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BIOCATALYSIS - ORIGINAL PAPER

Biotransformation of menadione to its prenylated derivative MK-3 using recombinant *Pichia pastoris*

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Abstract Prenylated quinones, especially menaquinones, have significant physiological activities, but are arduous to synthesize efficiently. Due to the relaxed aromatic substrate specificity and prenylation regiospecificity at the ortho- site of the phenolic hydroxyl group, the aromatic prenyltransferase NovQ from *Streptomyces* may be useful in menaquinone synthesis from menadione. In this study, NovQ was overexpressed in *Pichia pastoris*. After fermentation optimization, NovQ production increased by 1617%. Then the different effects of metal ions, detergents and pH on the activity of purified NovQ were investigated to optimize the prenylation reaction. Finally, purified NovQ and cells containing NovQ were used for menadione prenylation in vitro and in vivo, respectively. Menaquinone-1 (MK-1) was detected as the only product in vitro with γ,γ-dimethylallyl pyrophosphate and menadione hydroquinol substrates. MK-3 at a concentration of 90.53 mg/L was detected as the major product of whole cell catalysis with 3-methyl-2-buten-1-ol and menadione hydroquinol substrates. This study realized whole cell catalysis converting menadione to menaquinones.

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Keywords Aromatic prenyltransferase · *Pichia pastoris* · Menadione prenylation · Chemoenzymatic synthesis · Whole cell catalysis

Introduction

To date, a considerable number of prenylated products with one or more prenyl groups have been isolated from organisms, especially from higher plants and microorganisms. These prenylated products originate from multiple natural product classes, such as the flavonoids, phenylpropanoids, polyketides, and prenylated quinones. Such compounds often possess physiological and pharmacological properties, such as anti-microbial, anti-oxidant, anti-inflammatory or anti-cancer activities, and have been widely used as health care products and drugs [29]. Prenylated quinones (or their derivatives) play a crucial role in cell physiology, in particular electron transfer pathways [2, 21, 35, 50]. Vitamin K is one of the most valuable as it can play a role in hepatic blood anticoagulation, bone health, cardiovascular health, prevention of cancer, suppression of inflammation, prevention of brain oxidative damage, sphingolipid synthesis, osteoporosis and even as a feasible treatment for mitochondrial pathologies such as Parkinson's disease and amyotrophic lateral sclerosis [2, 8, 23, 31, 37, 43].

Natural vitamin K exists in two molecular forms, vitamin K_1 or phylloquinone (PK) and vitamin K_2 or menaquinone (MK). MKs are a series of compounds with the general structure of 2-methyl-3-(poly)prenyl-1,4-naphthoquinone. According to the number of prenyl units contained in the side chain, the forms of MKs are designated as MK-*n*, where "*n*" ranges from 1 to 14 [49]. Vitamin K_3 or menadione lacks a side chain, but is believed to be

biologically active in animals [15]. It can be obtained by chemosynthesis at low cost [6].

MKs are the final active form of vitamin K in the human body. Both PK and menadione need to be converted into MKs before becoming active. Recent research has revealed that all forms of vitamin K, including MKs, are processed by a membrane-associated protein UBIAD1, after being absorbed into the body [27, 28]. This process consists of removal of an integral side chain and prenylation with a new geranylgeranyl chain. Although all forms of vitamin K would be biologically active, MKs have more advantages than PK and menadione in practical use. For example, MKs manifest a much greater activity in blood anticoagulation than PK [36], and menadione has a higher toxicity than MKs and PK, due to reactive oxygen species production and the subsequent induction of cell damage [1, 24]. Therefore, MKs are the most promising and valuable among all forms of vitamin K.

MKs can be obtained by chemical synthesis or bacterial fermentation methods in industrial production. From 1958, methods such as one-flask synthesis and the Grignard reagent and Diels–Alder reactions have been proposed for MKs syntheses, as they afford the advantages of fast and high yields [10, 33, 48]. However, these methods have shortcomings, such as the high expense of the catalysts, production of isomers, and environmental pollution. Bacterial fermentation is an economical and practical method for production of MKs. It has mostly focused on *Flavobacterium* spp. and *Bacillus subtilis* [34, 44]. Although fermentation is associated with low production costs, high biological activity and environmental friendliness, the disadvantages may include a longer synthesis pathway, lower production and higher cost of purification. An enzymatic semi-synthesis of MKs would be advantageous.

In both chemical and microbial syntheses of MKs, the prenylation of the naphthoquinone ring is the critical step. To enhance the fermentation of MKs, the biosynthetic pathways of MKs in bacteria were studied, and MenA, a membrane-associated 1,4-dihydroxy-2-naphthoate (DHNA) prenyltransferase, was found to be a key enzyme [5, 19, 25, 38]. However, the membrane-associated characteristics of MenA are not suited to industrial use. Moreover, prenylated DHNA requires a further enzymatic methylation and oxidation to transform it to MKs. A better MKs synthetic strategy may be enzymatic prenylation of menadione. To achieve this goal, we selected the ABBA family of aromatic prenyltransferases.

The ABBA aromatic prenyltransferase enzyme family is so designated because of the α–β–β–α architecture of the fold in the active site [32]. These enzymes are mainly involved in the biosynthesis of aminocoumarin antibiotics, prenylated polyketides and prenylated phenazines in some actinomyces [51]. CloQ, NphB, SCO7190 and NovQ are family members that have been previously investigated [3, 22, 29, 32, 51]. These intracellular soluble enzymes manifest a relaxed specificity for aromatic molecular substrates due to the spacious aromatic substrate binding pocket in the protein structure [22, 32]. NovQ comes from *Streptomyces niveus* or *S. spheroids*, and catalyzes the biosynthesis of novobiocin, an aminocoumarin antibiotic. It displays relaxed aromatic substrate specificity but prenylation regiospecificity at the ortho- site of the phenolic hydroxyl group [29]. Based on the catalytic performance of NovQ with other substrates and an analysis of possible substituent positioning on menadione, we speculated that, catalytic NovQ menadione prenylation may be a possible approach to obtaining MKs.

In preliminary studies, *S. niveus* showed limited menadione and MKs tolerance of less than 10 mg/L, which meant, it is unsuitable for whole cell catalysis. Construction of an engineered strain for NovQ heterologous expression would be a solution. *Escherichia coli*, *Saccharomyces cerevisiae* and *Pichia pastoris* are three of the most popular hosts for heterologous protein expression. The *S. cerevisiae* system has disadvantages such as low fermentation density and over-glycosylation. Therefore, it is not suitable for the expression of prokaryotic proteins such as NovQ. *E. coli* is a successful system for expressing heterologous prokaryotic proteins. However, we found *E. coli* has limited menadione and MKs tolerance, as did *S. niveus*. *P. pastoris* could be an appropriate host due to its superior ability to express prokaryotic proteins [9] and much higher menadione and MKs tolerance.

The present study was carried out with a focus on establishing a prenylation system in engineered *P. pastoris* for the transformation of menadione to its prenylated derivative MKs.

