

Improving coenzyme Q₈ production in *Escherichia coli* employing multiple strategies

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Abstract Coenzyme Q (CoQ) is a medically valuable compound and a high yielding strain for CoQ will have several benefits for the industrial production of CoQ. To increase the CoQ₈ content of *E. coli*, we blocked the pathway for the synthesis of menaquinone by deleting the *menA* gene. The blocking of menaquinone pathway increased the CoQ₈ content by 81 % in *E. coli* ($\Delta menA$). To study the CoQ producing potential of *E. coli*, we employed previous known increasing strategies for systematic metabolic engineering. These include the supplementation with substrate precursors and the co-expression of rate-limiting genes. The co-expression of *dxs-ubiA* and the supplementation with substrate precursors such as pyruvate (PYR) and parahydroxybenzoic acid (pHBA) increased the content of CoQ₈ in *E. coli* ($\Delta menA$) by 125 and 59 %, respectively. Moreover, a 180 % increase in the CoQ₈ content in *E. coli* ($\Delta menA$) was realized by the combination of the co-expression of *dxs-ubiA* and the supplementation with PYR and pHBA. All in all, CoQ₈ content in *E. coli* increased 4.06 times by blocking the menaquinone pathway, *dxs-ubiA* co-expression and the addition of sodium pyruvate and parahydroxybenzoic acid to the medium. Results suggested a synergistic effect among different metabolic engineering strategies.

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Introduction

Coenzyme Q_n (CoQ_n) is a medically valuable bioactive compound [1–3], and microbial fermentation is the preferred method for its production to ensure its bioactivity (all trans-isomer). *E. coli* is a competitive species for industrial applications because of its short multiplication time, growth using inexpensive substrates and its amenability to genetic modifications. Although it naturally synthesizes CoQ₈ rather than CoQ₁₀, the biochemical pathway leading to the biosynthesis of CoQ₁₀ in *E. coli* has been almost entirely deciphered [4–6]. For improving the yield of CoQ, researchers have attempted to introduce a new metabolic pathway to the host [4, 7]. However, “pathway-blocking engineering” has not yet been developed for CoQ production, which is one of the well-known important approaches of metabolic engineering.

Menaquinone (MK) and Demethylmenaquinone (DMK) are two derivatives of naphthoquinone (NQ). They function as electron transporters in the anaerobic respiratory chain in *E. coli* [8]. Although the MK and DMK have no essential function for aerobiosis of *E. coli*, they are synthesized at a high concentration under aerobic condition [9]. As shown in Fig. 1, DMK/MK synthesis occurs as a branched pathway of CoQ₈ synthesis in *E. coli* and both MK and CoQ pathways share octaprenyl diphosphate (OPP) and chorismate. Octaprenyl transferase (MenA) directs OPP to MK pathway using 1,4-dihydroxy-2-naphthoic acid (DHNA) DHNA and OPP as substrates [10]. A previous study indicates that CoQ₈-deficient mutant enhanced MK-8 content by 30 % [11], suggesting that MK pathway and CoQ₈

