

# Inhibition of Coenzyme Qs Accumulation in Engineered *Escherichia coli* by High Concentration of Farnesyl Diphosphate

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## Abstract

**Background:** Coenzyme Q<sub>10</sub> (CoQ<sub>10</sub>) is an isoprenoid component used widely in nutraceutical industries. Farnesyl diphosphate synthase (FPPS) is a responsible enzyme for biosynthesis of farnesyl diphosphate (FPP), a key precursor for CoQs production. This research involved investigating the effect of FPPS over-expression on CoQs production in engineered CoQ<sub>10</sub>-producing *Escherichia coli* (*E. coli*).

**Methods:** Two CoQ<sub>10</sub>-producing strains, as referred to *E. coli* Ba and *E. coli* Br, were transformed by the encoding gene for FPPS (*ispA*) under the control of either the *trc* or *P<sub>BAD</sub>* promoters.

**Results:** Over-expression of *ispA* under the control of *P<sub>BAD</sub>* promoter led to a relative increase in CoQ<sub>10</sub> production only in recombinant *E. coli* Br although induction by arabinose resulted in partial reduction of CoQ<sub>10</sub> production in both recombinant *E. coli* Ba and *E. coli* Br strains. Over-expression of *ispA* under the control of stronger *trc* promoter, however, led to a severe decrease in CoQ<sub>10</sub> production in both recombinant *E. coli* Ba and *E. coli* Br strains, as reflected by reductions from 629±40 to 30±13 and 564±28 to 80±14 µg/g Dried Cell Weight (DCW), respectively. The results showed high level of FPP reduces endogenous CoQ<sub>8</sub> production as well and that CoQs are produced in a complimentary manner, as the increase in production of one decreases the production of the other.

**Conclusion:** The reduction in CoQ<sub>10</sub> production can be a result of Dds inhibition by high FPP concentration. Therefore, more effort is needed to verify the role of intermediate metabolite concentration and to optimize production of CoQ<sub>10</sub>.

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**Keywords:** Coenzyme Q<sub>10</sub>, Decaprenyl diphosphate synthase, Farnesyl diphosphate, Isoprenoid

## Introduction

Ubiquinone, also known as CoQ, is an isoprenoid component and lipid-soluble molecule which plays various roles in both eukaryotic and prokaryotic cells. The most important function of CoQ is in energy generation, where it is responsible for electron transfer in the respiratory chain. In addition, CoQ has also been found to have an antioxidant function and is involved in various biological processes including gene expression, formation of disulfide bonds in proteins and generation of cellular signals<sup>1-7</sup>. The lack of CoQ has been associated with various diseases<sup>8</sup>.

Ubiquinone has been used as a medicine, but is now widely used as a nutritional supplement<sup>9,10</sup>. The effect of ubiquinone on the lifespan of humans is currently being studied extensively while studies on other roles

of ubiquinone are also underway<sup>11</sup>. Ubiquinone is generally composed of a benzene ring and an isoprenoid side chain, which is a homopolymer of Isopentenyl diphosphate (IPP), also known as the isoprene unit. The length of the side chain determines the type of CoQ in various organisms. For example, the CoQ side chain in human cells is comprised of ten isoprene units and forms CoQ<sub>10</sub>, while *Escherichia coli* (*E. coli*) contains CoQ<sub>8</sub> which possesses eight isoprene units in its side chain<sup>12-16</sup>. The synthesis of the CoQ<sub>10</sub> side chain is catalyzed by Dds, whereas Octaprenyl diphosphate synthase (Ods) is the enzyme responsible for biosynthesis of the corresponding side chain in *E. coli*. Therefore, the expression of the heterologous gene encoding for Dds can lead to the production of CoQ<sub>10</sub> in trans-

