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ScienceDirect
Journal of Biotechnology 128 (2007) 120–131
Journal of BIOTECHNOLOGY
www.elsevier.com/locate/jbiotec

Phenotypes and fed-batch fermentation of ubiquinone-overproducing fission yeast using *ppt1* gene

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Received 8 July 2006; received in revised form 12 September 2006; accepted 13 September 2006

Abstract

Ubiquinone (UQ), a component of the electron transfer system in many organisms, has been widely used for pharmaceuticals and cosmetics. In this study, we cloned and overexpressed the full-length *ppt1* (MTppt1) gene, which encodes *p*-hydroxybenzoate:polyprenyltransferase and ERppt1 gene, which was modified to be localized on endoplasmic reticulum in fission yeast. The yeast MTppt1 and ERppt1 transgenic lines showed about 3.7 and 5.1 times increment in UQ content and the recombinant yeasts with a higher UQ level are more resistant to H_2O_2, Cu^{2+} and NaCl, and interestingly their growth was also faster than the wild type at lower temperature. For large-scale cultivation, the direct feedback control of glucose using an on-line ethanol concentration monitor for ubiquinone production of yeast ERppt1 by high-cell-density fermentation was investigated and the fermentation parameters (e.g., dissolved oxygen, pH, ethanol concentration, oxygen uptake rate, carbon dioxide evolution rate and respiration quotient) were also discussed. After 90 h cultures, the yeast dry cell weight reached 57 g l^{-1} and the ubiquinone yield reached 23 mg l^{-1}. In addition, plasmid stability was maintained at high level throughout the fermentation.

Keywords: Cloning; Subcellular localization; Fission yeast; Oxidative stress; Fed-batch fermentation; Ubiquinone-10

1. Introduction

Ubiquinone (UQ) is a lipid component of the membrane-bound electron transport system in both prokaryotes and eukaryotes, and has been successfully used as the therapy for various diseases (Eerrante et al., 2002; Muller et al., 2003). The enzymes identified by the previous works involved in UQ biosynthesis are found to be localized in mitochondria (Marbois et al., 2005; Okada et al., 2004, 1998; Uchida et al., 2000), and it is generally believed that UQ biosynthesis take place in the mitochondria.

UQ biosynthesis consists of 10 sequential steps including methylation, decarboxylation, hydroxylation, and isoprenoid transfer (Meganathan, 2001). In UQ biosynthesis, prenylation which is the condensation reaction between the 4-hydroxybenzoate and isoprenoid side chain is presumed to be the

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doi:10.1016/j.jbiotec.2006.09.012

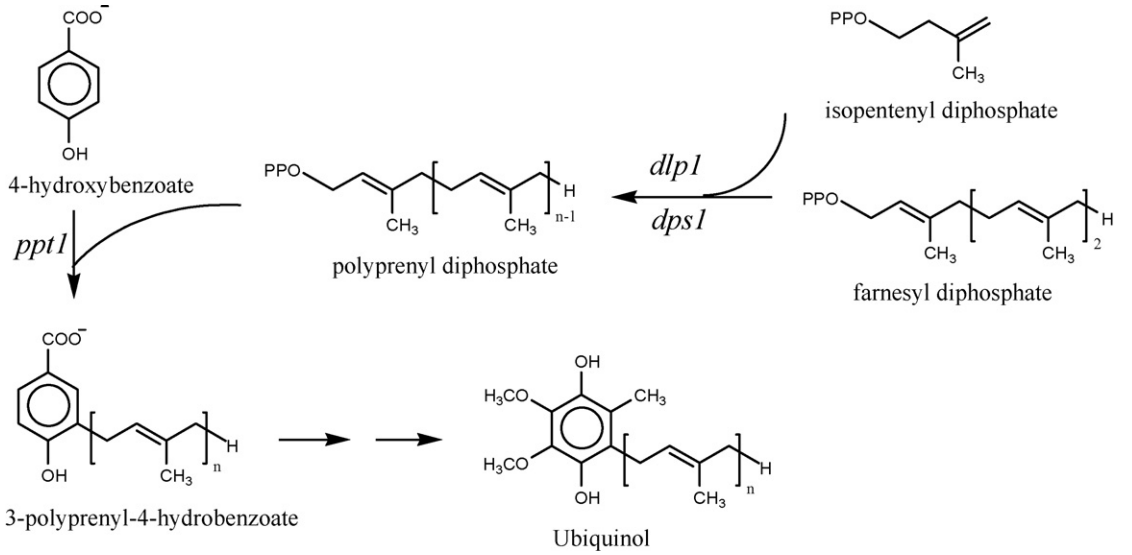

Fig. 1. Ubiquinone biosynthesis pathway in *S. pombe*.

rate-limiting step (Fig. 1), and is catalyzed by 4-hydroxybenzoate:polyprenyl diphosphate (4-HB:PPP) transferase, encoded by *ubiA* gene in *E. coli* (Siebert et al., 1992), by *coq2* gene in *Saccharomyces cerevisiae* (Ashby et al., 1992) and by *ppt1* gene in *Schizosaccharomyces pombe* (Uchida et al., 2000). *E. coli ubiA* gene and *S. cerevisiae coq2* gene seems to have higher activity when fused with both N-terminal and C-terminal signal peptides for ER targeting in *Lithospermum erythrorhizon* (Boehm et al., 2000) and tobacco (Ohara et al., 2004), respectively. 4-HB:PPP transferase is a membrane-bound enzyme, and has been reported to accept a broad array of polyprenyl diphosphates (PPP) as side chain precursors (Suzuki et al., 1994; Okada et al., 1998).

