

Effect of iron and silver nanoparticles on coenzyme Q₁₀ production by *Gluconobacter japonicus* FM10

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ABSTRACT

Background and Objectives: Coenzyme Q₁₀ is an anti-aging agent whose demand is increasing progressively. There are various strategies used for increasing coenzyme Q₁₀ production by microorganisms. In this study, for the first time, we investigated the effect of iron oxide and silver nanoparticles on coenzyme Q₁₀ production by *Gluconobacter japonicus* FM10.

Materials and Methods: In the first step, a preliminary experiment was set and carried out to obtain the minimum inhibitory concentrations of the nanoparticles on the strain FM10. Then the sub-MIC concentrations were used to investigate their effect on coenzyme Q₁₀ production in the stationary and exponential phases of the growth, separately.

Results: The results showed that coenzyme Q₁₀ production increased in the presence of the iron oxide and silver nanoparticles. The silver nanoparticles induced 1.9 times higher coenzyme Q₁₀ production. The highest level of coenzyme Q₁₀ was induced when the silver nanoparticles were added to the culture medium at the stationary phase.

Conclusion: This should be noticed that so far nanoparticles have been considered as antibacterial agents, rather than being considered to cause probable beneficial effects on the induction of useful products in the microbial world. In this regard, their potential for increasing coenzyme Q₁₀ production has received no attention. However, our present results showed that the nanoparticles can be used to increase the production efficiency of coenzyme Q₁₀ in *Gluconobacter*.

Keywords: Coenzyme Q₁₀; *Gluconobacter*; Nanoparticles; Exponential phase; Stationary phase

INTRODUCTION

Coenzyme Q is a vitamin-like lipophilic component that is part of the respiratory chain of pro-

karyotes and eukaryotes. Coenzyme Q has two main parts; the functional part of the molecule, the quinone ring, and the structural part, an isoprenoid side chain made of several isoprene units, which keeps the coenzyme Q molecule in the membrane (1). Isoprenoid side chain can vary in chain length (6-10 isoprene units), depending on the species. Coenzyme Q that contains 10 isoprene units is called coenzyme Q (CoQ₁₀) (2). It has many roles in the cell due to its high tendency for electron absorption. Coenzyme Q participates as membrane-bound redox-active molecule in several cellular functions such as the formation of disulfide bonds in proteins, detoxification of harmful reactive oxygen species, controlling the cellular redox status and gene expression (3). It is widely used as a supplement to increase energy and immunity, as well as an anti-inflammatory and anti-aging sup-

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plement. It is also used to treat Parkinson's disease, Huntington's, cancer, AIDS and muscular dystrophy. This molecule is also effective in the health of teeth and gums, lowering blood sugar and protecting lipids in cosmetic, medicine and food industries (4). The coenzyme Q₁₀ is one of the secondary metabolites and its biosynthetic pathway is very complex and varies in different organisms, but generally involves two separate steps: the biosynthesis of the quinone ring, and the isoprenoid chain. The two components conjugate to form the main molecule (5). Coenzyme Q₁₀ production is currently performed mainly by chemical synthesis, semi-chemical synthesis (extraction from plants and structural modification) and microbial fermentation processes. The cost of chemical and semi-chemical synthesis is high. Therefore, the microbial fermentation process is more attractive since only the Trans isomer is produced (6). Thus far, many investigations have been performed to produce and enhance the production yield of coenzyme Q₁₀ by microorganisms (7). Isolation of microbial strains is the most successful strategy in the strain development for coenzyme Q₁₀ production (8). A number of bacteria including *Agrobacterium tumefaciens*, *Rhodobacter sphaeroides*, *Paracoccus denitrificans*, *Pseudomonas* and engineered *E. coli* have been reported as coenzyme Q₁₀ producers (5). The enhancement of coenzyme Q₁₀ production has been achieved by mutagenesis, using the precursors, the metabolic engineering and increasing of coenzyme Q₁₀ yield through improving growth conditions (8).

