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Construction of a novel MK-4 biosynthetic pathway in *Pichia pastoris* through heterologous expression of *HsUBIAD1*

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Abstract

Background: With a variety of physiological and pharmacological functions, menaquinone is an essential prenylated product that can be endogenously converted from phyloquinone (VK₁) or menadiene (VK₃) via the expression of *Homo sapiens* UBIAD1 (*HsUBIAD1*). The methylotrophic yeast, *Pichia pastoris*, is an attractive expression system that has been successfully applied to the efficient expression of heterologous proteins. However, the menaquinone biosynthetic pathway has not been discovered in *P. pastoris*.

Results: Firstly, we constructed a novel synthetic pathway in *P. pastoris* for the production of menaquinone-4 (MK-4) via heterologous expression of *HsUBIAD1*. Then, the glyceraldehyde-3-phosphate dehydrogenase constitutive promoter (P_{GAP}) appeared to be most suitable for the expression of *HsUBIAD1* for various reasons. By optimizing the expression conditions of *HsUBIAD1*, its yield increased by 4.37 times after incubation at pH 7.0 and 24 °C for 36 h, when compared with that under the initial conditions. We found *HsUBIAD1* expressed in recombinant GGU-23 has the ability to catalyze the biosynthesis of MK-4 when using VK₁ and VK₃ as the isopentenyl acceptor. In addition, we constructed a ribosomal DNA (rDNA)-mediated multi-copy expression vector for the fusion expression of *SaGGPPS* and *PpIDI*, and the recombinant GGU-GrIG afforded higher MK-4 production, so that it was selected as the high-yield strain. Finally, the yield of MK-4 was maximized at 0.24 mg/g DCW by improving the GGPP supply when VK₃ was the isopentenyl acceptor.

Conclusions: In this study, we constructed a novel synthetic pathway in *P. pastoris* for the biosynthesis of the high value-added prenylated product MK-4 through heterologous expression of *HsUBIAD1* and strengthened accumulation of GGPP. This approach could be further developed and accomplished for the biosynthesis of other prenylated products, which has great significance for theoretical research and industrial application.

Keywords: *Pichia pastoris*, Aromatic prenyltransferase, *HsUBIAD1*, Geranylgeranyl diphosphate synthase, Menaquinone

Background

Predominant Most research on vitamin K has been devoted to its physiological function as an essential cofactor for γ -glutamyl carboxylase (GGCX), an enzyme

that catalyzes the posttranslational modification of proteins involved in blood coagulation and bone metabolism and prevention of cardiovascular calcification [1–5]. Natural vitamin K has two molecular forms: the plant form, phyloquinone (PK) or vitamin K₁, and the bacterial form, known as the menaquinones (MKs) or vitamin K₂ [6, 7]. The common structure of a 2-methyl-1,4-naphthoquinone nucleus is present in both PK and MKs, which are structurally different in the length and degree of saturation of the isoprene side-chain. At the 3-position of the

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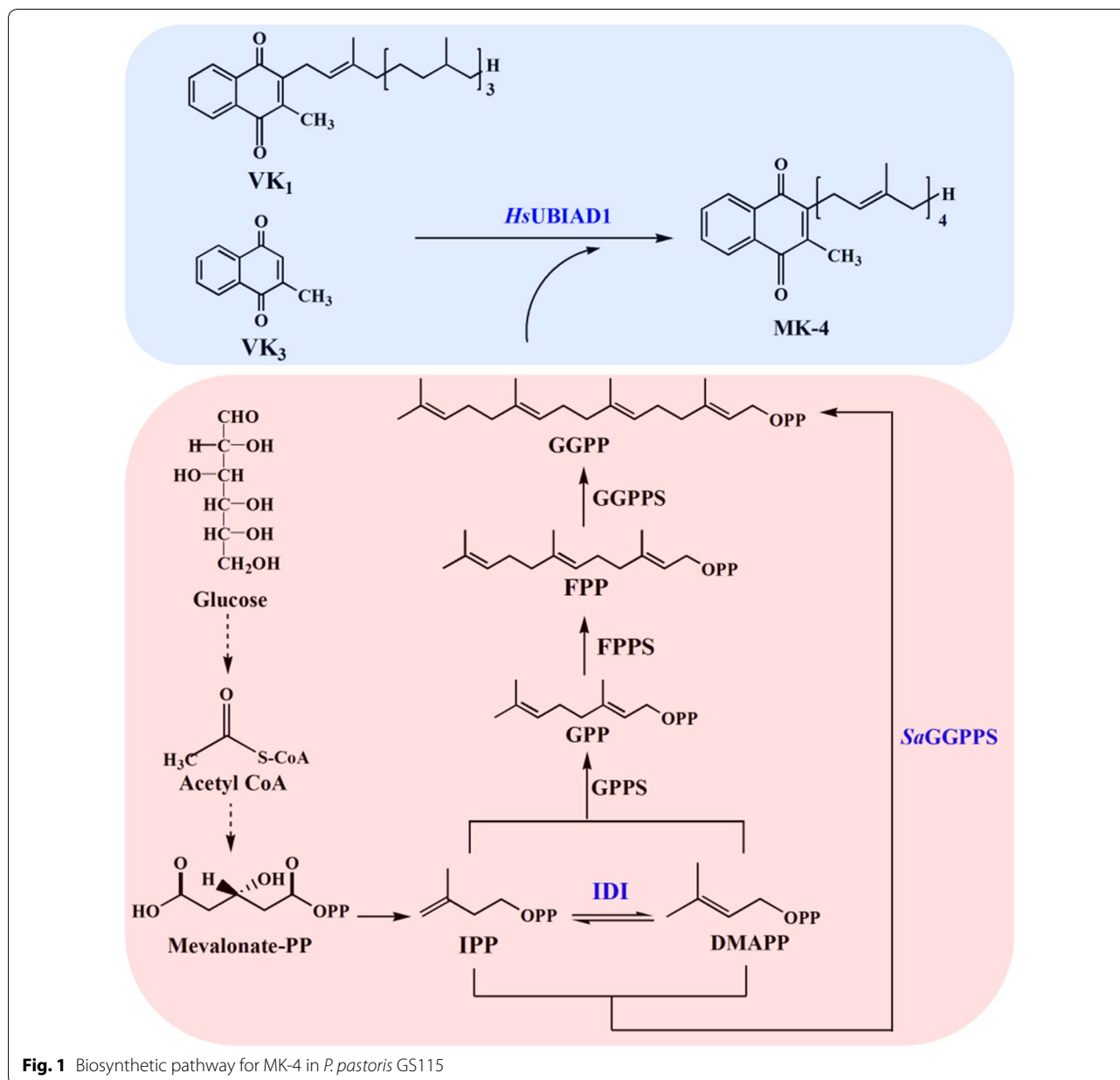


common nucleus, PK has a monounsaturated side chain of four isoprenyl residues. In contrast, MKs are classified into 14 types, which are named MK-n according to the number of repeating unsaturated isoprenyl units. Over the past few years, numerous studies have indicated that MKs can play a role in treating mitochondrial pathologies such as Parkinson's disease and amyotrophic lateral sclerosis, and even exhibit anticancer activity in several types of cancer cells, including liver cancer, lung cancer, bladder cancer, prostate cancer and ovarian cancer [8–11]. PK is primarily obtained from leafy green vegetables and is endogenously converted to menatetrenone (MK-4) by cleavage of the side chain in the intestine, then menadione (VK₃) is delivered through the mesenteric lymphatic system and blood circulation to tissues such as the brain, kidney and pancreas [1, 4, 12]. Recent research has found that *Homo sapiens* UbiA prenyltransferase containing 1 (*HsUBIAD1*), a human homologue of *Escherichia coli* prenyltransferase menA and mammalian mitochondrial prenyltransferase COQ₂ catalyzes the conversion of PK to its prenylated derivative MK-4 [12–14]. As a member of the membrane prenyltransferase family, the structure of *HsUBIAD1* has been resolved in many studies; it contains eight transmembrane helices and two characteristic conserved motifs (NDxxDxxxD and DxxxD) [15, 16]. In particular, the isoprenyl substrate is located in the membrane embedded active site between the two aspartate-rich motifs. In previous studies, *HsUBIAD1* was usually expressed in animal cell expression systems to investigate its related properties [6, 17]. This may be because animal cell expression systems have certain advantages in expressing desired proteins with near-native functional properties. Nevertheless, we cannot ignore the shortcomings of low expression levels, high cost and time-consuming and complicated culture conditions. The components of the medium used for animal cell culture are relatively complex and expensive. In addition, animal cell culture systems are sensitive to the environment and often present the risk of infection with bacteria, fungi and viruses, resulting in relatively high requirements for the operating environment and equipment. Furthermore, it is relatively difficult to screen stable high expression strains using animal cell expression systems and often requires a longer time period due to their low recombination rate. The methylotrophic yeast, *Pichia pastoris* has become an attractive workhorse for biotechnology, especially for the production of both secreted and intracellular heterologous proteins [18–21]. It is an important heterologous protein expressing system that has several advantages over other eukaryotic and prokaryotic expression systems. Firstly, *P. pastoris* not only has the ability to grow high cell densities with a rapid growth rate, it is also capable of metabolizing