Ubiquinone not only plays a vital role as a component of the electron transfer system but also acts as an antioxidant, which was investigated in many previous reports (Frei et al., 1990; Ernster and Dallner, 1995; Grant et al., 1997). A strain of *S. pombe* unable to produce ubiquinone because of a deficiency of decaprenyl diphosphate synthase or Dlp1 protein is sensitive to H_2O_2 and requires an antioxidant to grow on glucose-containing medium (Suzuki et al., 1997; Saiki et al., 2003b). Similarly, another *S. pombe* which does not produce ubiquinone is also sensitive to H_2O_2 and Cu^{2+}, suggesting that ubiquinone protects against oxidants (Saiki et al., 2003a). However, there are few reports on the phenotypes of the yeasts containing higher ubiquinone content.

Fed-batch fermentation is generally applied to achieve a more efficient production of intracellular products by *S. cerevisiae* (Shang et al., 2006a,b). Ethanol concentration can be measured effectively for the feedback control of glucose-feeding rate and high concentration of ethanol during fermentation is unfavorable for the growth of yeast cells. On-line analysis of glucose and indirect parameters, such as oxygen uptake rate (OUR), carbon dioxide evolution rate (CER) and respiratory quotient (RQ), during fermentation are also important variables, which require careful regulation. An efficient production of bakers' yeast has been traditionally performed by applying an indirect feedback control of RQ data to control glucose-feeding rate (Mendoza-Vega et al., 1994). Yeast expression systems are commonly used for the production of industrial and pharmaceutical proteins. *S. pombe* possessing many merits, is a popular model system for producing a variety of proteins and is also expected to establish a high-cell-density fed-batch culture system to achieve large-scale cultivation. So *S. pombe* fed-batch culture should be studied and the application of datum such as OUR, CER, RQ and ethanol concentration as sensitive control variables requires a careful design.

In the present study, we overexpressed PPT1 on mitochondria and ER in *S. pombe*, separately. The yeasts with higher ubiquinone content were more resistant to oxidants than the wild types. In an attempt to study for a large-scale production of UQ-10, fed-batch fermentation was performed using the recombinant yeasts and the fermentation parameters were studied.

2. Materials and methods

2.1. Materials

Restriction enzymes and other DNA-modifying enzymes were purchased from Takara Bio, Inc. (Dalian China) and Beijing Sunbiotech Co. Ltd. (PR China). Ubiquinone-10 was purchased from Sigma Chemical Co. All other chemicals were of analytical grade from standard suppliers.

2.2. Strains, vectors and media

E. coli DH5α was used as a host for cloning experiments and pMD18-T (Takara) as a cloning vector. *E. coli* DH5α was grown in LB liquid medium (10 g l^{-1} bacto-tryptone, 5 g l^{-1} bacto-yeast extract, 10 g l^{-1} NaCl) or on LB agar, supplemented with 100 μg ml^{-1} ampicillin, whenever required, to maintain the plasmids. Isopropyl-β-D-thiogalactopyranoside (IPTG) (Sigma) and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) (Sigma) were added to the culture media whenever required (Sambrook and Russel, 2001).

The fission yeast Leu^- strain SPQ-01 and the pESPM vector used for the *ppt*1 gene expression were kindly provided by Dr. Xia (Wang et al., 2004), the Chinese Academy of Sciences, Beijing, China. Yeast cells were grown in YE (5 g l^{-1} yeast extract, 30 g l^{-1} glucose) or EMM minimal media (Invitrogen). YEA and EMMA contain 150 mg l^{-1} of adenine in YE and EMM, respectively. The concentration of the supplemented Leu was 225 mg l^{-1}.

2.3. General methods of molecular biology manipulations

Plasmid DNA was isolated by using a mini-prepared DNA Purification System (Omega). Generation of DNA by the polymerase chain reaction (PCR), restriction enzyme digestion, agarose gel electrophoresis, ligation and transformation were carried out according to standard protocols (Sambrook and Russel, 2001).

2.4. Preparation of genome DNA

Yeast cells, grown aerobically in YEA medium with Leu at 30 °C, were harvested in the late log phase and the chromosomal DNA was isolated according to the method of Sambrook and Russel (2001).

2.5. Construction of expression cassettes for yeast

The PCR primers of full-length *ppt1* gene (MTppt1) carrying a natural mitochondrial-targeting signal at the N-terminus were designed from the reported *S. pombe* *ppt1* gene with its genomic DNA as template (Uchida et al., 2000). The forward primer sequence was S1, 5′-atat ctcgag atg ataa tta agcc tat agc-3′ containing *Xho*I site, and the site also followed the start codon region. The reverse primer sequence was S2 5′-aatt ggatcc ttaa gaa tcgt aaa taa agg-3′ containing *Bam*HI site and harboring the stop codon region of *ppt1* gene. Oligonucleotides were synthesized in the Applied Biosystems DNA Synthesizer at Beijing Sunbiotech Co. Ltd. PCR program was performed on a Thermocycler (MJ Research PTC-200, USA), and consisted of: 1 cycle at 94 °C for 5 min, 30 cycles at 94 °C for 30 s, 54 °C for 30 s, and 72 °C for 1 min 10 s, as well as a final extension step of 72 °C for 7 min. PCR products were analyzed by 0.8% (w/v) agarose gel electrophoresis. After a recovery by a gel extraction kit (Omega), the *ppt1* gene was cloned into the pMD18-T vector. The resulting recombinant plasmid pMD18-T-ppt1 was then transformed into competent *E. coli* DH5α. On LB agar plate containing IPTG, X-gal and 100 μg ml^{-1} ampicillin, white colonies were selected as positive transformants, and were further identified by plasmid extraction and PCR. The nucleotide sequence of the *ppt1* gene was verified by the dideoxyribonucleotide chain termination method at Beijing Sunbiotech Co. Ltd.