Gluconobacter is a Gram-negative bacterium belonging to the family *Acetobacteraceae* (9). It has been shown that *Gluconobacter* is well adapted for industrial uses (10). The main industrial important applications of *Gluconobacter* are the production of vitamin C, dihydroxyacetone, 6-amino-L-sorbose, shikimate and 3-dehydroshikimate (11). These products are the results of incomplete oxidation performing by this genus (12). It is indicated that there are several membrane-bound dehydrogenases located in the cytoplasmic membrane oxidizing sugars and sugar alcohols through one or more steps, and the CoQ₁₀ is the part of the *Gluconobacter* respiratory chain (13). In our previous study, *Gluconobacter japonicus* FM10 was shown to be a CoQ₁₀ natural producer (14).

Nevertheless, to the best of our knowledge, there is no study on the effect of nanoparticles on coenzyme Q₁₀ production by bacteria. Thus, we investigated the effect of iron oxide and silver nanoparticles on co-

enzyme Q₁₀ production by *Gluconobacter japonicus* FM10. To study the effect of nanoparticles on coenzyme Q₁₀ production, at first, the minimum inhibitory concentrations of these nanoparticles were determined. Then the sub-MIC concentrations were used to study their effect on coenzyme Q₁₀ production in the stationary and exponential phases, separately.

MATERIALS AND METHODS

Chemicals. The iron oxide nanoparticle (Fe₃O₄) and silver nanoparticles with dimensions of 20-30 and 20 nm, respectively were purchased from US Research Nanomaterials Co (USA). The reference CoQ₁₀ was purchased from Sigma-Aldrich Co. with CAS number 303-98-0 (≥98% - HPLC). All other chemicals were of analytical grade from standard suppliers.

Microorganism and media. The microorganism used in this study, *Gluconobacter japonicus* FM10, was isolated and identified previously (9). This strain was maintained on the GYC medium (glucose 50 g/L, yeast extract 10 g/L, CaCO₃ 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 CoQ₁₀ production culture contained 110 g/L sorbitol, 25 g/L yeast extract, 35 g/L peptone, 0.5 g/L KH₂PO₄ and 0.55 g/L MgSO₄ (10). All experiments were performed in 250-mL flasks containing 100 mL of the medium with pH 6.5, agitation speed of 180 rpm and incubation temperature of 30°C. Extraction of coenzyme Q₁₀ and measurement of dry cell weight was performed after 40 h of incubation.

Extraction and analysis of coenzyme Q₁₀. The cells from 1 mL of *Gluconobacter japonicus* FM10 cultures were harvested at 12000 × rpm for 15 min. The pellets were washed with 1 mL distilled water and suspended in 0.5 mL the CelLytic™ B cell lysis reagent (Sigma- Aldrich). After 30 min incubation at 30°C and shaking well, 1 mL hexane: 2-propanol (5:3) was added to the solution and mixed well. The upper phase was transferred into new tube and after adding 0.5 mL of 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 coenzyme Q₁₀ was performed by HPLC; (Agilent 1120, USA) with a Thermo scientist C18 col-

umn (250 mm × 4.5 mm × 5 μm) coupled to a UV detector with ethanol: methanol (70:30 V/V) as the mobile phase at a flow rate of 1 mL/min and coenzyme Q₁₀ was detected at 275 nm. Analysis of coenzyme Q₁₀ was also performed by Liquid chromatography-mass spectrometry (LC-MS). The Agilent LC-MS system (Waldbronn, Germany) included, a microcolumn C18 (150 mm × 1 mm × 5 μm) and a ThermoFisher Scientific (Bremen, Germany) ion trap mass spectrometer (model LCQ, mass range m/z 10-2000). The mobile phase was ethanol: methanol (70:30 V/V) at a flow rate of 1 mL/min.

Measurement of dry cell weight. For the dry cell weight (DCW) determination, 1 mL of the cultures was centrifuged at 12000 × rpm for 15 min, washed twice and dried at 60°C overnight to reach a constant weight.

