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Effect of Limited Oxygen Supply on Coenzyme Q_{10} Production and Its Relation to Limited Electron Transfer and Oxidative Stress in *Rhizobium radiobacter* T6102

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Coenzyme Q_{10} (CoQ_{10}) 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 CoQ_{10} production was enhanced by limited oxygen supply. To investigate whether the CoQ_{10} production is associated with its physiological functions of electron carrier and antioxidant, the effects of sodium azide and hydrogen peroxide on the CoQ_{10} production were studied, showing that the CoQ_{10} 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 CoQ_{10}, providing the relationship between the CoQ_{10} physiological functions and its regulation system.

Keywords: Coenzyme Q_{10}, hydrogen peroxide, limited oxygen supply, *Rhizobium radiobacter*, sodium azide

Coenzyme Q_{10} (CoQ_{10}) 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 CoQ_{10} has been widely used as pharmaceutical and cosmetic materials, because of its antioxidant properties, preventing cardiovascular disease and aging [4].

The CoQ_{10} 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 CoQ_{10} production, there have been various methods; mutagenesis of CoQ_{10}-producing microorganisms, optimization of the fermentation process, and gene expression involved in the CoQ_{10} biosynthesis. The mutant strains have been selected mostly by chemical mutagenesis based on the structural analogs of CoQ_{10} such as daunomycin, menadinone, and L-ethionine [19]. Recently, the high CoQ_{10}-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 CoQ_{10} 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 CoQ_{10} biosynthesis [4]. The CoQ_{10} 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 CoQ_8, to induce the production of CoQ_{10} instead of CoQ_8 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*

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to increase the CoQ_{10} flux through the isoprenoid pathway [15]. However, the CoQ_{10} production level from recombinant *E. coli* was lower compared with that from naturally CoQ_{10}-producing microorganisms.

In this paper, the CoQ_{10}-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 CoQ_{10} production were investigated. Additionally, the relationships between the CoQ_{10} 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 CoQ_{10} 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 CoQ_{10}-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 CoQ_{10} production by HPLC. In order to extract the CoQ_{10}, 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 CoQ_{10} production from the mutants by HPLC, we selected one mutant highly producing CoQ_{10}. To compare the CoQ_{10} production from the mutant strain with that of the parent strain, the two strains were seperately 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 CoQ_{10} 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 CoQ_{10} production from mutant strain T6102 by controlling the agitation speeds and aeration rates. The highest CoQ_{10} 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 aeration rates from 1.0 to 1.5 vvm were found to be detrimental to CoQ_{10} production, consistent with previous studies showing that the CoQ_{10} 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 CoQ_{10} production. Thus, it was necessary to investigate the effects of DO levels on the CoQ_{10} production. The increase of DO levels from 5% to 20% slightly increased the final DCW, whereas the CoQ_{10} concentrations and contents gradually decreased, showing the highest corresponding values under the condition of 5% DO level (Table 2). Overall, the accumulation of CoQ_{10} 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 CoQ_{10} production in *A. tumefaciens* and *P. denitrificans* [7, 13]. The enhanced production of CoQ_{10} by limited oxygen supply prompted us to study the DO-stat feeding fed-batch fermentation, which significantly improved the DCW and CoQ_{10} concentration from *R. radiobacter* WSH2601 [18].

Table 1. Effects of agitation speed and aeration rate on the cell growth and CoQ_{10} 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, K_2HPO_4 1 g/l, KH_2PO_4 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 OD_{600} and the cell growth (g-DCW/l). One OD_{600} unit was considered to be equal to 0.623 g-DCW/l. Experiments were carried out in triplicate.

In order to study whether the increase of CoQ_{10} production by the limited oxygen supply is associated with its physiological function, the electron transfer factor possible to regulate CoQ_{10} accumulation in a respiratory chain was investigated. To study the effects of electron transfer on the CoQ_{10} 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 CoQ_{10} 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.

