



Mutagenesis of *Rhodobacter sphaeroides* using atmospheric and room temperature plasma treatment for efficient production of coenzyme Q10

Rong-Song Zou,^{1,2} Suyan Li,^{1,*} Le-Le Zhang,³ Chong Zhang,⁴ Yi-Jun Han,² Ge Gao,² Xiangyang Sun,¹ and Xiaoqiang Gong¹

College of Forestry, Beijing Forestry University, Beijing 100083, China,¹ R&D Center, Shenzhen Biology & Technology Co., Ltd., Nei Monggol 010206, China,² Biobreeding Research Center, Wuxi Research Institute of Applied Technologies, Tsinghua University, Wuxi, Jiangsu 214000, China,³ and Department of Chemical Engineering, Tsinghua University, Beijing 100084, China⁴

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Coenzyme Q10 (CoQ10) plays an important role in the human respiratory chain and is widely used as medicine and dietary supplement. To improve the fermentation efficiency of CoQ10, a modified version of atmospheric and room temperature plasma (ARTP) treatment was used to mutate *Rhodobacter sphaeroides*. Meanwhile, Vitamin K3, a structural analog of CoQ10, was used as an inhibitor for mutant selection. In the first round of screening in 24-well plates, three mutants were obtained, with the production of CoQ10 at 311 mg/L, 307 mg/L, and 309 mg/L, which were increased from the parent's production at 265 mg/L. Furthermore, a second round of mutation and screening was performed based on the mutant strain with the highest production in the first round, leading to the identification of a mutant AR01 with the production of CoQ10 at ~330 mg/L. Finally, 590 mg/L CoQ10 was obtained for AR01 after 100 h fermentation, which was ~25.5% higher than that of the original parent strain. It is the first report of ARTP treatment usage for the selection of CoQ10 producing bacteria and the results show that plasma jet, driven by helium-based ARTP, can be a feasible strategy for mutation feeding.

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Coenzyme Q (CoQ), also called ubiquinone, is the only ubiquinone found in humans, playing an essential role in generating ATP in the respiratory chain (1,2). In addition, it's important in both bioenergetic and antioxidant processes and is widely used as a dietary supplement in medicine (3,4). Recent studies revealed that supplementation of 300 mg CoQ10 every day significantly enhances activities of antioxidant enzymes and lowers inflammation in patients with coronary artery disease who were subjected to statins therapy (5). Moreover, CoQ10 is effective in the treatment of asthenozoospermia (6). CoQ10 treatment improved Corneal Edema in children with Kearns–Sayre syndrome (7) and slowed the progressive deterioration of nervous system in Parkinson disease (8). Recently, CoQ10 was reported as a protective agent against trimethyltin chloride-induced hippocampal damage by diminishing neuronal necrosis and improving learning memory (9).

The purple bacterium *Rhodobacter sphaeroides* has been extensively studied for the production of CoQ10 (10–13). Therefore, screening of better strains with higher CoQ10 production is of vital importance. Microbial mutation breeding is an effective method to improve the outcome of fermentation, and it is widely used in industry (14). Physical and chemical mutagen treatments such as ultraviolet radiation, sodium azide, diethyl sulfate and ethyl methane sulphonate have been used for mutation breeding (15,16),

which have many disadvantages associated with safety or environmental protection (17). Ion beam implantation, a physical mutation method, is based on low-pressure plasma and widely used for generation of mutations (18). Plasma is ionized gas and requires a presence of complex vacuum system traditionally (19). Recently, this expensive and complicated vacuum system has been removed (20), allowing to easily obtain atmospheric and room temperature plasma (ARTP). ARTP treatment can cause more significant DNA damage to living cells in comparison with other mutation methods, leading to a higher mutation rate (21). The plasma jet was used to mutate *Streptomyces avermitilis* and yielded high total (over 30%) and positive (about 21%) mutation rates (22). A mutant with 66.3% higher production of ϵ -poly-L-lysine was also obtained after the treatment of bacteria with ARTP (23).