formed *E. coli* cells along with CoQ<sub>8</sub>. Accordingly, several CoQ<sub>10</sub>-producing *E. coli* strains have been developed by introducing Dds encoding gene from various sources either via plasmid-expression system or chromosomal integration<sup>4,15-18</sup>. The initial step in biosynthesis of isoprenoid side chain involves a condensation reaction between an IPP and its allelic isomer, Dimethylallyl diphosphate (DMADP) by Geranyl diphosphate synthase (GPPS) which results in the formation of Geranyl diphosphate (GPP) with ten carbons. FPPS adds another IPP to GPP and produces Farnesyl diphosphate (FPP) with 15 carbons. Likewise, Geranylgeranyl diphosphate synthase (GGPPS) uses FPP as a substrate and adds another IPP to form Geranylgeranyl diphosphate (GGPP) with 20 carbons. Finally, the polyprenyl diphosphate synthases such as Dds can use the above mentioned molecules (IPP, GPP, FPP and GGPP) as substrates for addition of more IPP molecules to make corresponding isoprenoid side chain. Finally, attachment of the synthesized side chain to benzene ring and subsequent modifications of the ring result in CoQ production<sup>3,16,17,19,20</sup>. Figure 1 illustrates the biosynthetic steps for CoQ<sub>10</sub> production.

Several Dds have been characterized and isolated from different sources<sup>7,21-23</sup>. FPP is found to be the most preferred substrate for all types of Dds in reactions taking place *in vitro*<sup>4</sup>. Accordingly, FPP can be an attractive precursor to control CoQ<sub>10</sub> biosynthesis in engineered CoQ<sub>10</sub>-producing *E. coli* strains. Indeed, this suggests a hypothesis that the over-expression of the heterologous gene encoding for FPPS (ispA) under the control of various promoters in CoQ<sub>10</sub>-producing *E. coli* strains can control the biosynthesis of CoQ<sub>10</sub>. This strategy allows whole biosynthetic pathway productivity to be controlled through the intermediate metabolites levels.

The current study was conducted to determine the effect of high FPP concentration on both CoQ<sub>10</sub> and CoQ<sub>8</sub> accumulation in engineered *E. coli* cells. Two CoQ<sub>10</sub>-producing strains previously constructed by introducing Dds encoding gene from two different organisms<sup>24</sup> were used for concomitant expression of FPPS using either the *trc* or *P<sub>BAD</sub>* promoter to control the level of expression.

## Materials and Methods

### Strain, media and culture condition

*E. coli* DH5α was selected as the host for construction of recombinant strains (Table 1). Luria-Bertani (LB) medium was used for cloning related cultures<sup>25</sup>. For CoQ<sub>10</sub> production, cells were initially grown in 5 ml of 2YT pre-culture medium (1% yeast extract, 1.6% tryptone and 0.5% NaCl) at 37 °C. For production phase, seven ml of 2YTG medium (2YT medium supplemented with 0.5% glycerol and 0.01% 4-hydroxybenzoic acid) was used to carry out the cultivation in 25 ml tubes. The 2YTG media were inoculated with the pre-culture to an initial OD<sub>600</sub> of 0.1, and were then

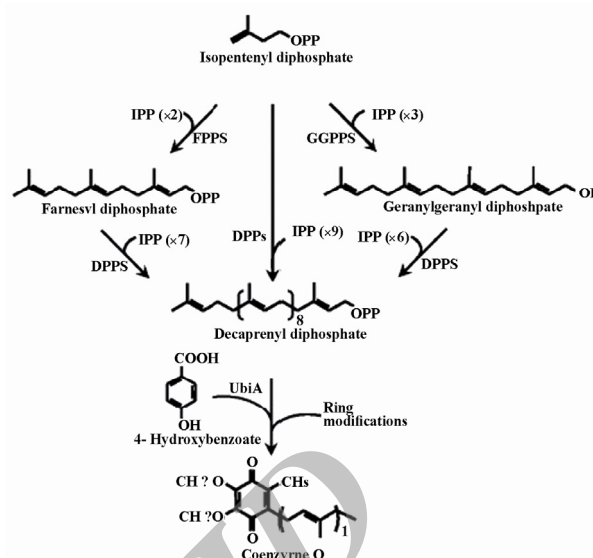


Figure 1. The tandem condensation reactions by decaprenyl diphosphate synthase result in the polymerization of isopentenyl diphosphate (IPP) molecules into decaprenyl diphosphate. The indicated enzymes are as follow: FPPS, farnesyl diphosphate synthase; GGPPS, geranylgeranyl diphosphate synthase; DPPS, decaprenyl diphosphate synthase and UbiA, 4HB-polyprenyl transferase (Zahiri *et al*, 2009).

incubated at 30 °C for 48 hr in a rotary shaking incubator (GFL, UK) at 200 rpm. Ampicillin (100 µg/ml) and kanamycin (30 µg/ml) were added to the culture media as required. The growth of cells was then monitored by measuring optical density of the culture samples at 600 nm. The dry cell weight (g DCW/l) of the culture samples was calculated from a standard curve. CoQs quantification was determined in triplicate culture of recombinant strains.

