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Is it possible to restore the reduced coenzyme Q_{10} production of a varied strain of *Gluconobacter*?

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ABSTRACT

Different strains of a bacterial culture can undergo phenotypic variation upon continuous passages. These changes often cause a reduction or loss of bacterial metabolic potential and ultimately lead to a decrease in production yield. The aim of this study was to address this question; is it possible to restore the reduced coenzyme Q_{10} production of a varied strain of *Gluconobacter* to a higher level? The main strain studied in this study was *Gluconobacter japonicus* FM10, from which the FM20 strain was obtained. FM20 strain was a varied strain whose ability on coenzyme Q_{10} production was reduced during some continuous passages. FM30 strain was obtained from FM20 strain by restricting the available oxygen. The amount of coenzyme Q_{10} produced by all three strains was measured. The activities of glycerol dehydrogenase and sorbitol dehydrogenase were also measured. The results showed that coenzyme Q_{10} production in FM30 strain that had been exposed to restricted oxygen was several times higher (6.3 mg/L) than the varied FM20 strain (0.8 mg/L), and even the original FM10 strain (2.7 mg/L). The investigation of the enzymes activities showed that glycerol dehydrogenase and sorbitol dehydrogenase activities of FM30 strain were higher than those of the others (0.66 and 0.52 U mg^{-1}, respectively). It can be concluded that by using some strategies, the metabolic potential of some varied strains can be restored. For strictly aerobic bacteria, *Gluconobacter*, the oxygen restriction may be an effective strategy for the restoration of coenzyme Q_{10} production ability.

Key words: Coenzyme Q_{10}, Dehydrogenase, *Gluconobacter*, Thermotolerant, Variation.

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INTRODUCTION

Gluconobacter is a genus of acetic acid bacteria, well known to have a strong ability to oxidize a broad range of sugars and sugar alcohols. These oxidations are called oxidative fermentation (Liu *et al.,* 2021). The presence of membrane-bound dehydrogenases located on the outer surface of the cytoplasmic membrane causes the oxidation of substrates. The reduced compounds are accumulated in the culture medium (Adachi *et al.,* 2016). These features of the microorganism lead to the applications in industry for fermentation of valuable products such as vitamin C, dihydroxyacetone, 6-amino-L-sorbose (a key mediator in the synthesis of the anti-diabetic drug miglitol), shikimate and 3-dehydrochikimate (Liu *et al.,* 2020). *Gluconobacter japonicas* was first described by Malimas in 2009 (Malimas *et al.,* 2009; Moghadami and Hosseini, 2020). There are a few studies concerning *Gluconobacter japonicas* to produce industrial products (Priya *et al.,* 2015; Moghadami *et al.,* 2021).

Phenotypic variation is a common phenomenon in the bacterial cultures. It may last for a few generations or may persist for many generations to come. This phenomenon has been studied by many researchers and several reasons have been proposed. These variations may be in response to stresses or be caused by genetic or epigenetic alterations (Smits *et al.,* 2006). Stochasticity in gene expression along with the constitute of the gene network modulating the cellular processes can induce phenotypic variation (Santillan *et al.,* 2007).

Coenzyme Q_{10} (CoQ_{10}) with ten isoprenoid units as the side chain, is the vitamin-like lipophilic antioxidant produced by human mitochondria. This molecule is an obligatory cofactor in the aerobic respiratory chain that produces ATP (Dieu Ndikubwimana and Lee, 2014). It is widely used as a supplement to increase energy and immunity, as well as an anti-inflammatory and anti-aging supplement. It is also used to treat Parkinson's disease, Huntington's, cancer, AIDS and muscular dystrophy (Kapoor *et al.,* 2013). The wide range of applications for CoQ_{10} in the pharmaceutical and cosmetic industries has led to a strong desire to produce it on a large scale (Lee *et al.,* 2017).

In our previous study (Moghadami *et al.,* 2019), it was shown that the thermotolerant *Gluconobacter japonicus* FM10 is a CoQ_{10} natural producer. Here, the characterization of *Gluconobacter japonicus* FM10 strain and its two derived strains were investigated for CoQ_{10}, glycerol dehydrogenase (GLDH) and sorbitol dehydrogenase (SLDH) activity and thermostability to address this question; is it possible to restore the reduced CoQ_{10} production potential of a varied strain of *Gluconobacter japonicus* FM10?