Vitamin K3 (VK3), a structural analog of CoQ10, can compete with CoQ10 for its combining proteins, leading to inhibition of bacterial growth. VK3 has been used as selection pressure to successfully identify mutants that can overcome the inhibition (24). After being treated with *N*-methyl-*N*'-nitro-*N*-nitrosoguanidine (NTG) and selected using VK3-resistant, the mutant of *Rhodobacter sphaeroides* produced CoQ10 with 16–20% higher production than the original strain was identified (25). In this study, using VK3 as a structural analog for selection, ARTP treatment was applied for the first time to generate *R. sphaeroides* mutants with higher CoQ10 production (Fig. 1). Excitingly, after two rounds of screening, a mutant AR01 with the production of CoQ10 at ~330 mg/L was identified. Finally, 590 mg/L CoQ10 was obtained for AR01 after

* Corresponding author. Tel.: +86 10 62338103; fax: +86 10 62336096.

E-mail address: lisuyan@bjfu.edu.cn (S. Li).

100 h fermentation, which was ~25.5% higher than that of the original parent strain. The results show that plasma jet, driven by helium-based ARTP, can be a feasible strategy for mutation feeding.

MATERIALS AND METHODS

Strain and culture medium Shenzhou6, a mutant strain of *R. sphaeroides*, was kindly provided by Space Biology R&D Center, China Academy of Space Technology. A single colony of Shenzhou6 was selected as the parent strain in this study. This strain was selected from a spaceflight mutation with Shijian-8 recoverable satellite. The CoQ10 production level of Shenzhou6 is 33% higher than that of the wild type strain.

The mutants generated by ARTP treatment were grown on plates with solid medium containing 2% agar, 1.5% glucose, 0.5% yeast extract, 4.2% NaCl, and 3.8% CaCO₃, with pH adjusted to 6.0. The culture plates were incubated at 32°C for 5 days for observation of colony morphology and size, and strains with the fastest growth rate were selected for fermentation. Each strain was put into a well with a hemispherical bottom on a 24-well plate that had a dimension of 10 mm (width) × 10 mm (width) × 50 mm (height). Each well contained 3 mL initiation medium consisted of 1% glucose, 1% corn steep powder, 1.2% NaCl, 3% CaCO₃, 0.15% yeast extract, 0.8% MgSO₄, and 5% (NH₄)₂SO₄ (pH 7). After 24 h incubation with shaking at 220 rpm at 32°C, 0.3 mL of the culture was transferred to 2.7 mL of production medium in another 24-well plate and incubated with shaking at 220 rpm at 32°C for 90 h. The production medium contained 2% glucose, 1% sucrose, 1% corn steep powder, 2% MgSO₄, 1% (NH₄)₂SO₄, 0.05% KH₂PO₄, and 0.05% K₂HPO₄, with a pH of 7.

Selection of mutants Vitamin K3 as a structural analog of CoQ10 was used to estimate mutagenesis efficiency of *R. sphaeroides*. To evaluate the optimal concentration, a series of VK3 concentrations were prepared on the culture plates. VK3 was diluted in *N,N*-dimethylformamide (DMF) and filtered through a sterile 0.22 μm filter. VK3 was added to the medium in a concentration of 0.5%, 1%, 1.5%, 2%, 2.5% (v/v), respectively. Each plate dish (90 mm in diameter) contained a 20 mL of the solid medium. Pre-cultured solution (200 μL) was spread on the solid medium to count the colonies and determine VK3 effect.

Helium-based ARTP breeding machine An ARTP-IIS model of ARTP breeding machine, provided by the Yuanqing Tianmu Company (Wuxi City, Jiangsu Province, China) was used in the study. A helium source was utilized and operated at room temperature. A parental strain was grown in the initiation medium for 24 h, then was diluted with sterile 0.8% NaCl saline to adjust cell density to an OD₆₀₀ range from 0.6 to 0.8. The 20 μL of resulting diluted solution was placed onto a sterilized metal plate, 7.0 mm in diameter, which was a component of the ARTP device. A mobile platform of the ARTP breeding machine, located under the plasma generator, was moved up and down during the experiment. The metal plate was placed on the mobile platform. The platform was adjusted so that the metal plate was within 2 mm underneath the generator. The ARTP machine was operated at 120 W with a helium gas flow rate of 10 standard liters (with one atmosphere) per minute. Exposure time was determined according to the mortality rate of the strain.