Materials and methods

Chemicals and reagents

High fidelity *Taq* Plus DNA polymerase (BBI Life Science Co., Shanghai, China) was used for DNA amplification. Restriction enzymes *Xho*I, *Not*I, *Stu*I were purchased from Sangon Biotech Co. (Shanghai, China). Ezfusion homologous recombination enzyme was supplied by Shanghai Generay Biotech. Co. (Shanghai, China). Anti-6× His-tag mouse monoclonal antibody and HRP-conjugated goat anti-mouse IgG were purchased from BBI Life Science Co. (Shanghai, China). Γ,γ-dimethylallyl pyrophosphate (DMAPP), geranyl pyrophosphate (GPP), farnesyl pyrophosphate (FPP), geranylgeranyl pyrophosphate (GGPP), MK-4, levulinic acid and phthalic acid

were purchased from Sigma-Aldrich Co. (Munich, Germany). All other commercially available chemicals and solvents were of analytical or higher grade. Chemical structures of some related compounds are shown in Table 1.

Microorganism strains and vector

Streptomyces niveus (CPCC 240348, China Pharmaceutical Culture Collection, Beijing, China) was used as *novQ* donor. *E. coli* DH5α, *P. pastoris* GS115 and pPIC9 vector (kindly provided by the laboratory of signaling transduction and transcription, University of Science and Technology of China, Hefei, China) were used for the construction of NovQ producing *P. pastoris*.

Construction and screening of recombinant NovQ producing *P. pastoris*

The two end sequences of *novQ* open reading frame (ORF) were not specific enough to obtain a single fragment from the genomic DNA of *S. niveus*. Therefore, a two-step PCR experiment was designed for the *novQ* ORF cloning. 3-kbp fragments containing the *novQ* ORF and the complete *novQ* ORF were successively obtained using the *novQF*/*novQR* and *novQF*′/*novQR*′ primer pairs listed in Table 2, respectively.

The *novQ* ORF was modified by PCR using the *novQ*(pPIC9 XhoI)F/*novQ*(pPIC9 *Not*I)R primers listed in Table 2. The modification included an insertion of 6× His-tag encoding sequences before the termination codon, and an addition of two extension homologous fragments of the up- and downstream sequences of the

Table 1 Chemical structures of related compounds in this manuscript

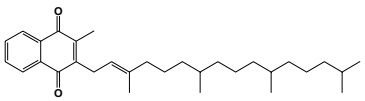
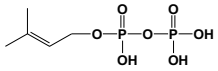
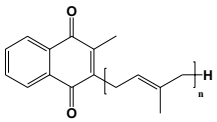
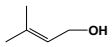
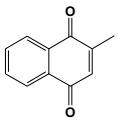
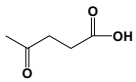
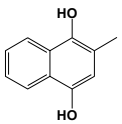
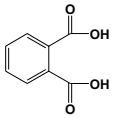
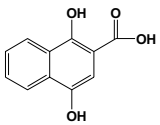
Compound	Chemical structure	Compound	Chemical structure
PK		DMAPP	
MKn		3-methyl-2-buten-1-ol	
menadione		Levulinic acid	
menadione hydroquinol		phthalic acid	
DHNA			

Table 2 Primers used in this study

Primer	Sequence (5′ → 3′)	Primer	Sequence (5′ → 3′)
*novQ*F	GTTCCCACGGTCAAATGCTT	*novQ*R	TAATCGGTGGAGAGGTGGAC
*novQ*F’	ATGCCCGCACTCCCGATG	*novQ*R’	TCATCGGGCACCTCCGGTG
novQ(pPIC9 *Xho*I)F	AGGGGTATCTCTCGAGAAAAGAATGCCCG-CACTCCCGATGAATC	*novQ*(pPIC9 *Not*I)R	AATTAATTCGCGGCCGCTTAATGATGA TGATGATGATGTCGGGCACCTCCGGTG
AOX1(5′)	GACTGGTTCCAATTGACAAGC	AOX1(3′)	GCAAATGGCATTCTGACATCC

insertion site in the pPIC9 vector. The modified *novQ* ORF was then homologously recombined with *XhoI* and *NotI* linearized pPIC9 vector using the EZfusion recombination system. In the recombinant pPIC9-*novQ* vector, ORF encoded 6× His-tag-linked NovQ protein was controlled by alcohol oxidase promotor 1 (AOX1) (Fig. 1). Then *StuI* linearized pPIC9-*novQ* vector was electroporated into *P. pastoris* GS115 cells with a MicroPulser™ Electroporator (Bio-Rad, Hercules, CA, USA).

The *P. pastoris* transformants were selected by MD histidine deficient plates and colony PCR using AOX1(5′)/AOX1(3′) primers listed in Table 2. Each positive colony was induced with BMMY media in 18 × 180 mm test tube at 28 °C, 250 rpm for 48 h. Then, dot-blot of the supernatants was conducted using anti-6 × His-tag mouse monoclonal antibody and HRP-conjugated goat anti-mouse IgG. GpN12 (CCTCC M 2016105) was selected as the relatively highest NovQ yielding strain.

Expression of recombinant NovQ

To optimize NovQ expression, the initial pH, culture temperature, and time of inductive fermentation were investigated. A single colony of GpN12 was cultured in 50 mL MGY histidine deficient media in a 500-mL flask at 28 °C, 250 rpm for 48 h. Then cells were transferred into 50 mL of BMMY media for inductive fermentation. The fermentation was carried out in the same 500-mL flask at 250 rpm with 3% methanol addition per 24 h. For optimization, the initial pH ranged from 4.5 to 8.5, culture temperature ranged from 22 to 30 °C, and induction time ranged from 24 to 168 h. The final biomass of each group of samples was measured by drying and weighing, and the extracellular NovQ level