### Plasmid construction and transformation

All restriction enzymes were purchased from Fermentas (Germany). T<sub>4</sub>DNA ligase was purchased from New England Biolab (UK). Plasmid isolation and PCR product purification kits were from QIAGEN (US). The Dds encoding gene (dds) had previously been isolated from *Agrobacterium tumefaciens* (*A. tumefaciens*) (ATCC 33970) and *Rhodobacter sphaeroide* (*R. sphaeroide*) 2.4.1 (ATCC 17023), as reported elsewhere<sup>23</sup>. The construction of plasmids harboring dds gene either from *A. tumefaciens* (atdds) or *R. sphaeroide* (rsdds) were described previously<sup>24</sup>. Briefly, atdds was obtained from the existing pTatdds plasmid and amplified by PCR using Atdds-3 and Atdds-4 primers (Table 1). The PCR product was then double digested by XmaI and ligated into the pBBR1MCS2 plasmid which had been digested by the same restriction enzyme, resulting in the formation of the pBatdds plasmid. Likewise, rsdds gene was amplified by PCR using existing pTrsdds plasmid as template and Rsdds-F and Rsdds-R primers (Table 1). Next, the PCR product was double digested with EcoRI and Sac I and inserted into the pBBR1MCS2 plasmid which had previously been digest-

Table 1. Strains, plasmids and primers used in this study

Name	Description	Reference
<b>Strains</b>		
<i>E. coli</i> DH5 $\alpha$	F/endA1 hsdR17 (rk- mk+) gin V44 thi-1 RecA1 gyrA (NalI) relA1 $\Delta$ (lacZYA-arg U169 deoR5 (Ψ80dlac $\Delta$ (lacZ) M15)]	(Gibco BRL)
<i>E. coli</i> Ba	<i>E. coli</i> DH5 $\alpha$ harboring pBatdds	(24)
<i>E. coli</i> Br	<i>E. coli</i> DH5 $\alpha$ harboring pBrdsdds	(24)
<i>E. coli</i> BaTi	<i>E. coli</i> Ba harboring pTispA	(This study)
<i>E. coli</i> BrTi	<i>E. coli</i> Br harboring pTispA	(This study)
<i>E. coli</i> BaDi	<i>E. coli</i> Ba harboring pDispA	(This study)
<i>E. coli</i> BrDi	<i>E. coli</i> Br harboring pDispA	(This study)
<b>Plasmids</b>		
pTrc99A	P <sub>trc</sub> expression vector, pBR322 origin, lac/q, Amp <sup>r</sup> , high copy	(Amersham, Bioscience)
pBBR1MCS2	Plac cloning vector, pBBR1 origin, lacZ $\alpha$ , Kan <sup>r</sup>	(37)
pBAD24	P <sub>BAD</sub> cloning vector, pBR322/ColE1 origin, araC, Amp <sup>r</sup>	(38)
pBatdds	pBBR1MCS2 with dds gene from <i>A. tumefaciens</i>	(24)
pBrdsdds	pBBR1MCS2 with dds gene from <i>R. sphaeroides</i>	(24)
pTispA	pTrc99A with ispA gene from <i>E. coli</i>	(This study)
pDispA	pBAD24 with ispA gene from <i>E. coli</i>	(This study)
<b>Primers</b>		
Atdds-3	5'-TCCCCCGGGCACAGGAAACAGACCATGGAGTTCTTG-3' (XmaI)	
Atdds-4	5'-TCCCCCGGGTTAGTTGAGACGCTCGATGCAGAAG-3' (XmaI)	
Rsdds-F	5'-ATAGAATTCAGGAGGTCATCGGGATGGGATTGGACGAGGTTTC-3' (EcoRI)	
Rsdds-R	5'-TAAGAGCTCAAGGGATCAGGCGATGCGTTCGAC-3' (SacI)	
ispA-F	5'- ATAGAATTCAGGAGGAATGAGTAATGGAC-3' (EcoRI)	
ispA-R	5'-ATAGGATCCGGTACCTCATTATTTATTACGC-3' (BamHI)	

