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SHORT COMMUNICATION

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Improving coenzyme Q_8 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 CoQ_8 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 CoQ_8 content by 81 % in *E. coli (ΔmenA)*. 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 CoQ_8 in *E. coli (ΔmenA)* by 125 and 59 %, respectively. Moreover, a 180 % increase in the CoQ_8 content in *E. coli (ΔmenA)* was realized by the combination of the co-expression of *dxs-ubiA* and the supplementation with PYR and pHBA. All in all, CoQ_8 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.

Keywords Coenzyme Q · Menaquinone · *menA* · Pathway engineering · Sodium pyruvate

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 CoQ_8 rather than CoQ_{10}, the biochemical pathway leading to the biosynthesis of CoQ_{10} 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 CoQ_8 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 CoQ_8-deficient mutant enhanced MK-8 content by 30 % [11], suggesting that MK pathway and CoQ_8

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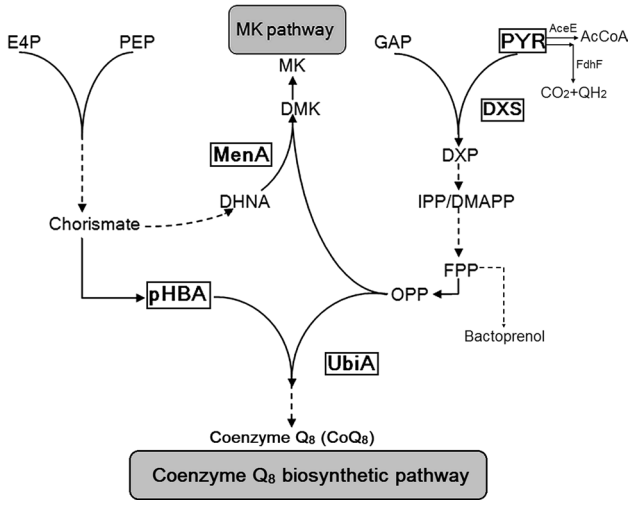

Fig. 1 Coenzyme Q_8 biosynthesis and branched pathways. Relevant abbreviations: *MK* menaquinone, *DMAPP* dimethylallyl diphosphate, *DXS* 1-deoxy-xylulose-5-phosphate synthase, *DMK* demethylmenaquinone, *DXP* 1-deoxy-xylulose 5-phosphate, *E4P* erythrose-4-phosphate, *FPP* farnesyl diphosphate, *GAP* 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 CoQ_8 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 CO_2 [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 CoQ_8 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). CoQ_{10} 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(*TCATCATTGTTTG ATGGGGCTGAAAGGCCCCATTTTTATTGGCGCGTATT* CTGTCAAACATGAG AATTAA) (homologous sequence) and *ΔmenA*-2 (*GCTATGTGGGCTGTTGGCAAAATCATCA ATTGTT AATTGATATTTGTCAG*GTGTAGGCTGGAGCT GCTTC) (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/CATTCAGTTG CTGCGAGA), which resulted in a 1.1 and 0.3 kb PCR product, respectively. After that, the help 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 (ΔmenA)* 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 (ΔmenA)* and the growth ability of this deficient strain was estimated using the growth curves by plotting OD_{600} 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 (G*GAATTC*GTGAATGAGTTTTGATTGCC AAATAC) (*EcoR I*) and *dxs*-2 (GG*GGTA CC*TTATATGC CAGCCAGGCCTTGAT) (*Kpn*I) 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(G*GAATTC*GTGAATGGAGTGGAGTCTGACGCA) (*EcoR I*) and *ubiA*-2 (GG*GGTACC*TCA GAAATGCCAGTA ACTCAT) (*Kpn*I) for amplification. Then, *ubiA* fragment was ligated into pMG-*dxs* to generate pMG-*dxs*-*ubiA* after amplification using primers *ubiA*-3 (GG*GGTACC*ATGGAG TGGAGTCTGACGCA) (*Kpn*I) and *ubiA*-4 (GC*TCTAGA*TC AGAAATGCC AGTAACTCAT) (*Xba*I). 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 OD_{600}·Vol. CoQ_8 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].