Mortality rate of *R. sphaeroides* after ARTP treatment A series of metal plates were placed on the operating platform for different exposure times to plasma. The metal plates were then washed with 1 mL 0.8% NaCl saline, 200 μL of which was spread on plates containing 20 mL solid medium. The 90 mm plates were incubated at 32°C for 3 days and colonies were counted.

Mortality rate was evaluated according to the following equation (22):

$$\text{Mortality rate} = (T/U) \times 100\% \quad (1)$$

where *U* is the number of total colonies grown on the solid medium for an ARTP-untreated sample, and *T* is the number of total colonies on the solid medium for an ARTP-treated sample.

Screening of mutants Diluted cultures treated by ARTP (as described above for mortality rate) (200 μL) was spread on plates with different concentrations of VK3 and incubated at 32°C for 6 days. The mutants were selected based on various colony size and growth rate from the untreated control and cultured as described above for 72 h before CoQ10 was extracted.

Fermentation of mutants Fermentation was carried in a 5 L fermentor (Biotech-5BG, Shanghai Baoxing Bio-engineering Equipment Co., Ltd., Shanghai, China), containing 3 L of production medium. The fermentation culture was incubated at 32°C with an agitation speed of 300 rpm and air flow rate: 110 L/min for 100 h. The feeding glucose solution was added into the culture medium automatically by the fermentor, to keep a glucose concentration of 6–8 g/L during the fermentation process. The dissolved oxygen (DO) was measured by the dissolved oxygen electrode of the fermentor. The oxygen uptake rate (OUR) of the fermentation process was analyzed by a process mass spectrometer (FWQ-1, Biology Institute of Shandong Academy of Sciences, Shandong Province, China).

Extraction and quantification of CoQ10 Currently, there is no method for the measurement of CoQ10 directly as it is an intracellular product (26). To extract CoQ10 from the 72-h-cultured broth, a 1 mL aliquot was sampled from each well of the 24-well plate and placed into a 15 mL centrifuge tube containing 9 mL ethanol. The tubes were then placed into an ultrasonic ice water bath for 1 h. The ultrasonic power was 1000 W. Next, the sonicated solution was filtered through a 0.22 μm filter, and 20 μL of filtered product was injected into a high-performance liquid chromatography (HPLC) column for CoQ10 quantification. The HPLC system (LC20A; Shimadzu, Kyoto, Japan) was equipped with a C-18 reverse-phase column (Inertsil ODS-SP, 5 μm, 4.6 × 150 mm, Shimadzu) and an SPD-M20A Photodiode Array Detector. The CoQ10 standard was purchased from U.S. Pharmacopeia (Rockville, MD, USA). A mixture of methanol:ethanol (1:1, v/v) was used as a mobile phase with a flow rate 1 mL/min. The absorbance was detected at 275 nm at 30°C.

Detection of mutants' genetic stability Genetic stability is a crucial parameter in industrial application and a reliability indicator of the mutation method. In this study, the mutant strain was first cultured on plates with a solid medium at 32°C for 6 days. Then, several colonies were streaked to new plates with the solid medium for the next 6-day cultivation as a subculture. Each selected mutant was subcultured for 15 times. Each subculture was cultured in a 24-well plate to evaluate the level of CoQ10 production.

