

Effect of Limited Oxygen Supply on Coenzyme Q₁₀ Production and Its Relation to Limited Electron Transfer and Oxidative Stress in *Rhizobium radiobacter* T6102

Seo, Myung-Ji[†] and Soon-Ok Kim*

Division of Bio-New Drug Development, Central Research Institute, Chem Tech Research Incorporation (C-TRI), Namyangju 472-030, Korea

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Coenzyme Q₁₀ (CoQ₁₀) production from *Rhizobium radiobacter* T6102 was monitored under various oxygen supply conditions by controlling the agitation speeds, aeration rates, and dissolved oxygen levels. As the results, the CoQ₁₀ production was enhanced by limited oxygen supply. To investigate whether the CoQ₁₀ production is associated with its physiological functions of electron carrier and antioxidant, the effects of sodium azide and hydrogen peroxide on the CoQ₁₀ production were studied, showing that the CoQ₁₀ contents were slightly enhanced with increasing sodium azide (up to 0.4 mM) and hydrogen peroxide (up to 10 μM) concentrations. These results suggest the plausible mechanism where the limited electron transfer stimulating the environments of limited oxygen supply and oxidative stress could accumulate the CoQ₁₀, providing the relationship between the CoQ₁₀ physiological functions and its regulation system.

Keywords: Coenzyme Q₁₀, hydrogen peroxide, limited oxygen supply, *Rhizobium radiobacter*, sodium azide

Coenzyme Q₁₀ (CoQ₁₀) is a benzoquinone containing a 10-unit isoprene side chain. It is distributed widely in the mitochondrial inner membrane, lysosomes, peroxisomes, and microsomes throughout the eukaryotic cells, and is located in the plasma membrane of the prokaryotic cells, transferring electrons from complex I/II to the cytochrome *bc*₁ complex during the oxidative phosphorylation for ATP generation [9, 10]. So far, the CoQ₁₀ has been widely used as pharmaceutical and cosmetic materials, because of its

antioxidant properties, preventing cardiovascular disease and aging [4].

The CoQ₁₀ production by fermentation of microorganisms including *Agrobacterium tumefaciens*, *Paracoccus denitrificans*, and *Rhizobium radiobacter* has been preferred to that by chemical synthesis owing to its complicated structure [13, 18, 19]. However, it has been needed to overcome the low yields of microbiological production for industrial purposes. In order to increase CoQ₁₀ production, there have been various methods; mutagenesis of CoQ₁₀-producing microorganisms, optimization of the fermentation process, and gene expression involved in the CoQ₁₀ biosynthesis. The mutant strains have been selected mostly by chemical mutagenesis based on the structural analogs of CoQ₁₀ such as daunomycin, menadinone, and L-ethionine [19]. Recently, the high CoQ₁₀-producing *A. tumefaciens* was selected by the irradiation of a nitrogen ion beam [6]. Despite the successful results by mutagenesis, the production levels have been still insufficient for industrial production, resulting in high production cost. Therefore, the fermentation process for CoQ₁₀ production has been optimized with temperature, pH, carbon and nitrogen sources, and dissolved oxygen (DO) levels [7]. Specifically, the DO levels have been reported as an important factor, since the oxidation–reduction potential can regulate the CoQ₁₀ biosynthesis [4]. The CoQ₁₀ production from *A. tumefaciens* was increased by the limited oxygen supply and addition of azide as the electron flux inhibitor [3]. More recently, there have been studies on the expression of the decaprenyl diphosphate synthase (DPS) gene involved in the biosynthesis of the 10-unit isoprene side chain in *Escherichia coli*, which originally produces the CoQ₈, to induce the production of CoQ₁₀ instead of CoQ₈ from *E. coli* [12]. In addition, the 1-deoxy-D-xylulose 5-phosphate synthase (DXS) that served as a rate-limiting enzyme of the 2-C-methyl-D-erythritol 4-phosphate pathway was co-expressed with DPS in *E. coli*

*Corresponding author

Phone: +82-31-557-0001; Fax: +82-31-557-6622;
E-mail: sokim@c-tri.co.kr

[†]Present address: Department of Chemistry, Box H, Brown University, Providence, RI 02912-9108, U.S.A.

to increase the CoQ₁₀ flux through the isoprenoid pathway [15]. However, the CoQ₁₀ production level from recombinant *E. coli* was lower compared with that from naturally CoQ₁₀-producing microorganisms.

