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1. Biological significance of Coenzyme Q10

Coenzyme Q or ubiquinone (CoQ) is a naturally occurring coenzyme formed from the conjugation of a benzoquinone ring and an isoprenoid chain of varying length. Coenzyme Q10, the main quinone species produced by human, provides therapeutic benefits in certain human diseases, such as cardiomyopathy, when administered orally. Increased consumer demand has led to the development of bioprocesses for the commercial production of coenzyme Q10, by using microbes that produce high level of coenzyme Q10 naturally.

However, as knowledge of biosynthetic enzymes and of regulatory mechanism modulating CoQ production increases, opportunities arise for the genetic engineering of CoQ10 production in hosts, such as *Escherichia coli*, that are better suited for commercial fermentation. Various strategies used to improve and/or engineer CoQ10 production in microbes and analyze yields obtained in the light of the current knowledge on the biosynthesis of this molecule. CoQ10 was first isolated from beef heart mitochondria by Dr. Frederick Crane of Wisconsin, U.S.A., in 1957 [1]. Professor Morton of England introduced the name Coenzyme Q, meaning the ubiquitous quinone [2]. In 1958 Karl Folkers determined the chemical structure of CoQ10 i.e., 2,3 dimethoxy-5 methyl-6 decaprenyl benzoquinone (Figure 1), synthesized it, and were the first to produce it by fermentation. In the mid-1960’s, Professor Yamamura of Japan was the first person who used CoQ in the treatment of congestive heart failure. In 1966, Mellor and Tappel found that the reduced CoQ6 is an effective antioxidant [3, 4]. In 1972 Gian Paolo Littarru of Italy along with Professor Karl Folkers documented a deficiency of CoQ10 in human heart disease [5]. By the mid-1970’s, the Japanese pharmaceutical companies started to produce pure CoQ10. Peter Mitchell received the Nobel Prize in 1978 for his contribution to understand biological energy transfer through the formulation of the chemiosmotic theory, which includes the vital proton motive role of CoQ10 in energy transfer systems [6, 7, 8, 9].
In the early 1980's, the technology was developed for directly measurement of CoQ10 in blood and tissue by high performance liquid chromatography and Lars Ernster of Sweden found the importance of CoQ10 as an antioxidant and free radical scavenger [10].

Coenzyme Q10 (2, 3-Dimethoxy-methyl-6-decaprenyl-l, 4-benzoquinone)

Figure 1 (A) Structure of Coenzyme Q10 (oxidized and reduced forms)

Coenzyme Q10 (2, 3-Dimethoxy-methyl-6-decaprenyl-l, 4-benzoquinone)
1.2 Why Coenzyme Q10?

Due to the stress conditions caused by the disease or by the busy life style in humans, ageing factors are continuously increasing and energy production of body system decreased as well. Mitochondria are known as the power houses of cell for energy production. Coenzyme Q10 is the main component of electron transport chain in mitochondria. During the stress condition energy production as well as CoQ10 levels in the body decreases. This can be compensated by giving coenzyme Q10 supplementation orally and health conditions can be improved greatly. Coenzyme Q10 supplementation energized the body and increase body energy production in the form of ATP.

Coenzyme Q10 is located predominantly at the mitochondrial inner membrane, where its primary role is in the transfer of electrons from membrane-bound dehydrogenases to complex III of the electron transport chain [11, 12, 13]. Coenzyme Q10 also acts as an antioxidant that protects membrane phospholipids and proteins from lipid peroxidation by scavenging free radicals directly and/or by regenerating levels of tocopherol [14, 15, 16]. Bacteria possess several structurally different quinones, among which Coenzyme Q (CoQ), menaquinone (MK) and de methyl menaquinone (DMK) are the most common. These quinones are found in the cytoplasmic membrane, where they participate as electron carriers in respiration and in disulfide-bond formation [17, 18]. CoQ participates in aerobic respiration, whereas MK and DMK have roles in anaerobic respiration [17,19]. CoQ molecules are classified based on the length (n) of their isoprenoid side chain (CoQ-n). For example, the main CoQ species in humans is CoQ10, in rodents it is CoQ9, in Escherichia coli it is CoQ8 and, in Saccharomyces cerevisiae, it is CoQ6. The CoQ biosynthesis pathway has been deciphered almost entirely in the two model organisms E. coli and S. cerevisiae [20-36]. Several reviews are available that describe those CoQ pathway(s) in detail and discuss their regulation as well as function(s) of the CoQ molecule [37-43].

1.3 Functions of Coenzyme Q10

Coenzyme Q10 is acts as a supporting medicine and a growing body of evidence suggests that the oral administration of CoQ10 is beneficial in the treatment of human conditions, such as cardio-myopathy, diabetes, Parkinson’s and Alzheimer’s disease and can also reduce the risks of myopathy associated with the use of statin drugs [44, 45, 46, 47, 48].
Moreover, human CoQ10 deficiency, either caused by mutations in CoQ biosynthetic enzymes or by mutations indirectly leading to low CoQ10 levels, has been associated with cases of encephalomyopathy, cerebellar ataxia and Leigh syndrome [49, 50, 51, 52]. Some of these coenzyme Q10 deficient patients showed clinical improvements following Coenzyme Q10 supplementation treatments [50, 53].

The use of CoQ10 is also gaining popularity in the cosmetic industry owing to its antioxidant properties [54]. Reports of these recent findings on the potential health benefits of CoQ10 have led to an increase in consumer demand for this molecule, which has been paralleled by intensified efforts in the development of bioprocesses for its commercial production. Up till now, successful approaches for the commercial production of CoQ10 have relied predominantly on bacterial mutants selected for their high CoQ10 content. Although this strategy resulted in strains with titers suitable for commercial application, further improvements will probably rely on a solid knowledge of the biosynthetic pathway of CoQ10 production in microbes, which could then be coupled with metabolic-engineering strategies.