RESULTS

Optimization of ARTP exposure time for effective mutagenesis

The active species in the ARTP plasma jet region caused the gene alteration (27). The effect of operation for the microbial mortality and mutation efficiency is affected by the dosage of the plasma jet. In the mutation system, the ARTP input power determined the plasma dosage, the distance between the metal sample plate and plasma generator and the treatment time (28). In this study, the power input was set at 120 W, and the

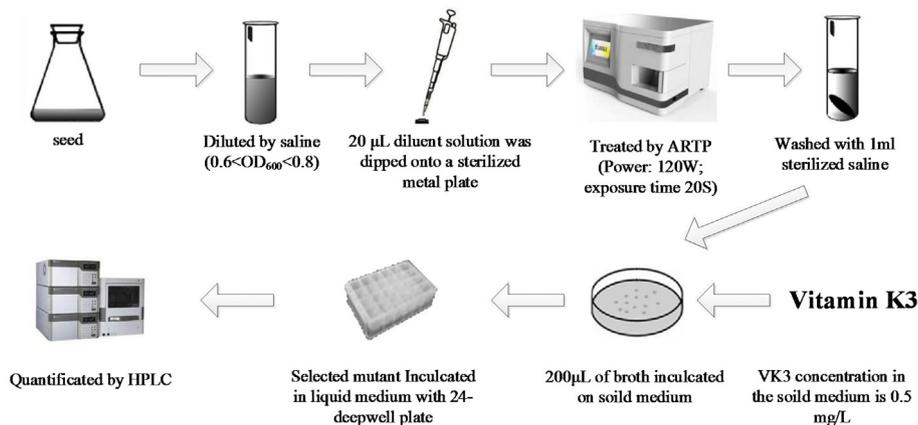


FIG. 1. A schematic diagram of the ARTP breeding for *R. sphaeroides*.

distance between the metal sample plate and plasma generator is around 2 mm (22), so the treatment duration (exposure time) was the primary parameter that was evaluated.

As the first step of mutation, the DNA damages caused by ARTP would display a complex response like the SOS response in the microorganisms (21,29–31). Previous studies showed the longer expose time, the higher level of the DNA damages can be caused by ARTP treatment (21), which would lead higher mutation rate. Meanwhile, the expose time is proportional to the mortality rate. However, too high mutation rate would lead few strains for screening. To identify an optimized exposure time for ARTP treatment, a series of time including 5 s, 10 s, 20 s, 30 s, 60 s, and 90 s were tested, the corresponding number of cells after ARTP treatment was around 1.1×10^7 , 4.0×10^6 , 8.4×10^5 , 4.2×10^5 , 4.0×10^4 , 1.1×10^3 , whereas the number before treatment was $\sim 2.0 \times 10^7$. Mortality rates of various exposure times are shown in Fig. 2. When *R. sphaeroides* were treated for 20 s, the mortality rate was 95.8%. In this study, setting the mortality rate at 95.8% is considered as a suitable rate for mutagenesis.

Determination of optimal VK3 concentration for mutation screening

VK3 was used as a structural analog of CoQ10 in this study. μ Culture (200 μ L) was inoculated on solid medium plates with different concentrations of VK3 (i.e., 0.1 g/L, 0.3 g/L, 0.5 g/L, 0.7 g/L and 0.9 g/L). The plates were incubated at 32°C for 6 days.

Average colony number on each solid medium plate with different concentrations of VK3 is shown in Table 1. When 0.1 g/L VK3 was added to the solid medium, the average number of colonies on the plate was 258, which was close to the control. With the increase of the VK3 concentration, the number of colonies was gradually decreased. When the concentration reached 0.7 g/L, a small number of colonies was grown (Table 1). The presence of 0.9 g/L of VK3 in the solid medium completely stopped the bacterial growth, suggesting VK3 was an effective inhibitor. In this study, the concentration of 0.5 g/L in the solid medium was chosen as the most suitable one for mutagenesis detection.

Screening of mutants A total of ~ 2000 mutant strains were screened. As a result, 88 mutant strains were identified with CoQ10 production higher than that of the control strain by more than 5% (data not shown). Among them, 23 mutant strains had CoQ10 production higher than that of the control strain by more than 10% (data not shown), and three strains increased CoQ10 production by more than 15% (Table 2). Among the three strains, the mutant strain 5-60 showed the highest CoQ10 production after 15 generations and were selected for the second round of ARTP mutation.