Determination of minimum inhibitory concentration (MIC) of silver and iron oxide nanoparticles. The microtiter plate assay was used to determine the minimum inhibitory concentration of silver and iron oxide nanoparticles. The iron oxide and silver nanoparticles were used separately with concentrations of 800, 400, 200, 100, 50, 25, 12, 6, 3 and 1.5 μg/mL. Since the nanoparticles create turbidity in the culture medium, the wells 1 to 10 were loaded with the same amount of nanoparticle without bacterial inoculation (15). Absorption (A) of the wells containing the bacterial cells and nanoparticles was subtracted from the absorption of the wells containing only the nanoparticles.

The effect of silver and iron oxide nanoparticles on coenzyme Q₁₀ production. Since 200 μg/mL of iron oxide nanoparticles and 100 μg/mL of silver nanoparticle were determined as MIC, the concentrations of sub-MIC were used to produce coenzyme Q₁₀. For this purpose, 50 μg/mL of silver and 100 μg/mL of iron oxide nanoparticles were added to the culture medium. The flasks were incubated at 30°C at 180 rpm for 40 h. Then the dry cell weight and coenzyme Q₁₀ were measured. At the next step, the effect of silver and iron oxide nanoparticles were determined on coenzyme Q₁₀ production at two phases of growth (exponential and stationary phases). To perform this experiment, the culture flasks were divided into three series:

Series 1- Before the inoculation (from the begin-

ning of the exponential phase of growth), 50 μg/mL of silver nanoparticles were added to the flasks; Series 2- After 40 h of incubation (when the cultures reached the stationary phase of growth), 50 μg/mL of silver nanoparticle was added to the flasks; Series 3- The third series of flasks was inoculated as the control, without adding the nanoparticles (Control culture).

The flasks were incubated at 30°C at 180 rpm for 40 h. The dry cell weight and coenzyme Q₁₀ were measured every 4 h (44 h, 48 h and 52 h). The same experiments were performed for iron nanoparticles by adding 100 μg/ml of iron oxide nanoparticles.

RESULTS

Production of coenzyme Q₁₀ by FM10 strain.

Fig. 1 shows the growth curve of *Gluconobacter japonicus* FM10 and the phases in which nanoparticle were added into the culture medium. The level of coenzyme Q₁₀ produced by the strain FM10 before adding silver and iron oxide nanoparticles was 2.7 mg/L, the dry cell weight was 5.3 g/L and its specific coenzyme Q₁₀ content was 0.5 mg/g DCW. Fig. 2 shows the chromatograms of the HPLC analysis of coenzyme Q₁₀ standard (A) and coenzyme Q₁₀ (B) obtained from *G. japonicus* FM10. The result of LC-MS analysis of coenzyme Q₁₀ obtained from *G. japonicus* FM10 showed the molar mass of 864 that represents coenzyme Q₁₀ protonated ion. The molar mass of coenzyme Q₁₀ (C₅₉H₉₀O₄) is 863 g/mol (Fig. 4).

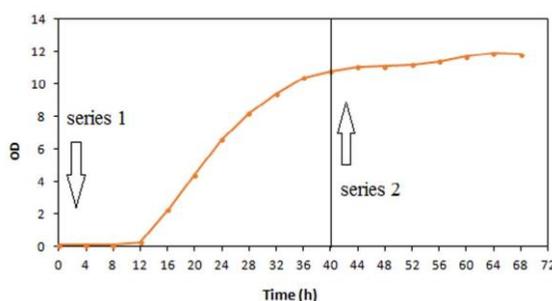


Fig. 1. The growth curve of *Gluconobacter japonicus* FM10 in 250 mL- baffled flasks. The initial pH was 6.5 and the temperature was set at 30°C. The cultivation was continued for 72 h. Forty hours after the initiation of culture was considered as the stationary phase. Series 1. Before the inoculation 50 μg/ml of silver nanoparticles were added to the flasks. Series 2. At the first of the stationary phase of growth, 50 μg/ml of silver nanoparticle were added to the flasks.