Figure 1 B. 3-D structure of Coenzyme Q10 with “head and tail”
<table>
<thead>
<tr>
<th>Condition</th>
<th>Reference(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cardiovascular disorders</td>
<td>Belardinelli <em>et al.</em> (2006)</td>
</tr>
<tr>
<td>Parkinson’s Disease</td>
<td>Shults, C.W. <em>et al.</em> (2002)</td>
</tr>
<tr>
<td>Age-related decline in immunity</td>
<td>Dhanasekaran, M. and Ren, J. (2005)</td>
</tr>
<tr>
<td>Mitochondrial cytopathies</td>
<td>Genova <em>et al.</em> 2005</td>
</tr>
<tr>
<td>Ischimia</td>
<td>Tiano, L. <em>et al.</em> (2007)</td>
</tr>
<tr>
<td>Immune disorders</td>
<td>Langsjoen P. H <em>et al</em> (1991)</td>
</tr>
<tr>
<td>Muscular dystrophy</td>
<td>Beal MF (2004)</td>
</tr>
<tr>
<td>Alzheimer’s Disease</td>
<td>Beal MF (2004), Soderberg, M <em>et al.</em> (1992)</td>
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Table-1 Use of Coenzyme Q10 in health care as a supporting medicine and antioxidants
2. CoQ10 production methods

Two manufacturing processes are currently being used in commercial production of Coenzyme Q10. One is chemical synthesis process and another is microbial fermentation process. In case of chemical synthesis, relatively higher rates of “cis” isomers in the side chain (pharmacologically less active form) and Coenzyme Q9 which has only 9 isoprene unites in the side chain are formed as bi-products which must be eliminated from the final product through a purification process. On the other hand, the natural fermentation process yields the “all trans” form of Coenzyme Q10 (pharmacologically active form), resulting in scarcely a trace of Coenzyme Q9.

2.1 Chemically Synthetic and semi-synthetic methods for Co Q10 production

For the chemical synthesis of coenzyme Q10, it is important to develop a highly stereoselective process, due to the all-C-conformation of the coenzyme Q10 tail. E-1,4-Diiodo-2-methyl-1-butene as an E-conformational isoprene synthon is prepared from 3-butyn-1-ol with over 99% selectivity and 77% yield in two reaction steps using Zr-catalyzed carboalumination of alkynes. All-E-conformation can be achieved by this unit for the lipophilic tail synthesis of coenzyme Q10. From E-1,4-diiodo-2-methyl-1-butene to coenzyme Q10 there are nine synthetic steps involving Pd-catalyzed homoallyl- and homopropargyl-alkenyl coupling. The overall yield is 26%, with 98% selectivity, without stereoisomeric separation [55]. Solanesol, nonaprenol isolated from tobacco leaves, is used as a starting material for isoprenoid side-chain synthesis in the semi-synthetic method. The quinonoid head can be prepared from 2,3,4-methoxy-6-methyl benzaldehyde. The overall yield is 64% without impurities detected in the fermentation products, such as the cis-isomer produced by the synthetic method and coenzyme Q9 but, these two methods are environmentally unfavorable and suffer from low overall yields [56]. By using this methodology, coenzyme Q10, (E,Z,E)-geranylgeranoil, and other natural or unnatural compounds have been synthesized efficiently [55].
Lipshutz et al. [56] reported the easy method for the production of CoQ10 by chemical synthesis using 2-alkoxy-3, 4-dimethoxy-6-methylbenzyl chloride, 1-TMSpropyne, and solanesol.

Figure 2 A. Chemical synthesis of Coenzyme Q10 [55]

Figure 2 B. Retro synthetic analysis of CoQ10
After this process of chemical synthesis came into existence various modifications and improved work done in the process such as [56] a new and novel route to CoQ10 has
been realized, which calls for six total operations (3 - 4 - 5 - 2 - 9 - 10 - CoQ10) to arrive at coenzyme in 64% overall yield, which constitutes the efficient synthesis. Synthetic CoQ10 was identical to authentic material in all respects. Particularly noteworthy is the fact that by virtue of the synthetic sequence outlined herein, typical impurities such as the corresponding cis-isomer and CoQ9, sometimes found in CoQ10 which is produced industrially almost entirely by fermentation, are completely averted [56].

A new route to the key coupling partner, chloromethylated CoQ10 (1), allows for direct formation of CoQ10 (3) via nickel-catalyzed crosscoupling with the side chain in the form of an in situ-derived vinyl alane (2).

![Chemical Structures]

*Figure 2 F. Single step Coenzyme Q10 synthesis [57]*

Lipshutz et al [57] describe an improved synthesis of key coupling partners chloromethylquinone 1 and vinylalane 2. As previously described, these reactive species combine under nickel catalysis to generate CoenzymeQ10 directly.

The most expensive ingredient in our CoQ10 synthesis is solanesol, notwithstanding its status as a waste product of tobacco. Isolation of this 45-carbon allylic alcohol in both quantity and in high (>90%) purity is challenging and can be costly depending upon the method of purification. Thus, an ideal synthesis would seek to minimize the extent to which intermediates based on solanesol are manipulated in route to CoQ10 [57].
Two new “generations” of methodological advances are reported for the Negishi et al. [55] carboalumination of terminal alkynes. Use of simple, inexpensive additives that alter the Al-Zr complex formed between Me$_3$Al and Cp$_2$ZrCl$_2$ give rise to an especially effective reagent mix that result in virtually complete control of regiochemistry upon carboalumination of 1-alkynes. One timely application to coenzyme Q10 is highlighted. Regioisomers from subsequent coupling, which would otherwise be very difficult to separate, are avoided [58].