ed with the same enzymes, leading to the formation of pBrdsdds. FPPS encoding gene (ispA) had been isolated from the *E. coli* (GenBank: D00694.1) as reported previously<sup>26</sup>. The ispA gene was obtained from the existing pBatdds ispA ubi CABG plasmid by PCR using ispA-F and ispA-R primers (Table 1). The PCR product was subsequently double digested by EcoRI and BamHI and ligated to pTrc99A plasmid under the control of the trc promoter, leading to the formation of pTispA plasmid. The double digested ispA fragment was also ligated to pBAD24 plasmids under the control of P<sub>BAD</sub> promoter, using the same enzymes, and led to the formation of pDispA plasmid. Both pTrc99A and pBAD24 plasmids had previously been digested by EcoRI and BamHI. The recombinant plasmids were introduced to *E. coli* cells using a modified chemical transformation method<sup>27</sup>.

#### CoQs extraction

CoQs content of cells was determined by extraction. Specifically, 500  $\mu$ l of culture was centrifuged at 10,000  $xg$  (Heraeus, Biofuge, UK). The pellet was washed with 1 ml of distilled water, then 1 ml of 20 mM Tris-HCl buffer (pH=7.6). 450  $\mu$ l of lysis buffer [8% sucrose, 5% Triton X-100, 50 mM Tris-HCl (pH=8), 50 mM EDTA (pH=8) and 1 mg/ml of lysozyme] was added to washed pellet and incubated at 37 °C for 30 min. 900  $\mu$ l of a hexane/propanol (5:3 v/v) mixture was added to lysed cells and centrifuged at

11,000  $xg$  for 2 min. The hexane organic phase containing CoQs was transferred to a clean tube. Hexane (500  $\mu$ l) was added to the aqueous phase and centrifuged at 11,000  $xg$  for 2 min. The second hexane phase was added to the first and dried in a vacuum evaporator (Speedvac®, AES 1010, US). The pellet was dissolved in 500  $\mu$ l of absolute ethanol (HPLC grade, Merck, US).

#### Determination and quantification of CoQs

The CoQ content was determined from 10  $\mu$ l injected sample by HPLC (Shimadzu 10A system, Japan) equipped with a Symmetry® C18 column (Waters, US). The mobile phase was the mixture of ethanol and methanol (70:30 v/v) with flow rate of 1 ml/min. CoQs were detected using a UV detector at 275 nm. The identification of corresponding peaks in the HPLC chromatograms of experimental samples was carried out using authentic standards, CoQ<sub>9</sub> and CoQ<sub>10</sub>. Quantification of CoQs were performed with the aid of an appropriate standard curve.

## Results

#### CoQs production in *E. coli*

As mentioned in our previous work<sup>24</sup>, two type of dds genes, rsdds and atdds, were inserted into the pBr1MCS2 plasmid, and resulted in the formation of plasmids pBrdsdds and pBatdds. The recent plasmids were transformed into *E. coli* DH5 $\alpha$ , and designated as

*E. coli* Br and *E. coli* Ba, respectively. The recombinant strains were grown in 2YTGH medium under the same experimental conditions. Their CoQs content was extracted and determined quantitatively by detection of the corresponding peaks in the HPLC chromatogram. The expression of rsdds or atdds in the transformed *E. coli* Ba and *E. coli* Br led to the production of CoQ<sub>10</sub> together with CoQ<sub>8</sub>. On average, *E. coli* Ba had CoQ<sub>10</sub> content of  $629 \pm 40$   $\mu\text{g/g}$  DCW while *E. coli* Br had  $564 \pm 28$   $\mu\text{g/g}$  DCW under the described experimental conditions. *E. coli* naturally has an Ods encoding gene. So, the CoQ<sub>8</sub> content was also measured in CoQ<sub>10</sub>-producing strains. Expression of dds led to a significant decrease in CoQ<sub>8</sub> production by both *E. coli* Ba (from  $990 \pm 32$  to  $360 \pm 23$   $\mu\text{g/g}$  DCW) and *E. coli* Br (from  $990 \pm 32$  to  $246 \pm 24$   $\mu\text{g/g}$  DCW), when compared to that in *E. coli* DH5 $\alpha$ <sup>24</sup>. An example of HPLC chromatogram for CQs identification in *E. coli* Ba is shown in figure 2. The recombinant *E. coli* strain produced CoQ<sub>10</sub> and slight amounts of CoQ<sub>9</sub> in addition to naturally occurring CoQ<sub>8</sub>. The CoQ<sub>9</sub> might be produced as a result of immature release of a fraction of the growing polyprenyl chains from the Dds<sup>17</sup>.