To express PPT1 protein in *S. pombe*, plasmid pMTppt1 was constructed. pMD18-T-ppt1 was digested with *Xho*I and *Bam*HI, and the resulting DNA fragment, after a recovery from agarose gel, was ligated into the corresponding sites of vector pESPM. The recombinant plasmid pMTppt1 was used to transform *S. pombe*.

To localize the PPT1 polypeptide on the ER membrane, an ER signal of the *S. pombe* BiP gene (Alison and John, 1992), which was ligated to the pMD18-T named pMD18-T-ER by *Xho*I site was synthesized by the Applied Biosystems DNA Synthesizer at Beijing Sunbiotech Co. Ltd. Then, the ER peptide signal was added to the 5′-end of the truncated *ppt1*, which was cloned into the pESPM to yield pERppt1. The truncated *ppt1* was amplified by PCR with the primers S3, 5′-a ctcgag atat ttc aagc ggtt aaaa tc-3′ containing *Xho*I site, and S4, 5′-a ggatcc tta aag ttc atc ggc a gaa tcgt aaa taa agg attt-3′ containing *Bam*HI site and also harboring the stop codon region and an ER retention signal (ADEL). PCR program was performed on a Thermocycler (MJ Research PTC-200, USA), and consisted of: 1 cycle at 94 °C for 5 min, 30 cycles at 94 °C for 30 s, 57 °C for 30 s, and 72 °C for 1 min, as well as a final extension step of 72 °C for 7 min. The PCR product was inserted to pMD18-T to yield pMD18-T-tppt1.

2.6. S. pombe transformation and screening

Expression vectors with the MTppt1 and ERppt1 gene were transformed into *S. pombe*, respectively, by electroporation (Prentice, 1991) and transformants were selected on EMM minimum nutrient agar plates with thiamine at 30 °C for 3 days. Cells from the selected clones were further determined by plasmid extraction and PCR.

2.7. Transcriptional expression analysis of MT-type and ER-type ppt1 gene in S. pombe

Total RNA was isolated with Yeast RNA Mini Kit (BioDev, China). One microgram of RNA in a total volume of 20 μl was used for each RT reaction, which was carried out at 42 °C for 1 h and chilled on ice for 2 min. The product was used as template for PCR.

MTppt1 primers were 5′-atg ataa tta agcc tat agc-3′ and 5′-a gaa tcgt aaa taa agg att-3′. ERppt1 primers were 5′-atat ttc aagc ggtt aaaa-3′ and 5′-aag ttc atc ggc a gaa tc-3′. As a control, primers of tubulin gene (5′-ggtcactaca cagaaggtgc-3′ and 5′-aaatcagagt tcaattggcc-3′) were used. The PCR was carried out with 23–30 cycles and the products were separated on a 1% agarose gel.

2.8. Extraction and measurement of ubiquinone

Ubiquinones extracted from *S. pombe* recombinants were analyzed by the method of Shan et al. (2001) and Qiu et al. (2004) with modifications. Cells treated by 3 M HCl were stirred at 90 °C for 80 min. After addition of NaOH solution to maintain the pH at 7, the cell pellet was extracted at room temperature for 2.5 h with acetone. The mixture was centrifuged and the upper layer of the solution was subjected to partial pressure distillation at 80 °C to evaporate to dryness and the residue was redissolved in ethanol. The ubiquinone was analyzed by HPLC on a C18 reverse-phase column (250 mm × 4.6 mm, Beifen Tianpu Technology Co. Ltd., Beijing, China) with ethanol as the mobile phase at a flow rate of 1 ml min^{-1}. Ubiquinone was detected at 275 nm.

2.9. Shake flask cultivation of the recombinants

The selected recombinant cells, MTppt1 and ERppt1, were grown to mid-exponential phase in minimal medium supplemented with 18 μM thiamine at 30 °C, and then cells were washed three times with the EMMA minimal medium without thiamine to derepress the *nmt1* promoter. Cells were further cultured at 30 °C in EMMA medium, and cell pellets were collected at apposite intervals to test for ubiquinone content. Cells with empty pESPM vector were used as a control.

2.10. Cultivation in bioreactor

Firstly, 300 ml yeast cells harboring pERppt1 plasmid was grown at 30 °C in EMMA medium with thiamine for 20–24 h. Secondly, the culture was centrifuged at 4000 × *g* for 10 min to collect the cell and the cell pellet, which was used as inoculum was resuspended in 50 ml EMMA medium without thiamine. Fermentation experiments for yeast cells were performed at 30 °C in a 5 l bioreactor (Biotech-5BG; Shanghai Baoxing Bioengineering Equipment, Shanghai, China) using 3 l initial complex medium (30 g l^{-1} glucose, 0.1 g l^{-1} $MgSO_4 \cdot 7H_2O$, 0.04 g l^{-1} Na_2HPO_4, 0.4 g l^{-1} KH_2PO_4, 4 g l^{-1} $(NH_4)_2SO_4$, 1 mg l^{-1} calcium pantothenate, 0.1 mg l^{-1} KI, 0.4 mg l^{-1} $MnSO_4 \cdot H_2O$, 0.5 mg l^{-1} $ZnSO_4 \cdot 7H_2O$, 0.15 mg l^{-1} $FeCl_3 \cdot 6H_2O$,