methanol as its sole carbon and energy source [22, 23]. In addition, the heterologous genes of interest have been stably integrated into the genome, ideally into a targeted locus via homologous recombination that eliminates segregational instability, which is different from expression via plasmid DNA [24, 25]. Also, *P. pastoris* has the subcellular structure of eukaryotes, so that it exhibits certain advantages in post-translational modifications, including polypeptide folding, phosphorylation, glycosylation and methylation [26, 27]. In order to achieve successful recombinant protein expression, choosing the appropriate expression vector is an important prerequisite as well as selection of the host strain. Thus far, the majority of heterologous proteins have been successfully expressed by methanol inducible alcohol oxidase promoter (P_{AOXI}) and glyceraldehyde-3-phosphate dehydrogenase constitutive promoter (P_{GAP}). In the presence of methanol, P_{AOXI} is induced to generate the enzyme alcohol oxidase [28], which catalyzes the first step of formaldehyde assimilation pathways, converting methanol to formaldehyde [29, 30]. It has been reported that the expression levels of heterologous proteins are tightly regulated by P_{AOXI} [31, 32]. However, large amounts of methanol are needed as inducer for large-scale fermentations, resulting in risks of toxicity and safety. From a security perspective, the constitutive expression vector controlled by P_{GAP} is more appropriate for recombinant protein production. Furthermore, recent studies have shown that the level of expression seen with the P_{GAP} can be slightly higher than that obtained with the P_{AOXI} [29, 31, 33, 34].

Geranylgeranyl diphosphate (GGPP), an important precursor for the biosynthesis of MK-4, consists of isoprenoid building blocks accumulated via the mevalonate (MVA) pathway in *P. pastoris* (Fig. 1). Research has shown that the geranylgeranyl diphosphate synthase from the archaeobacterium *Sulfolobus acidocaldarius* (*SaGGPPS*) is capable of synthesizing geranylgeranyl diphosphate through sequential condensations of isopentenyl diphosphate (IPP) with dimethylallyl diphosphate (DMAPP), thereby reducing competition for the common precursor farnesyl diphosphate (FPP) [35–37].

A previous study [38] found that *P. pastoris* has much higher menadione and MKs tolerance when compared with other host strains such as *E. coli*. As an appropriate host due to its superior ability to express eukaryotic proteins, *P. pastoris* has many biotechnological applications; however, the menaquinone biosynthetic pathway has not been discovered in *P. pastoris*. Considering all of the above, in the present study we have attempted to construct a novel synthetic pathway in *P. pastoris* for the production of MK-4 by heterologous expression of *HsUBIAD1*. Meanwhile, to increase MK-4 production, the *Sulfolobus acidocaldarius* geranylgeranyl diphosphate



synthase (*SaGGPPS*) was introduced into *P. pastoris* to improve GGPP supply, although *P. pastoris* does contain the genes that encode a GGPP synthase.

Materials and methods

Strains and plasmids

Escherichia coli DH5 α cells used for plasmid propagation were preserved in our laboratory. *P. pastoris* GS115 (kindly provided by the Laboratory of Signaling Transduction and Transcription, University of Science and Technology of China, Hefei, China) was used to construct the MK-4 production strain. Expression vectors

pPICZA and pGAPZA were purchased from YouBio (Hunan, China). All strains and plasmids used in this study are listed in Additional file 1: Tables S1 and S2.

Culture conditions

Escherichia coli were incubated at 37 °C in a low-salt LB medium consisting of 1% tryptone, 0.5% yeast extract, and 0.5% NaCl. YPD medium (2% tryptone, 1% yeast extract, and 2% glucose) was used for cultivating *P. pastoris* for further preparation of competent cells. BMGY medium used for activating recombinant *P. pastoris* consisted of 2% tryptone, 1% yeast extract, 1.34% yeast

nitrogen base (YNB) medium without amino acids, 1% glycerol, 4×10^{-5} % biotin, and 0.1 M potassium phosphate (pH 6.0). The constitutive recombinant *P. pastoris* with P_{GAP} promoter was incubated for approximately 24 h at 30 °C and 250 rpm in 250-mL flasks containing 25 mL of BMGY medium. For protein expression driven by P_{AOXI} in *P. pastoris*, the cells were harvested by centrifuging at $5000 \times g$ for 10 min at room temperature and resuspended in BMMY medium (2% tryptone, 1% yeast extract, 1.34% YNB, 4×10^{-5} % biotin and 0.1 M potassium phosphate (pH 6.0) and 0.3% methanol) to a final density at OD_{600} of 1.0, and methanol was added to the BMMY medium to a final concentration of 0.3% every 24 h. Solid medium was obtained by adding 2% agar to the liquid medium. The media were prepared by sterilization at 121 °C for 20 min, and glucose solution was sterilized separately at 115 °C for 15 min. In addition, the stock solution of $10 \times$ YNB and $500 \times$ biotin were added separately after filtration sterilization.

Bioinformatics analysis of *HsUBIAD1*

SOPMA (https://npsa-prabi.ibcp.fr/cgi-bin/npsa_automat.pl?page=npsa_sopma.html) was applied to predict the secondary structure of *HsUBIAD1* from the amino acid sequence, in particular to predict the rate of secondary structural elements [39]. Since the hydrophobicity of an amino acid sequence predicts the protein folding, we analyzed the hydrophobicity of *HsUBIAD1* using several tools including SOSUI, ProtScale and DNAMAN. SignalP 3.0 (<http://www.cbs.dtu.dk/services/SignalP-3.0/>) is commonly used to predict the presence and location of signal peptide, and it was used to discriminate the signal peptide of *HsUBIAD1* [40]. WoLF PSORT (<https://wolfpsort.hgc.jp/>) was used to predict the subcellular location of *HsUBIAD1* based on the PSORT II program [41]. The transmembrane topology of *HsUBIAD1* was predicted using a range of transmembrane topology predictors, such as Phobius, OCTOPUS and TMHMM. And the online analysis software, Protter, was used to visualize the transmembrane topology of *HsUBIAD1*. SWISS-MODEL, an automated protein homology-modelling server, was used to predict the tertiary structure of *HsUBIAD1* based on its amino acid sequence.

The homologous sequences of *HsUBIAD1* obtained from the National Center for Biotechnology Information (NCBI) database were aligned using Clustal X. Then, phylogenetic analyses were conducted using MEGA X program. The evolutionary history was inferred using the neighbor-joining method based on the Poisson model and bootstrap analysis was carried out with 1000 replicates. The phylogenetic tree was further edited by EvolView. Multiple sequence alignment of *ApUbiA*, *AtPPT1*, *EcUbiA*, *LePGT-1*, *OsPPT*, *ScCoQ₂*, *BIMenA*,

EcMenA, *KpMenA*, *PaUBIAD1*, *PtUBIAD1* and *HsUBIAD1* were performed using Clustal X and then analyzed by ESPript 3.0.

Construction of the expression vector

The codon of the *HsUBIAD1* gene, which was artificially synthesized by General Biosystems, Inc. (Anhui, China), was optimized based on codon preference to achieve high levels of protein expression in *P. pastoris*. The CAI and GC contents of the optimized sequence were analyzed by the GenScript Web server (<http://www.genscript.com>). The *HsUBIAD1* gene was amplified using primers UBIAD1-F-EcoRI and UBIAD1-R-NotI listed in Additional file 1: Table S3, which contained full-length UBIAD1. The PCR products were digested with *EcoRI* and *NotI* and inserted between the *EcoRI* and *NotI* sites of pGAPZA where *HsUBIAD1* is under the control of constitutive promoter P_{GAP} on the vector, and the resulting plasmid was named pGU. The *HsUBIAD1* fragment described above was cloned into multiple cloning sites of pPICZA, which thus generated pPU. In the recombinant pPU vector, *HsUBIAD1* protein was controlled by P_{AOXI} . The recombinant plasmids grown on low-salt LB agar plates with 50 µg/mL Zeocin were sequenced after colony PCR to confirm the presence and orientation of the insert.