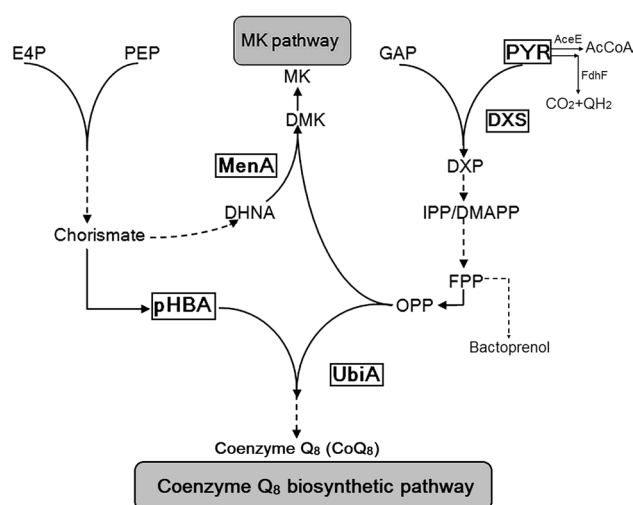


Fig. 1 Coenzyme Q₈ biosynthesis and branched pathways. Relevant abbreviations: MK menaquinone, DMAPP dimethylallyl diphosphate, DXS 1-deoxy-xylulose-5-phosphate synthase, DMK demethylmenaquinone, DXP 1-deoxy-xylulose 5-phosphate, E4P erythrose-4-phosphate, FPP farnesyl diphosphate, GAP D-glyceraldehyde-3-phosphate, IPP isopentenyl diphosphate, OPP octaprenyl diphosphate, PEP phosphoenolpyruvate, PYR pyruvate, DHNA 1,4-dihydroxy-2-naphthoic acid, MenA DHNA-octaprenyltransferase, OPP octaprenyl diphosphate, pHBA parahydroxybenzoic acid, UbiA 4-hydroxybenzoate octaprenyl transferase, AcCoA Acetyl-Coenzyme A, QH2 cytochrome c reductase, AceE pyruvate dehydrogenase, FdhF formate dehydrogenase

pathway inhibit each other by competing for the common precursors. Since *menA* is not an essential gene for *E. coli* aerobiosis [12], blocking the MK pathway by deleting *menA* gene may facilitate CoQ₈ accumulation without any negative effects on aerobic fermentation.

Pyruvic acid (PYR) is a precursor for the isoprenoid pathway (Fig. 1), but it can be catalyzed to form Acetyl-Coenzyme A (Acetyl-CoA) and CO₂ [13]. Therefore, isoprenoid pathway competes with other pathways for PYR. The supplementation of sodium pyruvate may provide enough PYR as a substrate for the isoprenoid pathway, thereby leading to an increase in the yield of CoQ.

Both 1-deoxy-D-xylulose-5-phosphate synthase (DXS) and *p*-hydroxybenzoate octaprenyl transferase (UbiA) are rate-limiting enzymes in CoQ biosynthesis [14]. Previous studies have shown that an overexpression of DXS or UbiA [15–17] can enhance CoQ content in *E. coli*. But the combined effect of co-expression of these two genes on the CoQ content has not been studied.

In this study, for the first time, we blocked MK pathway by deleting *menA* to enhance the content of CoQ₈ in *E. coli*. To further improve the yield of CoQ, the two rate-limiting enzymes DXS and UbiA were co-expressed in the *menA*-deficient strain of *E. coli*. Moreover, the effects of

supplying precursors, such as pHBA and PYR on CoQ content, were also studied. Finally, manipulations such as MK pathway blocking, addition of precursors (pHBA and PYR) and DXS-UbiA co-expression were combined to study the full potential of CoQ production by *E. coli*.

Materials and methods

General materials and methods

General molecular manipulations were performed according to standard protocols [18]. PCRs were performed using Ex Taq or PrimeSTAR HS DNA polymerase (TaKaRa, Dalian City, China). CoQ₁₀ and MK-8 standards were purchased from Sigma (Shanghai, China). Electroporator (Eppendorf, Hamburg, Germany) was used for the introduction of plasmids or DNA fragments into bacterial cells. High-performance liquid chromatography system (Hitachi, Tokyo, Japan) was used for the analysis of CoQ and NQ content.

Media, strains, plasmids and culture conditions

E. coli strain BW25113 was used for constructing a recombinant strain and DH5α was used for plasmids propagation. Plasmids, pKD46, pKD13 and pCP20 [19], were used as molecular tools in gene deletion. Plasmid pMG103 [19] was used for gene overexpression.