0.05 mg l^{-1} $CuSO_4 \cdot 5H_2O$, 0.2 g l^{-1} $CaCl_2 \cdot 2H_2O$, 0.5 mg l^{-1} H_3BO_3, 1 mg l^{-1} citric acid·H_2O, 15 mg l^{-1} nicotinic acid, 10 mg l^{-1} inositol, 4×10^{-5}% biotin, 0.1 g l^{-1} adenine). The feeding solution (600 g l^{-1} glucose, 20 g l^{-1} $(NH_4)_2SO_4$, 4 g l^{-1} KH_2PO_4, 5 g l^{-1} NaCl, 1.5 g l^{-1} $MgSO_4 \cdot 7H_2O$, 0.04 g l^{-1} Na_2HPO_4, 0.02 g l^{-1} $CaCl_2 \cdot 2H_2O$, 4×10^{-4}% biotin, 10 mg l^{-1} calcium pantothenate, 1 mg l^{-1} KI, 4 mg l^{-1} $MnSO_4 \cdot H_2O$, 5 mg l^{-1} $ZnSO_4 \cdot 7H_2O$, 1.5 mg l^{-1} $FeCl_3 \cdot 6H_2O$, 0.5 mg l^{-1} $CuSO_4 \cdot 5H_2O$, 0.5 g l^{-1} adenine) was added into the reactor using the feedback control system and pH was controlled between 4.5 and 5.5 by the automatic addition of 25% ammoniacal liquor and 10% H_2SO_4. Air-flow rate was maintained at 3 l min^{-1} during the batch period and shifted to 10 l min^{-1} for the fed-batch periods. The stirrer speed was increased from 300 to a maximum of 800 rpm, whenever necessary, to compensate for the increasing oxygen demand. The dissolved oxygen was measured using an autoclavable O_2 sensor (Mettler Toledo, Greifensee, Switzerland).

2.11. Analysis of fermentation parameters

Cell-density was determined by measuring the OD of the culture at 600 nm with a UV–vis spectrophotometer. Higher OD samples were diluted suitably to have an absorbance in the range of 0.2–0.6. Centrifuging the sample broth at 4000 × *g* for 20 min and drying the washed cell to a constant weight at 105 °C determined dry cell weight.

The residual glucose concentration in the medium was measured by an enzyme electrode biosensor of glucose and lactic acid (SBA-40C, Biology Institution of Shandong Academy of Science, Jinan, China). Ethanol concentration was determined on-line using an ethanol analysis instrument (East China University of Science and Technology, Shanghai, China). For on-line analysis, the oxygen and carbon dioxide contents of the exhaust gas produced from fermentation were analyzed using an exhaust gas analyzer (LKM2000-03; Lokas Automation Corp., Teajeon-City, South Korea). OUR, CER and RQ were calculated by following equations (Shang et al., 2006b):

$$\mathrm{OUR} = \frac{F_{\mathrm{in}}}{V}\left[C_{O_{2\mathrm{in}}} - \frac{C_{\mathrm{in}}^{\mathrm{interia}}\, C_{O_{2\mathrm{in}}}}{1-(C_{O_{2\mathrm{out}}}+C_{CO_{2\mathrm{out}}})}\right] \quad (1)$$

$$\mathrm{CER} = \frac{F_{\mathrm{in}}}{V}\left[\frac{C_{\mathrm{in}}^{\mathrm{interia}}\, C_{CO_{2\mathrm{out}}}}{1-(C_{O_{2\mathrm{out}}}+C_{CO_{2\mathrm{out}}})} - C_{CO_{2\mathrm{in}}}\right] \quad (2)$$

$$\mathrm{RQ} = \frac{\mathrm{CER}}{\mathrm{OUR}} \quad (3)$$

where F_{in} indicates inlet gas flux (mol h^{-1}); V the fermentation liquid volume (l); $C_{\mathrm{in}}^{\mathrm{interia}}$, $C_{O_{2\mathrm{in}}}$, and $C_{CO_{2\mathrm{in}}}$ the concentrations of inert gas, oxygen and carbon dioxide in inlet gas, respectively, %; $C_{O_{2\mathrm{out}}}$ and $C_{CO_{2\mathrm{out}}}$ indicate the concentrations of oxygen and carbon dioxide in outlet gas, respectively, %.

Plasmid stability was measured by direct plating of appropriately diluted culture onto selective EMM and nutrient-rich YEA agar plates supplemented with Leu, and was calculated by taking the ratio of the average number of colonies from three selective plates to the average from three nutrient-rich plates.

3. Results

3.1. Cloning of ppt1 gene and construction of ER-localized ppt1 gene

It is generally believed that UQ biosynthesis enzymes are localized in the mitochondria because their full-length polypeptide sequences, including PPT1 peptide, have natural mitochondrial signal peptides (Uchida et al., 2000), but some studies have reported PPT activity in microsomal fractions (Kalen et al., 1990; Ohara et al., 2004). We, therefore, investigated localizing membrane-bound PPT1 polypeptide in different subcellular compartments to see the effect on UQ production. The MT-signal peptide of *ppt1* gene was removed and the ER-signal of the *S. pombe* BiP gene and the ER-retention signal ADEL sequence were fused to the N- and C-termini in the truncated *ppt1* gene to yield ERppt1 for the precise targeting and retention of this polypeptide at the ER membrane (Fig. 2). ERppt1 gene, as well as full-length *ppt1* gene (MTppt1), was then subcloned in a shuttle vector under the thiamine repressible *nmt1* promoter (Siam et al., 2004).