#### Effect of *ispA* over-expression under the control of $P_{BAD}$ promoter in CoQ<sub>10</sub>-producing *E. coli*

The expression of *ispA* results in an increased level of FPP production. FPP is the most suitable substrate for all types of Dds in vitro reactions<sup>4</sup>. In order to study the effect of FPPs on CoQs production, the *ispA* gene was ligated into the pBAD24 plasmid, leading to the formation of pDispA plasmid (Figure 3A). This recombinant plasmid was used for transformation of *E. coli* Ba and *E. coli* Br. The resulting recombinant cells, designated as *E. coli* BaDi and *E. coli* BrDi, were cultured in 2YTG medium, and their CoQs were extracted and quantified by HPLC.

In the absence of inducer, the expression of *ispA* under the control of  $P_{BAD}$  promoter does not have significant effect on CoQ<sub>10</sub> production in *E. coli* BaDi, with negligible change from  $629 \pm 40$  to  $636 \pm 35$   $\mu\text{g/g}$  DCW (Figure 4), while relative increase was observed

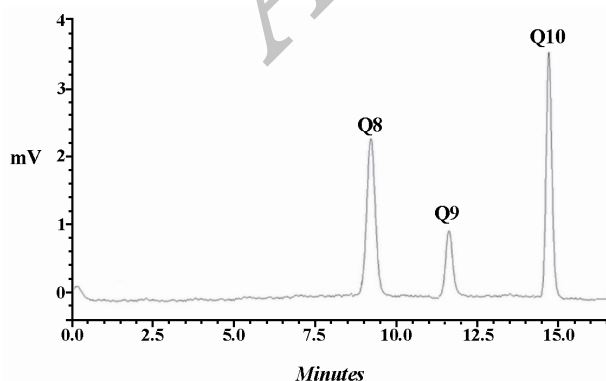


Figure 2. HPLC chromatogram for CQs identification in *E. coli* Ba. The recombinant *E. coli* produced CoQ<sub>10</sub> and slight amounts of CoQ<sub>9</sub> in addition to naturally occurring CoQ<sub>8</sub>.

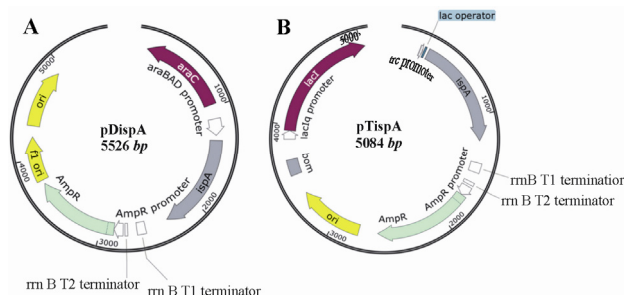


Figure 3. Schematic representation of recombinant pDispA plasmid, encoding for *ispA* gene under the control of  $P_{BAD}$  promoter (A) and pTispA plasmid, encoding for *ispA* gene under the control of *trc* promoter (B). The genetic maps are generated by SnapGene software (from GSL Biotech; available at [snapgene.com](http://snapgene.com)).

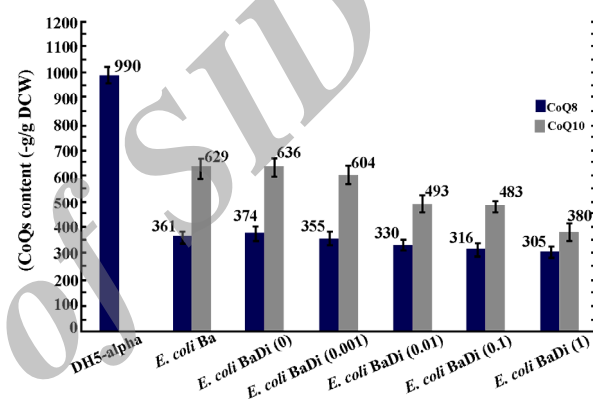


Figure 4. Plasmid pDispA was introduced into *E. coli* Ba, and the coenzyme Qs content was quantified in the resulting strain, as referred to *E. coli* BaDi. Numbers in brackets indicate arabinose mM concentration in cultures. Error bars indicate the standard error of the mean of three independent experiments.