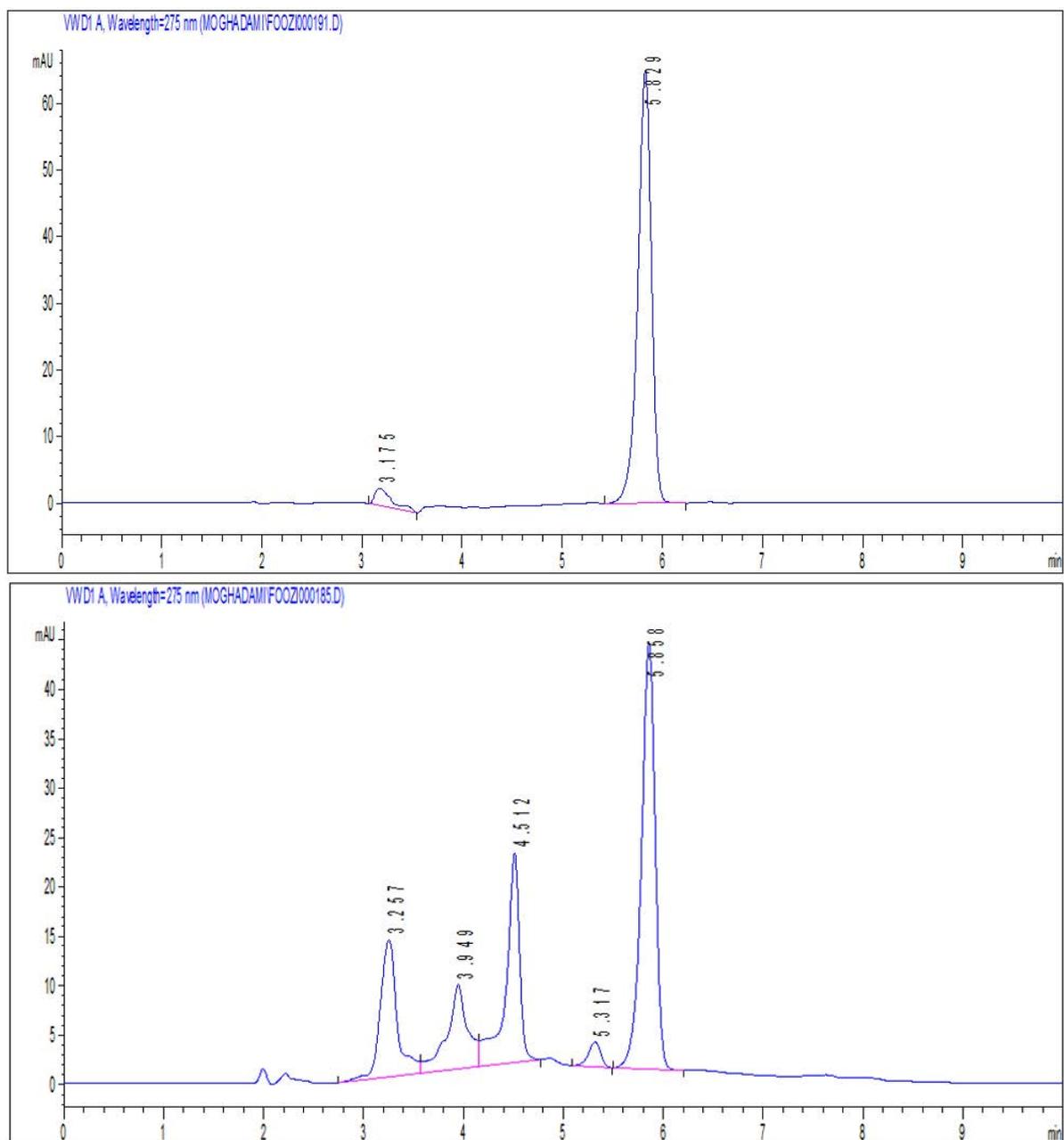


Fig. 2. A) The chromatogram of the HPLC analysis of CoQ₁₀ standard. The peak in the retention time of 5.8 represents CoQ₁₀. B) HPLC analysis of CoQ₁₀ obtained from *G. japonicus* FM10 produced in the presence of sorbitol, yeast extract and peptone at 30°C, pH 6.5 and 180 rpm. The peak in the retention time of 5.8 represents CoQ₁₀.

Minimum inhibitory concentration of silver and iron oxide nanoparticles. The results showed that the strain FM10 had a growth turbidity in the presence of 200 µg/mL of iron oxide nanoparticles and 100 µg/mL of silver nanoparticle (MIC). Therefore, the concentrations of 100 µg/mL of iron oxide nanoparticle and 50 µg/mL of silver nanoparticle were used for further studies as sub-MIC concentration.

Production of coenzyme Q₁₀ in the presence of silver and iron oxide nanoparticles. Coenzyme Q₁₀ production was evaluated in the control culture and in the presence of silver and iron oxide nanoparticles. They were added to the culture at the exponential and the stationary phases separately. The chromatograms of coenzyme Q₁₀ production in the presence of silver and iron nanoparticles are compared in Fig. 3. The results of coenzyme Q₁₀ production in the presence