In this paper, the CoQ₁₀-highly-producing *R. radiobacter* T6102 mutant was isolated after mutagenesis using *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG), and the effects of aeration rate, agitation speed, and DO levels on the CoQ₁₀ production were investigated. Additionally, the relationships between the CoQ₁₀ production and its physiological functions as an electron carrier and antioxidant were studied through investigations of the effects of sodium azide and hydrogen peroxide on the CoQ₁₀ production.

R. radiobacter ATCC4718 was cultured in a 500-ml flask containing 100 ml of the basal medium (glucose 20 g/l, peptone 5 g/l, yeast extract 3 g/l, malt extract 3 g/l) at 30°C and 200 rpm for 24 h and then harvested by centrifugation. The cells were washed twice with 0.1 M sodium citrate (pH 5.5) and the NTG was then added to the washed cells at 0.2 mg/ml. After incubation at 30°C for 1 h, the cells were washed twice with 0.1 M potassium phosphate (pH 7.0), diluted with the basal medium, and then spread on the basal medium agar plate, controlling the cell viability to be nearly 1%. After incubation at 30°C for 24 h, approximately 2,300 mutants had grown on the agar plate. To select the CoQ₁₀-highly-producing mutant, the mutants were cultured in a 50-ml flask containing 10 ml of the basal medium at 30°C and 200 rpm for 24 h, followed by extraction for the analysis of CoQ₁₀ production by HPLC. In order to extract the CoQ₁₀, the cells were extracted with acetone and centrifuged to decant the supernatants into the fresh tubes. After adding hexane, the upper phases were pooled and then concentrated by evaporating the organic solvents. The yellow-hued residues dissolved in the 100% ethanol were analyzed by the HPLC described in our previous study [14]. Following the screening of CoQ₁₀ production from the mutants by HPLC, we selected one mutant highly producing CoQ₁₀. To compare the CoQ₁₀ production from the mutant strain with that of the parent strain, the two strains were separately cultured in 500-ml flasks containing 100 ml of the basal medium at 30°C and 200 rpm for 24 h. No remarkable difference existed between the dry cell weights (DCW) of the parent strain ATCC4718 (6.64±0.12 g/l) and the selected mutant strain T6102 (6.50±0.06 g/l). However, the CoQ₁₀ concentrations and contents from T6102 were 10.49±1.32 mg/l and 1.61±0.22 mg/g, respectively, which were 1.5 times higher than those from ATCC4718 (7.08±0.53 mg/l and 1.07±0.10 mg/g).

We investigated the effects of oxygen supply on the CoQ₁₀ production from mutant strain T6102 by controlling the agitation speeds and aeration rates. The highest CoQ₁₀ concentrations and its contents showed with the agitation speed of 400 rpm and aeration rate of 1.0 vvm (Table 1). The increase of agitation speeds from 400 to 600 rpm and

Table 1. Effects of agitation speed and aeration rate on the cell growth and CoQ₁₀ production.