Aerobic cultivation was performed in 100 mL medium in a 300-mL shake flask at 250 rpm. Anaerobic cultivation was achieved by a static culture in the presence of nitrogen. Luria–Bertani broth (LB) (pH 7.0) medium was used for usual bacterial reproduction. M9 medium (0.2 % glucose or sodium succinate as required) was used to study the growth curve of *E. coli* (*ΔmenA*).

menA gene deletion

The gene deletion of chromosomal *menA* was performed using procedure described previously [19]. Firstly, for the homologous recombination of *menA* gene, *kanR* cassette was amplified using primers *ΔmenA*-1(TCATCATTTGTTGATGGGGCTGAAAGGCCCATTTTATTGGCGCGTATTCTGTCAAACATGAG AATTAA) (homologous sequence) and *ΔmenA*-2 (GCTATGTGGGCTGTTGGCAAAATCATCAATTGTT AATTGATATTTGTCAGGTGTAGGCTGGAGCTGCTTC) (homologous sequence) with plasmid pKD13 as the PCR template. Then, linear *kanR* cassette was introduced into *E. coli* BW25113 (pKD46) for homologous recombination. After curing pKD46 at 42 °C, the semi-engineered strain designated as *E. coli* (*menA::kanR*) was

subjected to PCR with two primer pairs test-1/2 (ATGGCA GGTGAACGAATC/CATCAGCCATGATGGATACT) and test-3/4 (CGTGATATTGCTGAAGAGCT/CATTGAGTTG CTGCGAGA), which resulted in a 1.1 and 0.3 kb PCR product, respectively. After that, the helper plasmid pCP20, encoding the FLP recombinase, was introduced into *E. coli* (*menA::kanR*) to facilitate the removal of *kanR* from chromosome. The candidate *E. coli* ($\Delta menA$) strain was tested by PCR with primers test-1 and test-4.

Physiological effects of blocking MK pathway

To examine the physiological effects of blocking MK pathway, the concentrations of MK-8 and DMK-8 were detected in *E. coli* ($\Delta menA$) and the growth ability of this deficient strain was estimated using the growth curves by plotting OD₆₀₀ against culture time. To avoid the anaerobic growth relying on the energy from the glycolysis pathway, cells were cultured in M9 minimal medium with succinate as the non-fermentable carbon source.

Plasmid construction

For gene overexpression, the coding regions of *dxs* and *ubiA* were amplified from *E. coli* BW25113 genomic DNA. The primers *dxs*-1 (GGAATTCGTGAATGAGTTTTGATTGCC AAATAC) (*EcoRI*) and *dxs*-2 (GGGGTA CCTTATATGC CAGCCAGGCCTTGAT) (*KpnI*) were used to amplify *dxs* gene followed by ligation into pMG103 [20] to generate the plasmid pMG-*dxs*. Similarly, the plasmid pMG-*ubiA* was generated by ligating *ubiA* into pMG103 using the primers *ubiA*-1 (GGAATTCGTGAATGGAGTGGAGTCTGACGCA) (*EcoRI*) and *ubiA*-2 (GGGGTACCTCA GAAATGCCAGTA ACTCAT) (*KpnI*) for amplification. Then, *ubiA* fragment was ligated into pMG-*dxs* to generate pMG-*dxs-ubiA* after amplification using primers *ubiA*-3 (GGGGTACCATGGAG TGGAGTCTGACGCA) (*KpnI*) and *ubiA*-4 (GCTCTAGATC AGAAATGCC AGTAACTCAT) (*XbaI*). The three plasmids pMG-*dxs*, pMG-*ubiA* and pMG-*dxs-ubiA* were sequenced to ensure their accuracy.

Coenzyme Q and naphthoquinone assay

Cell growth was measured using a spectrophotometer at 600 nm and converted to dry cell weight (DCW) using a prepared standard curve of DCW versus OD₆₀₀. Vol. CoQ₈ was extracted with an organic solvent from the saponified liquid before being subjected to high-performance liquid chromatography, according to the procedure described before [9]. The procedure used to extract, separate and analyze MK and DMK is described previously [10].

