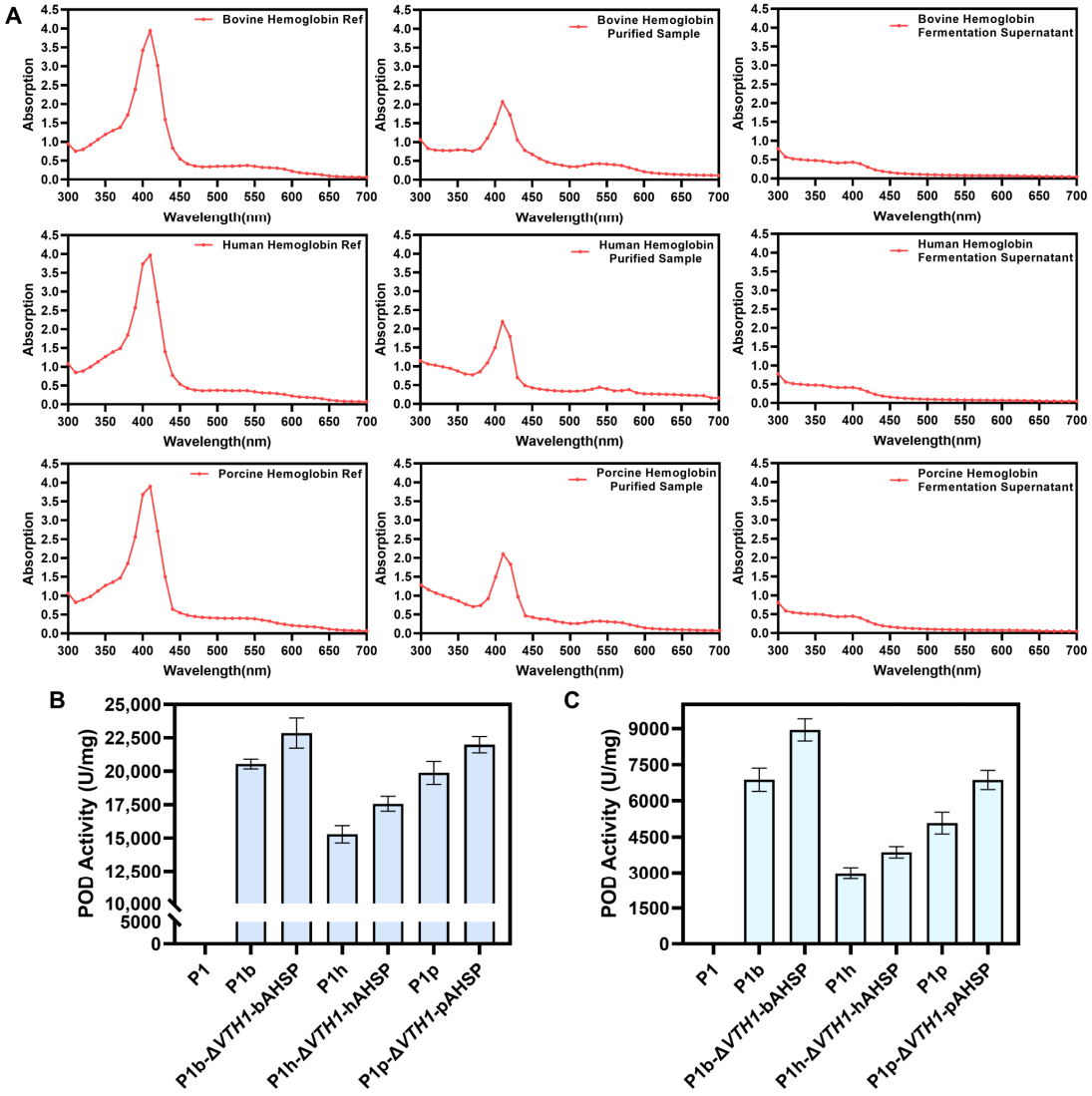

Figure 9. Biochemical properties and peroxidase activities of synthesized mammalian hemoglobins. (**A**) The analysis of absorption spectra. The absorption spectra of purified bHB, hHB, pHB, reference standards (bHB-ref, hHB-ref, pHB-ref), and the fermentation supernatant (bHB, hHB, pHB) were recorded between 300 to 700 nm, highlighting the Soret peak at 410 nm. (**B**) The measurement of peroxidase activities (POD) for purified bHB, hHB, pHB, and P1 (blank control). (**C**) The measurement of peroxidase activities (POD) for the fermentation supernatant (bHB, hHB, pHB), and P1 (blank control).

Moreover, verifying proteins via MALDI-TOF/TOF mass spectrometry is critical for accurate protein identification. The data encompasses peak lists, search results, decoy database analysis files, a FASTA file of the protein sequence, and summaries outlining protein coverage and matched peptides identified through MS/MS and decoy database analysis using Mascot [63]. Peptide identification of trypsin-digested hemoglobin samples from SDS-PAGE gels via MALDI-TOF MS confirmed accurate matches for bovine, human, and porcine hemoglobins, with detailed results available in Supplementary Notes S1–S3.

