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Association of Colony Morphology with Coenzyme Q_{10} Production and Its Enhancement from *Rhizobium radiobacter* T6102W by Addition of Isopentenyl Alcohol as a Precursor

Seo, Myung-Ji[1]*, Moo-Chang Kook[2], and Soon-Ok Kim[3]

[1]*Fermentation and Functionality Research Group, Korea Food Research Institute, Sungnam 463-746, Korea*
[2]*Department of Marine Biotechnology, Anyang University, Incheon 417-833, Korea*
[3]*Division of Research and Product Development, Union Korea Pharm., Co. Ltd., Seoul 138-878, Korea*

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***Rhizobium radiobacter* T6102 was morphologically purified by the aniline blue agar plates to give two distinct colonies; white smooth mucoid colony (T6102W) and blue rough colony (T6102B). The coenzyme Q_{10} (CoQ_{10}) was produced just by T6102W, showing 2.0 mg/g of CoQ_{10} content, whereas the T6102B did not produce the CoQ_{10}. All of the used CoQ_{10} biosynthetic precursors enhanced the CoQ_{10} production by T6102W. Specifically, the supplementation of 0.75 mM isopentenyl alcohol improved the CoQ_{10} concentration (19.9 mg/l) and content (2.4 mg/g) by 42% and 40%, respectively.**

Keywords: Aniline blue, coenzyme Q_{10}, isopentenyl alcohol, *Rhizobium radiobacter*

Coenzyme Q_{10} (CoQ_{10}) as an essential component of the membrane-bound electron transport system is typically synthesized from two major parts; a benzoquinone ring as its head group, and decaprenyl diphosphate as its tail group containing a 10-unit isoprenoid side chain [2]. Over the past several decades, CoQ_{10} has been increasingly used as a functional material for anti-aging cosmetics as well as pharmaceutical material preventing cardiovascular disease and mitochondrial respiratory chain disease [4, 11]. These various industrial applications of CoQ_{10} have demanded to develop its production level.

For the production of CoQ_{10}, three major procedures, namely the extraction from living organism, fermentation of microorganisms, and chemical synthesis, have been employed. However, the CoQ_{10} production level by extracting it from animal and plant tissues had not been satisfied in large scale owing to limited sources and low extraction yield. Moreover, the latter had not been desirable because of low yield resulted from its complicated structure and different starting materials from those used in microorganisms and human [5], even though the easy semichemical synthesis of CoQ_{10} was reported [9]. Therefore, the microbiological CoQ_{10} production by fermentation has been increasingly employed using bacteria such as *Agrobacterium* (*Rhizobium*) [5], *Rhodobacter* [18], and *Paracoccus* [10]. However, there is still limitation to the development of CoQ_{10}-containing products owing to the low yield of microbiological fermentation. Thus, various process have been attempted to increase the CoQ_{10} production; supplementation of CoQ_{10} biosynthetic precursors [7], mutant strain development [19], optimization of fermentation process [5], and genetically engineered strain development [13].

Recently, we selected *R. radiobacter* as a CoQ_{10} producer and developed its mutant strain named as T6102 [14]. During the studies on CoQ_{10} production with this mutant, we found that its stock culture formed the mixture of two types of colonies with different morphology, which might occasionally cause the significant reduction of CoQ_{10} with its repeated subculture. In this study, we investigated the correlation of colony type with CoQ_{10} production in *R. radiobacter* T6102. In addition, the effects of several precursors including isopentenyl alcohol on CoQ_{10} production were examined.

R. radiobacter T6102 formed two distinct colonies on the aniline blue agar plates; white smooth mucoid colony (T6102W) and blue rough colony (T6102B) (Fig. 1A and 1B). It has already been known that *Agrobacterium* sp. produce two types of polysaccharides; water-soluble succinoglycan and water-insoluble curdlan [6]. These polysaccharides have been simply detected on agar plates with trypan blue and aniline blue as indicators. The aniline

*Corresponding author
Phone: +82-31-780-9362; Fax: +82-31-709-9876;
E-mail: mjseo@kfri.re.kr