in *E. coli* BrDi from  $564 \pm 28$  to  $750 \pm 28$   $\mu\text{g/g}$  DCW (Figure 5). However, once induction by arabinose was applied, relative reduction in CoQ<sub>10</sub> production was obtained which was proportional to the concentration of arabinose in both strains. In the absence of induction, the CoQ<sub>8</sub> production demonstrated roughly a two-fold reduction in *E. coli* BrDi (from  $246 \pm 24$  to  $117 \pm 13$   $\mu\text{g/g}$  DCW), while no significant change (from  $361 \pm 23$  to  $373 \pm 28$   $\mu\text{g/g}$  DCW) was observed in *E. coli* BaDi. Insignificant reduction in CoQ<sub>8</sub> production was observed in both strains with increased arabinose concentration (Figures 4 and 5). As a whole, the results showed that CoQs production in both *ispA*-expressing strains is inhibited once the induction is applied.

#### Effect of *ispA* over-expression under the control of *trc* promoter in CoQ<sub>10</sub>-producing *E. coli*

In order to confirm that the reduction in CoQs production is due to the inhibitory effect of high FPP concentration, *ispA* was expressed under the control of *trc* promoter which is a stronger promoter than  $P_{BAD}$  and regulated alternatively by adding IPTG to the culture media. So, the *ispA* gene was ligated into the pTrec99A



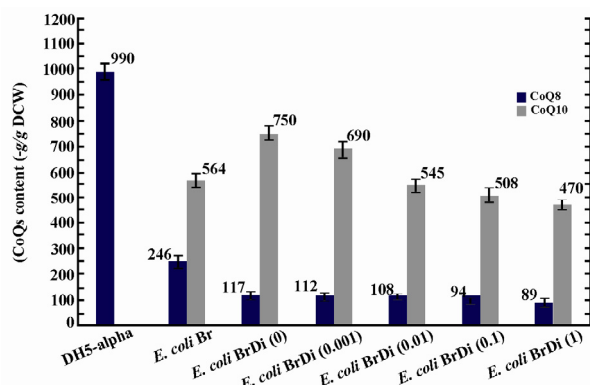


Figure 5. Plasmid pDisA was introduced into *E. coli* Br, and the coenzyme Qs content was quantified in the resulting strain, as referred to *E. coli* BrDi. Numbers in brackets indicate arabinose mM concentration in cultures. Error bars indicate the standard error of the mean of three independent experiments.

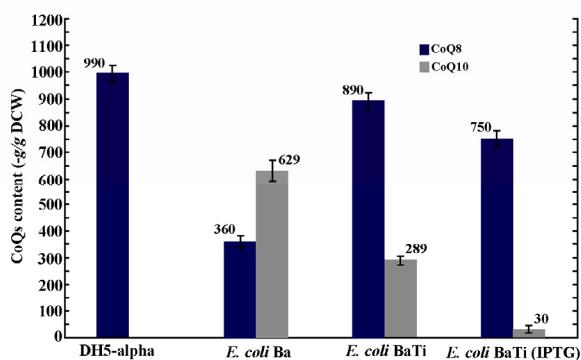


Figure 6. Plasmid pTispA was introduced into *E. coli* Ba, and the coenzyme Qs content was quantified in the resulting strain, as referred to *E. coli* BaTi. Error bars indicate the standard error of the mean of three independent experiments.

plasmid, leading to the formation of pTispA (Figure 3B). This recombinant plasmid was used for transformation of *E. coli* Ba and *E. coli* Br. The resulting recombinant cells, designated as *E. coli* BaTi and *E. coli* BrTi, were cultured in 2YTG medium under the same conditions, as described previously. Then, their CoQs were extracted and quantified by HPLC. The results of this study demonstrated that the over-expression of *ispA* was highly inhibiting for CoQ<sub>10</sub> biosynthesis in both *E. coli* BaTi (Figure 6) and *E. coli* BrTi (Figure 7) and reduced CoQ<sub>10</sub> production levels in the two strains from 629±40 to 289±16 µg/g DCW and from 564±28 to 110±20 µg/g DCW, respectively, even in the absence of an inducer. The presence of the inducer, IPTG (0.05 mM), led to a further decrease in CoQ<sub>10</sub> production to 30±13 µg/g DCW in *E. coli* BaTi and 80±14 µg/g DCW in *E. coli* BrTi. These results show that the inhibition of CoQ<sub>10</sub> production was more severe when compared to the *E. coli* BrDi and *E. coli* BaDi where the *ispA* gene is controlled by P<sub>BAD</sub> promoter.