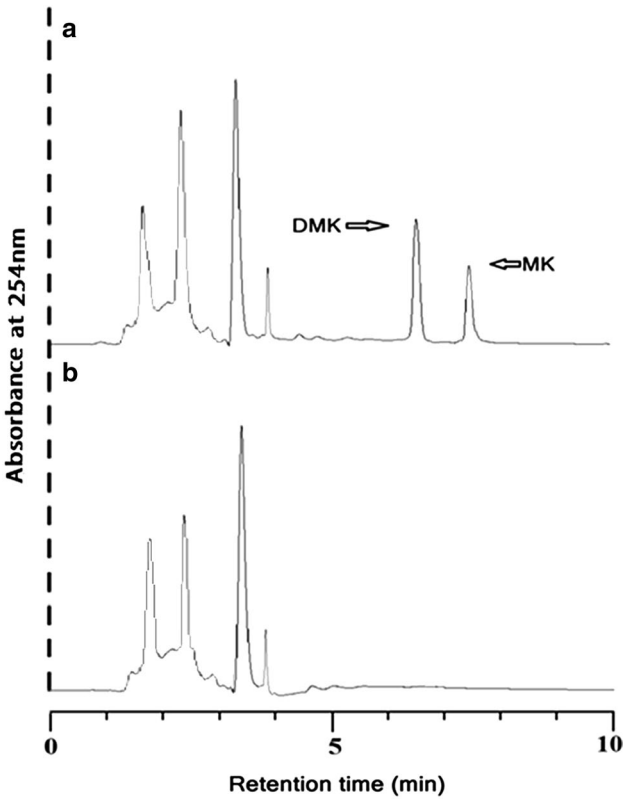

Fig. 2 HPLC chromatogram of DMK and MK; **a** extract from wild-type strain; **b** extract from *E. coli* (Δ*menA*)

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 (ΔmenA)*. 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

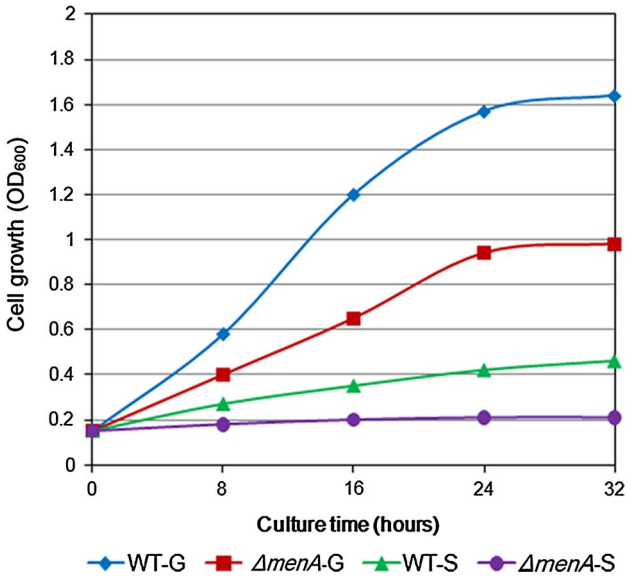

Fig. 3 Anaerobic growth ability of deficient strain grown in different carbon sources; *WT* wild-type strain, *ΔmenA E. coli (ΔmenA)*, *G* Glucose, fermentable carbon source, *S* succinate, non-fermentable carbon source, *WT-G* wild-type strain cultured with glucose, *WT-S* wild-type strain cultured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

Fig. 3) between the wild strain and *E. coli (ΔmenA)* 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 Q_8 in *E. coli (ΔmenA)*

To investigate the effect of the interruption of MK pathway on CoQ content in *E. coli*, CoQ_8 content of *E. coli (ΔmenA)* was measured. As shown in Fig. 4, CoQ_8 content was increased from 0.31 mg/g DCW in the wild-type strain to 0.56 mg/g DCW in *E. coli (ΔmenA)* 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 CoQ_8 increased from 0.59 to 1.04 mg/L.

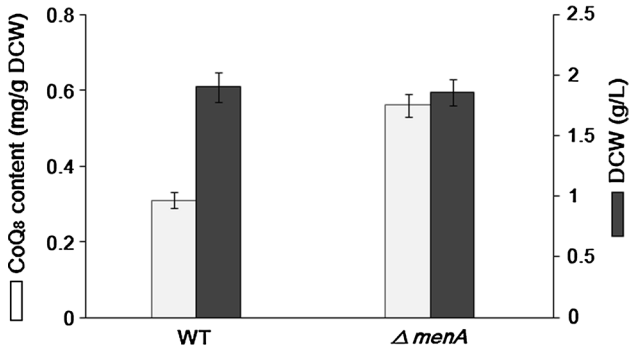

Fig. 4 Coenzyme Q_8 content in bacterial strain with a blocked MK pathway. *WT* wild-type strain, *ΔmenA menA* deleted strain, *DCW* dry cell weight. The results represent the mean value ± SD of duplicate samples in three independent experiments