To obtain stable multi-copy integrants in *P. pastoris*, we attempted to construct a multi-copy expression vector by integrating the nucleotide sequence of a portion of the 28S ribosome DNA (rDNA) amplified from *P. pastoris* into the expression vector pGAPZA. Concretely, the rDNA fragment was amplified from the genomic DNA of *P. pastoris* GS115 using primers rDNA-F and rDNA-R listed in Additional file 1: Table S3, and cloned into the *BamHI* site of pGAPZA to gain the desired expression vector pGAPZA-rDNA. The geranylgeranyl pyrophosphate synthase gene (*ggpps*) from *Sulfolobus acidocaldarius* used in this study was artificially synthesized by General Biosystems, Inc. (Anhui, China). The coding region of *ggpps* was amplified by PCR using primers GGPPS-F-EcoRI and GGPPS-R-NotI listed in Additional file 1: Table S3, and cloned into the expression vector pGAPZA-rDNA using the same methods, thus generating pGrG. Subsequently, the expression cassettes of *SaGGPPS* were integrated into the rDNA locus by homologous recombination.

The IPP isomerase gene (*idi*) was PCR amplified from the genomic DNA of *P. pastoris* GS115 using primers IDI-F-EcoRI and IDI-R (Additional file 1: Table S3), while the *ggpps* fragment was PCR amplified from the plasmid pGrG by primers GGPPS-F and GGPPS-R-*NotI* listed in Additional file 1: Table S3. The *idi-ggpps* fusion gene was constructed by fusing the *ggpps* gene to the 3'-end of

the *idi* gene as follows, and the flexible linker (GGGG)₂ sequence GGTGGCGGTGGCTCGGGCGGTGGTGGGTCG was inserted between the *idi* and *ggpps* genes. These two fragments were purified separately and then fused by overlap extension PCR using the primer pair of IDI-F-*EcoRI* and GGPPS-R-*NotI* (Additional file 1: Table S3), which contained *EcoRI* at the 5'-end and *NotI* at the 3'-end. The *idi-ggpps* fusion gene was digested with *EcoRI* and *NotI* and cloned into multicloning sites of pGAPZA-rDNA as described earlier, and the corresponding plasmid of pGrIG was then constructed.

Transformation and screening of recombinant *HsUBIAD1*-producing *P. pastoris*

The electrocompetent cells of *P. pastoris* GS115 were prepared according to the following protocol. The single colony of *P. pastoris* was grown in 250-mL flasks containing 25 mL of YPD medium and incubated at 30 °C and 250 rpm overnight (16–18 h). 50 mL of fresh YPD medium were inoculated in a 250-mL flask with 0.5 mL of the overnight culture until it reached an OD₆₀₀ of 1.1–1.3. The culture was centrifuged at 1500×g for 5 min at 4 °C, then the cells were suspended in 20 mL of ice-cold, sterile water. The cells were centrifuged at 1500×g for 5 min at 0 °C, then resuspended the pellet with 20 mL of ice-cold sterile water, repeatedly. After three centrifugations, the pellet was resuspend in 5 mL of ice-cold 1 M sorbitol. After centrifugation again at 1500×g for 5 min at 0 °C, the cells were finally suspended in 0.2 mL of ice-cold 1 M sorbitol.

The recombinant vectors pGU and pPU were linearized by the restriction enzymes *BspHI* and *SacI* separately, then electroporated into the disarmed *P. pastoris* GS115 by electroporation. Transformants were selected on MD histidine deficient plates (1.34% YNB, 4 × 10⁻⁵ % biotin, 2% glucose, and 2% agar) containing Zeocin (100 µg/mL), and the presence the of expression cassette was confirmed by colony PCR using pGAP-F/3'AOX1 and 5'AOX1/3'AOX1 primer pairs listed in Additional file 1: Table S3. After initial selection, a range of concentrations (200, 400, 500, 1000 µg/mL) of Zeocin was used to select multi-copy transformants.

Expression of the recombinant *HsUBIAD1*

The colony that was positive for GGU and GPU was cultured for 24 h at 30 °C and 250 rpm in an 18 × 180 mm test tube containing 5 mL of BMGY medium for the expression of recombinant *HsUBIAD1*. After 24 h of cultivation, activated GPU cells were harvested and resuspended in 5 mL of BMMY medium in the same test tube for inductive fermentation at 22 °C for 48 h with the addition 2% methanol every 24 h. The intracellular proteins of recombinant *P. pastoris* were extracted using Yeast

Total Protein Extraction Kit (Sangon Biotech, Shanghai, China). Then, the expression levels of recombinant *HsUBIAD1* were detected by dot-blot to screen the high-yield *HsUBIAD1*-producing strains.

To obtain the optimal expression conditions in *P. pastoris* GS115, the culture temperature ranged from 20 to 30 °C, and the initial pH values ranged from 4 to 9. The recombinant *P. pastoris* was grown at 250 rpm in 250-mL flasks containing 50 mL of BMGY medium, and samples were taken every 12 h during the fermentation. Once the culture was completed, the samples were centrifuged at 13,000×g for 5 min at 4 °C, then the recombinant *HsUBIAD1* was extracted from the wet cell mass using the Yeast Total Protein Extraction Kit. Next, the expression levels were determined by western blot analysis, and ImageJ was used to process and analyze the results with β-actin as the reference. At the same time, another portion of the wet cells after centrifugation was dried in a vacuum freeze dryer for 6–8 h, and the biomass of the recombinant GGU-23 was detected by measuring the dry cell weight (DCW). All values reported are the averages of triplicate trials (± standard error).

Western blot detection of *HsUBIAD1*

The samples were mixed with 5× SDS-PAGE sample loading buffer and boiled for 5 min. Protein bands were separated by SDS-PAGE with a 12% separating gel and a 5% stacking gel, and transferred to nitrocellulose membranes (Beyotime, Shanghai, China). The membranes were incubated with 5% nonfat dried milk in TBS with 0.1% Tween-20 (TBST) for blocking, and then incubated with 6× His-tag mouse monoclonal antibody (1:2500 dilution, EnoGene Biotech, Nanjing, China). After washing with TBST, the membranes were incubated with appropriate horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (1:5000 dilution, EnoGene Biotech, Nanjing, China). Finally, the immunoreactive proteins were detected with BeyoECL Star (Beyotime, Shanghai, China).

Purification of the recombinant *HsUBIAD1*

The intracellular proteins previously extracted were concentrated and subjected to subsequent purification using Ni-NTA affinity chromatography. The crude enzyme solution was purified with a Ni-NTA Sefinose™ Resin Kit (BBI, UK) according to the manufacturer's specifications. Then the eluent was passed through a desalting gravity column (BBI, UK) to remove the salts. The purified proteins of *HsUBIAD1* were frozen and stored at -80 °C in preparation for subsequent experiments.

Enzymatic assay of *HsUBIAD1*

The enzymatic reaction for *HsUBIAD1* was conducted in vitro and the conditions were adjusted according to

the method of Hirota et al. [1]. Reactions were initially performed in 1 mL of 100 mM Tris-HCl (pH 8.0) containing 1 mM DTT, 10 μ M GGPP, 10 μ M VK₁ or VK₃ and 10 mg of *HsUBIAD1* protein. Reaction mixtures were incubated at 37 °C for 1 h, and then stopped by the addition of 1 mL of methanol, and the organic phase was used for HPLC analysis. The activity of *HsUBIAD1* was measured at different pH and temperature values in the presence of different metallic ions, and the initial activity of *HsUBIAD1* was measured without metal ions at pH 8.0 and 37 °C. The reported values of relative activity are the averages of triplicate trials.

Additionally, we analyzed the enzymatic reaction conditions of *HsUBIAD1* in vivo using the whole-cell catalytic system. After 36 h of fermentation, VK₃ (10 mg/L, in methanol) and MgCl₂ (10 mM) were added to the catalysis mixture, which was incubated at 31 °C for to realize the subsequent catalytic reaction. Subsequently, the catalytic products were extracted and detected.

Transformation and screening of high-yield MK-4 producing *P. pastoris*

The recombinant plasmids pGrG and pGrIG were linearized by the restriction enzyme *SpeI* for integration into the rDNA locus, and electroporated into the electrocompetent cells of the high-yield strain GGU-23 to generate GGU-GrG and GGU-GrIG, respectively. Transformants were selected on histidine-deficient MD agar plates containing 500 μ g/mL Zeocin. Then, colony PCR analysis was rapidly performed to identify the presence of the expression cassette using the pGAP-F/3'AOX1 primer pairs listed in Additional file 1: Table S3. To screen the high-yield MK-4 producing *P. pastoris*, the positive transformants GGU-GrG and GGU-GrIG were inoculated and subsequently treated as described previously.