Agitation (rpm)	Aeration (vvm)	Cell growth (g-DCW/l)	CoQ ₁₀ Production	
			Concentration (mg/l)	Content (mg/g)
200	0.5	12.18±0.75	18.35±1.09	1.51±0.01
300	0.5	13.53±0.64	20.10±1.44	1.49±0.07
400	0.5	15.23±0.69	26.93±2.42	1.77±0.08
500	0.5	15.71±0.71	16.48±1.31	1.05±0.04
600	0.5	16.79±1.11	14.96±1.12	0.89±0.02
400	1.0	15.60±1.06	28.43±1.84	1.82±0.01
400	1.5	15.81±1.07	18.31±1.53	1.16±0.04

R. radiobacter T6102 cultured in the basal medium at 30°C for 24 h was transferred with 10% (v/v) of the inoculums to an 8-l jar fermentor (Biotron) containing a 4-l working volume of enriched basal medium (glucose 20 g/l, peptone 10 g/l, yeast extract 10 g/l, malt extract 5 g/l, K₂HPO₄ 1 g/l, KH₂PO₄ 0.5 g/l, pH 7.0) and then cultured at 30°C for 48 h. Cell mass was determined using a calibration curve showing the relationship between the OD₆₀₀ and the cell growth (g-DCW/l). One OD₆₀₀ unit was considered to be equal to 0.623 g-DCW/l. Experiments were carried out in triplicate.

aeration rates from 1.0 to 1.5 vvm were found to be detrimental to CoQ₁₀ production, consistent with previous studies showing that the CoQ₁₀ production level from *A. tumefaciens* ATCC4452 was gradually decreased as agitation speeds (from 450 to 600 rpm) and aeration rates (1.0 to 1.5 vvm) were increased [3]. These results strongly supported that a limited oxygen supply can enhance the CoQ₁₀ production. Thus, it was necessary to investigate the effects of DO levels on the CoQ₁₀ production. The increase of DO levels from 5% to 20% slightly increased the final DCW, whereas the CoQ₁₀ concentrations and contents gradually decreased, showing the highest corresponding values under the condition of 5% DO level (Table 2). Overall, the accumulation of CoQ₁₀ stimulated by the limited oxygen supply was ascertained in this study, consistent with some previous studies. The low oxygen level was found to be effective to enhance the CoQ₁₀ production in *A. tumefaciens* and *P. denitrificans* [7, 13]. The enhanced production of CoQ₁₀ by limited oxygen supply prompted us to study the DO-stat feeding fed-batch fermentation, which significantly improved the DCW and CoQ₁₀ concentration from *R. radiobacter* WSH2601 [18].

In order to study whether the increase of CoQ₁₀ production by the limited oxygen supply is associated with its physiological function, the electron transfer factor possible to regulate CoQ₁₀ accumulation in a respiratory chain was investigated. To study the effects of electron transfer on the CoQ₁₀ production, sodium azide was used as the electron transfer inhibitor. Sodium azide inhibits the heme group of cytochrome in cytochrome *c* oxidase, which acts as a rate-limiting enzyme in oxidative phosphorylation, limiting the electron transfer from cytochrome to oxygen [1]. The final DCW gradually decreased according to increases of the

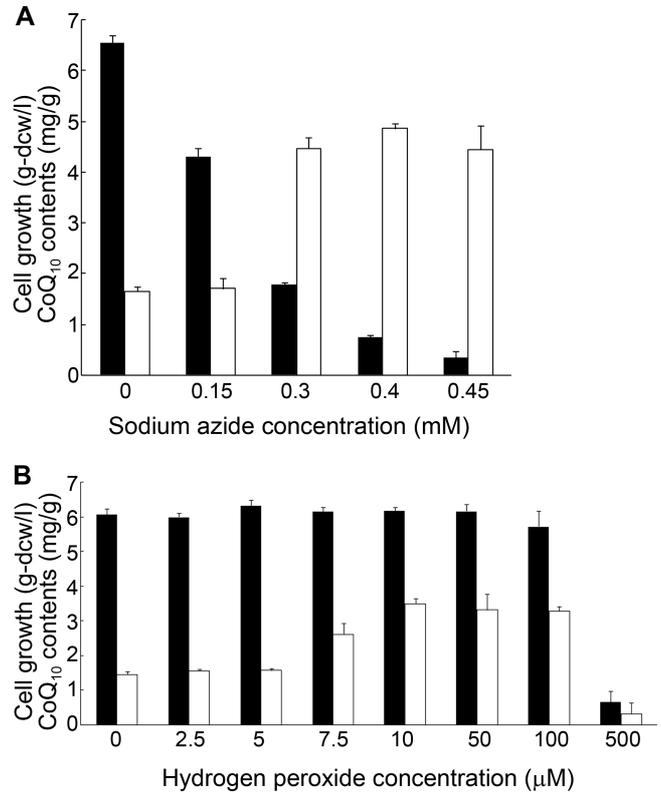
Table 2. Effect of DO level on the cell growth and CoQ₁₀ production.