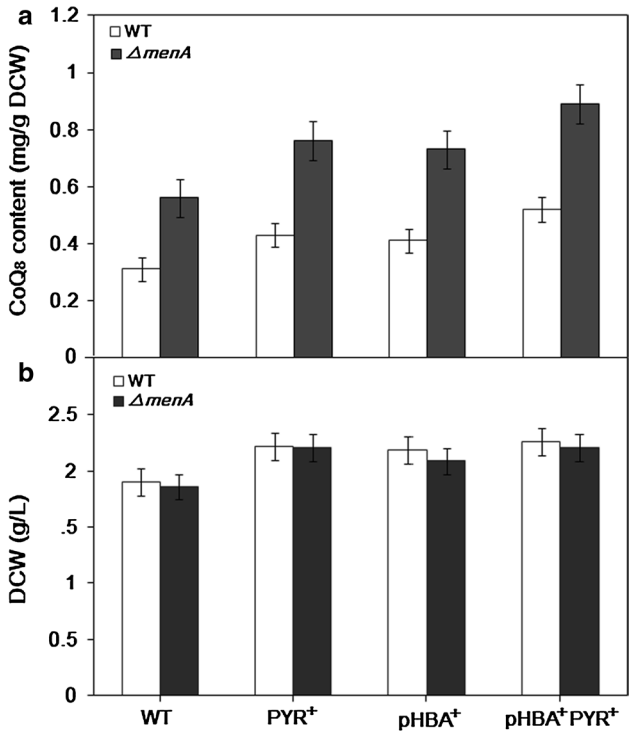

Fig. 5 Coenzyme Q_8 yields upon supplementation with precursors. *CK* the data from the culture in LB medium served as control; **a** CoQ_8 contents of the two strains, **b** Cell mass; *DCW* dry cell weight, "+" means supplementation; the results represent the mean value ± SD of duplicate samples in three separate experiments

The increase in CoQ_8 content suggested that precursors such as OPP and chorismic acid were directed toward the biosynthesis of CoQ_8 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 CoQ_8. A further increase in CoQ yields could possibly be achieved by blocking other branched pathways,

Table 1 Content of CoQ_8 under multiple strategies

Strategies	CoQ_8 content (mg g^{-1} DCW)		DCW (g/L)	
	WT	*ΔmenA*	WT	*ΔmenA*
CK	0.31 ± 0.02	0.56 ± 0.03	1.90 ± 0.12	1.86 ± 0.13
ubiA$^+$	0.54 ± 0.04	0.95 ± 0.06	1.59 ± 0.08	1.57 ± 0.09
dxs$^+$	0.61 ± 0.03	0.83 ± 0.05	1.61 ± 0.11	1.55 ± 0.10
dxs$^+$*ubiA*$^+$	0.84 ± 0.06	1.26 ± 0.06	1.58 ± 0.10	1.54 ± 0.11
dxs$^+$*ubiA*$^+$*pHBA*$^+$*PYR*$^+$	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, *ΔmenA E. coli(ΔmenA)*, *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 Q_8 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 CoQ_8 in both the wild-type strain and the *menA*-deficient stain. Moreover, yields were even better when both pHBA and sodium pyruvate were supplemented together than when they were supplemented individually. The CoQ_8 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 (ΔmenA)* 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 CoQ_8 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 CoQ_8 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 CoQ_8. 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 CoQ_8.

dxs-ubiA co-expression maximized the content of coenzyme Q_8 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, CoQ_8 content of *E. coli (ΔmenA)* increased by 0.7 mg/g while the increment for the wild-type stain was 0.53 mg/g. To increase CoQ_8 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 CoQ_8 to 1.57 mg/g for *E. coli* (*ΔmenA*), which was 4.06 times higher than the parent strain (Table 1). The CoQ_8 production of *E. coli* (*ΔmenA*) 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 CoQ_8 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 CoQ_8 content in *E. coli*.

Although the inherent ubiquinone is CoQ_8 in *E. coli*, CoQ_{10} 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 CoQ_{10} producing *E. coli* strains and supplemented pHBA or PYR in culture medium, the production of CoQ_{10} should be higher. Because of that, more precursors would be directed to CoQ_{10} biosynthesis pathway.

Several microorganisms have been employed as the producers of CoQ_{10}, Ha et al. [24] and Gu et al. [25] achieved high CoQ_{10} yields (626.5 and 320 mg/L) by fed-batch fermentations using *Agrobacterium tumefaciens*. Yoshida et al. [26] could achieve a maximal CoQ_{10} production by fed-batch

fermentation using *Rhodobacter sphaeroides*. Although the yields of CoQ_{10} 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 CoQ_{10} showed normal physiological status as the wild-type strains. Although there is no evidence that the accumulation of a high amount of CoQ_{10} has negative effects on the physiological functions of the bacteria, it is unpredictable if the content of CoQ_{10} reaches a very high level. Fortunately, the internal CoQ_{10} 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 CoQ_{10} on the physiological functions of the bacteria.

The strategies employed in this study, branched pathway blocking and precursors supplementation, are suitable for improving CoQ_{10} 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 bactoprenol 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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