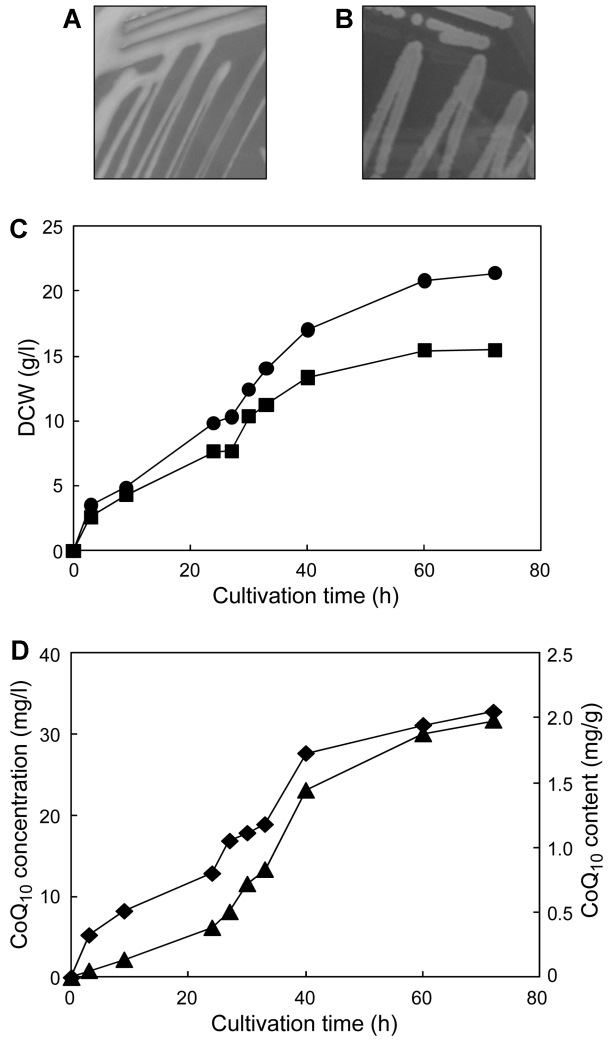

Fig. 1. Effects of colony types of *R. radiobacter* T6102 isolated on aniline blue plates on CoQ_{10} production.
(**A**) Strain T6102W. (**B**) Strain T6102B. (**C**) Comparison of DCW profiles between T6102W (squares) and T6102B (circles). (**D**) Time profiles of CoQ_{10} concentration (triangles) and content (diamonds) produced by T6102W. *R. radiobacter* T6102 was streaked on aniline blue agar medium (1% glucose, 0.5% yeast extract, 0.005% aniline blue, and 2% agar) and incubated at 30°C for 48 h [12]. For pre-seed culture, the resulted two distinct colonies (T6102B and T6102W) were each cultured in a 50 ml flask containing 10 ml of basal medium (20 g/l glucose, 5 g/l peptone, 3 g/l yeast extract, and 3 g/l malt extract) at 30°C and 200 rpm for 24 h. Then, the seed culture was performed in a 500 ml flask containing 100 ml of the same basal medium and culture conditions. To compare the profiles of CoQ_{10} production by each strain, the seed culture was transferred with 5% (v/v) of the inoculums to a 5 l jar fermentor (KF-5, Ko-biotech, Korea) containing a 2 l working volume of enriched basal medium (20 g/l glucose, 10 g/l peptone, 10 g/l yeast extract, 5 g/l malt extract, 1 g/l K_2HPO_4, and 0.5 g/l KH_2PO_4). The fermentor was operated with 300 rpm and 0.5 vvm at 30°C for 72 h. Cell mass was determined by using a predetermined calibration curve relating optical density at 600 nm (OD_{600}) and DCW. One OD_{600} unit was considered to be equal to 0.62 g-DCW/l.

blue method can detect the blue colony producing curdlan- and the white colony producing succinoglycan-type polysaccharides, although the strain purely isolated from the white colony may be unstable to be spontaneously mutated to a blue colony [6].