The basic disadvantage of this chemical synthesis needs to be performed at -70 °C and, even a 90% yielding reaction is at a disadvantage, since an additional 5-10% will be lost as the regioisomeric product and the difficulties in separation of isomeric form of CoQ10.

2.2 Microbial production

2.2.1 CoQ10 producing microorganisms

Natural high producers of CoQ10 include strains of *Agrobacterium tumefaciens*, *Paracoccus denitrificans* and *Rhodobacter sphaeroides* [59]. Notably, CoQ is the only class of quinone present in all these species as opposed to quinones present in *E. coli*, which produces CoQ, MK and DMK [60]. The productivity of these strains ranges from 30 to 130 mg g$^{-1}$ CoQ10 [59]. A commercially viable strain should produce yields higher than 500 mg g$^{-1}$, which means that titers from natural high producers are insufficient for industrial purposes. Growth-conditions optimization and metabolic regulation mechanisms have been investigated in natural producers of CoQ10 with the aim to improve their CoQ biosynthesis.
The oxidation-reduction potential (ORP) of the fermentation medium is an important factor that regulates CoQ10 biosynthesis in these strains. Low ORP, which is established by limiting the O₂ supply to growing cultures, increases CoQ10 production in *A. tumefaciens* and *R. sphaeroides* [59, 61, 62, 63].

One hypothesis that has been proposed to explain this increase is that the reduction of ORP might shift the ratio between oxidized CoQ and reduced CoQH₂ towards CoQH₂. This might trigger cells to synthesize more CoQ10 to compensate for this imbalance, to restore respiratory functions and/or to scavenge toxic electrons in the membrane [61].

### 2.2.2 Overproduction of Coenzyme Q10

**A. Improved production in microorganisms by mutagen treatment**

Selection studies of wild-type strains have shown *A. tumefaciens* ATCC 4452, *Rhodobacter sphaeroides* FERM-P4675 and *P. denitrificans* ATCC 19367 are excellent coenzyme Q10 producers. Further strain development by chemical mutagenesis was performed for the above strains. N-Methyl-N-nitro-N-nitroso-guanidine was applied as a mutagen and several drugs such as L-ethionine (an analogue of L-methionine, which is a precursor for the methoxy moiety of coenzyme Q), daunomycin and menadinone (vitamin K₃) were used as selection pressure. A series of mutagenesis using different selection markers and drug resistance resulted in the construction of a mutant strain, *A. tumefaciens* AU-55, which could produce up to 180 mg l⁻¹ of coenzyme Q10 in 58 h with a specific coenzyme Q10 content of 4.5 mg g⁻¹ of dry cell weight. Another mutant strain, *R. sphaeroides* Co-22-11, produced 346.8 mg l⁻¹ of final coenzyme Q10 concentration with 8.7 mg g⁻¹ of dry cell weight under a limited supply of air in Erlenmeyer flasks without baffle plates. In low-aeration conditions, multilayer structures of inner membranes which retained coenzyme Q10 were highly developed [59]. After strain development, optimization of fermentation strategies and environmental parameters such as temperature, carbon/nitrogen ratio, oxygen supply and oxidation-reduction potential (ORP) were done in order to improve the product yield of coenzyme Q10 in the mutant strains. Temperature and carbon/nitrogen ratio were optimized for a mutant strain, *Agrobacterium* sp. derived from *A. tumefaciens* ATCC 4452, for high productivity.
and reduction of culture broth viscosity due to the formation of extra cellular polysaccharides [115].

The optimal conditions for low viscosity and high coenzyme Q10 production were 8% sugar, 0.16–0.26% ammonium nitrogen and a temperature of 32–34°C. The optimal oxygen absorption rates (OAR) for the above mutant were determined at 0.84 mmol O\textsubscript{2} l\textsuperscript{-1} min\textsuperscript{-1} for a maximum coenzyme Q10 concentration of 66 mg l\textsuperscript{-1} and at 0.58 mmol O\textsubscript{2} l\textsuperscript{-1} min\textsuperscript{-1} for a maximum specific coenzyme Q10 content of 3.2 mg g\textsuperscript{-1} dry cell weight [115]. Besides the increases in CoQ10 biosynthesis described earlier, further improvements in CoQ10 production were achieved by chemical mutagenesis, enabling CoQ10 yields up to 770 mg l\textsuperscript{-1}[62]. Mutant strains of \textit{R. sphaeroides} displayed higher CoQ10 contents of up to 8.7 mg g\textsuperscript{-1} dry cell weight (DCW) when aeration of the culture was reduced and this corresponded with the development of an invaginated inner membrane structure [59].

High CoQ10 producing mutants were selected based on several indirect phenotypes, such as growth on structural-analogue inhibitors of the pathway or alteration in pigment production. The rationale was to find mutants with an up regulated CoQ10 pathway by selecting for growth on pathway or respiration inhibitors. \textit{Agrobacterium tumefaciens} mutants with higher CoQ10 content than the parent strain were identified based on their ability to grow in the presence of the structural analogues of CoQ, daunomycin and menadinone. Mutants were also selected based on growth in the presence of L-ethionine, which is an analogue of methionine, the precursor that supplies the three methyl groups modifying the Coenzyme Q10 benzoic ring [59].

Analogues of aromatic amino acids and inhibitors of respiration could also be used to improve Coenzyme Q10 yields in these \textit{Agrobacterium tumefaciens} mutants [59]. \textit{R. sphaeroides} mutants were selected based on their pigment-production phenotype in which the appearance of green colonies was used as an indicator of reduced carotenoid content [59]. Increased Coenzyme Q10 levels in mutant strains of \textit{Protomonns extorquens} and \textit{Rhodobacter sphaeroides} KY8598 were also associated with low carotenoid content [62,64].