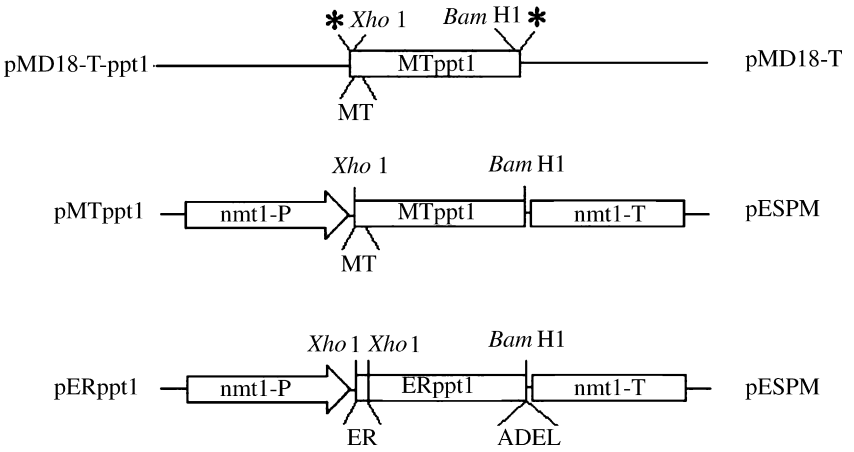

Fig. 2. Expression cassettes of PPT1 were designed to target the mitochondria (MTppt1) or ER (ERppt1) in a shuttle vector for yeast expression. These *ppt1* genes were driven by a nmt1 promoter for fission yeast. MT, natural mitochondrial signal peptide of PPT1. ER, signal peptide for ER-targeting from *S. pombe* BiP gene. ADEL, ER-retention signal.

3.2. Transcriptional expression analysis of ppt1 gene in S. pombe

A series of yeast lines were identified by using an approach of quantitative RT-PCR analysis. Different specific primers summarized in Section 2 were used to check the transcripts of exogenous MT-type and ER-type *ppt1* gene in all recombinant yeast cells. The results showed that transcriptional messages of the exogenous MTppt1 genes amplified by MTppt1 primers at 23 PCR cycles were detected in transformed MTppt1 yeast lines, but none is presented in yeast lines with an empty vector (Fig. 3). The same result of transcriptional information of ERppt1 gene amplified by ERppt1 primer at 23 PCR cycles was also obtained (Fig. 3). When PCR was performed using MTppt1 primers for 30 cycles, *ppt1* gene was also amplified to a detectable level in the yeast strain with empty vector, and all the transgenic lines showed much higher expression levels (Fig. 3).

3.3. Shake flask cultures of the transgenic yeast cells

To test the effect of PPT1 peptide in yeast cells, a system of *S. pombe* MTppt1, ERppt1, as well as the empty pESPM vector as a control, was used in the experiment. The cultures were performed as described in Section 2 and UQ contents were standardized by HPLC. HPLC analyses showed that the wild-type and recombinant yeasts contained solely UQ-10 as UQ (data not shown). There was no difference in the growth pattern of MTppt1, ERppt1 and the pESPM cells in EMMA at 30 °C (Fig. 4). However, removal of thiamine from the medium after 24 h, the maximal UQ10 contents in MTppt1 and ERppt1 obtained were 3.7 and 5.1 times higher than the control pESPM vector, respectively (Fig. 4).

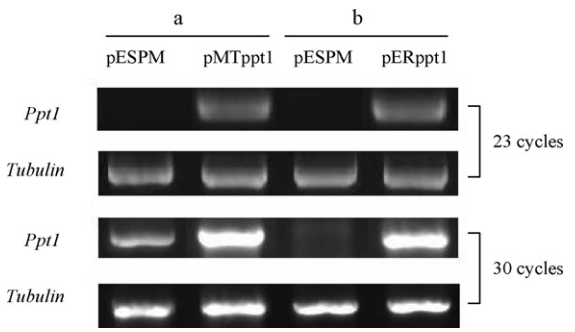

Fig. 3. Expression of pMTppt1 and pERppt1 gene in *S. pombe*. mRNA was detected using quantitative RT-PCR with tubulin as an internal control. a and b was performed by MTppt1 and ERppt1 primers, respectively. The PCR products of pMTppt1, pERppt1, and tubulin gene was about 1074, 1020, and 440 bp, respectively.

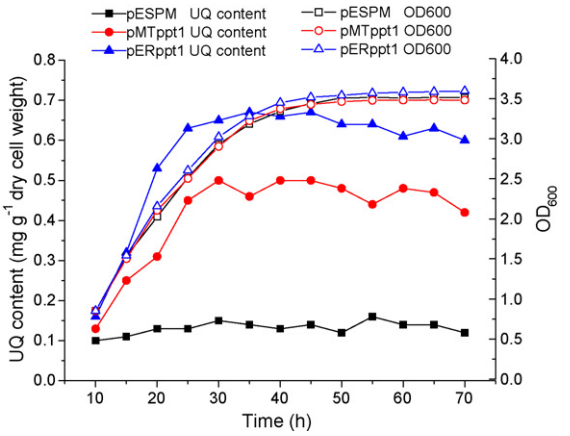

Fig. 4. UQ content of fission yeast containing plasmids of pESPM, pMTppt1 and pERppt1, respectively, in shake flask culture. pESPM, wild-type cells with empty vector pESPM; pMTppt1, mitochondria-targeting clone; pERppt1, ER-targeting clone.

3.4. Phenotype analyses of UQ10-overproducing yeasts

It was previously reported that KS10 (*Δdps1:ura4*), a strain of *S. pombe* whose decaprenyl diphosphate synthase encoding *dps1* gene has been disrupted, and NU609 (*Δppt1::ura4*), a strain of *S. pombe* whose 4HB:PPP encoded by *ppt1* gene has been disrupted, is unable to produce ubiquinone and have some notable additional phenotypes (Suzuki et al., 1997; Uchida et al., 2000). These include H_2O_2 and Cu^{2+} sensitivity and a requirement of cysteine, glutathione or α-tocopherol for growth on minimal medium (Uchida et al., 2000). These facts support that ubiquinone acts as an antioxidant in *S. pombe*. But little is known about the phenotypes of the yeasts producing higher ubiquinone, we thus tested the phenotypes of recombinant yeasts which may produce more ubiquinone. UQ10-overproducing yeasts, MTppt1 and ERppt1, do not show any growth rate differences from wild-type yeast when cultured at 30 °C (Fig. 4). However, transformants having high UQ10 grew faster than the wild type when suffered from 3 mM H_2O_2 (Fig. 5A) and 2 mM Cu^{2+} (Fig. 5B). These results strongly suggest that ubiquinone can serve as an antioxidant in fission yeast cells.