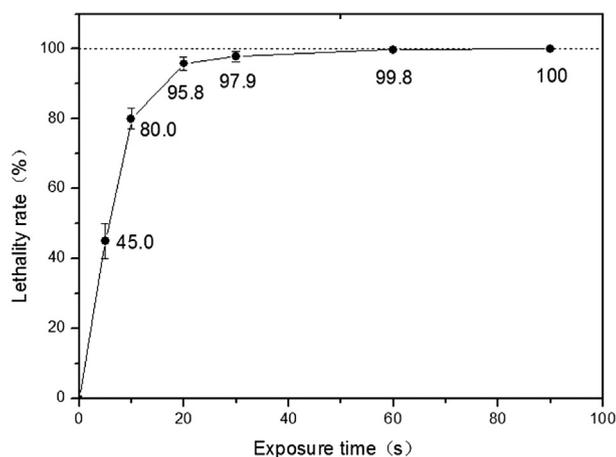


FIG. 2. The mortality rate at different exposure time (n = 5).

TABLE 1. The number of colonies grown on a 90 mm solid medium plate containing VK3 (n = 5).

Concentration of VK3 (g/L)	0 Without DMF	0 With DMF	0.1	0.3	0.5	0.7	0.9
Number of colonies	265 \pm 17	262 \pm 13	258 \pm 15	105 \pm 8	27 \pm 5	3 \pm 2	0 \pm 1

When 0.1 mg/L VK3 was added to the solid medium, the average number of colonies on the plate was 258, which was close to the control. With the increase of the VK3 concentration, the number of colonies was gradually decreased. When the concentration reached 0.7 g/L, a small number of colonies was grown. The presence of 0.9 g/L of VK3 in the solid medium completely stopped the bacterial growth.

TABLE 2. CoQ10 productivity of the parental and mutant strains during generations (mg/L, n = 5).

Strain	Generation					
	1	3	6	9	12	15
Parent	265 \pm 10	273 \pm 9	269 \pm 12	275 \pm 8	267 \pm 13	271 \pm 9
8-29	311 \pm 9	318 \pm 10	312 \pm 13	329 \pm 14	308 \pm 12	316 \pm 8
6-2	307 \pm 11	316 \pm 8	310 \pm 8	324 \pm 10	310 \pm 12	320 \pm 10
5-60	309 \pm 8	320 \pm 12	316 \pm 12	318 \pm 11	307 \pm 9	322 \pm 14

The CoQ10 production for all mutant strains was $\sim 15\%$ higher than that for the parental strain, and this level stayed stable during the 15 generations.

Second round of mutation and screening Using the mutant strain 5-60 as a new parent strain, a second round of ARTP mutation was further performed. Similarly, a total of ~ 1000 mutant strains were screened. Unfortunately, most of the mutant strains didn't show significant increase on CoQ10 production than 5-60 (less than 3%, data not shown). Nevertheless, a mutant strain, AR01 exhibited $\sim 7\%$ increase on Q10 production than that of 5-60 after fifteen generations, which was $\sim 22\%$ higher than the original parent strain Shenzhou6 (Table 3). Therefore, AR01 was selected for the further fermentation cultivation to measure the CoQ10 content.

Fermentation of the mutants and the CoQ10 content measurement

To finally evaluate the productivity for CoQ10 of AR01 in the large-scale production, fermentation cultivation was performed for AR01 and the original parent strain Shenzhou6. During the 100 h cultivation, the differences in dissolved oxygen (DO), oxygen uptake rate (OUR) and the CoQ10 production between AR01 and the parent strain were measured every 5 h. As shown in Fig. 3A and B, OUR of AR01 was faster than the parent. Consistently, production of CoQ10 in the AR01 stain was higher than that of the parent (Fig. 3C). Notably, 590 mg/L CoQ10 was obtained for AR01 after 100 h fermentation, which was $\sim 25.5\%$ higher than that of the original parent strain, suggesting the superiority of AR01 in CoQ10 production that ARTP was quite a feasible strategy for mutation feeding.