DO level (%)	Cell growth (g-DCW/l)	CoQ ₁₀ Production	
		Concentration (mg/l)	Content (mg/g)
Uncontrolled	13.08±0.85	21.64±1.55	1.65±0.06
5	14.60±0.60	28.55±1.87	1.95±0.05
10	15.07±0.70	24.12±0.92	1.60±0.02
15	15.67±0.80	20.49±1.30	1.30±0.04
20	17.29±1.00	15.84±1.23	0.92±0.02

The fermentation process of *R. radiobacter* T6102 and the determination of cell mass are described in Table 1. The DO levels were monitored by a DO electrode (Hamilton). Various DO levels from 5% to 20% were automatically maintained by controlling the agitation speeds from 100 to 600 rpm, purging the pure oxygen with the settled aeration rate of 1.0 vvm. Under the uncontrolled condition, the DO level reached as low as 0% after 24-h fermentation. Experiments were carried out in triplicate.

sodium azide concentration from 0 to 0.45 mM; however, the CoQ₁₀ contents significantly increased, showing the CoQ₁₀ contents with 0.4 mM sodium azide was about 3-times higher than the corresponding values without the sodium azide (Fig. 1A). Based on the results, it seems highly likely that the partial limit of electron transfer to oxygen by sodium azide stimulates the environments of limited oxygen supply [3]. In the respiratory chain, the cytochrome transfers an electron from the cytochrome *bc*₁ complex, receiving it from complex I/II by CoQ₁₀, to cytochrome *c* oxidase, which then transfers it to an oxygen molecule. The inhibition of cytochrome *c* oxidase by sodium azide results in the accumulation of the reduced cytochrome *bc*₁ complex, which subsequently leads the reduced CoQ₁₀ (CoQ₁₀H₂) to be accumulated. The imbalance of essential redox component of CoQ₁₀ in the respiratory chain might shift the ratio between the oxidized form (CoQ₁₀) and reduced form (CoQ₁₀H₂) toward CoQ₁₀H₂ [11]. Finally, the cells might be forced to synthesize more CoQ₁₀ to fix the imbalance *via* feedback regulation [3].

CoQ₁₀ has also been known to be involved in the protection of cells against oxidative stress in prokaryotic as well as eukaryotic cells [2, 5]. Therefore, this prompted us to investigate the effects of oxidative stress on the CoQ₁₀ production using hydrogen peroxide, one kind of reactive oxygen species (ROS). The CoQ₁₀ contents were slightly enhanced with the low concentrations (up to 10 μM) of hydrogen peroxide, although there were no significant differences of the DCW with corresponding concentrations. However, it should be mentioned that the high range of hydrogen peroxide concentration from 50 to 100 μM had no significant influence on either DCW or CoQ₁₀ contents. In addition, the higher concentration (500 μM) inhibited cell growth because of its toxicity (Fig. 1B). These results indicated that the accumulation of CoQ₁₀ could be stimulated by the presence of low concentrations of hydrogen peroxide.