In addition, it was observed that the reduction of CoQ<sub>10</sub> production in both *E. coli* BaTi and *E. coli* BrTi

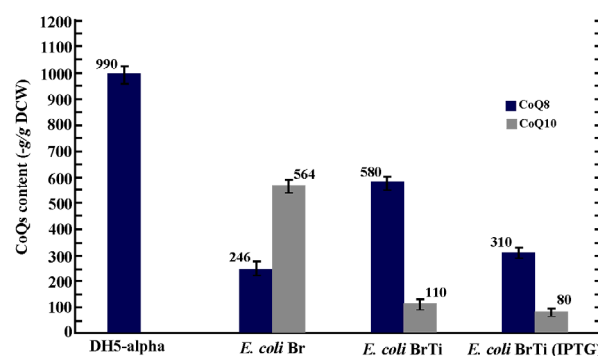


Figure 7. Plasmid pTispA was introduced into *E. coli* Br, and the coenzyme Qs content was quantified in the resulting strain, as referred to *E. coli* BrTi. Error bars indicate the standard error of the mean of three independent experiments.

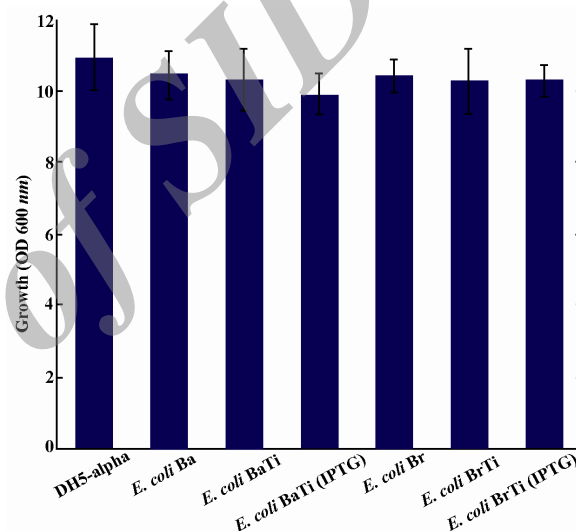


Figure 8. No significant changes were observed in growth associated with *ispA* over-expression. Error bars indicate the standard error of the mean of three independent experiments.

occurred simultaneously with an increase in CoQ<sub>8</sub> production when compared to *E. coli* Ba and *E. coli* Br (from 360 to 890 µg/g DCW in *E. coli* BaTi and 246 to 580 µg/g DCW in *E. coli* BrTi). However, induction by IPTG (0.05 mM) decreased the CoQ<sub>8</sub> biosynthesis as well in both *E. coli* BaTi (to 750 µg/g DCW) and *E. coli* BrTi (to 310 µg/g DCW) as shown in figures 6 and 7. The growth of recombinant strains was also studied and showed no significance changes by *ispA* over-expression (Figure 8).

### Discussion

Previous isoprenoid research has implicated the critical role of IPP levels in the biosynthesis of isoprenoid including CoQs<sup>28-31</sup>. Accordingly, various strategies have been used to improve CoQ<sub>10</sub> production through IPP availability by introducing genes that encode for mevalonate biosynthetic pathway enzymes<sup>28,32-35</sup>. Although these efforts have led to an increase in CoQ<sub>10</sub>

accumulation, there has not yet been an effective method developed<sup>17</sup>. Specifically, it has been demonstrated that other intermediate metabolites alongside IPP contribute to the control of ubiquinone biosynthesis<sup>36</sup>.

