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Research Article

Increased Production of Coenzyme Q_{10} from Genetic Engineered *Rhodobacter sphaeroides* Overexpressing UbiG

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Abstract: The aim of this study was to increase the Coenzyme Q_{10} (CoQ_{10}) yield from *Rhodobacter sphaeroides* via genetic engineering pathway. CoQ_{10} plays important roles in many biological processes and has been proven to be effective in the treatment of many diseases. In the present study, the *ubiG* gene located in CoQ_{10} biosynthesis pathway was effectively overexpressed in *Rb. sphaeroides* to increase CoQ_{10} production. The growth of host cells was slightly influenced by overexpressing *ubiG*. The crude CoQ_{10} production was enhanced by 58.31% compared to that from the control. The *ubiG* mRNA level was significantly increased compared to the wild type harboring empty vector as measured by qRT-PCR. Moreover, the crude CoQ_{10} exhibited strong anti-oxidant activity as measured *in vivo* by zone of inhibition assay.

Keywords: Anti-oxidation, CoQ_{10}, genetic engineering, hydroxyl radical scavenging activity, *Rb. sphaeroides*, *ubiG*

INTRODUCTION

CoQ_{10} (2,3-dimethoxyl, 5-methyl, 6-decaisoprene parabenzoquinone) is a lipid-soluble material wide-spread in prokaryotes and eukaryotes, which could be used in the treatment of many diseases. It has been proposed that CoQ_{10} could effectively protect rat cardiomyocytes against cisplatin-induced cardiotoxicity via attenuating oxidative stress (Zhao, 2019). Yousef and co-workers suggest that CoQ_{10} has beneficial effects against neuronal damage induced by lead acetate (PbAc) through its antioxidant, anti-inflammatory, anti-apoptotic and neuromodulatory activities (Yousef *et al*., 2019). Jahangard and co-workers suggest that CoQ_{10} is considered a safe and effective strategy for treatment of patients with Bipolar disorder during their depressive phase (Jahangard *et al*., 2019). Treatment by CoQ_{10} will reduce p53, Puma and Bax mRNA expression levels and increase Bcl-2 mRNA expression levels and thus mitigates ionizing radiation-induced testicular damage through inhibition of oxidative stress and mitochondria-mediated apoptotic cell death (Said *et al*., 2019). Moreover, CoQ_{10} possesses strong anti-oxidant capacity and thus can protect phosphate, lips, proteins and DNA (Cluis *et al*., 2007; Kaci *et al*., 2018; Rizvi *et al*., 2015).

Production of adequate and low cost CoQ_{10} is required because of its applications in many fields related to people's health. Currently, CoQ_{10} is normally produced by three approaches including chemical synthesis, semi-chemical synthesis and microbial fermentation. Compared to other two ways, microbial fermentation is becoming more and more popular. *Rb. sphaeroides* is considered a promising microorganism for producing natural functional CoQ_{10} (Zahiri *et al*., 2006; Zhu *et al*., 2017). The whole genome of this bacterium has been completely sequenced. The biosynthesis pathway for the formation of CoQ_{10} in *Rb. sphaeroides* includes three pathways, the 2-C-Methyl-$_D$-Erythritol 4-Phosphate (MEP) pathway, the shikimate pathway and the Quinine Modification Pathway (QMP). UbiG is an oxygen-methyltransferase, participating in two steps for the synthesis of CoQ_{10} in *Rb. sphaeroides*. UbiG will catalyzes 2-Decaprenyl-6-hydroxyphenol into 2-Decaprenyl-6-methoxyphenol and catalyzes the formation of CoQ10 from 2-Decaprenyl-3-methyl-5-hydroxy-6-methoxy-1,4-benzoquinone, which is the last step for the biosyntheisi of CoQ_{10} in *Rb. sphaeroides* (Lu *et al*., 2015).

Rb. sphaeroides is considered an excellent model for studying photosynthesis and membrane development (Kiley and Kaplan, 1987). LH1 is one of the most important photosynthetic apparatus encoded by *puf* operon (Hu *et al*., 2002). *puf* operon promoter is normally regulated by oxygen tension and light intensity (Hu, *et al*., 2010). A powerful promoter and optimal growth conditions are very important for largest production of CoQ_{10} in genetic engineered *Rb. sphaeroides*.