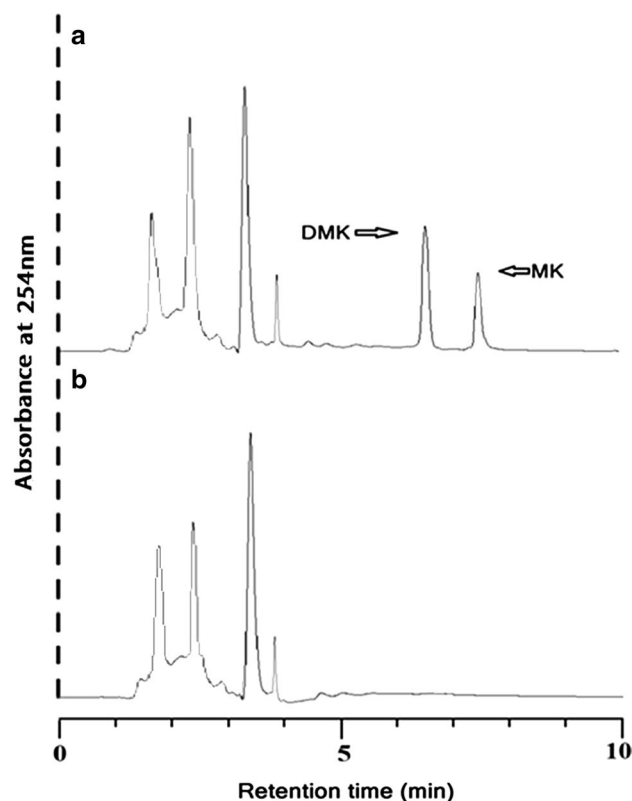


Fig. 2 HPLC chromatogram of DMK and MK; **a** extract from wild-type strain; **b** extract from *E. coli* ($\Delta menA$)

Results and discussion

Physiological effects of MK pathway blocking on the mutant strain

After confirming the deletion of *menA* by PCR and sequencing, the physiological effects of blocking the MK pathway were investigated. To further confirm the deletion of *menA*, the biosynthesis of MK and DMK in the constructed strain was determined. The analysis results (Fig. 2) demonstrated the presence of DMK and MK in wild-type strain. However, both DMK and MK were not detected in the newly constructed strain. This indicated that DMK and MK synthesis is blocked because of *menA* deletion. It also indicated that the synthesis of MK and DMK in *E. coli* is dependent on *menA* gene as it is the only pathway for the synthesis.

To investigate the physiological effects of *menA* gene deletion, growth ability, sensitivity to pH and temperature were examined for *E. coli* ($\Delta menA$). Results showed that there were no remarkable differences in the cell morphology (Online Resource Fig. 1) and the sensitivity to pH (Online Resource Fig. 2) and temperature (Online Resource

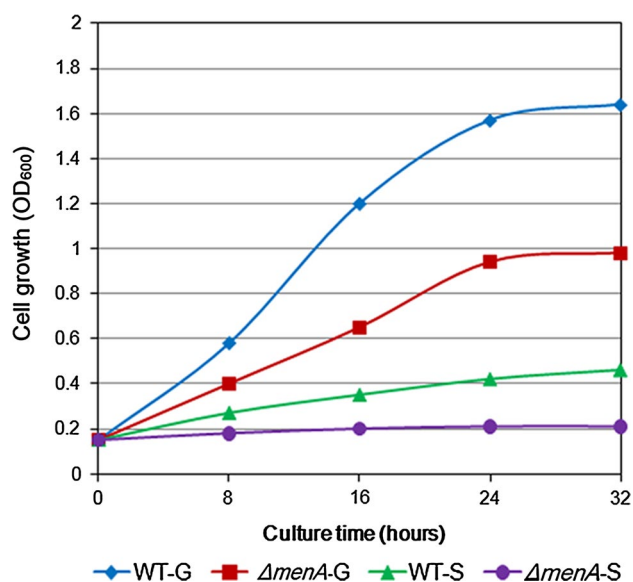


Fig. 3 Anaerobic growth ability of deficient strain grown in different carbon sources; WT wild-type strain, $\Delta menA$ *E. coli* ($\Delta menA$), G Glucose, fermentable carbon source, S succinate, non-fermentable carbon source, WT-G wild-type strain cultured with glucose, WT-S wild-type strain cultured with succinate, $\Delta menA$ -G *E. coli* ($\Delta menA$) cultured with glucose, $\Delta menA$ -S, *E. coli* ($\Delta menA$) cultured with succinate