Genetic stability validation To validate the genetic stability of AR01 for CoQ10 production, the productivity of AR01 was consecutively measured during the continuous passages. Notably, the productivity of AR01 was quite stable during the long-term passages (3 months). The average level of CoQ10 production in

TABLE 3. CoQ10 productivity of the parental and mutant strains after two rounds of ARTP treatment during generations (mg/L, n = 5).

Strain	Generation					
	1	3	6	9	12	15
Parent	265 \pm 6	273 \pm 10	271 \pm 11	261 \pm 8	266 \pm 10	270 \pm 12
5-60	308 \pm 9	314 \pm 9	315 \pm 9	308 \pm 8	311 \pm 10	311 \pm 9
AR01	325 \pm 6	335 \pm 9	340 \pm 8	334 \pm 12	328 \pm 8	330 \pm 9

The CoQ10 production for AR01 was $\sim 22\%$ higher than that for the parental strain, and this level stayed stable during the 15 generations.

AR01 is around 580–600 mg/L, which is higher than the parent strain whose fermentation level is around 470–490 mg/L, indicating its stability and value for industrial production (Fig. 4).

DISCUSSION

CoQ10 is composed of a quinone ring and trans-conformation of a hydrophobic side chain of polyisoprene. Mutation breeding technology is still the most effective method to improve microbial production of CoQ10. The acting force of plasma jet affecting naked DNA is the active chemical species (27). ARTP caused greater DNA damage and higher mutation rate than traditional mutation methods such as UV, 4-nitroquinoline-1-oxide and NTG treatment (21). Consistently, the article demonstrated the feasibility of ARTP treatment in generating mutants with improved CoQ10 productivity. Moreover, VK3 is a structural analog of CoQ10 and was demonstrated effective in mutant screening in this study. VK3 was selected as an inhibitor in the selection of mutants due to its high efficiency and safety in comparison with other inhibitors, such as benzoic acid and sodium azide (32).

CoQ10 is an intracellular product of bacterial organisms that cannot be detected directly (26). Thus, CoQ10 was extracted from the fermentation broth. We used 24-well plates in this study instead of fermentation tank or flask for bacteria incubation, which greatly improved the screening efficiency. Three mutants, 8-29, 6-2, and 5-60, with more than 15% productivity increase in comparison with the parental strain Shenzhou6, were identified in the first round and AR01 with more than 22% productivity increase was obtained in the second round based on the strain 5-60. The genetic stability of the mutants was maintained after long-term subcultures. During subcultures, the yield of CoQ10 in each generation was higher for mutants than it for the parent, demonstrating the high reliability of the mutation method with ARTP.

In this study, using VK3 as a structural analog for selection, ARTP treatment was applied for the first time to generate *R. sphaeroides* mutants with higher CoQ10 production. After two rounds of screening, a mutant AR01 with higher production of CoQ10 obtained which was ~25.5% higher than that of the original parent strain. Meanwhile, genomic and metabolic changes in AR01 compared to the parent strain responsible for increasing CoQ10 production is worthy of elucidation in the future work as it can