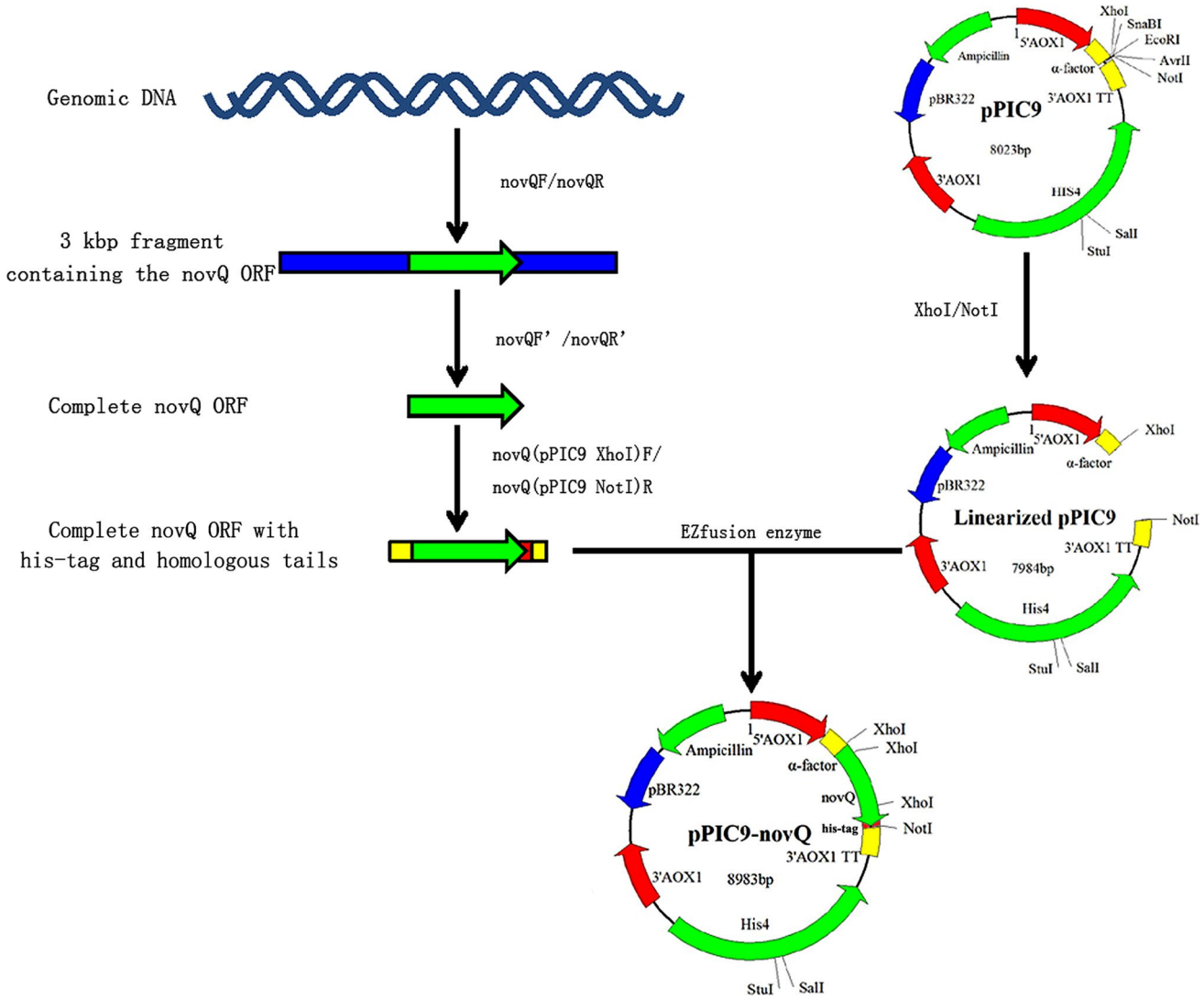


Fig. 1 Construction of recombinant vector pPIC9-*novQ*

was determined by grey level analysis of the western blot, in which anti-6 × His-tag mouse monoclonal antibody and HRP-conjugated goat anti-mouse IgG were used.

Substrate feeding fermentation of GpN12 was carried out in a 30-L automatic fermenter (Biotech-5BGZ-30BS-3000PLC, Shanghai Baoxing Bio-Engineering Equipment co. ltd., Shanghai, China). A single colony of GpN12 was inoculated in a 500-mL flask containing 50 mL MGY histidine deficient media, and cultured at 28 °C, 250 rpm for 48 h as inoculum culture. Next, 1.5 L of the inoculum culture were inoculated into 15 L of BMGY media at a pH of 8.0. The cells grew with glycerol continuous feeding in response to dissolved oxygen level, at 28 °C, 2.5 vvm, 800 rpm for 48 h. Then inductive fermentation was conducted at 25 °C, 5.0 vvm, 800 rpm for 132 h. Methanol was added as the only carbon source and inducer in response to the dissolved oxygen level. Ammonia solution was used to maintain the pH at 8.0. During the whole fermentation, the culture was sampled every 12 h.

Purification of recombinant NovQ

The culture broth from the feeding fermentation was used for NovQ purification. The crude enzyme solution was purified with a Ni–NTA His Bind Resin column (5 mL, 7sea Biotech., Shanghai, China), and then dialyzed extensively against Tris–HCl–$MgCl_2$ buffer (10 mM Tris, 1 mM $MgCl_2$, pH 8.0) to remove salts.

Protein characterization of recombinant NovQ

Western blot using anti-6× His-tag mouse monoclonal antibody and HRP-conjugated goat anti-mouse IgG was conducted to identify the target protein and a prestained protein marker was used to determine the molecular weight. Expasy online tools were used for protein molecular analysis and prediction of posttranslational modification.

Enzyme characterization of recombinant NovQ

According to previous research, NovQ catalyzes prenylation at an aromatic site, but not on the quinone ring. Therefore, in this study, we reduced menadione to the hydroquinol form using KBH_4 to facilitate prenylation at the 3′ site. The reduction was performed as Fu et al. described [7].

A standard prenylation system of 50 μL contained 1 mM menadione hydroquinol, 1 mM DMAPP, 10 mM $MgCl_2$, 50 mM Tris–HCl, and 0.2 g/L purified NovQ. To investigate the effect of pH on NovQ activity, the reaction was conducted in the pH range of 3.0–10.0. To investigate the effect of detergents on NovQ activity, 0.5% (w/v) SDS, CTAB, Triton X-100, Tween 80, Tween 100, or polyoxyethylene 20 oleyl ether (POE-20) was added to the standard synthesis system. To investigate the effects of metal ions, deionized enzyme was prepared by dialyzing extensively against Tris–HCl–EDTA buffer (10 mM Tris, 10 mM EDTA, pH 8.0). Different metal chlorides ($CaCl_2$, $MnCl_2$, $ZnCl_2$, $CuCl_2$, $CoCl_2$, $NiCl_2$, $FeCl_2$, and $FeCl_3$) or EDTA solution were used to replace $MgCl_2$ in the standard system at the same concentration. To investigate the specificity of the side chain donor, GPP, FPP, and GGPP were used to replace DMAPP in the standard system.

The prenylation systems were incubated at 30 °C for 24 h in PCR tubes, under the protection of nitrogen. Then 1 μL of 5 M $FeCl_3$ was added to each tube to oxidize hydroquinol to the quinone form. The oxidation was conducted at 30 °C for 4 h, and then the products were extracted three times with 150 μL of ethyl acetate. The organic phases were combined and subjected to high-performance liquid chromatography (HPLC) and LC-TOF (6224 TOF, Agilent Technologies, Santa Clara, CA 95051, USA) analysis. The relative activity of recombinant NovQ was calculated according to the reduction of menadione (hydroquinol form).