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Up to date, enhanced production of CoQ_{10} from *Rb. sphaeroides* by overexpression of *ubiG* under *puf* operon promoter and micro-aerobic growth conditions has not been reported. In the present study, the *ubiG* was overexpressed in *Rb. sphaeroides* initiated by *puf* operon promoter. The production of CoQ_{10} from the genetic engineered strain was enhanced by over 58%, which was increased much higher than reported literature (Lu *et al*., 2015). The present study will promote the application of *Rb. sphaeroides* for large scale production of functional CoQ_{10}.

MATERIALS AND METHODS

Bacterial strains and growth conditions: *Rb. sphaeroides* strains were grown at 30°C in malate minimal medium (Remes *et al*., 2014). Growth under micro-aerobic conditions was performed as described in our previous study (Hu *et al*., 2010). *E. coli* strains were cultivated vigorously in flasks at 37°C in Luria-Bertani medium. Antibiotics were added to the growth media at the following concentrations when necessary: 200 μg/mL ampicillin, 20 μg/mL tetracycline for *E. coli* and 1.5 μg/mL tetracycline for *Rb. sphaeroides*.

Construction of DNA plasmids: The *ubiG* was amplified from *Rb. sphaeroides* by PrimeSTAR HS DNA polymerase (TAKARA) with the primers of *ubiG*-F (5'-GCTCTAGAATGGAATCGTCCAGCACC ATCGACC-3') and *ubiG*-R (5'-CGGGATCCTCAGC TGCGCCGCACGC-3') and ligated into cloning vector pMD18-T (TAKARA) and subsequently sequenced. The *ubiG* fragment was cut from pMD18-*ubiG* plasmid by *Xba*I and *Bam*HI and purified by gel extraction and ligated into pRK*puf* (Hendrischk *et al*., 2009) digested by *Xba*I-*Bam*HI, producing pRK*ubiG* overexpression vector.

Construction of genetic engineered *Rb. sphaeorides*: The constructed plasmid pRK*ubiG* was transferred into *Rb. sphaeroides* 2.4.1 by using the *E. coli* S17-1 as the donor as described in the previous study (Hu *et al*., 2010).

Production of crude CoQ_{10} from the genetic engineered strain: Colonies of the conjugant were selected and cultivated under micro-aerobic conditions in the dark at 30°C until OD_{660} reached approximately 0.6. Pre-cultures were respectively inoculated into 100-mL flasks containing malate minimal media with 1.5 μg/mL tetracycline at the ratio of 1% and grown under micro-aerobic conditions in the dark at 30°C for 48 h. Crude CoQ_{10} was extracted and quantified as described by Chen *et al*. (2006), respectively.

Quantitative RT-PCR: Total RNA was isolated from cell cultures by using the Tiangen Bacteria RNA Isolation Kit (#DPN430) according to the manufacturer's instructions. mRNA from genetic engineered strain and wild type strain harboring empty vector was considered sample mRNA and control mRNA, respectively. To further confirm the absence of DNA, PCR was performed targeting *gloB* (RSP_0799). qRT-PCR was performed as described previously (Remes *et al*., 2014) in a Bio-Rad CFX96 Real Time system. Primers used for qRT-PCR were *ubiG*-real-F (5'-GCAAAGCTCCATGC CGAG-3') and *ubiG*-real-R (5'-GTCGAGCAGATCATCAGG-3'). Relative mRNA expression levels were normalized to the reference gene *rpoZ* (Zeller *et al*., 2007) according to the formula given by Pfaffl (2001).

Zone of inhibition assay: Zone of inhibition assay was performed as described in our previous study (Zhao *et al*., 2019). Filters soaked with 5 μL of 700 mM H_2O_2 were placed on the top of the plates.

Data analysis: All experiments were repeated for 3 times. Turkey test and GraphPad Prism software were used to analyze the data trend.