MATERIALS AND METHODS

Microorganisms

The microorganism used in this study, *Gluconobacter japonicus* FM10 was isolated and identified previously (Moghadami *et al.,* 2019). Two strains were created in this study; FM20 and FM30. FM20 strain is a varied strain created from FM10 strain during some continuous passages on GYC medium. The FM30 strain was obtained from the FM20 strain through restricting available oxygen. The suspension of FM20 strain was inoculated into the tubes containing 5-mL of the seed culture. Then, 1 mL of sterile liquid paraffin was added to the tubes. The tubes were incubated at 30 °C for 72 h. and 0.1 mL of the suspension was struck on the GYC medium.

Chemicals and media

The reference CoQ_{10} standard was purchased from Sigma-Aldrich Co. with CAS number 303-98-0 (≥98%- HPLC). All other chemicals were of analytic-al grade from standard suppliers. This strain was maintained on the GYC medium (glucose 50 g/L, yeast extract 10 g/L, $CaCO_3$ 30 g/L, Agar 25 g/L) for 2-3 months, in a frozen state at -70 °C as stock. The seed culture contained 20 g/L sorbitol, 3 g/L yeast extract, and 3 g/L peptone, and the CoQ_{10} production culture contained 110 g/L sorbitol, 25 g/L yeast extract, 35 g/L peptone, 0.5 g/L KH_2PO_4, and 0.55 g/L $MgSO_4$ (Moghadami *et al.,* 2021). All experiments were performed in 250-mL flasks containing 100 mL of medium with pH: 6.5, agitation speed of 180 rpm, and incubation temperature of 30 °C. Extraction of CoQ_{10} and measurement of dry cell weight were performed after 40 h of growth.

Extraction and analysis of CoQ_{10}

The cells in 1 mL culture of *Gluconobacter japonicus* FM10 were harvested at 9000×g for 15 min. The pellets were washed with 1 mL of distilled water and suspended in 0.5 mL of the Cell Lytic B (Sigma- Aldrich). After 30 min incubation at 30 °C and shaking well, 1 mL of hexane: 2-propanol (5:3) was added to the solution and mixed well. The upper phases were transferred into new tubes and after adding 0.5 mL hexane and mixing vigorously, the upper phase was re-transferred into the tube. After evaporation, 0.5 mL ethanol was added to the dried residue. Analysis of CoQ_{10} was performed by high-performance liquid chromatography (Agilent 1120, USA) with a Thermo scientist C18 column (250 mm×4.5 mm×5 µm) coupled to a UV detector with ethanol: methanol (70:30) as the mobile phase at a flow

Figure 1. The colonies of different strains of *Gluconobacter japonicus*.

rate of 1 mL/min and CoQ_{10} was detected at 275 nm.

Measurement of dry cell weight

For the Dry Cell Weight (DCW) determination, 1 mL of the cultures was centrifuged at 9000×g for 15 min, washed twice and dried at 60 °C overnight to reach a constant weight.

Thermo stability of the strains

Since FM10 strain was found to be a thermotolerant bacterium (Moghadami *et al.,* 2019), to study the thermo stability of FM20 and FM30 strains, three strains were cultured in GYC medium at 37, 38, 39 and 40 °C for 48 h. Then, the colony formation was studied (Moghadami *et al.,* 2018).

Preparation of membrane fractions

Cells grown in the shaking incubator were harvested after 40 h by centrifugation at 9000×g for 15 min. The preparation of membrane fractions was performed by the method described previously (Shinagawa and Ameyama, 1989) with a modification, i.e. the cells were lysed by the sonicator (Misonix, USA) 10 min with 5 intervals for 4 times.

GLDH and SLDH enzymes assay

The GLDH and SLDH activities were measured with ferricyanide as an electron acceptor as described previously (Shinagawa and Ameyama, 1989; Ameyama *et al.,* 1985). One unit of enzyme activity was defined as the amount of enzyme catalyzing the oxidation of 1 µmol of substrate per min. Four absorbance units equaled 1 µmol of the oxidized substrate. Protein concentration was measured by a modified Lowry method with bovine serum albumin as the standard (Shinagawa and Ameyama, 1989).

RESULTS

Morphological characteristics and acetic acid formation from ethanol

The difference of colony sizes in FM10, FM20, and FM30 was examined. The colonies of three strains are shown in Figure 1. The size of FM20 colonies was larger than FM10 and the size of FM30 colonies was smaller than FM10 colonies.

Growth curves of FM10, FM20, and FM30 strains

The Growth curves of FM10, FM20 and FM30 strains were studied in 250 mL- baffled flasks for 72 h. The results showed that the FM30 reached the stationary phase in 32 h., while the FM20 strain reached the stationary phase in 44 h (Figure 2).