Through an unclear kinase system in both prokaryotic and eukaryotic cells, exogenous isoprenols such as 3-methyl-2-buten-1-ol can be effectively used as precursors for isoprenoid diphosphate production [12, 17, 42, 45]. Therefore, in the whole cell catalysis experiment, 3-methyl-2-buten-1-ol was used as a side chain precursor of low cost. The culture broth of the feeding fermentation was used for whole cell catalysis. Fifty milliliters of broth were accurately adjusted to pH 8.0 and placed into a 250-mL flask. Ten milligrams of menadione hydroquinol and 50 μL of 3-methyl-2-buten-1-ol were added as substrate and 1.5 mL of methanol were added as energy and carbon source for the living yeast cells. $MgCl_2$ (10 mM) and 0.5% POE were added to activate the prenylation. The catalysis mixture was incubated at 25 °C for 24 h, and centrifuged at 4000 g for 10 min to separate the supernatant and cell pellet. Twenty milliliters of ethyl acetate were used to extract the products from the supernatant three times, and the extracts were combined. The cell pellet was lyophilized at −80 °C. Then 30 mL of methanol were used to extract the products from the pellet for 24 h at 25 °C in the dark. All of the extract was used for HPLC and LC-TOF analysis.

Degradation of the prenylation products

The major product of whole cell catalytic prenylation was purified using an LC purification system (218 Purification Systems, Agilent Technologies) with a Pursult XRs 10 C18 column (250 × 21.2 mm, Agilent Technologies). Methanol was used as the mobile phase. The purified product was oxidatively degraded using potassium permanganate

as Hammond et al. described [11], and then analyzed by HPLC.

HPLC and LC-TOF conditions

The products of the prenylation reaction in vitro and in vivo were analyzed by HPLC on a Shimadzu Essentia Lc-6 using a Shim-pack VP-ODS reversed-phase column (250 L × 4.6 mm, Shimadzu) with detection at 248 nm, which was the typical UV absorption of MKs, and a flow rate of 1 mL/min with a mobile phase of methanol with 10% (v/v) dichloromethane. MK-4 was the standard for product quantification. The degradation products of the prenylation products were analyzed at 228 nm and a flow rate of 1 mL/min. Methanol with 0.1% (v/v) methanoic acid was the mobile phase. The molecular analysis was processed by LC-TOF on a 6224 TOF system using eclipse plus a C18 column (Agilent Technologies) at a flow rate of 1 mL/min. Acetonitrile with 0.1% (v/v) methanoic acid was the mobile phase.

Results

Construction and screening of recombinant NovQ producing *P. pastoris*

The sequence of *novQ* ORF obtained in this study (NCBI No.KX579043) displayed 10 mutations compared to the NCBI sequence AF170880.4. However, all the 10 mutations are synonymous mutations.

The recombinant NovQ was expressed in *P. pastoris* GS115 strain, and GpN12 (CCTCC M 2016105) was selected as the relatively highest NovQ yielding strain. NovQ expressed by GpN12 in shaking flask showed double positive bands with molecular weights of 40 and 45 kDa in the western blot (Fig. 2).

Expression of recombinant NovQ

To improve NovQ expression, the initial pH, induction temperature, and induction time were optimized in shaking flask culture (Fig. 3). Fermentation with an initial pH of 8.0 and induced at 25 °C for 96 h was regarded as optimized. Under these conditions, GpN12 produced a dramatic increase in the extracellular NovQ level of 1032%, compared to the initial condition(initial pH of 6.0 and induced at 28 °C for 48 h).

Substrate feeding fermentation based on the optimized shaking flask culture was used to further enhance NovQ expression. The profiles of the biomass and extracellular NovQ content of GpN12 are shown in Fig. 4a. After 48 h of cell growth culture and 96 h of inductive fermentation,

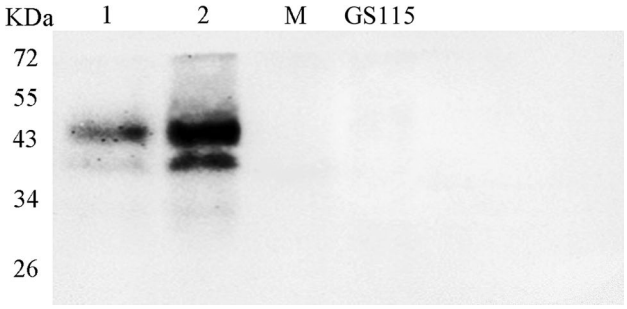


Fig. 2 Western blot analysis of recombinant NovQ in shaking flask culture. Anti-6 × His-tag mouse monoclonal antibody and HRP-conjugated goat anti-mouse IgG were used in this western blot. *Lane M*, Prestained protein marker; *lane 1*, 5 μL supernatant of GpN12; *lane 2*, 20 μL supernatant of GpN12

the biomass of GpN12 reached 112.72 g/L, and the extracellular NovQ level was 51.7% higher than in the optimized shaking flask culture. The western blot results showed that, the secretion of recombinant NovQ started after 24 h of induction and NovQ kept accumulating until 96 h. Compared with the NovQ expressed in shaking flask culture, NovQ from the feeding fermentation supernatant displayed only one positive band of 40 kDa in western blot (Fig. 4b). We also found that, considerable NovQ with a molecular weight of 45 kDa existed inside GpN12 cells after feeding fermentation.

Effect of pH, detergents and metal ions on the activity of NovQ

The effects of pH value, detergents, and metal ions on the activity of NovQ were investigated. The recombinant NovQ presented a higher activity in slightly acidic (pH 3.0–4.0) and alkaline (pH 8.0–9.0) solutions, but less catalytic activity in a neutral environment (Table 3).

Different kinds of detergent had various effects on NovQ activity (Table 4). The anionic detergent SDS strongly inhibited the prenylation reaction, while the cationic detergent CTAB, nonionic detergent Tween-60, and POE-20 significantly activated NovQ. POE-20 had the greatest stimulatory effect on NovQ, and the activity of NovQ in 0.5% POE-20 was 418.9% higher than in the control group.

The effects of metal ions on the activity of NovQ are shown in Table 5. Mg^{2+}, Zn^{2+}, Fe^{2+}, and Mn^{2+} could activate NovQ, while Ca^{2+}, Ni^{2+}, Fe^{3+}, Co^{2+}, and Cu^{2+} inhibited the prenylation reaction. Cu^{2+} almost totally inhibited the reaction by causing precipitation in the reaction system. In the absence of divalent cations but with 10 mM EDTA, recombinant NovQ exhibited considerable activity.