As another stress, the response of recombinant yeast to NaCl, was evaluated by growth rate. Fig. 5C shows that the transgenic yeasts grown much faster than the wild type when exposed to 200 mM NaCl, indicating that UQ10-overproducing yeasts had tolerance for salinity stress. Interestingly, both MTppt1 and ERppt1 types were more suitable to the low temperature when they were cultured at 22 °C (Fig. 5D).

3.5. Fed-batch experiment of S. pombe pERppt1

It is well known that high-cell-density fermentation is one of the most effective production processes in the biotechnology industry, so a fed-batch fermentation of *S. pombe* pERppt1 in a 5 l bioreactor was performed.

In the early phase of fermentation, DO decreased rapidly whereas OUR increased gradually with an increased consumption of oxygen (Fig. 6). After agitation speed was increased at 19 h, DO decreased again at 19–33 h with the consumption of glucose and OUR increased from 0.32 to 1.9 mol h^{-1} l^{-1} at the beginning of 33 h. OUR continuously increased at the 33–43 h and was maintained at approximately 2–3 mol/(h l) during the yeast rapid growth phase. CER firstly increased then decreased from 0 to 16 h and it continuously increased with OUR at 16–42 h. Whereafter both air-flow rate and agitation speed was required to be increased to maintain DO above 10%. DO varied inversely in proportion to OUR under well-ventilated conditions throughout the fermentation.

For the first 15th hour, RQ increased firstly and decreased rapidly to 2 at 15th hour and then decreased gradually to 1.2 at 34th hour. At the following time, RQ data maintaining about 1–1.2 (Fig. 6) from 34th to 80th hour to be taken as the feedback variable to control glucose-feeding rate indicated the high conversion rate of glucose to CO_2 and H_2O, and the production of yeast cells by the aerobic metabolic pathway. After 87.5 h of cultivation, the maximum amount of biomass (56.5 g DCW l^{-1}) and UQ-10 yield (23.17 mg l^{-1}) were obtained. The maximum specific UQ-10 content was 0.42 mg g^{-1} at 60 h. When the RQ data increased with ethanol concentration at the 90th hour, fermentation should be stopped because of decreasing metabolism and cell lysis.

At the beginning of culture, the yeast cells grown anaerobically had a relatively low growth rate coupled with a high rate of conversion of glucose to ethanol (Fig. 7). Ethanol concentration reached maximum value of about 26 g l^{-1} at the 14th hour and gradually decreased to 2.5 g l^{-1} at 46th hour due to carbon deficiency. Feeding solution started immedi-