The correlation between reduced carotenoid content and increased CoQ10 production is not entirely clear but it might be possible that excess isoprenoid precursors resulting
from mutations in the carotenoid pathway are redirected to CoQ10 synthesis. Another possible explanation for the increase in CoQ10 synthesis observed in carotenoid-mutant strains is that the cells are compensating for the loss of carotenoids as antioxidants by increasing the levels of a different antioxidant, CoQ10, to protect themselves from oxidative damage.

B. Improved Coenzyme Q10 production by recombinant E. coli

E. coli, which makes coenzyme Q8 naturally, can be manipulated to synthesize coenzyme Q10 by introducing the decaprenyl diphosphate synthase gene from various organisms. Recently, fermentation studies on the production of coenzyme Q10 were undertaken with recombinant E. coli containing the *Gluconobacter suboxydans* decaprenyl diphosphate synthase gene [88]. Fed-batch fermentation with a glucose-limited feeding strategy yielded a final concentration of 103 g l\(^{-1}\) of cells, 25.5 mg l\(^{-1}\) of coenzyme Q10 and 0.29 mg l\(^{-1}\) of specific coenzyme Q10 content. Such a low level of specific coenzyme Q10 content could be improved by metabolic engineering of the precursor synthesis pathways with the introduction of the key enzymes involved in the non-mevalonate pathway.

The results of metabolic modification for the production of isoprenoids such as terpenes and carotinoids can be applied to coenzyme Q10 production, which also uses the same building blocks, including IPP and DMAPP. It was reported that individual or combinational overexpression of the *dxs* and *dxr* genes in the non-mevalonate pathway and the *idi* and the *ispA* genes concerning FPP synthesis improved the concentrations of target compounds [96, 130, 131].

3. Metabolic regulation of Coenzyme Q10 of biosynthesis in prokaryotes and eukaryotes

Coenzyme Q10 is present in cell membrane and in mitochondrial respiratory chain in eukaryotes. It has also several other functions of great importance for the cellular metabolism. The isoprenoid side chain of coenzyme Q10 is synthesized from acetyl co-A by a series of enzymatic reaction, while the benzoquinone portion is synthesized from amino acids. Coenzyme Q10 shares a common biosynthetic pathway with the cholesterol, Isopentenyl pyrophosphate and its isomer di-methyl allyl pyrophosphate is linked alternately to form poly prenyl chain, which is also called isoprene.
The biosynthesis of isoprenoids occurs via two distinct pathways — the mevalonate pathways and non-mevalonate pathways (deoxy-xylulose 5-phosphate) DXP pathway. Mevalonate pathways are associated with archaeobacteria and eukaryotes whereas DXP pathway is characteristic of eubacteria and higher organisms. [65] The biosynthesis of CoQ10 has been extensively studied in prokaryotes and eukaryotes organisms. The terminal part of the prokaryotic CoQ10 biosynthesis differs from eukaryotic in order of synthetic events. In prokaryotes the modifications of ring after attachment of the side chain proceed via a decarboxylation prior to hydroxylation and methylation whereas in eukaryotes decarboxylation occurs after hydroxylation and methylation.

\[
\text{Acetyl-CoA} \xrightarrow{HMG-CoA \text{ reductase}} \text{Isopentenyl-PP} \\
\quad \xrightarrow{} \text{Dimethylallyl-PP} \\
\quad \xrightarrow{} \text{Geranyl-PP} \\
\quad \xrightarrow{} \text{Farnesyl-PP} \\
\quad \xrightarrow{\text{trans-Prenyltransferase}} \text{Decaprenyl-PP} \\
\quad \xrightarrow{\text{Squalene synthase}} \text{Squalene} \\
\quad \xrightarrow{\text{cis-Prenyltransferase}} \text{Geranylgeranyl-PP} \\
\quad \xrightarrow{} \text{Cholesterol} \\
\quad \xrightarrow{} \text{Dolichol} \\
\quad \xrightarrow{} \text{Dolichyl-P} \\
\]

Figure 3 A. Biosynthesis of Coenzyme Q10 in microorganism [132]
Figure 3 B. Biosynthetic pathways for the synthesis of isopentenyl diphosphate and dimethylallyl diphosphate. [133]

A: Mevalonate pathway. 10, acetyl-CoA; 21, acetoacetyl-CoA; 22, HMG-CoA; 23, mevalonate; 24, mevalonate 5-phosphate; 25, mevalonate 5-diphosphate; 26, isopentenyl diphosphate; 27, dimethylallyl diphosphate. B: 2-C-Methyl-D-erythritol 4-phosphate (MEP) pathway. 28, pyruvate; 29, hydroxyethyl-TPP anion; 30, D-glyceraldehyde-3-phosphate; 31, 1-deoxy-D-xylulose 5-phosphate (DXP); 32, 2-C-methyl-D-erythritol 4-phosphate (MEP); 33, 4-diphosphocytidyl-2-C-methylerythritol; 34, 4-diphosphocytidyl-2-C-methyl-D-erythritol 2-phosphate; 35, 2-C-methyl-D-erythritol 2,4-cyclodiphosphate; 26, isopentenyl diphosphate; 27, dimethylallyl diphosphate
Figure 3 C. Metabolic pathway for the biosynthesis of Coenzyme Q10 from p-hydroxy benzoic acid in gram negative bacteria [134, 135].

Figure 3 D. Compartmentalization of the mevalonate pathway and associated reactions after the branch-point.