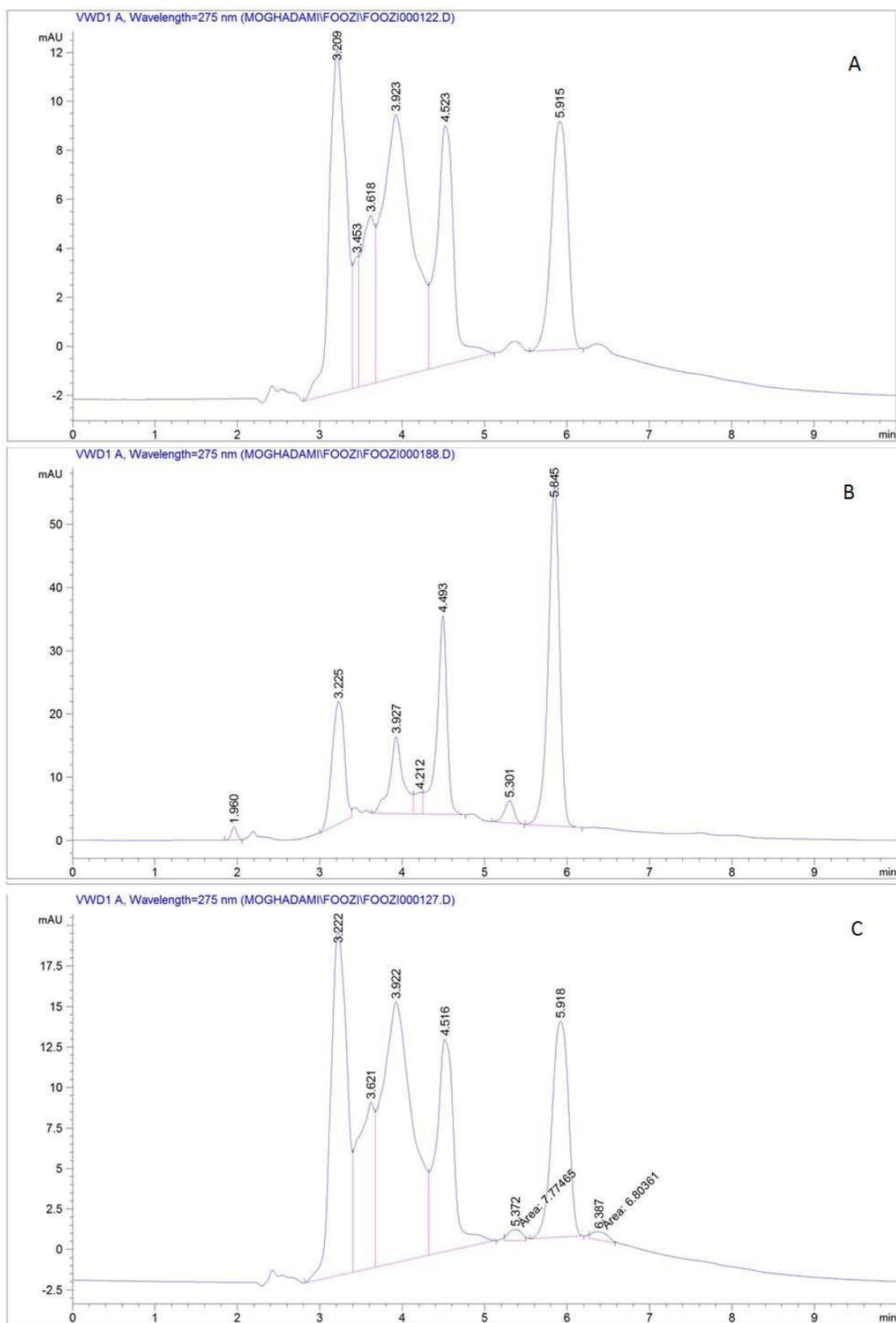
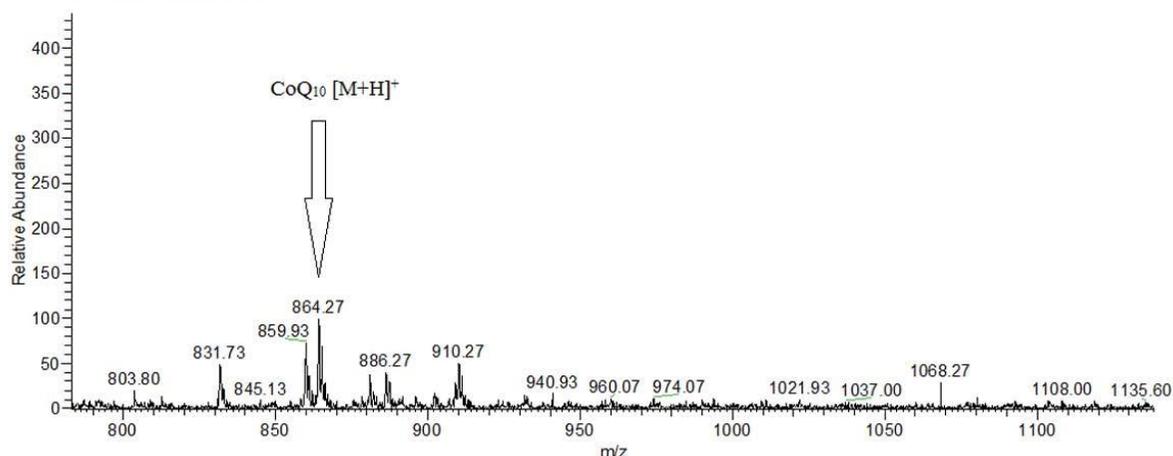


Fig. 3. HPLC chromatograms of coenzyme Q₁₀ production A) without nanoparticle treatment B) treated by silver nano particles and C) treated by iron oxide nanoparticles.

MOGHADDAMI 170905134240 #713 RT: 19.41 AV: 1 NL: 6.63E5
T: + p ESI Full ms [150.00-2000.00]



MOGHADDAMI 170905134240 #714 RT: 19.44 AV: 1 NL: 7.17E5
T: + p ESI Full ms [150.00-2000.00]

Fig. 4. LC-MS analysis of CoQ₁₀ produced by *G. japonicus* FM10 in the presence of sorbitol, yeast extract and peptone at 30°C, pH 6.5 and 180 rpm. The molar mass of CoQ₁₀ (C₁₀H₅₉O₉) is 863. The molar mass of 864 is visible that represents CoQ₁₀ [M+H]⁺. LC-MS, Agilent (Waldbronn, Germany), C4 column (250 mm×4.6 mm×5μm×100 Å°).