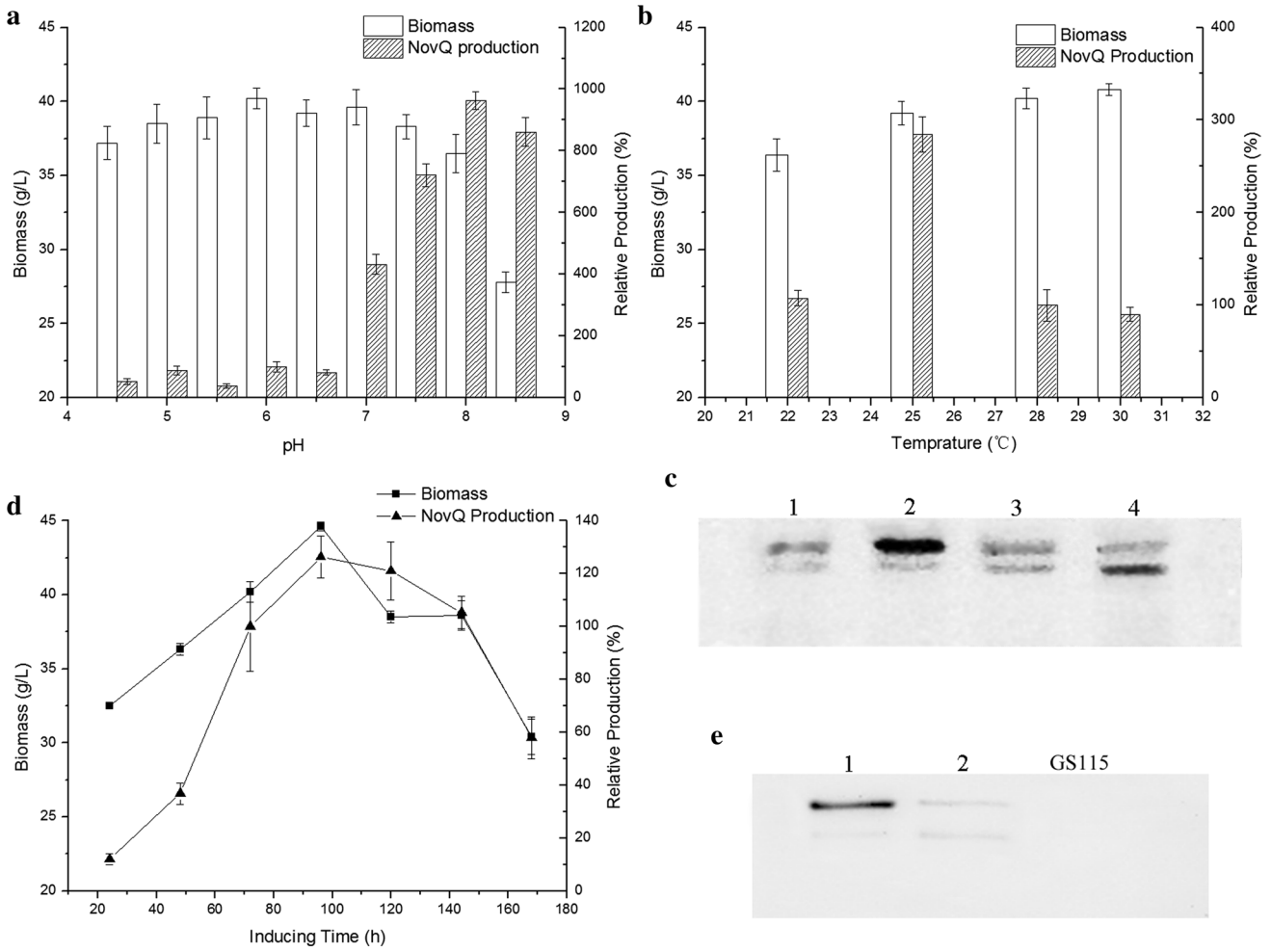


Fig. 3 NovQ fermentation of GpN12 at different initial pH values, induction temperatures and induction times. **a** NovQ fermentation of GpN12 at different initial pH values. **b** NovQ fermentation of GpN12 at different induction temperatures. **c** Western blot analysis of extracellular NovQ produced at different induction temperatures. Anti-6 × His-tag mouse monoclonal antibody and HRP-conjugated goat anti-mouse IgG were used in this western blot. *Lane 1*, recombinant NovQ expressed at 22 °C; *lane 2*, recombinant NovQ expressed at 25 °C; *lane 3*, recombinant NovQ expressed at 28 °C; *lane 4*, recombinant NovQ expressed at 30 °C. **d** NovQ fermentation of GpN12 with different induction times. **e** Western blot analysis of NovQ produced in initial and optimized shaking flask culture. Anti-6 × His-tag mouse monoclonal antibody and HRP-conjugated goat anti-mouse IgG were used in this western blot. *Lane 1*, NovQ produced in optimized shaking flask culture (initial pH 8.0, 10% volume of liquid, 250 rpm, 3% methanol addition per 24 h, 25 °C inducing for 96 h); *lane 2*, NovQ produced in initial shaking flask culture. The extracellular NovQ level in the initial group (initial pH 6.0, 10% volume of liquid, 250 rpm, 3% methanol addition per 24 h, 28 °C induction temperature for 72 h) was taken as 100%

Enzymatic prenylation of menadione

When menadione hydroquinol and recombinant NovQ were incubated with DMAPP, GPP, or FPP in vitro, respectively, the formation of prenylation products could be detected, while if GGPP or the NovQ enzyme was heat-denatured, these products were absent (Fig. 5a). The products were analyzed by LC-TOF (Fig. 5c). Cations with an $m/z = 241.14$, $m/z = 309.22$, and $m/z = 377.26$, were obtained with DMAPP, GPP, and FPP, which were consistent with the predicted m/z ratio of a hydrogen adduct cation of single prenylated menadione or MK-1 ($m/z = 241.14$), double prenylated menadione or MK-2 ($m/z = 309.18$), and triple prenylated menadione or MK-3 ($m/z = 377.24$), respectively. All the three products displayed a strong UV absorption around 248 nm, which was typical of menaquinones.

GpN12 cells containing NovQ were used for whole cell catalysis. In preliminary experiment, GpnN12 cells dead when pH decreased from 8.0 to 5.0. Therefore, we chose pH 8.0 instead of pH 3.0, which was optimum for purified NovQ, as a whole cell catalysis parameter. After incubating with menadione hydroquinol, methanol, and 3-methyl-2-buten-1-ol, one prenylation product and three prenylation