Abbreviations: CoA, coenzyme A; CoQ, coenzyme Q; FPP, farnesyl pyrophosphate; GGPP, geranylgeranyl pyrophosphate; GPP, geranyl pyrophosphate; IPP, isopentenyl pyrophosphate.
3.1 HMG-Co A reductase regulation

The main regulatory enzyme of the mevalonate pathway is HMG-Co A reductase, preferentially affecting Coenzyme Q synthesis [66]. Statin are the main inhibitors of HMG-Co-A reductase [67]. The initial part of the mevalonate pathway is a sequence of reactions that form farnesyl-PP (FPP) from acetyl-CoA. FPP is the last common substrate for the biosynthesis of several end products including CoQ10 [68]. In addition to HMG-CoA reductase, the branch point enzyme after FPP synthase e.g. squalene synthase for cholesterol, Cis phenyl transferase for delicol and trans-prenyl transferase for CoQ10 are the main regulatory enzymes in the biosynthesis of lipids. [69]. (Figure 3B & 3E)

![Changes in farnesyl-PP pool](image)

**Figure 3 E. Regulation of FPP pool in CoQ10 biosynthesis [69]**

3.2 Inhibitors and stimulator of metabolic biosynthetic pathway

*Antimycin*

Antimycin is the most potent inhibitors of the ubiquinone reduction site (Qi site) of complex III. This inhibitor consists of salicylic acid and dilactane moiety. Salicylic acid moiety is recognized by the enzyme. The dilactane ring moiety play a supporting role in inhibition binding to the Qi site by increasing the hydrophobicity of the molecules [70].

*Stigmatellin*

Stigmatellin is one of the most potent inhibitors of the ubiquinone oxidation site (Qo site) of complex III [70]

*Paraquat*

Paraquat (1, 1 di methyl -4, 4 bi pyridium di chloride) is an oxidative stressor by the process of lipid per-oxidation [71].
**Para-hydroxy benzoic acid**

The biosynthesis of ubiquinone involves two pathways one for the formation of benzaquinone nucleus and another for the formation of isoprenoid side chain. The precursor for the benzaquinone nucleus is PHB derived from tyrosine. The isoprene pathway probably up to the formation of isopentenyl pyrophosphate is shared by ubiquinone and sterol [72].

**Lovastatin**

Lovastatin inhibits the synthesis of cholesterol and other non sterol end products such as CoQ10. Lovastatin treatment diminish ubiquinone and alpha tocopherol content [73].

4. **Metabolic engineering**

4.1 **Metabolic engineering of genes responsible for CoQ10 production**

The isolation of natural producers and their chemical mutants has been the most successful strategy so far in the development of microbial strains for the commercial production of CoQ10. However, as knowledge of the biosynthetic enzymes and of the regulatory mechanisms of CoQ10 production increases, opportunities have arisen for the metabolic engineering of CoQ10 production in microbes. Most efforts to engineer a CoQ10 pathway are focused on *E. coli* currently because this organism is well suited for genetic modifications and large-scale fermentation. Metabolic engineering of *E. coli* for high CoQ10 yield requires several biochemical steps to be modified and/or optimized. Foremost, *E. coli* requires the expression of an exogenous decaprenyl diphosphate synthase (DPS) to condense the two precursors isopentenyl diphosphate and famesyl diphosphate into decaprenyl diphosphate (DPP) instead of octaprenyl diphosphate produced by wild-type *E. coli*. In addition, increasing the availability of metabolic precursors is necessary to improve Coenzyme Q10 production in *E. coli*. Two approaches have been explored so far to this end increasing flux of the isoprenoid precursors and overexpressing selected ubi genes involved in the Coenzyme Q10 biosynthetic pathway [74, 75].

4.2 **Heterologous gene expression for improved CoQ10 production**

The length of the isoprenoid tail of Coenzyme Q10 is dictated by the polyprenyl diphosphate synthase present in the host organism [76]. This enzyme catalyzes the sequential condensation between isopentenyl diphosphate and allylic diphosphates to form a polyprenyl diphosphate of a relatively defined chain length. In *E. coli*, an
octaprenyl diphosphate synthase, IspB, catalyzes the formation of octaprenyl diphosphate (OPP) and small amounts of shorter prenyl diphosphates. Accordingly, the main CoQ form present in *E. coli* is CoQ-R as well as some minor forms, including CoQ1 to CoQ7 [77]. CoQ species with longer side chains are typically produced by certain Gram-negative aerobic rods, such as *Agrobacterium tumefaciens* and *Gluconobacter suboxydans*, some Gram-negative such as *Paracoccus denitrificans* some Gram-negative phototrophic bacteria, including *Rhodobacter sphaeroides*, and also some fungi [60, 78]. The current strategy used to engineer *E. coli* to produce CoQ10 is to introduce a gene coding for a DPS cloned from a CoQ10 producing microbe. DPS from *Saitoella complicata*, *Bulleromyces albus* and *Agrobacterium sp*. KNK712 have been patented for the production of CoQ10 [79, 80, 81]. *E. coli* strains expressing a foreign DPS accumulate CoQ10, in addition to varying levels of CoQ8 and CoQ9, depending on the DPS expressed [82, 83, 87, 88]. From an industrial point of view, this is undesirable because reduced purity translates into an increase in downstream processing and purification steps, which further translates into increased product costs. Most bacteria surveyed to date produce minor amounts of shorter CoQ forms in addition to their main CoQ species, suggesting the presence of more than one prenyl synthase or, probably, that DPS enzymes generate shorter prenyl diphosphates in addition to DPP [60].

Nevertheless, the DPS of certain bacteria appear to be more specific towards DPP. For instance, when DPS from *Rhodobacter sphaeroides* was expressed in *E. coli*, it displayed more specificity towards DPP than DPS from *Agrobacterium tumefaciens* [83]. Isolating DPS enzymes with stringent product specificity with the goal of obtaining recombinant *E. coli* strains that are highly enriched for CoQ10 might therefore be a good approach to increase yields and reduce the costly purification of CoQ10 from unwanted CoQ species during the manufacturing of high-purity formulations.