We compared the profiles of CoQ_{10} production as well as cell growth between T6102W and T6102B strains purified morphologically in 5 l jar fermentors. The produced CoQ_{10} was analyzed by HPLC, as described in our previous study [12]. Although both types were derived from the same mutant, T6102, the cell growth and CoQ_{10} production were significantly different between them. The T6102B strain exhibited a high cell growth level compared with the T6102W, showing an increase of 38% on final dry cell weight (DCW) (Fig. 1C). However, the CoQ_{10} production from T6102B was not detected, whereas the final CoQ_{10} concentration and content for T6102W were 31.6 mg/l and 2.0 mg/g, respectively (Fig. 1D). During CoQ_{10} production, it was observed that the culture broth became viscous after 30 h of fermentation, maybe due to the accumulation of succinoglycan as a by-product [5]. Similarly, it has been reported that one strain (named as Rw) purely isolated from *Agrobacterium* sp. KY-8589 by the trypan blue method showed the relatively high CoQ_{10} productivity, high broth viscosity, and low cell growth levels compared with the other strain (named as Sy) [8].

According to the CoQ_{10} biosynthetic pathway, we selected several precursors for the enhancement of CoQ_{10} production. Isopentenyl alcohol and 3-methylcrotonic acid can be produced by the dephosphorylation of isopentenyl diphosphate and by the dephosphorylation of dimethylallyl diphosphate followed by oxidation, respectively [3, 16]. Therefore, we used both precursors for CoQ_{10} production associated with isoprenoid side chain synthesis. We also selected shikimic acid as a potential precursor because it has been known that the *p*-hydroxybenzoic acid, which is used as a precursor of benzoquinone ring, may be produced from chorismatic acid, which is derived from shikimic acid [2]. Since solanesol, an unsaturated nonaprenol isolated from tobacco leaves, is widely used as the starting material for isoprenoid side chain in semichemical CoQ_{10} synthesis, we also used it as another potential precursor in this study [2].

None of the precursors significantly altered the final DCW. However, comparing with the T6102W as a control without any precursor, the CoQ_{10} concentrations increased 38% by adding isopentenyl alcohol, 16% by 3-methylcrotonic acid, 15% by shikimic acid, 21% by solanesol, and 28% by isopentyl alcohol. Accordingly, the CoQ_{10} contents showed the increase of 38%, 21%, 19%, 23%, and 26% by addition of the corresponding precursors mentioned above. Overall, all precursors were effective for the enhancement of CoQ_{10} production, which was the highest (18.3 mg/l of concentration and 2.3 mg/g of content), especially when isopentenyl alcohol was used as a precursor (Fig. 2A). To evaluate the effect of the concentration of isopentenyl alcohol on CoQ_{10} production, various concentrations of isopentenyl alcohol

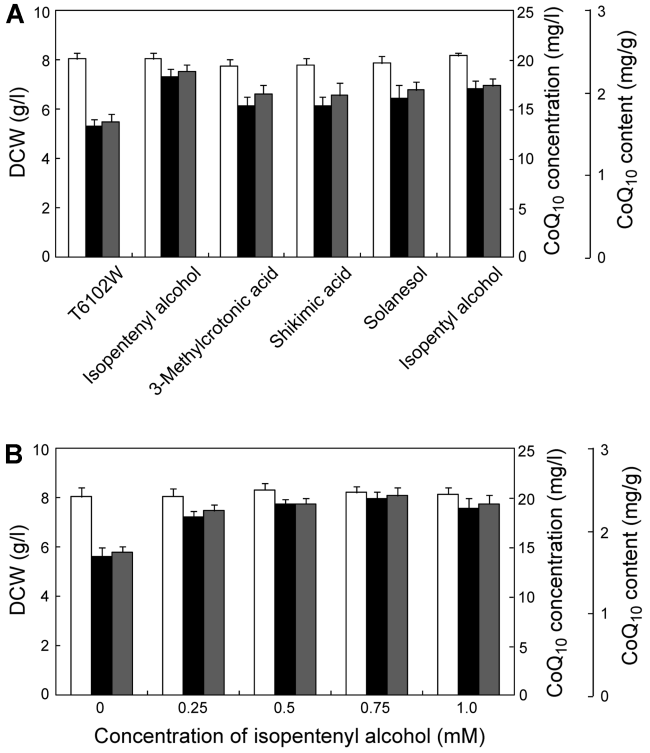

Fig. 2. Effects of precursors on CoQ_{10} production by *R. radiobacter* T6102W.