**Fig. 1.** Effects of sodium azide (A) and hydrogen peroxide (B) on the cell growth and CoQ₁₀ contents.

R. radiobacter T6102 was grown in a 500-ml flask containing 100 ml of the basal medium at 30°C and 200 rpm for 24 h after adding the sodium azide (A) and hydrogen peroxide (B). Black and white bars indicate the cell growth and CoQ₁₀ contents, respectively. Experiments were carried out in triplicate.

In fact, the reduced CoQ₁₀ (CoQ₁₀H₂) is the antioxidant to be depleted to protect against the oxidative cellular damage mediated by lipid peroxidation [16]. The previous study on the effect of hydrogen peroxide on the CoQ₁₀ and CoQ₁₀H₂ concentrations in lymphocytes had shown that the hydrogen peroxide decreased the CoQ₁₀H₂ content; however, the total CoQ₁₀ content (CoQ₁₀ plus CoQ₁₀H₂) was not affected, suggesting that CoQ₁₀H₂ was oxidized to CoQ₁₀ [17]. Recently, an interesting study has reported that the oxidative stress derived from Ca²⁺ supplementation induced lipid peroxidation, and the increased CoQ₁₀ content decreased the oxidative stress to protect the membrane against lipid peroxidation in *A. tumefaciens* [8]. Overall, it could be suggested that the oxidative stress by hydrogen peroxide might cause the decrease of antioxidant CoQ₁₀H₂, and the depleted CoQ₁₀H₂ could be probably oxidized to the CoQ₁₀, resulting in the increase of CoQ₁₀ content.

In conclusion, this study demonstrated that the CoQ₁₀ production from *R. radiobacter* was enhanced under limited oxygen supply, limited electron transfer, and oxidative stress. Considering the fact that sodium azide limits the

electron transfer to oxygen in the respiratory chain, it could be suggested that the sodium azide can stimulate the environments of limited oxygen supply. Besides this, the CoQ₁₀ enhancement by sodium azide could be ascribed to the feedback regulation of CoQ₁₀ to adapt the imbalance of the ratio between CoQ₁₀H₂ and CoQ₁₀, because sodium azide can result in the accumulation of reduced cytochrome *bc*₁ complex, which subsequently led to the accumulation of CoQ₁₀H₂. This study also suggests that the antioxidant CoQ₁₀H₂ could be depleted to protect against the oxidative stress by hydrogen peroxide and was probably oxidized to CoQ₁₀. Finally, our results can provide the regulation of CoQ₁₀ biosynthesis by its physiological functions of electron carrier and antioxidant.