4. Conclusions

In this study, the synthesis of human, bovine, and porcine hemoglobins in *P. pastoris* was achieved for the first time by enhancing the secretory pathway of heterologous proteins. In conclusion, the engineered *P. pastoris* strain capable of secreting mammalian hemoglobins was constructed by the screening of signal peptides, the knock-out of vacuolar protease-related genes, and the co-expression of AHSP. In addition, based on the optimized fermentation conditions, the highest titers of bovine, human, and porcine hemoglobins were achieved at the fermenter level (Table 1).

Table 1. The latest research on the biosynthesis of animal hemoglobins.

Sources	Strategy	Titer	Refs.
E. coli as host			
Crocodylus siamensis	Intracellular inducible expression by vector	3.0 mg/L (shaking-flask level)	[64]
Peromyscus maniculatus	Intracellular inducible expression by vector	/	[65]
Homo sapiens	Intracellular inducible expression by vector	20% of total protein (shaking-flask level)	[66]
S. cerevisiae as host			
H. sapiens	Intracellular integrated expression by vector; improving heme biosynthesis; reducing heme and hemoglobin degradation	18% of total protein (shaking-flask level)	[25]
Bos taurus	Intracellular inducible expression by vector; heme synthetic pathway engineering	25.2 ± 1.9 mg/L (shaking-flask level)	[26]
Sus scrofa		16.7 ± 0.5 mg/L (shaking-flask level)	
P. pastoris as host			
C. siamensis	Extracellular inducible integrated expression	6.0 mg/L (shaking-flask level)	[64]
B. taurus	Extracellular inducible integrated expression; signal peptide screening; knock-out of vacuolar protease related genes; fermentation conditions optimization;	96.1 ± 1.6 mg/L (shaking-flask level) 376.9 ± 13.3 mg/L (5-L fermenter)	This study
H. sapiens		55.7 ± 1.4 mg/L (shaking-flask level) 101.1 ± 6.7 mg/L (5-L fermenter)	
S. scrofa		72.6 ± 0.5 mg/L (shaking-flask level) 119.2 ± 7.3 mg/L (5-L fermenter)	

In previous studies, *S. cerevisiae* achieved maximal titers of 25.2 ± 1.9 mg/L for bovine hemoglobin and 16.7 ± 0.5 mg/L for porcine hemoglobin [26]. When normalized to the cell density, these yields correspond to approximately 0.55 mg/L/OD_{600} and 0.36 mg/L/OD_{600}, respectively. In contrast, the hemoglobin synthesized from engineered P1b/P1h/P1p-Δ*Vth1-AHSP* strains, the yields were 1.70 mg/L/OD_{600} for bovine, 0.98 mg/L/OD_{600}

for human, and 1.27 mg/L/OD_{600} for porcine hemoglobins. This comparative analysis demonstrates the potential of *P. pastoris* as a more efficient host for the production of mammalian hemoglobins.

This achievement paves a new way for the future industrial applications of mammalian hemoglobins, including the creation of hemoglobin-based oxygen carriers (HBOCs) and the production of cultured meat [9,14,15,67]. Furthermore, the strains and strategies described in this study can be utilized to produce other high-active hemoproteins. However, the challenge in the synthesis of mammalian hemoglobin is to balance the expression of two different globin subunits with the supply of sufficient heme to obtain the active hemoglobin ($\alpha_2\beta_2$). This imbalance of globin expression could be addressed to improve the production of hemoglobins. In addition, there were significant differences in the expression of hemoglobins from various animal sources in both shaking-flask and fermenter cultures. This difference highlights the complexity of the expressional system. Therefore, a customized optimization for fermenter conditions should be carried out separately for each protein to accommodate their distinct expressional profiles in the future.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/fermentation10040208/s1, Figure S1. The SDS-PAGE analysis for optimization of bHB fermentation conditions at shaking-flask level; Figure S2. The SDS-PAGE results of purified hemoglobin samples at 5-L fermenter scale; Table S1. Plasmids, genes, and promoters used in this study; Table S2. Strains used in this study; Table S3. Gene knock-in/out cassettes used in this study; Table S4. sgRNA sequences used in this study; Table S5. Primers used in this study; Table S6. Endogenous signal peptides were used in this study. Notes S1–S3. Identification results by MALDI-TOF mass spectrometry. Refs. [47,49,55] are cited in the supplementary materials.

Author Contributions: X.Z., T.Z., Z.L., J.Z., J.L., G.D. and J.C. conceived the idea and design for this work. C.L. carried out the experiments and data analysis. C.L. and X.Z. drafted and revised the paper. All the authors approved the final manuscript for publication. All authors have read and agreed to the published version of the manuscript.

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