Decaprenyl diphosphate synthase (ddsA) catalyzes the condensation reaction of isopentenyl diphosphate (IPP) with dimethylallyl diphosphate and hence plays a crucial role in synthesizing the decaprenyl tail in CoQ10 biosynthesis [37] the recombinant Escherichia coli system harboring the decaprenyl diphosphate synthase gene (ddsA) from *Gluconobacter suboxydans* was constructed to produce the maximum CoQ10 concentration of 26 mg l⁻¹ in glucose-limited fed-batch cultivation. [88]
IPP is a five-carbon building block in the synthesis of carotenoids and produced in \textit{E. coli} through the nonmevalonate pathway by Kuzuyama [84]. The first reaction in this pathway to synthesize IPP is catalyzed by 1-deoxy-d-xylulose 5-phosphate (DXP) synthase encoded by the \textit{dxs} gene [103], which is considered one of the rate-limiting enzymes for isoprenoid synthesis [104]. The enlargement of IPP pool by coexpression of DXP synthase might increase production of CoQ10, which uses IPP as a precursor like lycopene. Co-expression of the DXP synthase gene enhanced CoQ10 production in recombinant \textit{E. coli} [88].

CoQ10 has a ‘head and tail’ structure. In accordance, in vivo biosynthesis of both building blocks is essential for microbial production of CoQ10. p-Hydroxybenzoic acid to be used as a precursor of the ‘quinone head’ is derived from the shikimate pathway, which is a key pathway for the synthesis of aromatic amino acids through chorismate. The isoprenoid side chain of CoQ10 is produced through the nonmevalonate metabolic pathway in \textit{E. coli}. The first reaction of this pathway is mediated by DXP synthase encoded by the \textit{dxs} gene catalyzing the condensation reaction between \textit{d}-glyceraldehyde 3-phosphate and pyruvate [103]. DXP synthase has been shown to play an important regulatory role in the nonmevalonate pathway for the synthesis of IPP. Overexpression of \textit{dxr} (1-deoxy-d-xylulose-5-phosphate reductoisomerase), \textit{idi} (isopentenyl diphosphate isomerase), and \textit{ispA} (farnesyl diphosphate synthase) genes might result in an enhancement in CoQ10 production. All the enzymes involved in the nonmevalonate pathway might be amplified by constructing the bacterial artificial chromosome. IPP, one of the crucial precursors produced in the nonmevalonate pathway, plays an important role in all living organisms for the biosynthesis of diverse classes of products such as isoprenoids and terpenes [20].

Overexpression of the \textit{ddsA} gene for production of CoQ10 caused depletion of the IPP pool in \textit{E. coli}, i.e., the IPP pool size in the CoQ10 producing strain was less than one-third of the control strain. However, the diminished IPP level was recovered by coexpression of the DXP synthase. In conclusion, the productivity and relative content of CoQ10, which are both important economic yardsticks, were significantly enhanced by amplification of the DXP synthase level in recombinant \textit{E. coli}.
4.3 Role of precursors on Coenzyme Q10 production

Coenzyme Q10 is formed from a benzoic ring derived from para-hydroxybenzoate (PHB), itself; a product of the chorismate pathway. Other products of this pathway include DMK, MK, and aromatic amino acids and foliate. The hydrophobic tail, however, is an isoprenoid formed through the 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway, which is also used in the formation of DMK, MK, bactoprenol and the prenylation of some tRNAs [42,89,90]. The flux through the chorismate and MEP pathways are known to be limiting [91, 92] and are expected to become rapidly restrictive in a system in which the carbon flow is pulled towards CoQ10 synthesis. Therefore, to achieve maximum yields of Coenzyme Q10 in engineered microbes, it will be necessary to increase the flux of both of these precursors. Two approaches have been used to increase the flux through the isoprenoid pathway. The first approach is based on the over-expression of 1-deoxy-D-xylulose 5-phosphate synthase (DXS), which is a rate-limiting enzyme of the MEP pathway [93, 94, 95, 96, 97]. In *E. coli*, the overexpression of heterologous DXS isolated from *Pseudomonas aeruginosa*, *Bacillus subtilis* or *Synechocystis sp.* 6803 resulted in small increases in Coenzyme Q10 content [94, 97].

In the high Coenzyme Q10 producer strain *Agrobacterium tumefaciens* KCCM 10413, an increase in DXS activity lead to the accumulation of approximately 500 mg l\(^{-1}\) CoQ10 (CoQ10 8.3 mg g\(^{-1}\) DCW), a yield close to what was already obtained from the KCCM 10413 strain but nevertheless closer to what is desirable for commercial production of Coenzyme Q10 [95]. The second approach for increasing flux to isoprenoids is based on the heterologous expression of the typically eukaryotic mevalonate pathway in *E. coli*. Such a strain was first developed to enable high production of the antimalarial terpenoid precursor, amorphadiene [91]. Using this engineered mevalonate pathway in conjunction with the expression of a codon-optimized amorphadiene synthase, a yield of 500 mg l\(^{-1}\) was obtained [98]. The expression of a foreign mevalonate pathway for the production of isoprenoid precursors has therefore the potential to improve CoQ production in bacteria significantly. This strategy was indeed used recently to engineer high Coenzyme Q10 biosynthesis in *E. coli* [74]. Six genes of *Streptococcus pneumoniae* as well as acetoactyl-CoA thiolase from *Ralstonia eutropha* and IPP isomerase from *E. coli* were used to bypass the endogenous MEP pathway. Using this strategy, an *E. coli* strain producing 2.4 Coenzyme Q10 mg g\(^{-1}\) DCW was obtained [74].
This represents the highest CoQ content achieved so far in *E. coli* and makes this strain comparable with the natural high producers of Coenzyme Q10.