Fig. 3) between the wild strain and *E. coli* ($\Delta menA$) both in M9 and LB mediums. These results suggested that the deletion of *menA* had no negative effects on cell mass collection for CoQ production.

However, the deficient strain grew slowly when compared to the wild-type strain, even in the presence of fermentable glucose as carbon source in M9 medium in anaerobic condition (Fig. 3). Furthermore, the deficient strain could not grow when succinate (a non-fermentable sugar) was used as the only source of carbon in the M9 medium (Fig. 3). These results suggested that DMK and MK are absolutely essential for anaerobic respiration, which is the only way to produce energy for *E. coli* in M9 medium with non-fermentable succinate as the sole carbon source.

Increased production of coenzyme Q₈ in *E. coli* ($\Delta menA$)

To investigate the effect of the interruption of MK pathway on CoQ content in *E. coli*, CoQ₈ content of *E. coli* ($\Delta menA$) was measured. As shown in Fig. 4, CoQ₈ content was increased from 0.31 mg/g DCW in the wild-type strain to 0.56 mg/g DCW in *E. coli* ($\Delta menA$) in 48 h after fermentation in LB medium. It is worth mentioning that the absence of MK and DMK had no negative effects on cell mass (Fig. 4). And the production of CoQ₈ increased from 0.59 to 1.04 mg/L.

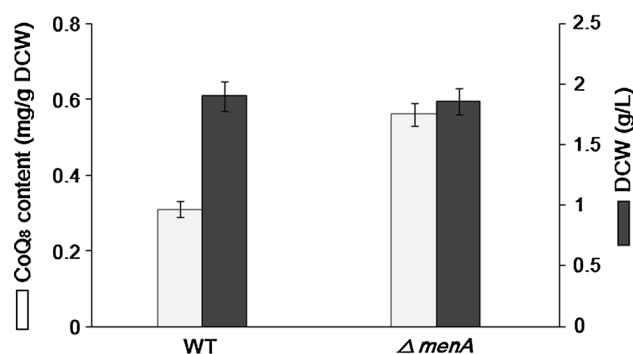


Fig. 4 Coenzyme Q₈ content in bacterial strain with a blocked MK pathway. WT wild-type strain, $\Delta menA$ *menA* deleted strain, DCW dry cell weight. The results represent the mean value $\pm$ SD of duplicate samples in three independent experiments