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REFERENCES

- Berndt, J. D., N. L. Callaway, and F. Gonzalez-Lima. 2001. Effects of chronic sodium azide on brain and muscle cytochrome oxidase activity: A potential model to investigate environmental contributions to neurodegenerative diseases. *J. Toxicol. Environ. Health A* **63**: 67–77.
- Britta, S. and K. P. Robert. 2000. Ubiquinone limits oxidative stress in *Escherichia coli*. *Microbiology* **146**: 787–796.
- Choi, G.-S., Y.-S. Kim, J.-H. Seo, and Y.-W. Ryu. 2005. Restricted electron flux increases coenzyme Q₁₀ production in *Agrobacterium tumefaciens* ATCC4452. *Process Biochem.* **40**: 3225–3229.
- Cluis, C. P., A. M. Burja, and V. J. J. Martin. 2007. Current prospects for the production of coenzyme Q₁₀ in microbes. *Trends Biotechnol.* **25**: 514–521.
- Do, T. Q., J. R. Schultz, and C. Clarke. 1996. Enhanced sensitivity of ubiquinone-deficient mutants of *Saccharomyces cerevisiae* to products of autoxidized polyunsaturated fatty acids. *Proc. Natl. Acad. Sci. USA* **93**: 7534–7539.
- Gu, S.-B., J.-M. Yao, Q.-P. Yuan, P.-J. Xue, Z.-M. Zheng, L. Wang, and Z.-L. Yu. 2006. A novel approach for improving the productivity of ubiquinone-10 producing strain by low-energy ion beam irradiation. *Appl. Microbiol. Biotechnol.* **72**: 456–461.
- Ha, S.-J., S.-Y. Kim, J.-H. Seo, D.-K. Oh, and J.-K. Lee. 2007. Optimization of culture conditions and scale-up to pilot and plant scales for coenzyme Q₁₀ production by *Agrobacterium tumefaciens*. *Appl. Microbiol. Biotechnol.* **74**: 974–980.
- Ha, S.-J., S.-Y. Kim, J.-H. Seo, M. Jeya, Y.-W. Zhang, T. Ramu, I.-W. Kim, and J.-K. Lee. 2009. Ca²⁺ increases the specific coenzyme Q₁₀ content in *Agrobacterium tumefaciens*. *Bioprocess Biosyst. Eng.* **32**: 697–700.
- Kalen, A., B. Norling, E. L. Appelkvist, and G. Dallner. 1987. Ubiquinone biosynthesis by the microsomal fraction from rat liver. *Biochem. Biophys. Acta* **926**: 70–78.
- Kawamukai, M. 2002. Biosynthesis, bioproduction, and novel roles of ubiquinone. *J. Biosci. Bioeng.* **94**: 511–517.
- Lorence, R. M., K. Carter, G. N. Green, and R. B. Gennis. 1987. Cytochrome *b*₅₅₈ monitors the steady state redox state of the ubiquinone pool in the aerobic respiratory chain of *Escherichia coli*. *J. Biol. Chem.* **262**: 10532–10536.
- Okada, K., T. Kainou, K. Tanaka, T. Nakagawa, H. Matsuda, and M. Kawamukai. 1998. Molecular cloning and mutational analysis of the *ddsA* gene encoding decaprenyl diphosphate synthase from *Gluconobacter suboxydans*. *Eur. J. Biochem.* **255**: 52–59.
- Petr, K., K. Igor, and D. Vladimír. 1993. Effect of oxygen on ubiquinone-10 production by *Paracoccus denitrificans*. *Biotechnol. Lett.* **15**: 1001–1002.
- Seo, M.-J., E.-M. Im, J.-H. Hur, J.-Y. Nam, C.-G. Hyun, Y.-R. Pyun, and S.-O. Kim. 2006. Production of coenzyme Q₁₀ by recombinant *E. coli* harboring the decaprenyl diphosphate synthase gene from *Sinorhizobium meliloti*. *J. Microbiol. Biotechnol.* **16**: 933–938.
- Seo, M.-J., E.-M. Im, J.-Y. Nam, and S.-O. Kim. 2007. Increase of CoQ₁₀ production level by the coexpression of decaprenyl diphosphate synthase and 1-deoxy-D-xylulose 5-phosphate synthase isolated from *Rhizobium radiobacter* ATCC 4718 in recombinant *Escherichia coli*. *J. Microbiol. Biotechnol.* **17**: 1045–1048.
- Stocker, R., V. W. Bowry, and B. Frei. 1991. Ubiquinol-10 protects human low density lipoprotein more efficiently against lipid peroxidation than does α -tocopherol. *Proc. Natl. Acad. Sci. U.S.A.* **88**: 1646–1650.
- Tomasetti, M., G. P. Littarru, R. Stocker, and R. Alleva. 1999. Coenzyme Q₁₀ enrichment decreases oxidative DNA damage in human lymphocytes. *Free Radic. Biol. Med.* **27**: 1027–1032.
- Wu, Z., G. Du, and J. Chen. 2003. Effects of dissolved oxygen concentration and DO-stat feeding strategy on CoQ₁₀ production with *Rhizobium radiobacter*. *World J. Microbiol. Biotechnol.* **19**: 925–928.
- Yoshida, H., Y. Kotani, K. Ochiai, and K. Araki. 1998. Production of ubiquinone-10 using bacteria. *J. Gen. Appl. Microbiol.* **44**: 19–26.