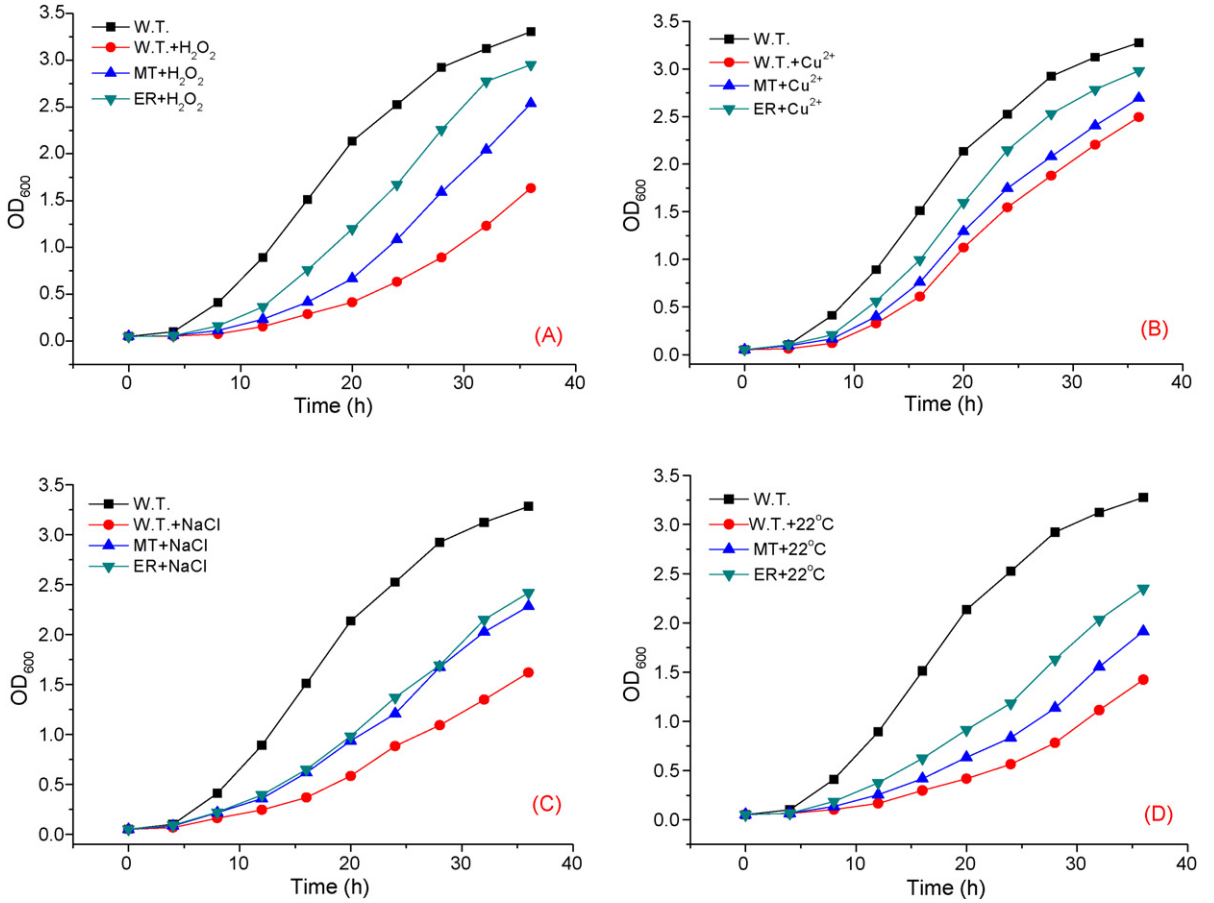

Fig. 5. Phenotypes of the transgenic yeasts under differential stress conditions. Yeasts were pre-grown in EMMA liquid medium to saturation. Then, yeasts were inoculated on fresh EMMA medium at 30 °C with 3 mM H_2O_2 (A), 2 mM Cu^{2+} (B), 200 mM NaCl (C) and low temperature 22 °C (D). W.T., pESPM strain; MT, pMTppt1 strain; ER, pERppt1 strain. Cell growth was measured at 4 h intervals in terms of OD600.

ately after depletion of the initial glucose at 9 h, and its feeding rate depended on the speed of ethanol concentration fell down during the culture of 15–42 h. When the speed of ethanol consumed fell down quickly, the feeding rate should be fast; when it increased, the feeding rate should be decreased for a period of time. At 42–90 h the ethanol concentration was maintained at a low level (2–3 g l^{-1}) (Fig. 7) by controlling the feeding solution feeding rate which did not distinctively inhibit the cell growth and UQ-10 content.

Fed-batch culture with addition of ammonia for pH control was shown in Fig. 7. The pH increased from 7th to 10th hour and decreased from 10th to 18th hour of fermentation. With the depletion of the initial glucose during the early phase of fermentation, the yeast cells continued to grow by consuming ethanol and organic acid produced during the early phase of growth. The consumption of organic acid increased the pH. And then pH was ranged between 4.6 and 4.9 from 20 to 42 h and maintained about 5.2 at 42–86 h. When the fermentation was finished, the yeast cells autolyzed and inclusions were released, causing an increase in pH.

4. Discussion

S. pombe is a popular model system for cell biology problems. It is genetically tractable and a wide variety of fission yeast-specific plasmids have been developed to facilitate molecular manipulation.

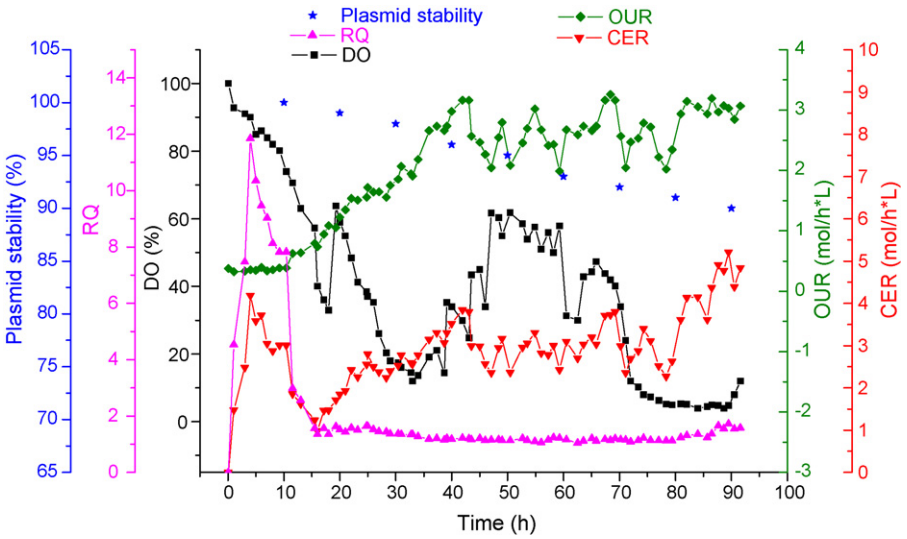

Fig. 6. The DO trend and DO relationship with OUR and CER during fed-batch culture of *S. pombe* pERppt1. The culture temperature was controlled at 30 °C.

In this study, pESPM was used as the expression vector which is pUC-based plasmid containing *S. pombe ars 1* and the *nmt1* promoter with *LEU* as the selectable marker. The *nmt* promoter is repressed by presence of thiamine in media. During shake flask culture, peak ubiquinone yield after induction, which occurs when thiamine is removed, takes approximately 20–24 h, presumably reflecting depletion of pools of thiamine in the cell (Fig. 4). Importantly, induction requires minimal media because rich media with yeast extract contain sufficient thiamine to repress the promoter.

Zhu et al. (1995) used all available genes, including *ubiCA*, *ubiB*, *ubiG*, *ubiH* and *ispB*, involved in