Nevertheless, the relatively modest increase in CoQ10 compared with 500 mg l\(^{-1}\) for amorphadiene with a comparable strategy is probably an indication that other bottlenecks in the pathways leading to CoQ10 biosynthesis are present. In addition to limited flux to DPP, it is probable that increasing the availability of the aromatic precursor PHB is required to enhance the production of CoQ10 appreciably. The addition of 0.01 % PHB as well as mevalonate to *E. coli* that expresses the lower part of the mevalonate pathway almost doubled Coenzyme Q10 production, indicating that PHB availability is indeed limiting CoQ10 synthesis in these recombinant strains [74]. However, the production of Coenzyme Q10 decreased in *P. extorquens*, whereas carotenoid and sterol biosynthesis increased on the addition of increasing amounts of PHB [73]. This indicates that increased PHB concentration, produced endogenously or not, might not necessarily result in increased CoQ10 synthesis in hosts other than *E. coli*. Although it is not clear to what extent *E. coli* can assimilate extracellularly fed PHB, the exogenous addition of PHB is not a commercially viable option owing to the increase in the cost of production. As an alternative to providing PHB in the medium; it is also possible to engineer *E. coli* with a higher flux through chorismate biosynthetic pathway. High PHB yields in recombinant *E. coli* were achieved by Barker and Frost [92], who maximized the carbon flow to PHB by the overexpression of selected enzymes of the chorismate pathway, while eliminating the production of the aromatic amino acids, which competes for the same precursors.

This strategy led to the production of considerably high levels of PHB (≤12 g l\(^{-1}\)) compared with levels produced normally by *E. coli* [92]. Combining such an engineered PHB pathway with isoprenoid overproduction would be expected to relieve bottlenecks caused by limited precursor availability and could therefore improve Coenzyme Q10 production significantly.

Blocking the biosynthesis of competing molecules, in particular of MK and DMK, might also increase CoQ10 yields in *E. coli*. MK and DMK share the quinone pool with CoQ and are formed of OPF and a naphtoquinone derived from chorismate [42]. Under aerobic conditions, the biosynthesis of CoQ is favoured over that of DMK and MK because it forms 65% of the quinone pool [99].
However, the ratio between the different quinones is expected to shift rapidly with changes in oxygen availability, even under standard batch conditions [100]. Therefore, blocking or down-regulating the biosynthesis of MK and DMK might be used as a metabolic engineering strategy to favour the production of CoQ in *E. coli*. Other pathways favour competing for chorismate and isoprenoid precursors could be considered in a similar manner.

4.4 Over expression of Coenzyme Q10 producing genes

These low Coenzyme Q10 contents are probably a reflection of limited precursor (PHB and OPP/DPP) flux. Although not adequate for the industrial production of Coenzyme Q10 such strains are informative with regards to which biochemical steps are most rate-limiting in the CoQ pathway. Notably, the overexpression of the PHB prenyltransferase UbiA (with DPS) from a plasmid resulted in a 3.5-fold increase in CoQ10 compared with the control strain (DPS), whereas the chorismate lyase UbiC, along with UbiA and DPS, resulted in a 5.1-fold increase (compared with DPS alone), the highest increase obtained so far through the optimization of the Coenzyme Q10 pathway [101].

This suggests that PHB prenyl transferase (UbiA) activity is a rate-limiting factor in UQ synthesis. This is not unexpected because PHB prenylation catalyzed by UbiA represents the branch point at which the isoprenoid precursor becomes committed to CoQ10 biosynthesis. However, UbiA is an integral-membrane protein and, as has been shown for most integral-membrane proteins, overexpression might not necessarily result in increased activity [102] and could explain previous reports of a more modest increase in CoQ8 observed on UbiA overexpression [75].

The effect of UbiC Overexpression on CoQ yields is also expected to be important because this enzyme is responsible for committing the precursor chorismate to Coenzyme Q10. The overexpression of the methyl transferase UbiG and of the hydroxylases UbiB and UbiH, however, had little impact on CoQ10 production, indicating that they are not bottlenecks in the pathway [75,101]. Other candidates for over expression include the decarboxylases UbiD and UbiX because a good correlation was shown between their expression and CoQ10 production in *E. coli* [103]. The effect of overexpressing these two enzymes on CoQ yields has not been reported so far but such a study will clarify whether decarboxylation is indeed a limiting step in the pathway.
To develop a successful metabolic-engineering strategy for CoQ biosynthesis in microbes, it is also important to investigate the cellular location of the enzymes and to determine if they are located in protein complexes that are necessary for their activity. All Coenzyme Q10 pathway intermediates possess a hydrophobic isoprenoid side chain that localizes them to the cytoplasmic membrane. Consequently, several CoQ10 biosynthetic enzymes have been demonstrated [22,104,105] or are predicted [106,107] to be membrane-associated. Furthermore, strains with mutations in UbiB, UbiG or UbiH accumulate the intermediate 2-octaprenylphenol [24, 36, 108], indicating that a loss of activity of any of these enzymes blocks the hydroxylation of this intermediate. This phenotype suggests interdependency or a complex among these three proteins. The presence of a protein complex is further supported by a study that reported the isolation, from the cytoplasmic membrane of *E. coli*, of a soluble complex that was able to convert 2-octaprenylphenol to CoQ10 in the presence of cytosolic proteins [109]. Moreover, there is increasing evidence for the existence of such a complex in *Saccharomyces cerevisiae* and, given the similarities between the two pathways, a related complex might be functional in *E. coli* [110, 111, 112, 113].