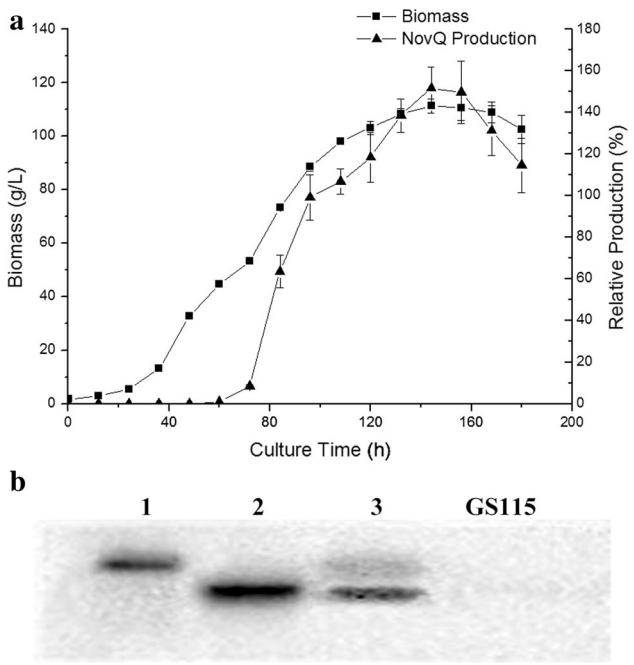


Fig. 4 NovQ fermentation of GpN12 in substrate feeding fermentation. **a** The NovQ fermentation profile of GpN12 in the substrate feeding fermentation. Extracellular NovQ in optimized shaking flask culture (initial pH 8.0, 10% volume of liquid, 250 rpm, 3% methanol addition per 24 h, 25 °C induction temperature for 96 h) was taken as 100%. **b** Western blot analysis of NovQ produced in shaking flask culture and feeding fermentation. Anti-6 × His-tag mouse monoclonal antibody and HRP-conjugated goat anti-mouse IgG were used in this western blot. *Lane 1*, Intracellular NovQ produced during feeding fermentation; *lane 2*, Extracellular NovQ produced during feeding fermentation; *lane 3*, Extracellular NovQ produced during shaking flask culture

Table 3 NovQ activity at different pH values

pH	Relative activity (%)[a]	pH	Relative activity (%)[a]
3	100 ± 2.6	7	37.8 ± 3.3
4	86.7 ± 4.8	8	87.3 ± 1.0
5	47.6 ± 2.7	9	84.7 ± 4.2
6	31.3 ± 5.2	10	54.1 ± 4.1

The activity was measured in the presence of 10 mM $MgCl_2$ without detergent

[a] The activity of NovQ at pH 3.0 was taken as 100%. All determinations were repeated three times

products could be detected in the supernatant and the cell pellet, respectively (Fig. 5b). These products were absent from incubation with GS115 control, except for a small peak at 9.8 min close to a huge peak at 9.6 min in the GpN12 group. Molecular masses of the products were analyzed by LC-TOF (Fig. 5c). The analysis of products from the supernatant revealed a peak with $m/z = 241.14$, consistent with

Table 4 Effects of detergents on NovQ activity

Detergent	Relative activity (%)[a]	Detergent	Relative activity (%)[a]
Control	100 ± 1.1	Tween-60	371.1 ± 12.1
SDS	0 ± 0.2	POE-20	518.9 ± 22.6
Triton-100	70.4 ± 2.8	CTAB	221.7 ± 18.5

The activity was measured in the presence of 10 mM $MgCl_2$ and pH 8.0

[a] The activity of NovQ in the control group without detergent was taken as 100%. All determinations were repeated three times

Table 5 Effects of metallic ions on NovQ activity

Metallic ion	Relative activity (%)[a]	Metallic ion	Relative activity (%)[a]
Mg^{2+}	185.4 ± 2.0	Fe^{3+}	62.4 ± 3.9
Ca^{2+}	78.6 ± 6.3	Mn^{2+}	125.8 ± 4.1
Zn^{2+}	126.9 ± 2.6	Cu^{2+}	0 ± 0.4
Ni^{2+}	67.1 ± 5.1	Co^{2+}	20.6 ± 1.1
Fe^{2+}	140.9 ± 3.2	EDTA	100 ± 3.5

The activity was measured without detergent at pH 8.0

[a] The activity of NovQ in the presence of EDTA without metal ions was taken as 100%. All determinations were repeated three times

the hydrogen adduct cation of single prenylated menadione or MK-1. Analysis of products from GpN12 cells generated three peaks with $m/z = 241.14$, $m/z = 309.22$, and $m/z = 377.26$. The peak with $m/z = 241.14$ was consistent with the hydrogen adduct cation of single prenylated menadione or MK-1 ($m/z = 241.14$). The peak with $m/z = 309.22$ was consistent with the hydrogen adduct cation of geranylated menadione or MK-2 ($m/z = 309.18$). The peak with $m/z = 377.26$ was consistent with the hydrogen adduct cation of farnesylated menadione or MK-3 ($m/z = 377.24$). The peak at 9.8 min in the GS115 group generated a cation peak with $m/z = 705.59$, which was not consistent with any form of MK.

Oxidative degradation was used for structure analysis of the prenylated product (Fig. 5d). Phthalic acid was obtained as a characteristic oxidation product of 1,4-naphthoquinone from all three prenylated products. Levulinic acid was obtained from double and triple prenylated products as a characteristic oxidation product of polyprenyl groups such as the geranyl and farnesyl group.

According to the positioning groups analysis for this enzymatic electrophilic aromatic substitution and oxidative degradation analysis, the products with $m/z = 241.14$, $m/z = 309.22$, and $m/z = 377.26$ were identified as MK-1, MK-2, and MK-3, respectively. Among the three prenylated products from the cell precipitation, the concentration of MK-3 reached 90.53 mg/L using MK-4 as

a standard, which was much higher than that of the other two prenylated products.

The HPLC analysis of both supernatant and cell pellet from the whole cell catalysis mixture showed that the menadione substrate mostly remained in the supernatant while less than 20% (menadione itself and its derivatives) could be detected in the cell pellet.

Discussion

Western blot of NovQ expressed by GpN12 in shaking flask showed double positive bands. The molecular weight of the 40 kDa band was consistent with NovQ obtained from recombinant *E. coli* [29]. We calculated the molecular weight of unmodified recombinant NovQ using Expasy online tools, and found the molecular weight of recombinant NovQ with α-factor was 45.1 kDa, which was consistent with the 45 kDa band we observed.

To improve expression, the initial pH, induction temperature, and induction time were optimized in shaking flask culture. The highest NovQ yield was obtained at pH 8.0 rather than pH 6.0, which was best for GpN12 growth. Similar results were obtained by Hu et al., who found the best pH for methionine adenosyltransferase expression in recombinant *P. pastoris* was 8.0, while the best pH for cell growth was 6.0 [14]. They ascribed the higher protein productivity in a slightly alkaline environment to enhancement in protein stability. In this study, the gene donor *S. niveus* grew much better in slightly alkaline media than in media with a pH value below 7.0. An acidic environment may inhibit correct folding of the NovQ protein or decrease protein stability. We calculated the isoelectric point (pI) of unmodified recombinant NovQ protein, and found it is 5.44. It is possible that the acidic NovQ protein may aggregate in a slightly acidic environment, affecting the folding or secretion process.

In this study, recombinant NovQ was better expressed at 25 °C rather than 28 or 30 °C, when GpN12 grew better. Low temperature cultivation has frequently been reported to improve the expression of extracellular heterologous proteins [4, 13, 18, 26, 39, 53]. The increase in target protein expression might be due to an enhanced protein folding pathway at lower temperatures, as well as increased cell viability [52]. Moreover, relatively high temperatures may activate extracellular proteases and reduce the stability of the target protein [4, 13, 18]. Western blot results showed that, as the induction temperature increased, the optical density ratio between the 45 and 40 kDa bands decreased. This result indicates that hydrolase activity might increases as the induction temperature is raised.