CoQ_{10} production

The chromatograms of CoQ_{10} production by three different strains are compared in Figure 3. The results of CoQ_{10} production by three strains of FM10, FM20 and FM30 are shown in Table 1. The result showed that the highest level of CoQ_{10} production was related to strain FM30 (6.3 mg/L) and the lowest level was related to FM20 strain (0.8 mg/L). The highest level of the specific content of Q_{10} was related to the FM30 strain (1.4 mg/g DCW).

Thermo stability of FM10, FM20, and FM30 strains

Thermo stability of FM10, FM20, and FM30 strains was investigated by culturing three strains in GYC medium at 37, 38, 39, and 40 °C for 48 h. Three strains were also able to grow at 39 °C. They did not show any changes in thermostability.

GLDH and SLDH activities

In order to clarify the relationship between CoQ_{10} production and GLDH and SLDH activities, the activity of these two enzymes were investigated in FM10, FM20, and FM30 strains in membrane fractions. The results showed that GLDH and SLDH activities of FM30 were higher than those of the others (0.66 and 0.52 U mg^{-1}, respectively). The lowest activities of GLDH and SLDH were observed in FM20 (0.37 and 0.22 U mg^{-1}, respectively). The GLDH activity was higher than SLDH in the strains (Figure 4). Positive correlations were found between CoQ_{10} level and GLDH (r=0.95)

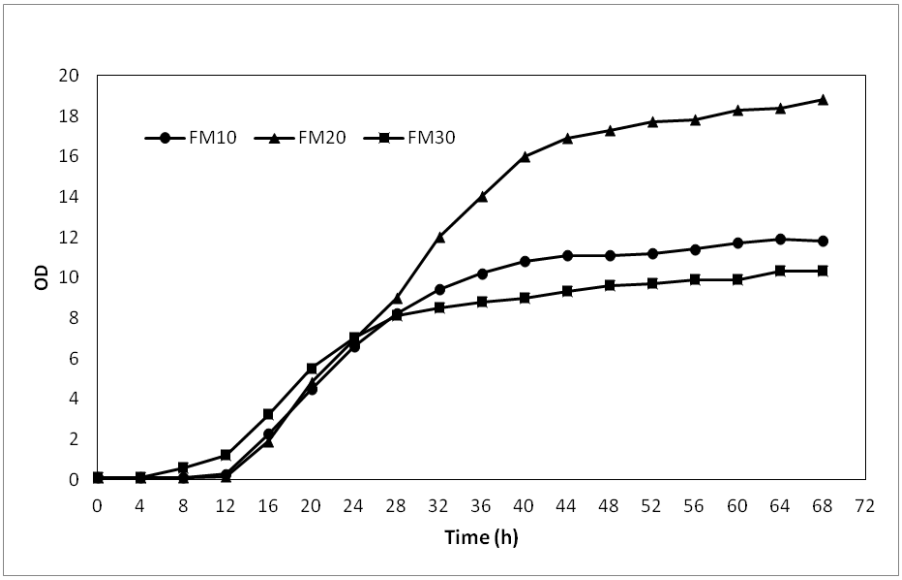

Figure 2. The Growth curves of FM10, FM20, and FM30 strains in 250 mL- baffled flasks. The initial pH was 6.5 and the temperature was set at 30 °C. The cultivation was continued for 72 h. Forty hours after the initiation of culture was considered as the stationary phase in the FM10 strain.

and SLDH (r=0.91) activities in different strains of FM10, FM20, and FM30.

DISCUSSION

The ability of bacteria to survive in diverse environments very much depends on their genetic makeup (Hallet *et al.,* 2001; Low *et al.,* 2001). However, environmental conditions including pH, salinity, temperature, and the availability of nutrients can strongly fluctuate the cells' conditions. Besides the genetic makeup, the origin of phenotypic variation may reside in the modifications of the DNA (Smits *et al.,* 2005). These alterations are epigenetic in nature since they have nothing to do with changes in DNA sequence, yet the phenotypes they cause to emerge can be inherited by daughter cells after division (Smits *et al.,* 2008).