R. radiobacter T6102W was cultured in a main 500 ml flask containing 100 ml of enriched basal medium at 30°C and 200 rpm for 72 h after adding each precursor purchased from Sigma-Aldrich, including isopentenyl alcohol (CAS no. 556-82-1), 3-methylcrotonic acid (CAS no. 541-47-9), shikimic acid (CAS no. 138-59-0), solanesol (CAS no. 540-03-4), and isopentyl alcohol (CAS no. 123-51-3) at the final concentration of 1.0 mM (**A**) or different concentrations of isopentenyl alcohol (**B**). White, black, and gray bars indicate DCW, CoQ_{10} concentration, and CoQ_{10} content, respectively.

(0.25, 0.5, 0.75, and 1.0 mM) were supplemented into the culture medium. The CoQ_{10} concentrations and contents were slightly enhanced with the increased final concentrations of isopentenyl alcohol (up to 0.75 mM), although there were no significant differences of the DCW with the corresponding concentrations. However, the isopentenyl alcohol of 1.0 mM did not seem to be effective on CoQ_{10} production. The optimal concentration of isopentenyl alcohol for CoQ_{10} production was found to be 0.75 mM, resulting in 19.9 mg/l of CoQ_{10} concentration and 2.4 mg/g of its content (Fig. 2B).

Isopentenyl diphosphate and dimethylallyl diphosphate are the important precursors for the isoprenoid side chain in CoQ_{10} biosynthesis. However, since these charged precursors had been thought to be difficult to permeate through the cell membrane, it has not been actively studied for the direct effects of these precursors on CoQ_{10} production, although it was reported that *E. coli* cells could be permeable to isopentenyl diphosphate in the presence of Mg^{2+} for isoprenoid compound biosynthesis [15]. On the other hand, the prenyl alcohols such as geraniol and farnesyl farnesol as well as isopentenyl alcohol and dimethylallyl alcohol have been studied for the enhancement of CoQ production [7]. In addition to these precursors for isoprenoid side chain biosynthesis, *p*-hydroxybenzoic acid and its biochemical precursors such as tyrosine, shikimic acid, and chorismatic acid had been thought to be possible precursors for the benzoquinone ring in CoQ_{10} biosynthesis [2]. However, not all of these possible precursors activate the CoQ_{10} production [7]. The CoQ_{10} production from *R. radiobacter* T6102W used in this study was improved by adding the precursors of all types tested, especially isopentenyl alcohol, suggesting that the supplementation of isopentenyl alcohol stimulates the biosynthetic pathway of the isoprenoid side chain to increase CoQ_{10} biosynthesis. Little is known about the effects of precursors on CoQ_{10} production using *Rhizobium* strains. It was recently reported that the addition of isopentyl alcohol increased the CoQ_{10} production by 17–18% compared with the control (with no precursor) from *R. radiobacter* WSH2601, showing 15.0 mg/l of CoQ_{10} concentration and 1.58 mg/g of its content [17]. However, it enhanced the CoQ_{10} production from *R. radiobacter* T6102W by 28% of concentration (17.1 mg/l) and 26% of content (2.1 mg/g) in this study (Fig. 2A). Recently, natural precursors such as carrot juice and tomato juice were also studied to enhance the CoQ_{10} production by *P. diminuta* because these plants contain active isoprenoid precursors of polyprenyl diphosphate [1]. More recently, the direct CoQ_{10} production by gel-entrapped *Sphingomonas* sp. using solanesol and *p*-hydroxybenzoic acid as precursors in a two-phase conversion system was reported [20].

In conclusion, *R. radiobacter* was morphologically purified to two distinct colonies on aniline blue plates. Only in the white smooth mucoid colony was CoQ_{10} production detected with viscosity possibly due to succinoglycan as a by-product of *R. radiobacter*. It was also established that the CoQ_{10} biosynthetic precursors including isopentenyl alcohol are effective to increase the CoQ_{10} production by *R. radiobacter* T6102W. In this study, the CoQ_{10} production (19.9 mg/l of concentration and 2.4 mg/g of content) was significantly enhanced by morphological purification and biosynthetic precursor addition, compared with our previous trial to improve CoQ_{10} production (10.5 mg/l of concentration and 1.6 mg/g of content) by chemical mutagenesis of wild-type strain T6102 (7.1 mg/l of concentration and 1.1 mg/g of content), suggesting the effectiveness of these approaches for CoQ_{10} production [14]. Finally, our results could be helpful to understand the association of morphologically different colonies with CoQ_{10} production from *R. radiobacter* and will contribute to the usefulness of precursors for the enhancement of CoQ_{10} production.

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