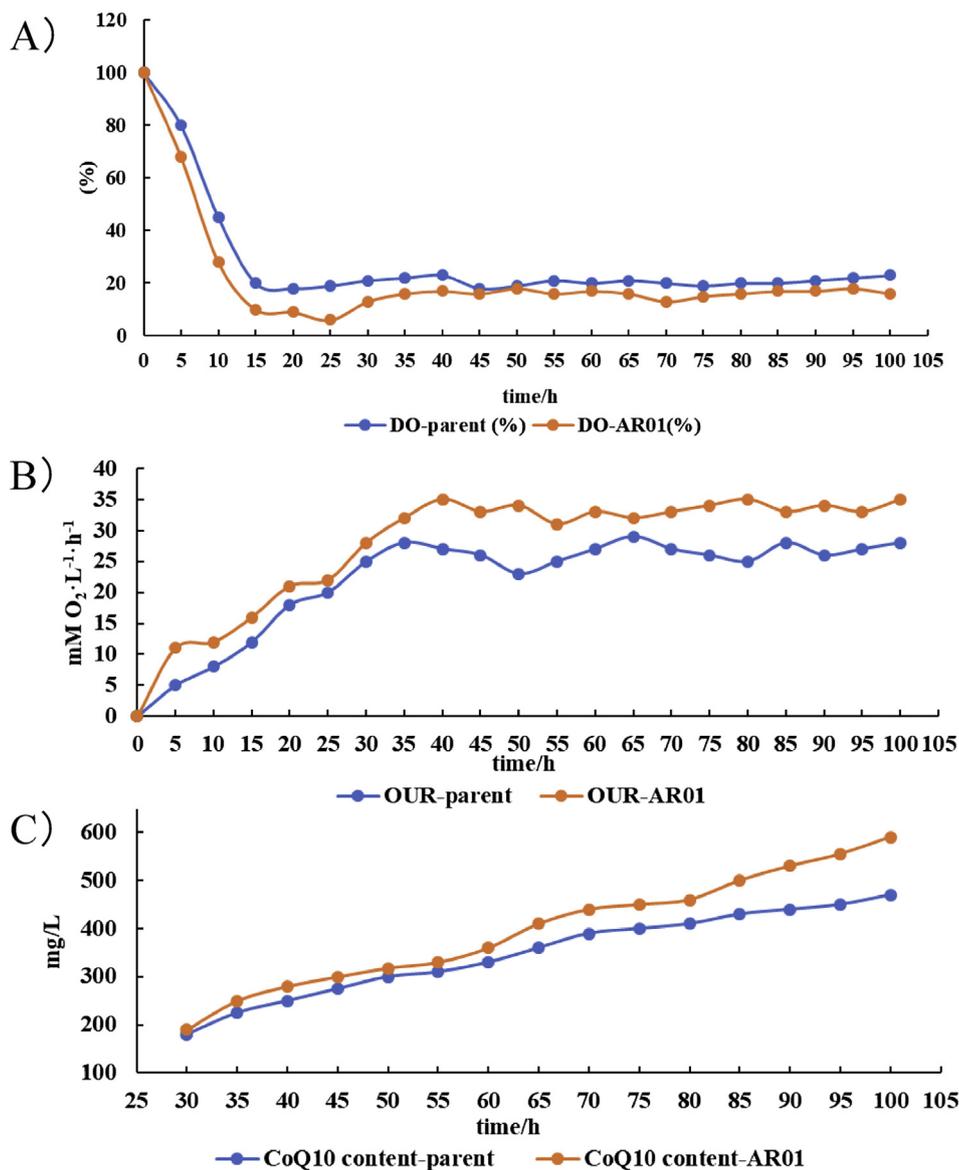


FIG. 3. The differences in dissolved oxygen (DO) (A), oxygen uptake rate (OUR) (B) and the CoQ10 production (C) between AR01 and the parent strain. During the fermentation process, DO and OUR was measured every 5 h from 0 h. Samples for CoQ10 quantitation was collected every 5 h from 30 h.

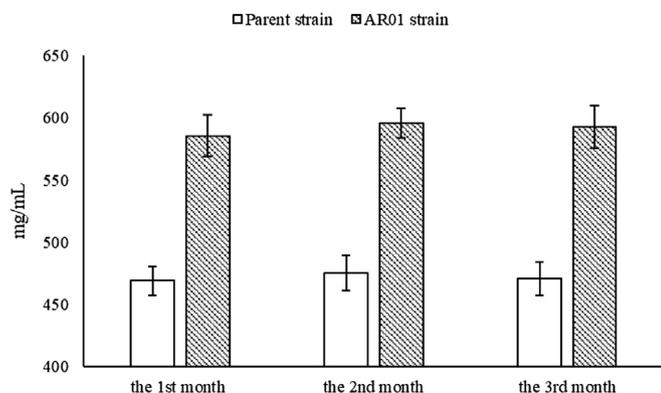


FIG. 4. The average level of CoQ10 fermentation of the parent and AR01 strain for 3 months ($n = 5$).

provide useful information for the further strain optimization. Nevertheless, the results in this study demonstrated ARTP can be a feasible strategy for mutation feeding.

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