In order to discover any possible variations in the original culture of FM10 strain, some characters were investigated including the CoQ_{10} production, SLDH and GLDH enzymes activity and thermostability. The FM10 strain had a great variation so that after a few passages, it changed morphologically. CoQ_{10} production by FM20 was less than FM10 strain. In this study, we added liquid paraffin to the tubes containing FM20 for restricting the oxygen availability. The FM30 strain colonies were smaller than the other two strains, which can mean that this strain produced less biomass than the other two strains. For this reason, the specific content of CoQ_{10} produced by FM30 was higher compared to the other two strains. In fact, the specific content of CoQ_{10} of the FM30 strain was about 2.8 times higher than that of the FM10 strain. Probably the bacterium increased the production of CoQ_{10} when it was under oxygen depletion stress for compensating its effect. The effect of aeration and O_2 level on CoQ_{10} production has been the subject of several studies (Sakato *et al.,* 1992; Seo and Kim, 2010). When the dissolved oxygen (DO) level decreased from 20 to 5% (Choi *et al.,* 2005) and the DO level was kept between 0 to 10 % (Ha *et al.,* 2007) both in *Agrobacterium tumefaciens* and also anaerobic culture of *Paracoccus denitrificans* (Matsumura *et al.,* 1983) an increase in the specific content of CoQ_{10} was observed. A mechanism has been proposed that as the final acceptor of electron (oxygen) in the respiratory chain decreases, electrons are trapped in quinone pool, leading to the accumulation of the reduced form of CoQ_{10} ($CoQ_{10}H_2$). Thus, in order to repair the imbalance in $CoQ_{10}/CoQ_{10}H_2$, more CoQ_{10} is synthesized (Cluis *et al.,* 2012).

Table 1. The amount of CoQ_{10} and the dry cell weight (DCW) in three strains.

Strain	CoQ_{10} (mg/L)	DCW (g/L)	SC of CoQ_{10} (mg/g DCW)
FM10	2.7±0.03	5.3±0.03	0.50
FM20	0.8±0.09	7.7±0.06	0.10
FM30	6.3±0.05	4.5±0.09	1.40

SC: Specific Content of CoQ_{10}.