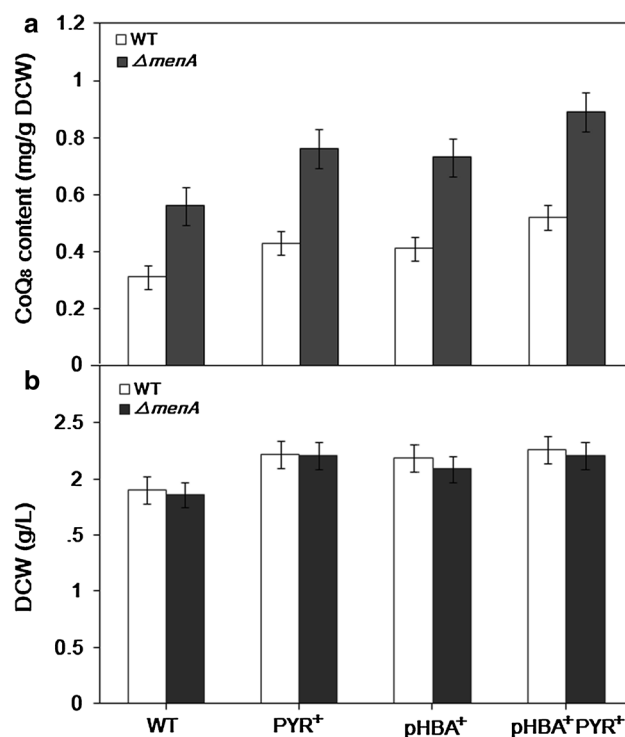


Fig. 5 Coenzyme Q₈ yields upon supplementation with precursors. CK the data from the culture in LB medium served as control; **a** CoQ₈ contents of the two strains, **b** Cell mass; DCW dry cell weight, “+” means supplementation; the results represent the mean value $\pm$ SD of duplicate samples in three separate experiments

The increase in CoQ₈ content suggested that precursors such as OPP and chorismic acid were directed toward the biosynthesis of CoQ₈ when the MK pathway was blocked. In this study, we demonstrate that the blocking of the branched pathway is an effective approach to improve the production of CoQ₈. A further increase in CoQ yields could possibly be achieved by blocking other branched pathways,

Table 1 Content of CoQ₈ under multiple strategies

Strategies	CoQ ₈ content (mg g ⁻¹ DCW)		DCW (g/L)	
	WT	$\Delta menA$	WT	$\Delta menA$
CK	0.31 ± 0.02	0.56 ± 0.03	1.90 ± 0.12	1.86 ± 0.13
<i>ubiA</i> ⁺	0.54 ± 0.04	0.95 ± 0.06	1.59 ± 0.08	1.57 ± 0.09
<i>dxs</i> ⁺	0.61 ± 0.03	0.83 ± 0.05	1.61 ± 0.11	1.55 ± 0.10
<i>dxs</i> ⁺ <i>ubiA</i> ⁺	0.84 ± 0.06	1.26 ± 0.06	1.58 ± 0.10	1.54 ± 0.11
<i>dxs</i> ⁺ <i>ubiA</i> ⁺ <i>pHBA</i> ⁺ <i>PYR</i> ⁺	1.06 ± 0.08	1.57 ± 0.08	1.78 ± 0.12	1.71 ± 0.10

Data represent the mean value ± SD of duplicate samples from three separate experiments

CK the data from the culture in LB medium served as control, “+”, supplementation or overexpression, WT wild type, $\Delta menA$ *E. coli*($\Delta menA$), DCW dry cell weight

such as the bactoprenol biosynthesis pathway (Fig. 1) which competes for FPP with CoQ pathway [21].

Effects of precursor supplementation on coenzyme Q₈ content

To study the effects of precursor supplementation on CoQ₈ accumulation, optimal concentrations of the precursors were adopted on the grounds of pre-experiments (Online Resource Fig. 4). As seen in Fig. 5a, the addition of pHBA (80 mg/L) and sodium pyruvate (60 mg/L) increased the content of CoQ₈ in both the wild-type strain and the *menA*-deficient strain. Moreover, yields were even better when both pHBA and sodium pyruvate were supplemented together than when they were supplemented individually. The CoQ₈ content increased from 0.31 to 0.52 mg/g for wild-type strain and from 0.56 to 0.89 mg/g for *E. coli* ($\Delta menA$) strain (Fig. 5a). Significantly, the cell mass of the two strains was increased by about 15 % with the supplementation of pHBA and sodium pyruvate (Fig. 5b). The combined supplementation with the two precursors increased the CoQ₈ production of wild strain and *menA*-deficient strain by 97.65 and 89.4 %, respectively. These results from the combined supplementations of pHBA and PYR demonstrated a synergistic effect on the content of CoQ₈ compared to the single supplementation. The reason might be that the combined supplementation balanced the flux of precursor molecules from aromatic and isoprenoid pathways.