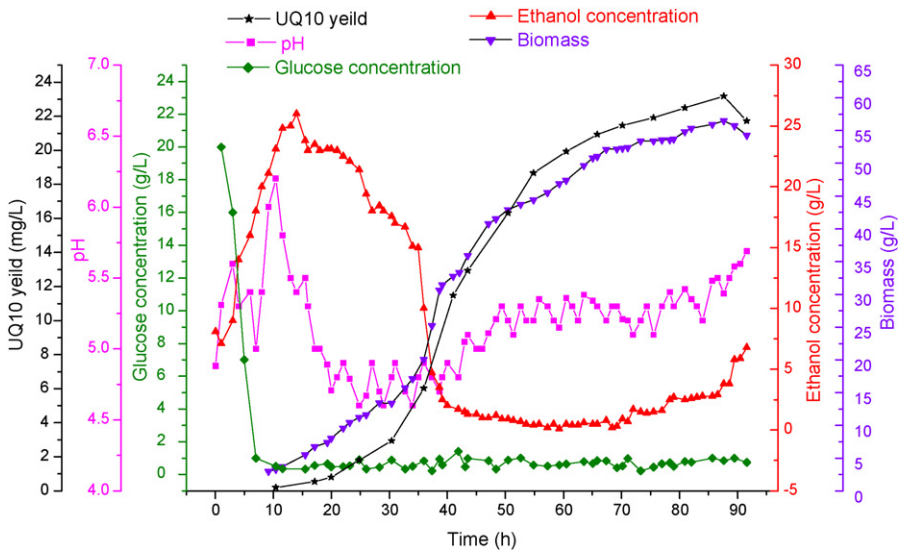

Fig. 7. Trends of biomass and UQ10 yield during fed-batch culture of *S. pombe* pERppt1. Glucose feeding started after 10 h. The culture temperature was controlled at 30 °C.

ubiquinone biosynthesis and succeeded in producing three times more ubiquinone than that produced by the wild-type *E. coli* strains. Their datum also suggest that 4HB:PPP transferase and PPP synthase are both important for UQ biosynthesis and that the original genes are more effective than exogenous genes. Our overexpression of the full-length *ppt1* gene lead to three times higher UQ yield in yeast, suggesting that 4HB:PPP, which couples the aromatic substrate and the prenyl chain, is a rate-limiting step in UQ biosynthesis. It is also indicates that prenylation of the PHB molecule leading to UQ takes place in the mitochondria. However, targeting the PPT1 polypeptide by fused with both N- and C-terminal signal peptides to the ER membrane had a markedly greater effect on UQ production than did the full-length PPT1 polypeptide targeted to the mitochondrial inner membrane, which suggests that UQ also can be synthesized in ER in *S. pombe*, at least in this step. In transformed rice plants, the production of UQ-9 was almost completely replaced with that of UQ-10 by targeting DdsA at the mitochondria, despite of the presence of endogenous UQ-9 synthesis (Takahashi et al., 2006). DdsA designed to express at the mitochondria increased accumulation of total UQ amount in seeds, which indicating that PPP synthase is a effective factor in UQ biosynthesis in rice plants (Takahashi et al., 2006). The previous reports also suggested that the PPP synthase is likely to be synthesized in the ER and the product is then transported to the mitochondria for the synthesis of UQ (Jun et al., 2004; Takahashi et al., 2006). Whether 4HB:PPP transferase exists in the same pathway need further analysis.

To date, many ubiquinone-less mutants of *S. pombe* due to the disruption of *dps1*, *ppt1*, *dlp1* and *abc1Sp* were reported. All the mutants displayed essentially the same phenotypes, namely sensitivity to H_2O_2 and Cu^{2+}, the need for an antioxidant such as glutathione for growth on minimal medium, and the production of H_2S. Our datum indicated that UQ-overproducing yeasts were more resistant to the active oxygen producing reagents, such as H_2O_2 and Cu^{2+}. Thus, these results further suggest that ubiquinone is not only important for respiration, but also appears to be necessary for protection against oxidative stress in fission yeast. This study also detected additional phenotypes that the ubiquinone-overproducing fission yeast strains had better tolerance for salinity and cold stress, however, the reasons still remain to be clarified.

The glucose effect is a major problem in industrially applicable fermentations where the microbial biomass yield is directly proportional to the product yield. A high-cell-density ($103\,g\,l^{-1}$) in fed-batch fermentation of *E. coli* BL21/pACDdsA increasing the UQ-10 concentration to $25.5\,mg\,l^{-1}$ was successfully achieved by controlling the glucose and acetate concentration (Park et al., 2005). Dissolved oxygen content is crucial factor in the high-cell-density fermentation of *E. coli*, so pure oxygen mixed with air was subjected for maintenance of respiratory activity (Park et al., 2005). However, oxygen consumption for *S. pombe* pERppt1 is not as much as *E. coli* BL21/pACDdsA does. Our successful fed-batch fermentation for the production of ubiquinone ($23\,mg\,l^{-1}$) by *S. pombe* pERppt1 was favored due to high-cell-density ($57\,g\,l^{-1}$) caused by the control of glucose feeding using an on-line ethanol concentration detector and an indirect feedback control of glucose via RQ data. Although we had examined other feeding control methods such as pH-stat and DO-stat, we found that pH and DO did not change quickly in response to substrate addition, and direct measurement of the ethanol concentration and indirect measurement of RQ were the most suitable techniques to monitor the cell growth. Variable pH value was used in this fermentation, which is better than the uniform value. In the prophase of fermentation, the pH was controlled at 4.75 which was facilitate to the yeast growth and then ubiquinone yield benefited from the pH value 5.2 during the anaphase of fermentation. Chemically defined medium was used in this fermentation because complex medium may contain the thiamine to repress the promoter, which also gives the selective pressure and profits the high-level plasmid stability throughout the fermentation.

Recently, with the increasing demand for ubiquinone, establishment of a mass production system of ubiquinone-10 in yeast is one of the most important subjects. Fed-batch fermentation demonstrated in this study may provide a process for culturing *S. pombe* in bioreactor by easily controlling the ethanol concentration and RQ thereby contributing to the high-cell-density fermentation and the biomass product. In addition, recombinant yeasts that have high levels of UQ will provide an appropriate model for further molecular and physiological studies.

Acknowledgements

Authors would like to express their thanks to financial supports from 863 High-Tech Program (2002AA217022), and National Natural Science Foundation of China (20576013, 20325622 and 50373003).

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