Extraction and analysis of MK-4

To extract a certain amount of MK-4 to be used for analysis, the recombinant *P. pastoris* was cultured according to the previous method. After fermentation and whole cell catalysis, 25 mL of fermented broth were centrifuged at 13,000 \times *g* for 5 min at 4 °C and dried in a vacuum freeze dryer for 6–8 h. The biomass of each recombinant was determined by weighing the freeze-dried cell mass, then methanol (5 mL) was added, followed by static extraction for 2 h. Then *n*-butyl alcohol (5 mL) was added to the supernatants and the extraction mixture was oscillated for 2 h at 150 rpm. The organic phase was centrifuged at 13,000 \times *g* for 10 min and filtered through 0.45 μ m pore organic membranes. Both intracellular and extracellular MK-4 levels were analyzed by high performance liquid chromatography (HPLC). Methanol and dichloromethane were selected to be the mobile phase at a ratio of 4:1

(v/v). The detector was operated at the wavelength of 248 nm for the quantitative detection of MK-4, at which the menaquinones exhibited a strong UV absorption. The contents of MK-4 were calculated from the peak areas based on standard curves of MK-4. Then, the prenylated products were analyzed using a liquid chromatography mass spectrometry (LC-MS) system equipped with LTQ Orbitrap XL ETD analyzer (Thermo Fisher Scientific, USA).

Results

Bioinformatics analysis of *HsUBIAD1*

The analysis results of SOPMA showed that *HsUBIAD1* consists of 49.70% alpha helices, 18.34% extended strands, 5.03% β -turns and 26.92% random coils (Additional file 1: Figure S1). The prediction results of ProtScale showed a GRVAY (Grand average of hydropathicity) value of 0.528, indicating that *HsUBIAD1* is a hydrophobin, which was consistent with the predictions of SOSUI (Additional file 1: Figure S2). In addition, the hydrophobicity plots generated by DNAMAN showed that significant hydrophobic domains appear in the potential transmembrane region predicted by Phobius (Additional file 1: Figure S3). The prediction results of SignalP 3.0 using neural networks (NN) and hidden Markov models (HMM) indicated that native *HsUBIAD1* has no signal peptide or signal anchor. In other words, *HsUBIAD1* is a non-secretory protein. Based on the results of WoLF PSORT, *HsUBIAD1* was predicted to contain a KKXX-like motif (SLPK) in the C-terminus, which was regarded as an endoplasmic reticulum (ER) membrane retention signal.

HsUBIAD1 was identified as a typical multi-spanning integral membrane protein according to the results of 13 transmembrane topology predictors, whereas different analysis software predicted a number of transmembrane helical segments (TMHs) ranging from 7 to 9 (Additional file 1: Table S4). Hence, the exact number of TMHs has not been determined from the current results. On the flip side, the forecasted results of most predictions indicated that the N-terminus of *HsUBIAD1* was located on the cytoplasmic side of the membrane (Additional file 1: Figure S4). And the tertiary structure of *HsUBIAD1* was predicted to contain 9 α -helices generated with the SWISS-MODEL program, using the *ApUbiA* (*Aeropyrum pernix*, 4-hydroxybenzoate octaprenyltransferase) structure as a template (Additional file 1: Figure S5).

The aromatic prenyltransferase (aPT) catalyzes the transfer of the isopentene group into the aromatic nucleus, and can be classified into two types, membrane-bound and soluble, according to bioinformatics analysis. The phylogenetic tree was constructed with the neighbor-joining method based on the Poisson model to show the relationship between *HsUBIAD1* and other aPTs. The

results showed that *HsUBIAD1* is located in the membrane-bound clade rather than the soluble clade. Moreover, *HsUBIAD1* should belong to the *menA* family rather than the *UbiA* family (Fig. 2a).

Next, multiple sequence alignments were carried out among several membrane-bound aromatic prenyltransferases and the results indicated that members of the *menA* family contain two aspartate-rich motifs (NxxxDxxxxxD and DxxxD) (Fig. 2b). Similar to the conserved motifs of the *UbiA* family, the conserved domains of *HsUBIAD1* were presumed to be the recognition site of the isoprenyl side chain.

Construction of the MK-4 biosynthetic pathway in *P. pastoris*

In order to improve the expression level of *HsUBIAD1* in *P. pastoris*, we performed a codon optimization of the original *HsUBIAD1*. After codon optimization, 242 bases were substituted and 210 codons were modified, and the codon adaptation index (CAI) was significantly increased from 0.59 to 0.85, which is considered to be suitable for good expression of *HsUBIAD1* according to the GenScript Web server. The original and codon-optimized synthetic sequences of *HsUBIAD1* are shown in Additional file 1: Figure S6. Furthermore, for efficient initiation of translation, the Kozak sequence (GCCACC) was introduced in front of the initiation codon by primer *UBIAD1-F-EcoRI*.

Moreover, to compare the effects of different promoters on protein expression levels, the expression vectors *pGAPZA* and *pPICZA* were used to produce recombinant *HsUBIAD1*, that contains the constitutive promoter P_{GAP} and the methanol inducible promoter P_{AOX1} , respectively. As shown in Additional file 1: Figure S7a, the PCR product was amplified and cloned into the *EcoRI* and *NotI* sites of *pGAPZA* and *pPICZA*, to generate recombinant *pGU* and *pPU* vectors, respectively. The results of sequencing verified that the presence and correct orientation of the *HsUBIAD1* ORF.

Transformation and screening of recombinant *HsUBIAD1* producing *P. pastoris*

The desired recombinant plasmids were obtained after selection and verification, and 5–10 μ g of plasmid DNA

were linearized and condensed, and resuspended in 10 μ L of ultrapure water, which was electroporated into the electrocompetent cells of *P. pastoris* GS115. The expression vectors *pGAPZA* and *pPICZA* contained the *HIS4* gene and ZeocinTM resistance gene, which can be used as selectable markers for screening of recombinant *HsUBIAD1*. The result of colony PCR and sequencing showed that the expression cassette was inserted successfully into the GS115 genome. The results of the dot-blotting assay showed that the relative expression level of *HsUBIAD1* in recombinant *GGU* was obviously superior to that of *GPU* (Additional file 1: Figure S8). In particular, *GGU-23* (E4) gave 105.8% higher *HsUBIAD1* production, when compared with that of *GPU-23* (E8), which was selected as the high-yield *HsUBIAD1*-producing strain.

Effects of culture conditions on *HsUBIAD1* expression level in recombinant *GGU-23*

The analysis of the western blot results showed a band between 35 and 45 kDa in samples from recombinant *GGU-23* that was not present in the negative control (Fig. 3). And the molecular weight of native *HsUBIAD1* was calculated based on the amino acid sequence as 37 kDa, which means that the exogenous *HsUBIAD1* was successfully expressed in the recombinant *P. pastoris* strains. To improve the expression level of *HsUBIAD1* in recombinant *GGU-23*, the initial pH and culture temperature during the fermentation processes were optimized. The production of *HsUBIAD1* increased by 4.37 times upon after incubation at pH 7.0 and 24 °C for 36 h, when compared with that under the initial conditions (Fig. 4).

Enzymatic assays of *HsUBIAD1*

In this study, the effects of pH, temperature and metal ions on the activity of *HsUBIAD1* in vitro were investigated. After optimization, the most appropriate pH was 7.0 and the most appropriate temperature was 31 °C for MK-4 production in the presence of Mg^{2+} (Fig. 5). Under the optimum conditions, the activity of *HsUBIAD1* was 229% higher than before. The results indicated that metal ions were not required for the synthesis of MK-4 catalyzed by *HsUBIAD1*, but the addition of Mg^{2+} and Na^+ improved the catalytic activity while Fe^{2+} , Ca^{2+} and Mn^{2+} inhibited the prenylation reaction to some extent.