5. Commercialization of CoQ10 and Market Demand

Commercial production of CoQ10 is largely by way of yeast fermentation and to a smaller extent by bacterial fermentation. There is also a semi synthetic process for producing CoQ10 using solanesol, a tobacco by product that provides the phytanyl side chain, and the amino acid tyrosine for the ring structure. At this time, most of the world supply of CoQ10 comes from Japan, with smaller quantities coming from China, India, South Korea and Italy. In the US, natural CoQ10 is now being produced in a large plant in Pasadena, Texas. CoQ10 occurs in two isomeric forms, namely the “trans” and the “cis” forms. The natural CoQ10 is in the trans form whereas the synthetic CoQ10 contains a mixture of both trans and cis isomers. The USP limits the presence of other CoQ analogs and the cis-isomer and related impurities to less than 1.5%. CoQ10 is industrially produced either by a semi synthetic process that uses a material originating in plants, or by fermentation process using effective microbes. Various strains, presently being used for commercial production of CoQ10 are: *Cryptococcus laurentii* FERM-P4834, *Cryptococcus luteolus* IFO-0611, *Rhodotorula glutinis* FERM-P4835, *Rhodotorula flava* IFO-0407, *Rhodotorula peneaus* IRO-0930, *Sporobolomyces salmonicolor* FERM-P4836 (AHU-3982), *Sporobolomyces roseus*
IFO-1037, *Sporobolomyces pararoseus* IFO-1103, *Sporobolomyces pararoseus* IFO-1103, *Oosporidium margaritiferum* ATCC-10676, *Aspergillus fumigatus* FERM-P4838(IAM-2004) *Cladosporium fulvum* FERM-P4839(IAM-5054) etc. As, commonly the microbes produce low amount of CoQ10 it is necessary to find out an economic fermentation process by using an inexpensive carbon source as a major component of broth to be used in CoQ10 production. Japanese patent application No.20,396/72 describes a method for producing CoQ10 by cultivating a microorganism of genus *Alcaligenes*, *Tricosporon* or *Aurreobasidium* in a nutrient culture medium. However, the production of CoQ10 was noticed to be in a small amount having less chance of commercialization. Methods of extraction of CoQ10 from microorganisms, especially from yeast and the like are disclosed in Japanese Patent Publication No.8836/1973, Japanese Patent Publication No.19034/1976, Japanese Patent Laid-Open Publication No.105288/1977, USA Patent No.4367288/1983 and USA patent No. 4447362/1984. Variety of yeast strain belonging from the genera *Sporobolomyces*, *Rhodototorula*, *Oosporidium* and *Sporidiobolus* are good examples of producing CoQ10. Specially, strains of the species *Sporidiobolus ruineniae* were recognized as good producers of Q10. (1978, U.S.Pat.No.4,070,244) having CoQ10 920 µg g⁻¹ of dry cells. In addition, microbes like *Rhodotorula mucilaginosa* and *Agrobacterium tumefaciens* were reported having the CoQ10 production about 128mg/480gm dry cell and 26mg/120 gm dry cell respectively (USA, Patent No. 4447362). *Paracoccus denitrificans* KY-3940 (ATCC19367) was reported having low yield (27mg l⁻¹) CoQ10 production as compared to other bacteria having relatively higher amount of CoQ10 Production [59].

The present research relates to a process for producing a mutagenized *Paracoccus denitrificans* strain having a CoQ10 productivity of greater than 500 mg l⁻¹ of CoQ10. The *Paracoccus denitrificans* strain is subjected to genetic manipulation by mutagenesis. The mutagenized *Paracoccus denitrificans* strain is subjected to a selection where the mutagenized *Paracoccus denitrificans* strain is cultivated under conditions which inhibit growth of the *Paracoccus denitrificans* strain so that the mutagenized strain overcomes the growth inhibition through higher amount of CoQ10 production.
In 2003 it was estimated that world market demand for CoQ10 was 150 tons. A market research study published forecasting demand for anti-aging products in the U.S. to 2007 and 2012 concluded that the demand for “age-defying appearance products” is anticipated to increase by 8% per annum. The study forecasted “stellar growth” for CoQ10. Another industry study with projections through to 2008 and 2013 concluded that “smaller-volume antioxidants, such as coenzyme Q10, are expected to record the fastest growth through 2008”. Since CoQ10 was deregulated in Japan in March 2001, sales have grown by 150% per year. In 2004 the market reached US$150 million and reached up to US$200 million by 2005.

6. FUTURE SCENARIO

So far, the isolation of strains by mutagenesis and selection on inhibitors has proven the most successful strategy to increase yields of CoQ10. However, the prospects of obtaining further improvements using this approach are unlikely because the mutations enabling growth on the selection media do not necessarily result in higher Coenzyme Q10 yields. Higher throughput screening strategies to detect increased CoQ10 production also need to be developed to pursue a random mutagenesis approach to strain improvement.

As opposed to random mutagenesis, metabolic engineering enables the targeting of genetic modifications to specific biochemical pathways. The first attempts to metabolically engineer CoQ10 production in \textit{E. coli} stand as important proofs of principle for the potential of rational design to improve CoQ10 production in biological systems.

Nevertheless, despite promising results obtained with metabolic engineering, current yields are not sufficient for commercial production and illustrate the need for a careful assessment of the physiological and metabolic bottlenecks limiting CoQ10 biosynthesis. Important questions that are still outstanding are if the yields are strictly limited by the flux through the pathways or if there are some additional, yet unidentified, physiological factors that limit CoQ accumulation in mutants and \textit{E. coli}.

When the CoQ yield of \textit{E. coli} and \textit{Paracoccus denitrificans} are compared, it is notable that the latter produces at least tenfold more Coenzyme Q10 [59].
From these results, it can be assumed that *Paracoccus denitrificans* and other high Coenzyme Q10 producers have added biochemical or physiological characteristics that enable them to produce and accumulate high levels of Coenzyme Q10. Differences that might be relevant to CoQ production include the composition and the efficiency of the electron-transport chain and the absence of other quinones, such as MK, in *Paracoccus denitrificans* [60,114]. We might learn valuable lessons from the careful analysis of strains producing high levels of Coenzyme Q10 naturally, some of which might also be implemented in engineering robust industrial strains.

7. REFERENCES


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