RESULTS AND DISCUSSION

Construction of the expression vector: The expression vector used in this study was constructed as shown in Fig. 1. UbiG is an important catalyzed enzyme in the ubiquinone pathway for the biosynthesis of CoQ_{10} in *Rb. sphaeroides* (Lu *et al*., 2015). It is an oxygen-methyltransferase, involved in catalyzing 2-Decaprenyl-6-hydroxyphenol into 2-Decaprenyl-6-methoxyphenol and catalyzing the 2-Decaprenyl-3-methyl-5-hydroxy-6-methoxy-1,4 benzoquinone into CoQ_{10}. *puf* operon is comprised of *pufB*, *pufA*, *pufL*, *pufM* and *pufX*, encoding the LH1 and reaction center in *Rb. sphaeroides* (Eisenhardt *et al*., 2018; Gong and Kaplan, 1996). The *puf* operon promoter initiates two transcripts, a 2.7-kb transcript for *pufBALMX* and a 0.5-kb transcript for *pufBA* (Gong *et al*., 1994). Under the micro-aerobic growth conditions or optimal light intensity, the *puf* operon promoter exhibits strongest activity. Moreover, the pRK415 vector (Chen *et al*., 2019) is a broad host range expression vector.

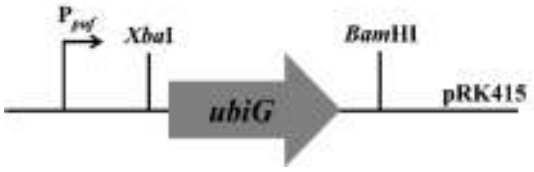

Fig. 1: Schematic representation of *ubiG* overexpression vector. The *ubiG* gene was activated by the strong *puf* operon promoter from *Rb. Sphaeroides*

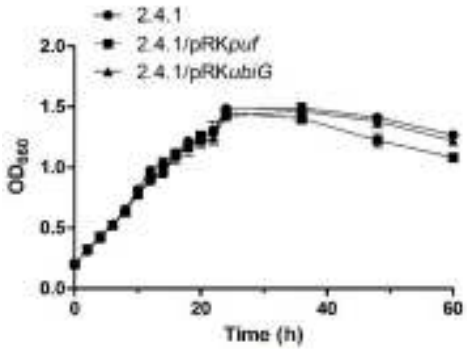

Fig. 2: Growth curve of wild type 2.4.1, 2.4.1/pRK*puf* and 2.4.1/pRK*ubiG*. Overexpression of *ubiG* slightly influenced the growth of host cells

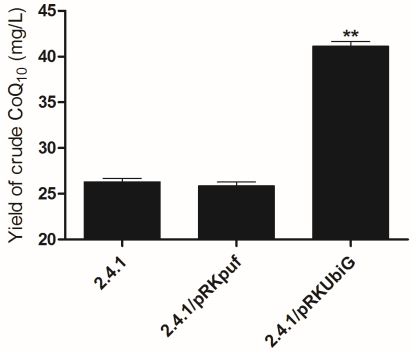

Fig. 3: Quantitative measurement of crude CoQ_{10}
The CoQ_{10} from 2.4.1/pRK*ubiG* was significantly higher than that in wild type 2.4.1 and 2.4.1/pRK*puf*; **: A highly significant change ($p \leq 0.01$)

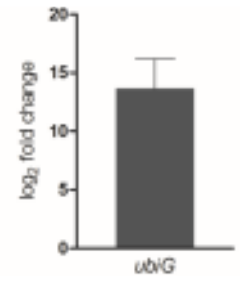

Fig. 4: Quantitative analysis of *ubiG* mRNA expression levels.
The *ubiG* mRNA expression level in 2.4.1/pRK*ubiG* was significantly increased with $\log_2$ fold change of 13.63 compared to that in the control 2.4.1/pRK*puf*

Construction of the genetic engineered strain overexpressing UbiG: The constructed expression vector was mobilized into *Rb. sphaeroides* by conjugation by using *E. coli* S17-1. The growth curve for the wild type, 2.4.1/pRK*puf* and 2.4.1/pRK*ubiG* were constructed to demonstrate whether the growth was influenced by overexpression of *ubiG*, as revealed in Fig. 2. It was obvious that overexpression of *ubiG* slightly influenced the growth of the host cells. In the first 20 h, growth rates for the three different strains were nearly the same since the growth curve was overlapped. However, at the stationary phase, significant difference between the wild type and 2.4.1/pRK*ubiG* at the time point of 48 h was observed. Similarly, remarkable differences between 2.4.1/pRK*puf* and 2.4.1/pRK*ubiG* at the time points of 22 and 48 h were observed. Although it has been proposed that the CoQ_{10} plays very important roles in energy generation and many other processes, which are important for cell's survival (Zahiri *et al*., 2006). In the present study, production of CoQ_{10} possibly did not strongly affect the growth of the host cells.