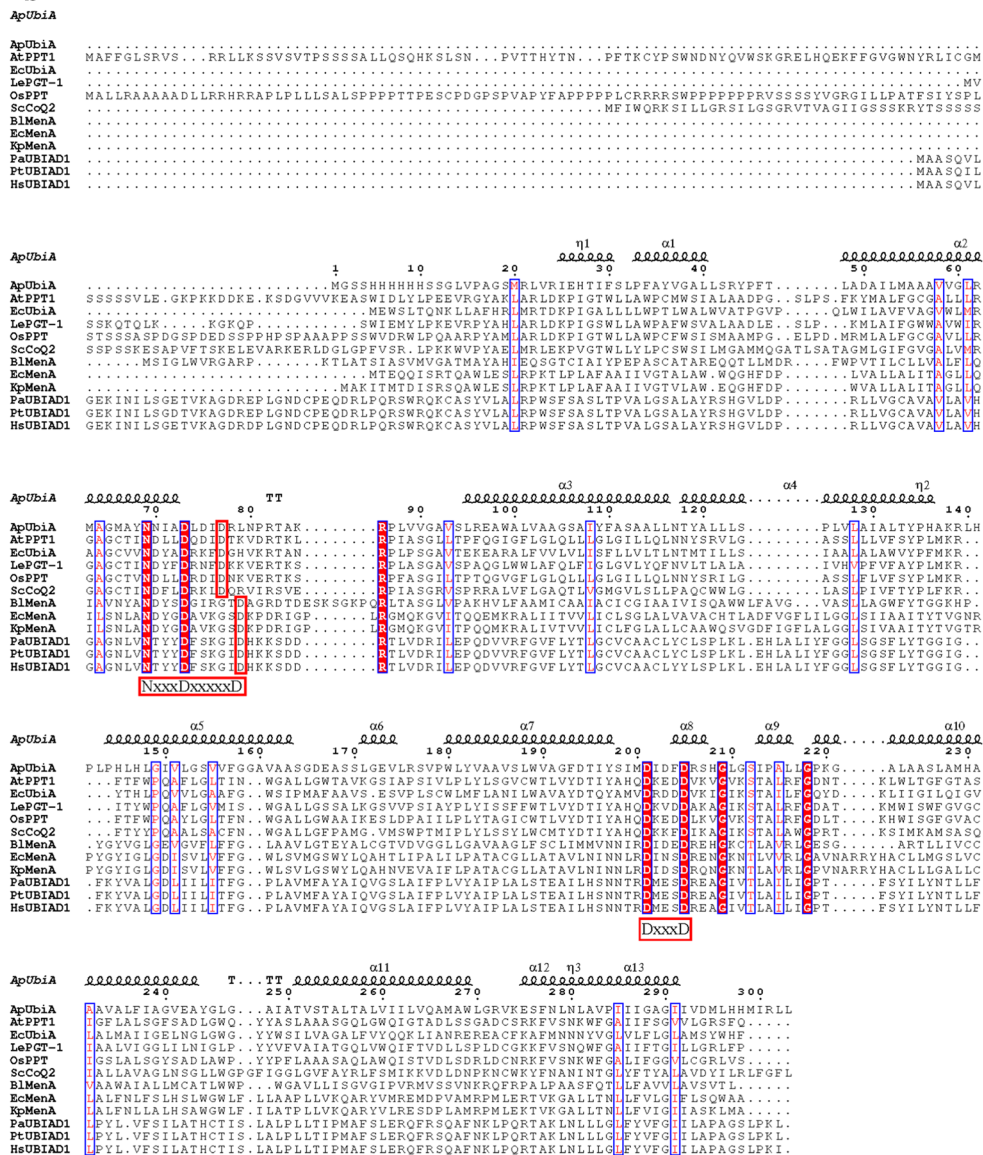
(See figure on next page.)

Fig. 2 Bioinformatic analysis of the aromatic prenyltransferase (aPT) *HsUBIAD1*. **a** Phylogenetic analysis of *HsUBIAD1* with the Mega X program. The phylogenetic tree was divided into two distinct clades: blue, *UbiA* family of membrane-bound aromatic prenyltransferases; green, *menA* family of membrane-bound aromatic prenyltransferases; yellow, soluble aromatic prenyltransferases. **b** Multiple sequence alignment of *HsUBIAD1*. The two conserved aspartate-rich motifs characteristic of the membrane-bound aromatic prenyltransferases, Ap, *Aeropyrum pernix*; At, *Arabidopsis thaliana*; Ec, *Escherichia coli*; Le, *Lithospermum erythrorhizon*; Os, *Oryza sativa*; Sc, *Saccharomyces cerevisiae*; Bl, *Bifidobacterium longum*; Kp, *Klebsiella pneumoniae*; Pa, *Papio anubis*; Pt, *Piliocolobus tephrosceles*; Hs, *Homo sapiens*

a



b



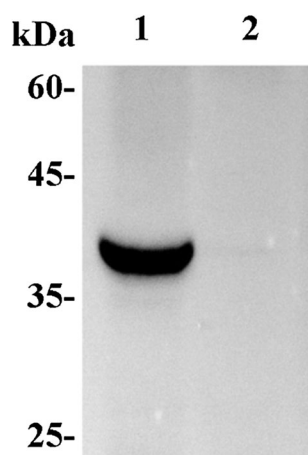


Fig. 3 Western blot analysis of *HsUBIAD1* in recombinant GGU-23. Lane 1, recombinant GGU-23; Lane 2, *P. pastoris* GS115

After 36 h of fermentation, VK_3 was directly added as the prenyl acceptor to the fermentation broth for prolonged cultivation without changing the medium, which was incubated at 31 °C for another 18 h. In this case, it is believed that Mg^{2+} promotes the binding of substrate GGPP to *HsUBIAD1*, although MK-4 could be biosynthesized in the absence of Mg^{2+} .

Improving GGPP supply to increase the yield of intracellular MK-4

To further increase the production of MK-4, we attempted to improve the supply of GGPP by heterologous expression of *SaGGPPS* and fusion expression of *PpIDI* and *SaGGPPS* (Additional file 1: Figure S7b). The expression cassettes were integrated into the rDNA locus rather than the GAP locus to increase the copy number. After transformation, recombinant strains of GGU-GrG and GGU-GrIG were cultured in test tubes prior to screening the high-yield strains for MK-4 production. The results of colony PCR confirmed that either pGrG or pGrIG was present in the genomic DNA of the recombinant GGU-23. According to the HPLC results, a novel prenylated product appeared at 7.1 min compared to wild-type GS115, which was basically consistent with MK-4 standards (Fig. 6a). The mass spectrometric analysis of the prenylated product displayed a peak with $m/z=445$, which corresponds to the hydrogen adduct cation of MK-4 (Fig. 6b). It was confirmed that MK-4 was predominantly biosynthesized intracellularly but was not detected in the supernatant. Furthermore, GGU-GrIG afforded 140% higher MK-4 production, when compared with that noted in GGU-GrG (Fig. 7). The yield of intracellular MK-4 was significantly improved to 0.24 mg/g DCW when additional GGPP was supplied.

Discussion

In recent decades, *P. pastoris* has become a mature and efficient expression system for recombinant exogenous proteins production. Above all, *P. pastoris* is easy to manipulate genetically, quickly grows to very high cell density in a relatively simple and inexpensive medium, and is able to secrete large amounts of recombinant proteins [21]. Furthermore, unlike the prokaryotic expression systems, *P. pastoris* has a eukaryotic post-translational modification system, which can facilitate expression of correctly-folded eukaryotic proteins [18, 42, 43]. And plasmids integrated into the genome by homologous recombination are not easily lost from their host, resulting in enhanced stability of the recombinant strain. However, due to the lack of native pathway for the biosynthesis of the naphthoquinone precursor, *P. pastoris* cannot synthesize menaquinone by itself. Moreover, if the de novo synthesis pathway of naphthoquinone ring is constructed, a series of enzymes need to be heterologously expressed, which involves complex regulation and time-consuming screening processes. Considering various aspects, it is more economically viable to exogenously add the naphthoquinone precursor analog VK_3 into the reaction system than to introduce an exogenous synthetic pathway in *P. pastoris*. Therefore, we attempted to achieve the synthesis of MK-4 by heterologous expression of the key enzyme that catalyzes the polymerization of the naphthoquinone ring and isopentenyl side chain rather than a series of enzymes. In the past, many papers and much research has suggested that *HsUBIAD1* is an efficient MK-4 biosynthetic enzyme that may have both side-chain cleavage and prenylation activities, as it is an aPT homologous to *EcMenA* [1, 2, 4, 6]. Previous studies have also suggested that *HsUBIAD1* should belong to the UbiA family. But with the deepening of research, *HsUBIAD1* is currently considered to belong to the menA family rather than the UbiA family, which is completely consistent with the analysis of the phylogenetic tree in this study. Besides, the discrepancy in prediction results was due to the different algorithms of the different transmembrane topology predictors. *HsUBIAD1* should contain 9 TMHs based on a comprehensive analysis of the transmembrane topology and hydrophobicity. This conclusion was reached for reference only, and the exact transmembrane topology of *HsUBIAD1* remain uncertain. Since the structure and function of proteins are closely related, predicting the physicochemical properties and structure of proteins, and analyzing their genetic relationships can lay a good theoretical foundation for the study of subsequent related protein functions and mechanisms. We confirmed that *HsUBIAD1* is a membrane-bound aromatic prenyltransferase by bioinformatics analysis, which affects the selection of subsequent