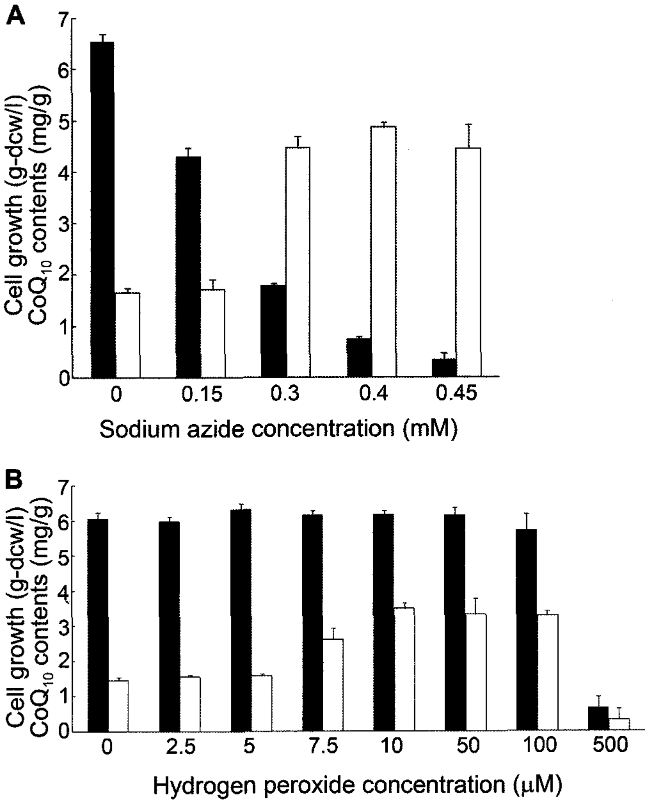

Fig. 1. Effects of sodium azide (**A**) and hydrogen peroxide (**B**) on the cell growth and CoQ_{10} contents.

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 CoQ_{10} contents, respectively. Experiments were carried out in triplicate.

sodium azide concentration from 0 to 0.45 mM; however, the CoQ_{10} contents significantly increased, showing the CoQ_{10} 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 CoQ_{10}, 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 CoQ_{10} ($CoQ_{10}H_2$) to be accumulated. The imbalance of essential redox component of CoQ_{10} in the respiratory chain might shift the ratio between the oxidized form (CoQ_{10}) and reduced form ($CoQ_{10}H_2$) toward $CoQ_{10}H_2$ [11]. Finally, the cells might be forced to synthesize more CoQ_{10} to fix the imbalance *via* feedback regulation [3].

CoQ_{10} 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 CoQ_{10} production using hydrogen peroxide, one kind of reactive oxygen species (ROS). The CoQ_{10} 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 CoQ_{10} contents. In addition, the higher concentration (500 μM) inhibited cell growth because of its toxicity (Fig. 1B). These results indicated that the accumulation of CoQ_{10} could be stimulated by the presence of low concentrations of hydrogen peroxide. In fact, the reduced CoQ_{10} ($CoQ_{10}H_2$) 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 CoQ_{10} and $CoQ_{10}H_2$ concentrations in lymphocytes had shown that the hydrogen peroxide decreased the $CoQ_{10}H_2$ content; however, the total CoQ_{10} content (CoQ_{10} plus $CoQ_{10}H_2$) was not affected, suggesting that $CoQ_{10}H_2$ was oxidized to CoQ_{10} [17]. Recently, an interesting study has reported that the oxidative stress derived from Ca^{2+} supplementation induced lipid peroxidation, and the increased CoQ_{10} 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 $CoQ_{10}H_2$, and the depleted $CoQ_{10}H_2$ could be probably oxidized to the CoQ_{10}, resulting in the increase of CoQ_{10} content.

In conclusion, this study demonstrated that the CoQ_{10} 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 CoQ_{10} enhancement by sodium azide could be ascribed to the feedback regulation of CoQ_{10} to adapt the imbalance of the ratio between $CoQ_{10}H_2$ and CoQ_{10}, because sodium azide can result in the accumulation of reduced cytochrome bc_1 complex, which subsequently led to the accumulation of $CoQ_{10}H_2$. This study also suggests that the antioxidant $CoQ_{10}H_2$ could be depleted to protect against the oxidative stress by hydrogen peroxide and was probably oxidized to CoQ_{10}. Finally, our results can provide the regulation of CoQ_{10} biosynthesis by its physiological functions of electron carrier and antioxidant.

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