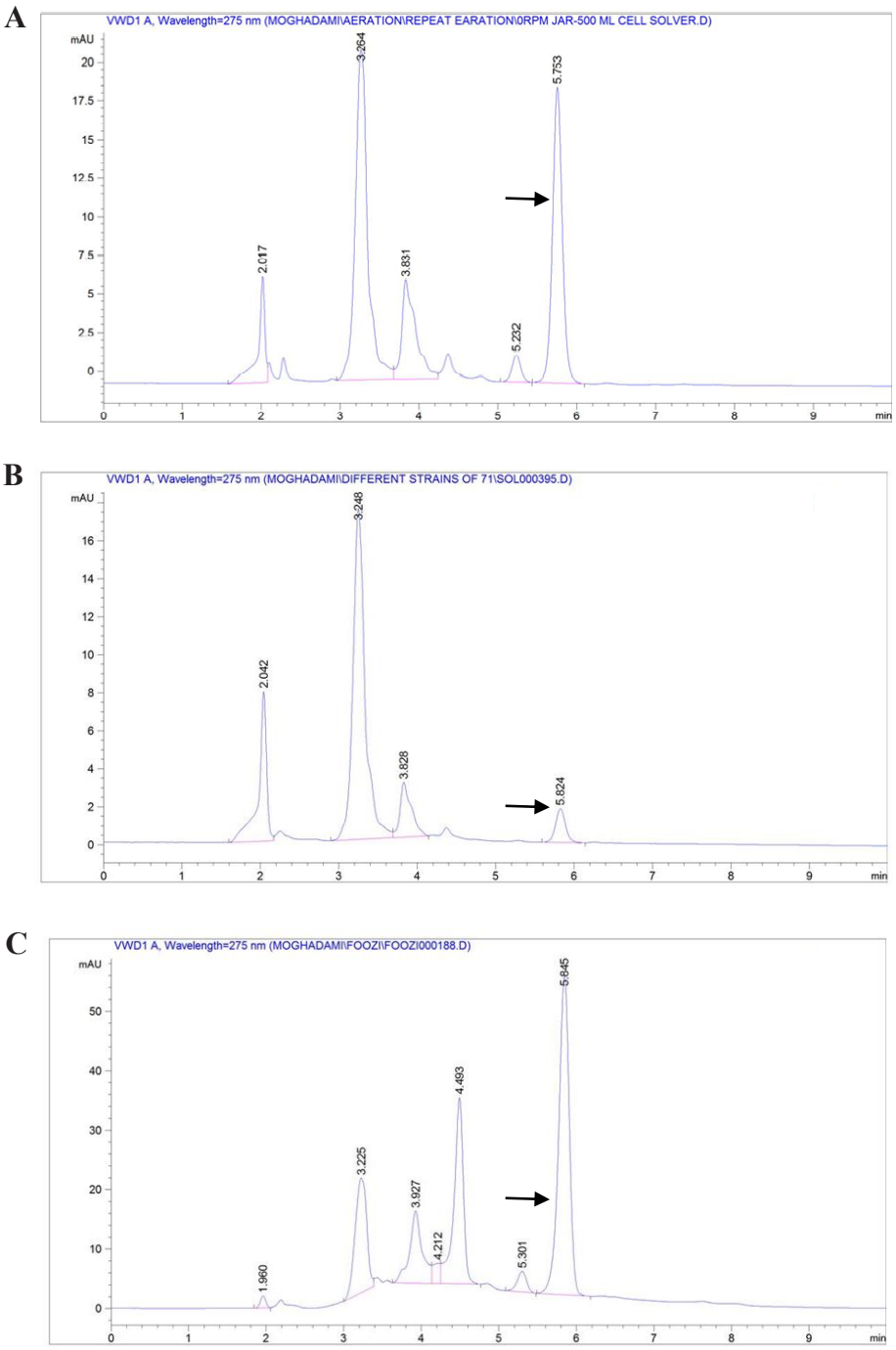

Figure 3. HPLC chromatograms of CoQ_{10} production by A) FM10 B) FM20 and C) FM30 strains produced at 30 °C, pH 6.5, and 180 rpm. The peak in the retention time of 5.8 represents CoQ_{10}.

In our previous study, it was found that the *Gluconobacter japonicus* FM10 strain was able to produce the highest amount of CoQ_{10} in the presence of sorbitol as the carbon source (Moghadami *et al.,* 2019). In the respiratory chain of *Gluconobacter*, D-Sorbitol is oxidized to L- sorbose through two membrane-bound dehydrogenases, glycerol dehydrogenase (GLDH) and sorbitol dehydrogenase (SLDH) (Matsushita, 1994). For this reason, the activity of two enzymes, glycerol dehydrogenase and sorbitol dehydrogenase was investigated, which are involved in sorbitol metabolism. The results showed that the highest and lowest activities of glycerol dehydrogenase and sorbitol dehydrogenase enzymes were observed in strains FM30 and FM20, respectively. Positive correlations between CoQ_{10} level and GLDH (r=0.95) and SLDH (r=0.91) activities in FM10, FM20, and FM30 strains were observed.

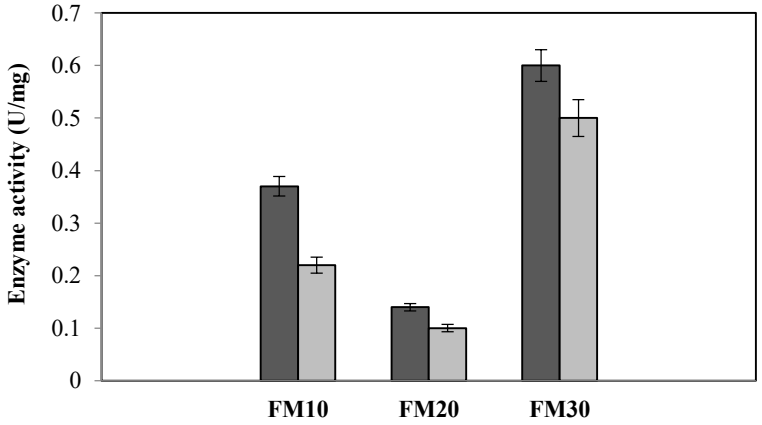

Figure 4. GLDH and SLDH activities of FM10, FM20, and FM30 strains. The cultivations were performed in baffled flasks at 33 °C, pH 6.5 and 180 rpm for 40 h. GLDH and SLDH activities were measured in membrane fractions. Black: GLDH, Grey: SLDH.

Strain FM10 was found to be a thermotolerant strain able to grow at 39 °C (Moghadami *et al.,* 2019). Strain FM20 was still able to tolerate 39 °C after tolerating 10 subcultures. In the case of strain FM30, the thermotolerance did not change and it was still able to tolerate 39 °C. This means that the thermostability of strain FM10 did not change during the variation or depletion of oxygen.

CONCLUSION

It can be concluded that by exposing the varied strains of *Gluconobacter* under stress such as oxygen restriction, the ability to produce CoQ_{10} could be restored. The CoQ_{10} production and GLDH and SLDH activities in the strain exposed to the restricted oxygen was higher than the varied strain. The CoQ_{10} production enhancement was so high that it was even greater than the original strain. *Gluconobacter* produces several industrially important metabolites. It seems that the oxygen restriction may be used for *Gluconobacter* strains, in which the fermentation ability is reduced in industry.

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Author's contributions

FM and RH designed the study. FM carried out the experiments. FM and RH wrote the manuscript.

Conflicts of interest

The authors declare no conflict of interest.

Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

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