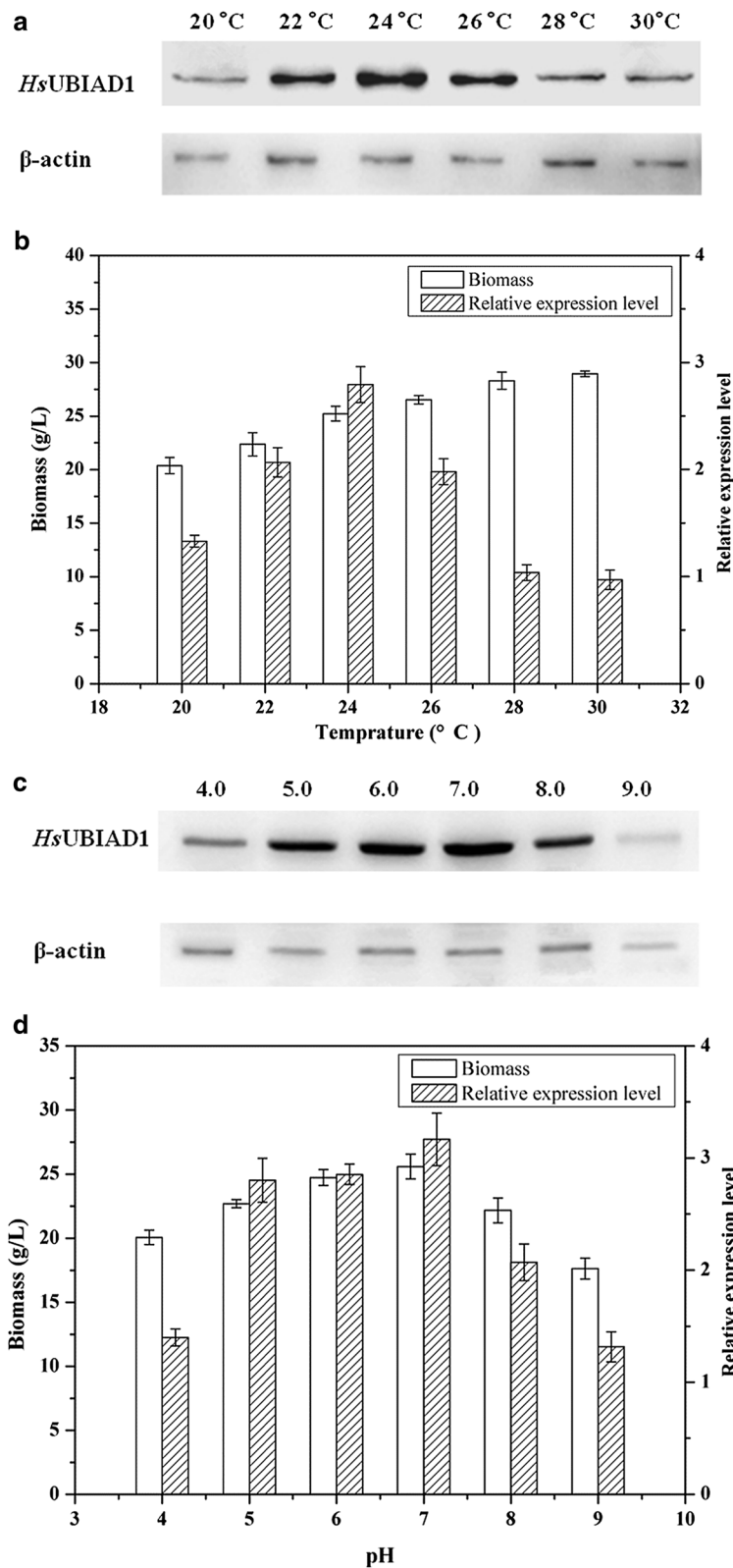


Fig. 4 Effects of culture condition on the expression level of *HsUBIAD1*. **a** Western blot analysis of *HsUBIAD1* at different temperature (at initial pH 6.0, for 24 h); **b** The expression of *HsUBIAD1* at different temperature (at initial pH 6.0, for 24 h); **c** Western blot analysis of *HsUBIAD1* at different initial pH (at 24 °C, for 24 h); **d** The expression of *HsUBIAD1* at different initial pH (at 24 °C, for 24 h); **e** Western blot analysis of *HsUBIAD1* for different time (at initial pH 7.0, at 24 °C); **f** The expression of *HsUBIAD1* for different time (at initial pH 7.0, at 24 °C)

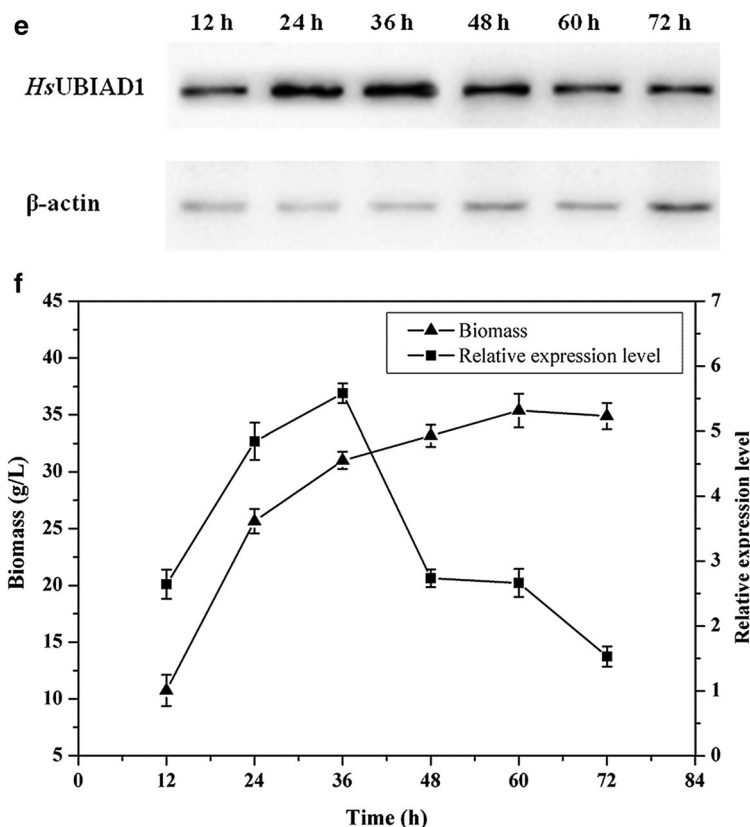


Fig. 4 continued

expression and purification strategies. In this study, we successfully constructed a heterologous expression vector for the efficient expression of *HsUBIAD1* in *P. pastoris*. The recombinant plasmids contain the *HIS4* gene and Zeocin[™] resistance gene as the selectable markers, and the *HsUBIAD1* fragment was integrated into the P_{GAP} locus of the chromosome by homologous recombination. At the same time, we also found that P_{GAP} was a strong constitutive promoter that can be used without methanol induction, which makes it possible to regulate the fermentation process safely and simply. Glycerol was used as the sole carbon source during the whole fermentation process to avoid adding methanol; as it was not necessary to shift cultures from one carbon source to another, strain growth was more straightforward. As an important regulatory element, the performance of the promoter directly affects the transcription level of the gene. It is gratifying that the expression level of *HsUBIAD1* was improved by 105.8% and the fermentation time was shortened by half under the control of P_{GAP} in this study.

On the one hand, the highest yield of *HsUBIAD1* was obtained at pH 7.0, which was suitable for biomass growth of GGU-23. The theoretical isoelectric point (pI) was calculated based on the *HsUBIAD1* amino acid

sequence as 8.4. Thus, it is assumed that the low expression level of *HsUBIAD1* may be caused by the alkaline environment, which may inhibit the correct folding and stability of the *HsUBIAD1* protein. On the other hand, the optimal culture temperature was 24 °C for the expression of *HsUBIAD1*, although higher temperatures were more conducive to the growth of the cells. It is possible that low temperature culture facilitated the expression and proper folding of *HsUBIAD1*. By optimizing the expression conditions, the production of *HsUBIAD1* increased by 4.37 times upon incubation at pH 7.0 and 24 °C for 36 h, when compared to the initial conditions (pH of 6.0 and induced at 28 °C for 24 h). Similar to these results, a low temperature culture has been shown to promote the expression of exogenous proteins in *P. pastoris* based on recent studies [42, 44, 45].