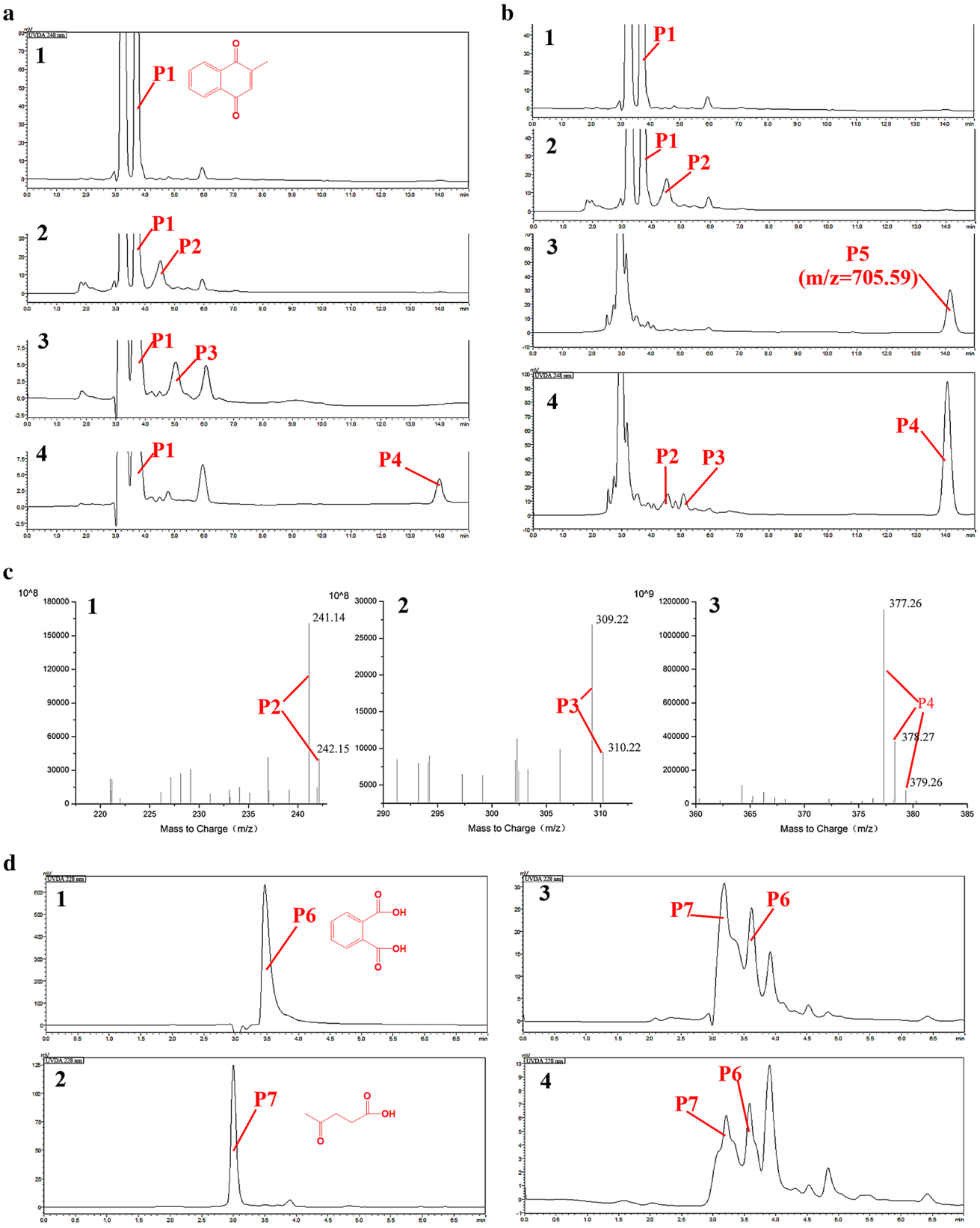
Both the biomass and extracellular NovQ peaked at 96 h in shaking flask experiments, and then decreased due to cytolysis and protein degradation. Further studies may focus on the enhancement of cell viability and protein stability. As a result of shaking flask optimization, inductive fermentation at an initial pH of 8.0 and 25 °C for 96 h was optimal for NovQ production by GpN12.

In substrate feeding fermentation experiments, the western blot of the supernatant showed only one positive band of 40 kDa. This might be due to the higher concentration of Kex2 protease produced by GpN12, which catalyzes the removal of α-factor from recombinant protein. Compared to shaking flask culture, GpN12 showed a much higher NovQ production and biomass. However, cytolysis and protease release still occurred after induction for more than 4 days. Fermentation strategies for enhancing GpN12 cell viability may be a research direction for NovQ production.

According to the pI analysis and posttranslational modification predictions, the unmodified NovQ is an acidic protein with a pI value of 5.44, while the pI value of the signal peptide digested NovQ was 6.04. In addition, five possible phosphorylated sites exist in the signal peptide digested NovQ. This suggests that recombinant NovQ protein may be electrically neutral and less polar in an acidic environment, and the affinity between NovQ and a fat-soluble substrate such as menadione hydroquinol might increase. Furthermore, the ionization and polarity of substrate DMAPP and menadione hydroquinol decreased in an acidic environment as well. As a result, the slightly acidic environment might promote contact among the reaction substrates and the catalyst NovQ. The high activity of NovQ in alkaline solution could be explained by dissociation of the pyrophosphoric group from DMAPP in an alkaline environment, which would contribute to the prenylation reaction.

Some detergents enhanced the NovQ prenylation, especially POE-20. This result is in agreement with Tani Yoshiki's study, in which the addition of POE played a crucial role in detergent-supplemented fermentation of MKs [46, 47]. Yoshiki attributed the successful enhancement of MKs production to the fact that POE could induce excretion of MKs, which would counteract the inhibitory effect of intracellular MKs on cell growth and MK production. Based on the activation effect of POE-20 on NovQ in vitro, it could be inferred that, POE-20 might activate the prenylation by interacting with the active center of the prenyltransferase or with the substrates in a special mechanism.