IPP, GPP, FPP and GGPP are potential substrates for the stepwise addition of IPP by Dds to make the long 50-carbon chain of CoQ<sub>10</sub>. Previous researches have indicated that FPP is the most suitable substrate for the Dds reactions *in vitro*<sup>4</sup>. In this study, FPPS encoding gene was introduced into two CoQ<sub>10</sub>-producing strains under the control of either the *trc* or *P<sub>BAD</sub>* promoters. The *ispA* gene was expressed under the control of *P<sub>BAD</sub>* promoter by which expression can be modulated over a wide range of inducer (arabinose) concentrations. The results showed relative increase in CoQ<sub>10</sub> production in *E. coli* BrDi when no induction was applied. Induction by arabinose, which results in higher FPP level, led to reduction in CoQ<sub>10</sub> accumulation in both strains. Thus, the higher FPP concentrations may have negative impact on CoQ<sub>10</sub> accumulation. The over-expression of *ispA* caused a large decrease in CoQ<sub>10</sub> production when the *trc* promoter was employed (*E. coli* BaTi and *E. coli* BrTi). In fact, the higher FPP concentrations from high-copy plasmid with strong *trc* promoter led to more reduction in CoQ<sub>10</sub> production, confirming the hypothesis that high concentration of FPP inhibits CoQ<sub>10</sub> accumulation; probably through inhibition of Dds enzymatic activity.

When it comes to monitor the simultaneous CoQ<sub>8</sub> production, it was observed that the production of CoQ<sub>10</sub> brings about a decline in CoQ<sub>8</sub> production and vice versa. It can be assumed that the introducing of *dds* gene leads the substrate flow to be conducted towards CoQ<sub>10</sub> production, thus decreasing CoQ<sub>8</sub> biosynthesis in *E. coli* Ba and *E. coli* Br. In contrast, due to the negative effects of *ispA* gene expression on CoQ<sub>10</sub> production in *ispA*-producing strains, the substrate flow might be conducted again towards CoQ<sub>8</sub> biosynthesis to some extent, because FPP can also function as a substrate for Ods as well as Dds<sup>4</sup>. However, higher level of FPP derived from induction by IPTG or higher level of arabinose reduced CoQ<sub>8</sub> production as well as that of CoQ<sub>10</sub> accumulation. It seems that high concentrations of FPP may have an inhibitory effect on the *E. coli* Ods enzyme activity as well as Dds, thus causing a substantial decrease in CoQ<sub>8</sub> biosynthesis. Growth was not significantly affected by the expression of *ispA*. It seems that the residual amount of CoQ<sub>8</sub> produced in *ispA*-expressing strains is still sufficient for respiration and consequently supporting the growth (Figure 8).

It seems that biochemical properties of various Dds from different organisms also play role in determining their enzymatic activities. For example, introduction of *pDispA* in *E. coli* BrDi resulted in relative increase in CoQ<sub>10</sub> production (from 546 to 750 µg/g DCW) while it had no significant effect on CoQ<sub>10</sub> production in *E. coli* BaDi (from 629 to 636 µg/g DCW). This may have resulted from the relatively low similarity between the

enzymes, which is reflected in differences in their biochemical characteristics<sup>23</sup>.

It seems that the biosynthesis of CoQs is controlled by intermediate metabolite concentration in a complex way that inhibition of polyprenyl diphosphate synthases by precursors is a major bottleneck and adjusting the precursor's concentrations is of great importance. Different strategies can be employed to achieve desirable precursor's concentration in engineered *E. coli*. For example, it is proposed that the type of expression vectors and the promoter strength are very important for CoQ<sub>10</sub> production in recombinant *E. coli*<sup>6</sup>. This idea was confirmed in the current study where *pTrc99A* plasmid, a high-copy number vector with strong promoter, was found to dramatically reduce CoQ<sub>10</sub> production while using *pBAD24* plasmid with an inducible weaker promoter has no severe, reducing effect on CoQ<sub>10</sub> production and even relatively increased CoQ<sub>10</sub> production in *E. coli* BrDi. It has also been observed that the co-production of other isoprenoid compounds which share same intermediate precursors may also contribute to control biosynthesis of CoQs through controlling metabolite flow rate<sup>24</sup>. These findings suggest that the over-expression of the *dds* gene with a high-copy plasmid, which leads to higher Dds concentration, alongside the *ispA* may enhance the CoQ<sub>10</sub> production via quenching the inhibition caused by the high concentration level of FPP.

## Conclusion

In summary, the regulation of the metabolite flow rate is a crucial step, and thus highly important from a biotechnological perspective. Therefore, more effort is needed to verify the role of intermediate metabolite concentration in the biotechnology strategy and to optimize the methods through which the production of CoQs can be elevated.

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## Conflict of Interest

None of the authors has any conflicts of interest to declare.

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