Herein, the results have demonstrated that *HsUBIAD1* expressed in *P. pastoris* has the ability to catalyze the biosynthesis of MK-4. Previous studies have shown that Mg^{2+} plays a key role in binding the side-chain of substrates to *HsUBIAD1* [1, 2]. However, Hirota et al. have reported that Mg^{2+} was necessary for recognizing and activating the short-chain isoprenyl substrates GPP or FPP, while the larger hydrophobic region of GGPP was

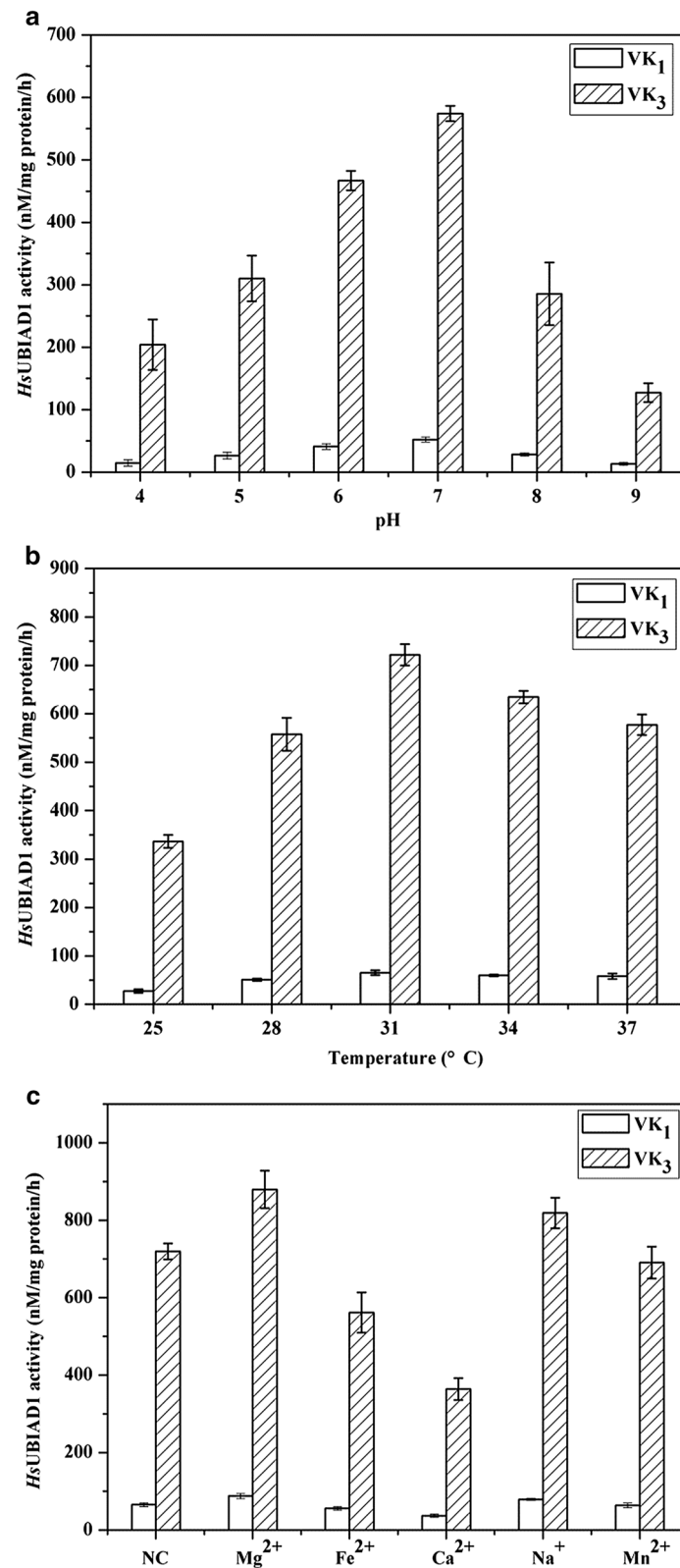
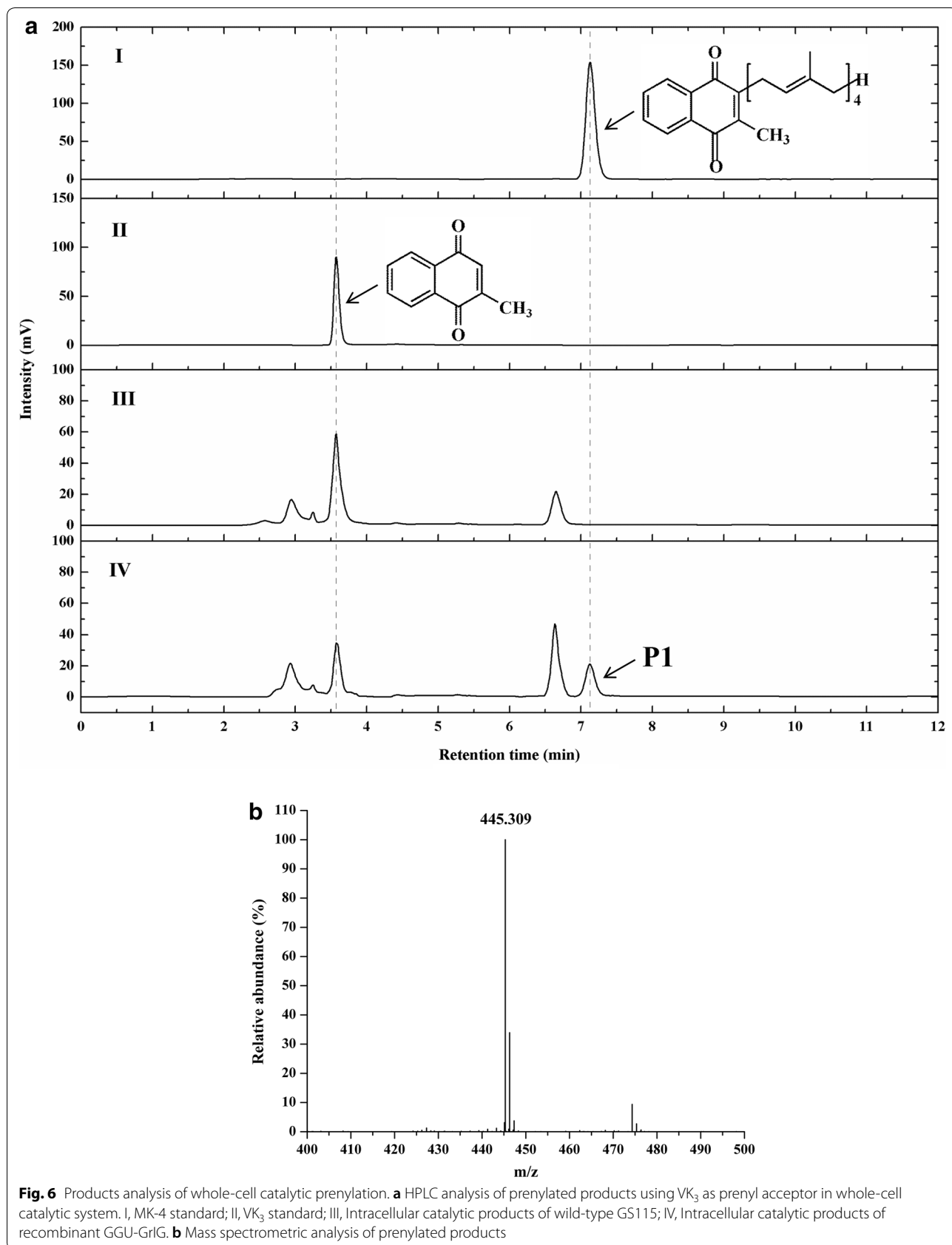
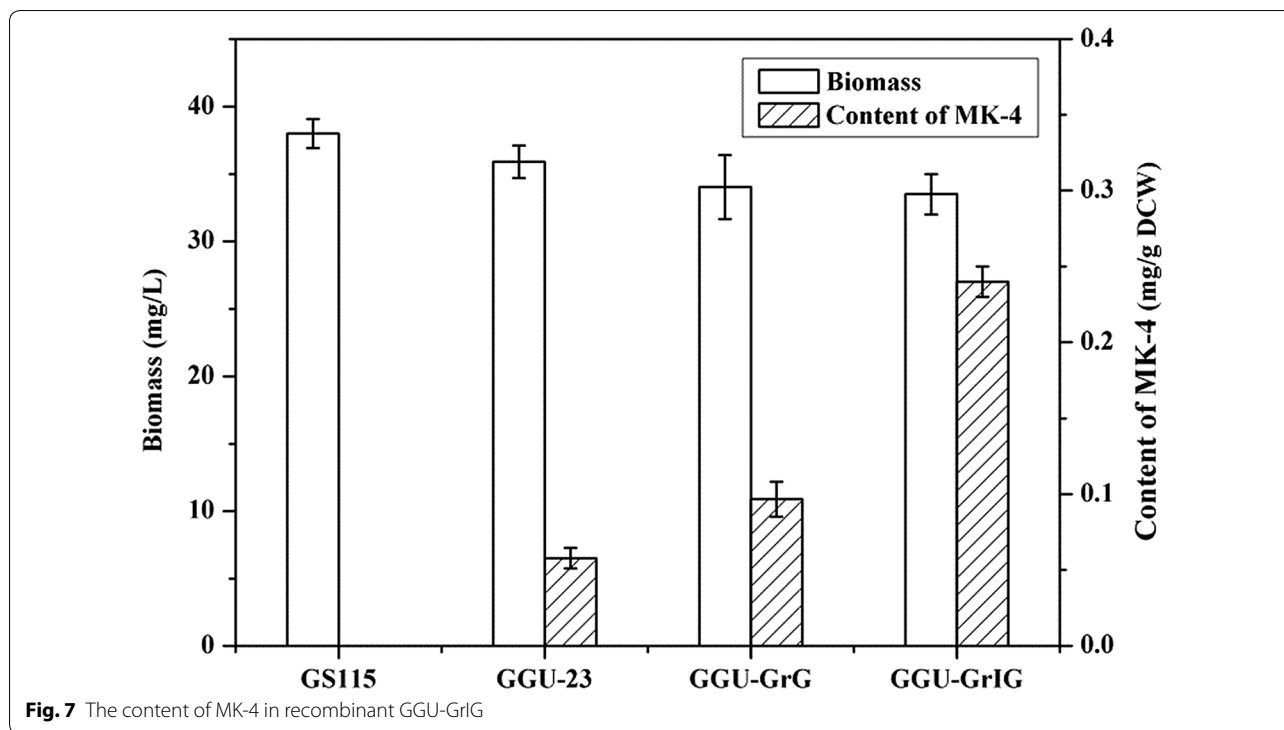