The crude CoQ_{10} was extracted from the genetic engineered strain, as observed in Fig. 3. The yield of crude CoQ_{10} from wild type 2.4.1 and 2.4.1/pRK*puf* was around 26.30 and 25.869 mg/L, respectively. However, the crude CoQ_{10} production from the genetic engineered 2.4.1/pRK*ubiG* was 41.47 mg/L. Compared to the wild type, the CoQ_{10} production in 2.4.1/pRK*ubiG* was increased by 58.31%, which was much higher than the rate described in reported study (Lu *et al*., 2015). It could be concluded that overexpression of the *ubiG* could significantly increase the crude CoQ_{10} production under micro-aerobic growth conditions initiated by the *puf* operon promoter.

Quantitative RT-PCR analysis for *ubiG*: qRT-PCR was employed to test the *ubiG* mRNA expression levels to further describe the reasons resulted in the enhancement of CoQ_{10} from the genetic engineered 2.4.1/pRK*ubiG*, as seen in Fig. 4. As expected, the *ubiG* mRNA level was significantly upregulated, with $\log_2$ fold change of approximately 13.63. UbiG is an oxygen-methyltransferase, involved in catalyzing 2-decaprenyl-6-hydroxyphenol into 2-decaprenyl-6-methoxyphenol and catalyzing the 2-decaprenyl-3-methyl-5-hydroxy-6-methoxy-1,4 benzoquinone into CoQ_{10} (Lu *et al*., 2015). The UbiG enzyme expression level should be upregulated because of the increased *ubiG* mRNA expression levels and crude CoQ_{10} production.

Anti-oxidant activity: From the zone of inhibition experiment, it could be concluded that the strain 2.4.1/pRK*ubiG* possessed much higher anti-oxidant activity than that of the control strain 2.4.1/pRK*puf*, as revealed in Fig. 5. The H_2O_2 is a normally used oxidant produced ·OH radical by Fenton reaction (Fischbacher *et al*., 2017). Obviously, the size of zone of inhibition for 2.4.1/pRK*ubiG* was much smaller than that of 2.4.1/pRK*puf*, indicating that the strain 2.4.1/pRK*ubiG* was less sensitive to H_2O_2 than that of the control strain 2.4.1/pRK*puf*. The genetic engineered strain 2.4.1/pRK*ubiE* was constructed in our previous study (Tang *et al*., 2019), which produced more CoQ_{10} than the present genetic engineered strain 2.4.1/pRK*ubiG*

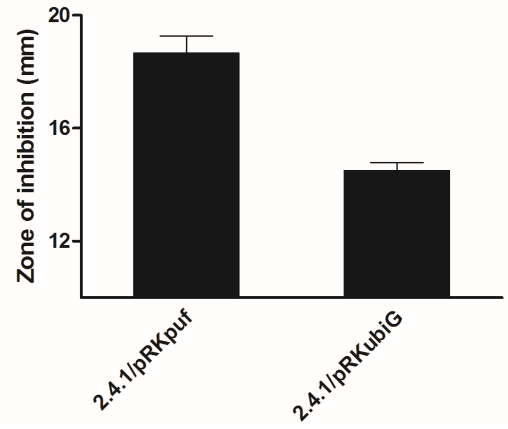

Fig. 5: Zone of inhibition assay for 2.4.1/pRK*puf* and 2.4.1/pRK*ubiG*.
The zone diameter for 2.4.1/pRK*ubiG* and 2.4.1/pRK*puf* was 14.500 and 18.667 mm, respectively; **: A highly significant change ($p \leq 0.01$)

and possessed a little bit smaller size of zone of inhibition. The zone of inhibition assay indicated that the crude CoQ_{10} was functional and thus possessed the potential for commercial utility in food, cosmetic and pharmaceutical industries after further purification.

CONCLUSION

In the present study, we constructed the genetic engineered *Rb. sphaeroides* strain 2.4.1/pRK*ubiG* to increase the CoQ_{10} production. Production of the crude CoQ_{10} from 2.4.1/pRK*ubiG* was increased by 58.31% and *ubiG* mRNA was significantly upregulated. Moreover, the genetic engineered strain 2.4.1/pRK*ubiG* exhibited more stronger anti-oxidant activity because of the much more production of crude CoQ_{10}.

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CONFLICT OF INTEREST

No potential conflicts of interest were disclosed.

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