Metal cations had different effects on NovQ prenylation activity. Mg^{2+} not only exhibited the strongest activation of NovQ in this study, but also did so for other ABBA prenyltransferases by taking part in the active centers [22, 30]. In our study, Ca^{2+} did not activate NovQ as Mg^{2+} does, and even partly inhibited prenylation. This result was different from that of Pojer's study, in which 2.5 mM Ca^{2+} activated CloQ 1.8-fold. This discrepancy might be due to a difference in posttranslational modification or the protein folding environment of *P. pastoris*. In the absence of divalent


a
1
P1
2
P1
P2
3
P1
P3
4
P1
P4
b
1
P1
2
P1
P2
3
P5
(m/z=705.59)
4
P2
P3
P4
c
1
241.14
P2
242.15
Mass to Charge (m/z)
2
309.22
P3
310.22
Mass to Charge (m/z)
3
377.26
P4
378.27
379.26
Mass to Charge (m/z)
d
1
P6
OH
OH
2
P7
OH
3
P7
P6
4
P6
P7

◂**Fig. 5** Product analysis of enzymatic menadione prenylation. **a** HPLC analysis of enzymatic menadione prenylation in vitro. 1, HPLC analysis of enzymatic menadione prenylation with DMAPP and denatured NovQ; 2, HPLC analysis of NovQ catalytic menadione prenylation with DMAPP; 3, HPLC analysis of NovQ catalytic menadione prenylation with GPP; 4, HPLC analysis of NovQ catalytic menadione prenylation with FPP. **b** HPLC analysis of whole cell catalytic menadione prenylation. 1, HPLC analysis of supernatant from GS115 control catalytic prenylation; 2, HPLC analysis of supernatant from GpN12 catalytic prenylation; 3, HPLC analysis of cell pellet from GS115 control catalytic prenylation; 4, HPLC analysis of cell pellet from GpN12 catalytic prenylation. **c** TOF–MS analysis of menadione prenylation products. 1, TOF–MS analysis of the P2 peak; 2, TOF–MS analysis of the P3 peak; 3, TOF–MS analysis of the P4 peak. **d** HPLC analysis of oxidative degradation products. 1, HPLC analysis of standard phthalic acid; 2, HPLC analysis of standard levulinic acid; 3, HPLC analysis of oxidative degradation products of standard MK4; 4, HPLC analysis of oxidative degradation products of the P4 peak

cations but with 10 mM of EDTA, recombinant NovQ exhibited considerable activity. This might be explained by the fact that, the catalytic activity of NovQ is independent of metal cations. Similar results were obtained by Ozaki et al., who found that recombinant NovQ could catalyze the prenylation of 4-hydroxyphenylpyruvate (4-HPP) independently of divalent cations to yield 3-dimethylallyl-4-HPP [29]. Pojer et al. studied the enzymatic characteristics of CloQ, a prenyltransferase with the highest homology with NovQ, and found it retains 25% of its original activity without divalent cations but with 2.5 mM of EDTA. The difference in effects on enzyme activity between Fe^{2+} and Fe^{3+} may due to the oxidation–reduction potential. Fe^{3+} is a specific oxidant or catalyst, which could oxidize hydroquinol or phenol to quinol and remove the aromaticity of the substrates [40]. Ozaki et al. suspected that the transformation of an aromatic substrate between keto and enol forms could prevent NovQ catalyzed prenylation [29].

Based on the LC-TOF analysis, information about the number of prenyl units connected to menadione was obtained. However, information about the positions of prenyl units on menadione was not clear. Only one prenylated product peak with $m/z = 241.14$ was obtained from the NovQ catalytic prenylation mixture in vitro, indicating that, if the single prenylation of menadione did not prevent a further prenylation, recombinant NovQ would catalyze the prenylation at a unique site or at least at a preferred site on menadione. Therefore, the product peaks with $m/z = 309.22$, and $m/z = 377.26$ from the whole cell catalysis were more likely to be geranylated and farnesylated menadione, rather than double or triple prenylated menadione with multiple prenylation sites. Related research has been conducted by Kumano et al., who analyzed the structural basis of ABBA prenyltransferase NphB and the kinetics of the prenylation reaction [20, 22]. They found an ABBA prenyltransferase catalytic prenylation process, in which the prenylation was described as an electrophilic aromatic substitution: during the prenylation, (poly) prenyl pyrophosphate and aromatic substrate bound to the active center of enzyme; (poly) prenyl pyrophosphate lost the pyrophosphate group and formed a carbocation; the carbocation electrophilically attacked the aromatic substrate to form a prenylated compound [44]. In a menadione hydroquinol molecule, there are three positioning groups on the same ring of naphthalene, two α hydroxyl groups and one β methyl group. All of them belong to ortho-positioning electron-donating groups, which could increase the electron cloud density and activate the substituted ring. According to substituent positioning effects, the 3′ site of menadione hydroquinol would be the most probable site for electrophilic substitution.

Oxidative degradation was performed for further product structure identification. Phthalic acid was detected as a degradation product of all prenylated menadiones. That meant that prenylation only occurred on the substituted ring of menadione. The empty 3′ site of menadione is the only choice for NovQ catalytic prenylation. Levulinic acid was detected as a degradation product of both double and triple prenylated menadione, confirming that the side chain forms were geranyl and farnesyl groups, respectively. Therefore, products with $m/z = 241.14$, $m/z = 309.22$, and $m/z = 377.26$ were regarded as MK-1, MK-2, and MK-3, respectively.

The major isoprenoid quinone in wild *P. pastoris* is CoQ-8 [41]. That means, *P. pastoris* has a complete synthesis pathway for polyprenyl groups, and can synthesize polyprenyl groups with different isoprene units (no more than eight) from DMAPP and isopentenyl pyrophosphate. In the NovQ catalytic prenylation in vitro, DMAPP was the optimum side chain donor, short polyprenyl such as GPP, and FPP were also accepted, while GGPP was not. NovQ showed a preference for short polyprenyl donors rather than long polyprenyl donors, which is in agreement with earlier research on ABBA prenyltransferases [20, 29, 30, 51]. The prenyl donor specificity of recombinant NovQ was studied by Ozaki et al. [29]. In their study, NovQ from engineered *E. coli* manifested a preference for the DMAPP substrate rather than GPP or FPP. Curiously, in this study, among the three prenylated products from the cell precipitation, the abundance of MK-3 was much higher than those of MK-1 or MK-2. This might be related to the higher concentration of farnesyl pyrophosphate among all of the short (poly)prenyl pyrophosphates in yeast cells [16]. Moreover, the broader specificity of the prenyl donor in recombinant NovQ may be a result of posttranslational modification in *P. pastoris*, such as phosphorylation or glycosylation.

The HPLC analysis results for both the supernatant and cell pellet from the whole cell catalysis mixture showed that, menadione substrate mostly remained in the supernatant, while less than 20% (menadione itself and the

derivatives) can be detected in the cell pellet. It could be inferred that, in whole cell catalytic prenylation, the transmembrane alignment of menadione might be an obstacle. Further studies could be focused on menadione modification, addition of detergents, and alteration of the permeability of GpN12 cells to enhance the cell catalysis efficiency.

Conclusion

In conclusion, the prenyltransferase NovQ was overexpressed in *P. pastoris*. After fermentation optimization, the NovQ production increased by 1617% in substrate feeding fermentation (pH 8.0, methanol continuous feeding at 25 °C for 96 h) compared to the initial shaking flask culture. Different effects of metal ions, detergents, and pH on purified NovQ were investigated, and Mg^{2+} and POE-20 displayed strong activation. Purified NovQ prenyltransferase and cells containing NovQ could be successfully used in menadione prenylation, and MK-1, MK-2, and MK-3 were detected as products. The concentration of the major product MK-3 in the whole cell catalysis system reached 90.53 mg/L. This study expands the application of NovQ prenyltransferase and provides a possible new approach to production of MKs.

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