The deletion of AceE and FdhF (Fig. 1) increased the content of lycopene by 4 and 9 %, respectively [22]. Therefore, deleting AceE and FdhF to direct PYR to isoprenoid pathway could possibly also increase the contents of CoQ₈. However, the increase in content of lycopene is low and the decrease in acetyl-coenzyme A (AcCoA) is extremely harmful to the bacterium. Therefore, the addition of sodium pyruvate might be a better method than the blocking of pathways by deleting AceE and FdhF to increase the yields of CoQ₈.

dxs-ubiA co-expression maximized the content of coenzyme Q₈ in combination with other strategies.

Overexpression of rate-limiting enzymes is an efficient strategy to increase the yields of CoQ [5–7, 17]. The co-expression of *dxs* and *ubiA* was attempted in this study to increase CoQ content. The co-expression resulted in better yields than the overexpression of *dxs* and *ubiA* alone (Table 1). More importantly, as shown in Table 1, CoQ₈ content of *E. coli* ($\Delta menA$) increased by 0.7 mg/g while the increment for the wild-type strain was 0.53 mg/g. To increase CoQ₈ content further under systematic metabolic engineering, *dxs-ubiA* co-expression was combined with addition of pHBA and sodium pyruvate. A combination of co-expression of *dxs* and *ubiA* and the addition of precursors resulted in maximum content of CoQ₈ to 1.57 mg/g for *E. coli* ($\Delta menA$), which was 4.06 times higher than the parent strain (Table 1). The CoQ₈ production of *E. coli* ($\Delta menA$) increased to 2.68 mg/L, which was 3.54 times higher than that of the parent strain. In this study, we observed a synergistic effect on the yield of CoQ₈ by blocking the MK pathway, addition of precursors and co-expression of *dxs* and *ubiA*. It demonstrates that a combination of multiple strategies is an efficient way to increase CoQ₈ content in *E. coli*.

Although the inherent ubiquinone is CoQ₈ in *E. coli*, CoQ₁₀ producing *E. coli* strains have been constructed by replacing octaprenyl diphosphate synthase (IspB) with decaprenyl diphosphate synthase (Dps) [5, 23]. If we deleted *menA* gene in the CoQ₁₀ producing *E. coli* strains and supplemented pHBA or PYR in culture medium, the production of CoQ₁₀ should be higher. Because of that, more precursors would be directed to CoQ₁₀ biosynthesis pathway.

Several microorganisms have been employed as the producers of CoQ₁₀, Ha et al. [24] and Gu et al. [25] achieved high CoQ₁₀ yields (626.5 and 320 mg/L) by fed-batch fermentations using *Agrobacterium tumefaciens*. Yoshida et al. [26] could achieve a maximal CoQ₁₀ production by fed-batch

fermentation using *Rhodobacter sphaeroides*. Although the yields of CoQ₁₀ achieved from *Escherichia coli* [5, 23, 27] were relatively lower compared to other organisms, *E. coli* is a competitive species for industrial applications because of its many advantages in production, such as short multiplication time, growth using inexpensive substrates, high-density fermentation and amenability to genetic modifications etc.

It is reported in previous studies [5–7, 27] that the strains containing high content of CoQ₁₀ showed normal physiological status as the wild-type strains. Although there is no evidence that the accumulation of a high amount of CoQ₁₀ has negative effects on the physiological functions of the bacteria, it is unpredictable if the content of CoQ₁₀ reaches a very high level. Fortunately, the internal CoQ₁₀ can be continually extracted into the culture medium by adding extracting agent in culture medium [28], which avoided the possible negative effects of excessive accumulation of CoQ₁₀ on the physiological functions of the bacteria.

The strategies employed in this study, branched pathway blocking and precursors supplementation, are suitable for improving CoQ₁₀ production, not only in *E. coli* but also in the other microorganisms. In future studies, we will be evaluating the blocking of other branched pathways such as baclophenol biosynthesis pathway [21] along with co-expression of genes such as *idi* [4, 29] on the yield of CoQ in *E. coli*.

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