Table 1. The amount of coenzyme Q₁₀ and the dry cell weight in the presence of 50 μg/mL of silver nanoparticles and 100 μg/mL of iron oxide nanoparticles in the exponential and the stationary phases.

Culture	Growth phase	CoQ ₁₀ (mg/L)	DCW (g/L)	SC of CoQ ₁₀ (mg/g DCW)
Silver nano particle (50 μg/mL) treated culture	exponential	1.9 ± 0.05	3.7 ± 0.1	0.51
	stationary	5.2 ± 0.1	5.3 ± 0.03	0.98
Iron nano oxide (100 μg/mL) treated culture	exponential	1.7 ± 0.09	4.2 ± 0.06	0.40
	stationary	3.5 ± 0.04	5.3 ± 0.03	0.66
Control culture		2.7 ± 0.03	5.3 ± 0.03	0.50

SC; Specific Content of CoQ₁₀

of silver and iron oxide nanoparticles (the exponential and the stationary phases) and control culture are shown in Table 1.

Coenzyme Q₁₀ production was higher in the presence of the silver nanoparticles than iron oxide nanoparticles. Coenzyme Q₁₀ production was higher when the nanoparticles were added to the culture medium at the stationary phase rather than the exponential phase. In the case of silver nanoparticle, coenzyme Q₁₀ production was 1.9 fold higher when nano particles were added at the stationary phase. However, this fold difference was 1.3 in the case of iron oxide nanoparticle treatment. The highest level of coenzyme Q₁₀ was produced when the silver nanoparticles were added to the culture medium at the stationary phase. The difference in coenzyme Q₁₀ production was almost two times between the

culture treated by silver nanoparticle at the stationary phase and the control culture. The cell growth (measured by dry cell weight) was reduced when the iron oxide and silver nanoparticles were added to the culture medium at the exponential phase. It showed slight decreases when the nanoparticles were added to the culture medium at the stationary phase of growth. The cultures treated with silver and iron oxide nanoparticles at the exponential phase of the growth showed 1.43 and 1.26 fold decrease in DCW compared to the control culture, respectively.

DISCUSSION

The antibacterial effects of nanoparticles on the various bacteria have been investigated so far and

the investigations have shown that most nanoparticles have antibacterial activity (16). The antibacterial effects of nanoparticles have been studied mostly on pathogenic bacteria (17-19). *Gluconobacter* species are not amongst pathogenic bacteria, therefore, there are a few studies performed on the antibacterial effect of nanoparticles on these bacteria. Garcia-Ruiz et al. showed that the concentrations higher than 45 µg/mL of various silver nanoparticles (silver-polyethylene glycol nanoparticles and silver-glutathione nanoparticles) inhibited the growth of *G. oxydans* (20).

There are many investigations published on the effect of nanoparticles on metabolites, particularly in plants (21-23). Some of these studies focused on the effect of nanoparticles on microbial biosurfactants (24, 25), microbial cellulose (26) and microbial polymers (27). It was revealed that, the addition of the Fe/SDS nanoparticles to the culture medium of *Pseudomonas aeruginosa* could increase the production of biosurfactant by 20% (24). The nanoparticle concentrations and addition time of nanoparticles to the culture medium are effective parameters on the enhancement of growth and rhamnolipid production (28). It has been proposed that the production of biosurfactant in the presence of iron nanoparticles was due to the effect of iron nanoparticle providing more nutrition to the organism by the activation of the medium (29). It has been also hypothesized that the produced electrons by nanoparticles may cause an increase in the enzymatic function of enzymes and cell membrane proteins or enhances the function of the electron transfer chain in the bacterial cell membrane and consequently facilitating cell metabolism, cell growth and increase of biosurfactant production (25, 29). This may be true for our case as well, i. e. the production of coenzyme Q₁₀ production, since coenzyme Q₁₀ is an essential part in the electron transfer chain in the cell membrane.