Fig. 5 Effects of reaction condition on *HsUBIAD1* activity using phyloquinone (VK₁) and menadione (VK₃) as the donor of naphthoquinone ring. **a** Effects of pH on *HsUBIAD1* activity (at 37 °C, without metallic ions); **b** Effects of temperature on *HsUBIAD1* activity (at initial pH 7.0, without metallic ions); **c** Effects of metallic ions on *HsUBIAD1* activity (at pH 7.0 and 31 °C), NC, negative control, without metallic ions





directly recognized by *HsUBIAD1* without the participation of Mg^{2+} [6]. In the present study, we found that GGPP could be recognized by *HsUBIAD1* and catalyze prenyl transfer to aromatic substrates with or without the participation of Mg^{2+} . In addition, *HsUBIAD1* displayed higher catalytic activity when VK_3 was used as the donor of a naphthoquinone ring. It is speculated that the isopentenyl side chain could be attached directly to the naphthoquinone ring of VK_3 , while the side chain of VK_1 needs to be removed to release VK_3 .

However, the yield of MK-4 was low, which was caused by the low content of the side-chain substrate GGPP. To improve the intracellular GGPP supply, we attempted to heterologously express *SaGGPPS* in recombinant GGU-23. In theory, there is a dose effect between the productivity of a heterologous protein and the copy number of the target gene [28]. In other words, we could improve the expression level of the protein by increasing the gene copy number within a certain range. In *P. pastoris*, the repeated sequence of rDNA has been used as the integration site of the expression cassettes of the target gene to obtain stable multicopy strains [46]. Similar approaches were used in the present study: we obtained stable multi-copy recombinant strains by integrating the expression cassettes of *SaGGPPS* into the 28S rDNA locus. Many studies have shown that *SaGGPPS* has the ability to biosynthesize GGPP by directly and continuously adding isoprenoid building blocks, which avoids

competition with other branched pathways for FPP [35, 47, 48]. The yield of MK-4 was indeed improved by heterologous expression of *SaGGPPS*. On this basis, we further increased the content of GGPP by expressing *SaGGPPS* fused with *PpIDI*. The purpose of the fusion expression strategy was to narrow the spatial distance between *SaGGPPS* and *PpIDI* and to reduce the steric hindrance of the substrates, which was able to improve the transfer efficiency of the isoprenoid building blocks and to facilitate the accumulation of the key precursor GGPP. Recently, research on multi-enzyme assembly strategies has furnished an attractive and worth-while method for promoting substrate transfer between multiple enzymes. Researchers hoped to achieve cascading amplification of target metabolites by eliminating interference from unrelated metabolic pathways. Other reported microbial strains including *Escherichia coli* and *Bacillus subtilis* have been well-documented in the biosynthesis of MKs. Liu et al. changed the state of the cell membrane in *Escherichia coli*, and the extracellular MK reached 10.71 ± 0.19 mg/L after 120 h of cultivation [49]. Wang et al. improved the redox potential and the state of the cell membrane of *B. subtilis* natto, the MK-7 concentration increased to 43 mg/L in a 5-L bioreactor for 144 h [50]. Ma et al. modified the MEP pathway in *B. subtilis* to enhance the biosynthesis of MK-7, resulting in 50 mg/L MK-7 after 120 h of cultivation [51]. Compared to other reported strains that synthesize MKs by de novo

synthesis, we first achieved whole-cell catalytic synthesis of MK-4 in *P. pastoris*, which took only 54 h. Ultimately, the yield of MK-4 was remarkably enhanced to 0.24 mg/g DCW using VK₃ as the prenyl acceptor.

Conclusion

In this study, we constructed a novel synthetic pathway in *P. pastoris* for the production of MK-4 by heterologous expression of *HsUBIAD1*. As a versatile and efficient expression system, *P. pastoris* has been successfully applied to the stable and efficient expression of *HsUBIAD1*. The effects of different promoters on *HsUBIAD1* expression levels were investigated: P_{GAP} was associated with a higher expression level than that obtained with P_{AOXI}. Moreover, P_{GAP} also offered significant advantages in safety and simplicity. By optimizing the expression conditions, the production of *HsUBIAD1* increased by 4.37 times upon incubation at pH 7.0 and 24 °C for 36 h. Moreover, we potentiated the biosynthesis of the key precursor GGPP in *P. pastoris* by fusion expression of *SaG-GPPS* and *PpIDI*, which improved the polymerization of isoprene units. The content of MK-4 was increased to 0.24 mg/g DCW by improving the GGPP supply when VK₃ was the isopentenyl acceptor. Thus, the concept of metabolic engineering has been successfully applied to the biosynthesis of a novel high value-added prenylated product MK-4 in *P. pastoris*, which has great significance for theoretical research and industrial application. This scheme could be further developed and refined for the biosynthesis of other prenylated products.

Supplementary information

Supplementary information accompanies this paper at <https://doi.org/10.1186/s12934-019-1215-9>.

Additional file 1: Figure S1. Predicted secondary structure of *HsUBIAD1* by SOPMA. **Figure S2.** Hydrophobicity plots of *HsUBIAD1* by ProtScale and DNAMAN. **Figure S3.** The topology of *HsUBIAD1* generated by Protter. **Figure S4.** Tertiary structure prediction of *HsUBIAD1*. **Figure S5.** Sequence comparison between of codon-optimized *HsUBIAD1* and original *HsUBIAD1* genes. **Figure S6.** Construction of recombinant vectors. **Figure S7.** Screening for *HsUBIAD1* high-yield recombinant *P. pastoris*. **Table S1.** Strain used in this study and information. **Table S2.** Plasmid used in this study. **Table S3.** Primers used in this study. **Table S4.** Prediction of transmembrane topology of *HsUBIAD1*.

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Authors' contributions

XS, WN, QY, HW and HT conceived and designed the research, XS carried out the majority of the experiments. XS analyzed the data and wrote the manuscript. HL, PW, LW, GZ and ZZ help to analyzed the data and revised the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

All data supporting the conclusions of this study are included in this article and the additional file.

Ethics approval and consent to participate

This article does not contain any studies with human participants or animals performed by any of the authors.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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