There are no studies on the effect of nanoparticles on coenzyme Q₁₀ production by bacteria. Thus, we investigated the effect of iron oxide and silver nanoparticles on coenzyme Q₁₀ production by *Gluconobacter japonicus* FM10. The results of this study showed that the iron oxide and silver nanoparticles had positive effects on the coenzyme Q₁₀ production and caused increases in coenzyme Q₁₀ level. The results also showed that the silver nanoparticles had a stronger antibacterial activity than the iron oxide nanoparticles. The similar results were obtained in

other studies. For example, the antimicrobial effect of silver nanoparticles was shown to be greater than other nanoparticles such as iron, zinc and gold (16). To clarify whether nanoparticles can increase the production of coenzyme Q₁₀ by the strain FM10, the effects of iron oxide and silver nanoparticles were studied on two growth phases, the exponential and the stationary phases, separately. When iron oxide and silver nanoparticles were added to the culture medium at the exponential phase of the growth, the cell growth showed a reduction. In fact, the resulting dry cell weight was less than that of the control (without adding nanoparticles), which subsequently reduced the production of coenzyme Q₁₀. When iron oxide and silver nanoparticles were added to the culture medium during the stationary phase, they did not affect the cell growth, but they caused an increase in coenzyme Q₁₀ level. The production of coenzyme Q₁₀ in the presence of 50 µg/mL silver nanoparticles was 5.2 mg/L, which is 1.9 times higher than that of the control culture. Since the stress in the respiratory chain on coenzyme Q₁₀-producing bacteria increases its production (30), it seems that using nanoparticles can increase production of coenzyme Q₁₀ by causing oxidative stress on the respiratory chain or producing the reactive oxygen species. The bacteria can reduce the harmful effects of oxidative stress and respiratory chain inhibitors with the help of the antioxidant activity of coenzyme Q₁₀ (31, 32). Therefore, *Gluconobacter japonicus* cells may increase the production of coenzyme Q₁₀ in response to the oxidative burst caused by nanoparticles.

Ha et al. showed that the oxidative stress imposing by addition of Ca²⁺ to the *Agrobacterium tumefaciens* culture increased the coenzyme Q₁₀ level. They rationalized that increase in coenzyme Q₁₀ level was due to the cell protective mechanisms against the impact of oxidative stress including the cell membrane damage and lipid peroxidation (30). Silver and iron nanoparticles play a role in oxidative stress in bacteria and produce a variety of reactive oxygen species that can have a lethal effect on bacteria (32). Choi et al. also showed that adding an electron flux inhibitor (Azide) to the *Agrobacterium tumefaciens* culture could increase coenzyme Q₁₀ level. However, H₂O₂ did not cause an increase in the intracellular coenzyme Q₁₀ content, and they concluded that oxidative stress does not affect the synthesis of coenzyme Q₁₀ (33).

The nanoparticles range from 1 to 100 nanome-

ters in diameter. Smaller particles more readily enter cells and interact with the cellular components. The exposure dose, particle size, coating, and aggregation state of the nanoparticles, as well as the cell type or organism on which it is tested, are all large determining factors on the effect and potential toxicity of nanoparticles. Various functions have been proposed for the effect of silver nanoparticles on cells, such as methylation of DNA, histone tail modification, post-transcriptional regulation by non-coding RNAs such as microRNAs and long non-coding RNAs (34).

CONCLUSION

Nanoparticles have been always considered as antimicrobial agents, while their usage for increasing the production of useful molecules such as coenzyme Q₁₀ has been received little attention. Nevertheless, the results of this study showed that nanoparticles at concentrations below their MICs could be used to increase the production of coenzyme Q₁₀ in *Gluconobacter*.

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