Increasing CoQ_{10} Production by *Rhodopseudomonas palustris* J001 Using a Two-Stage Fermentation Process

Shi-yun Jiang[a,b,*] and Long-jiang Yu[a,*]

[a] Institute of Resource Biology and Biotechnology, College of Life Science and Technology, Huazhong University of Science and Technology, Wuhan 430074, P. R. China.
Fax: +86 27 87 79 22 65. E-mail: jiangshiyun@126.com or yulongjiang@hust.edu.cn
[b] Department of Biotechnology, Guangxi University of Technology, Liuzhou 545006, P. R. China

* Authors for correspondence and reprint requests

Z. Naturforsch. **63c**, 884–888 (2008); received April 21/July 2, 2008

CoQ_{10} is used not only as a medicine but also as a food supplement due to its various physiological activities. The production of CoQ_{10} by microbes is a successful approach for generating large amounts of this natural product. The effects of dissolved oxygen (DO) contents and the two-stage fermentation process on cell growth and CoQ_{10} production by *Rhodopseudomonas palustris* J001 were investigated. The optimal DO contents for cell growth and CoQ_{10} production were 45% and 15%, respectively. A two-stage fermentation process, which consists of a 1st stage with 45% DO, a 2nd stage with 15% DO and a synchronous feeding of 2.0% NaAc at the switching time (42 h after inoculation), has proven to be the optimum fermentation process for the production of CoQ_{10}. The maximum biomass, CoQ_{10} production and CoQ_{10} production rate were 1.31 g l^{-1}, 89.1 mg l^{-1}, and 1.142 mg l^{-1} h^{-1}, respectively, increased by 28%, 585% and 426% as compared to the one-stage batch production with 45% DO. The DO level was the major factor to increase the CoQ_{10} production by the two-stage process.

Key words: CoQ_{10}, *Rhodopseudomonas palustris*, Two-Stage Process, Dissolved Oxygen

Introduction

CoQ_{10} (*i.e.* coenzyme Q_{10} or ubiquinone-10) is an electron transporter in the respiratory chains of prokaryotes and eukaryotes and functions as an effective intracellular antioxidant, redox control of cell signaling and gene expression (Crane, 2001; Mikael *et al*., 2004). It is involved in a variety of physiological activities (*e.g.* boosting energy, enhancing the immune system, and acting as an antioxidant to protect low-density lipoprotein from lipid peroxidation by scavenging peroxyl radicals and reducing α-tocopheryl radicals) and is used in the treatment of diseases such as hypertension, brain vascular injury, anemia, muscle dystrophy and alveolar pyorrhea (Crane, 2001; Mikael *et al*., 2004; Sasaki *et al*., 2005). Recently, CoQ_{10} is used not only as a medicine but also as a food supplement because of its various physiological activities. There are three means of commercial production of CoQ_{10}, extraction from plant and animal tissues, chemical synthesis and microbial fermentation (Eern and Keinan, 1988; Sakato *et al*., 1992; Park *et al*., 2005; Sasaki *et al*., 2005; Jiang and Yu, 2007). Fermentation methods have proven to be a successful approach for generating large amounts of this natural product.

In previous studies the contents of CoQ_{10} in microbial strains have been investigated. It has been found that photosynthetic bacteria contain the highest CoQ_{10} concentrations in nature (Carr and Exell, 1965). Recently, some studies on the production of CoQ_{10} by microorganisms have focused on the development of potent strains by conventional mutagenesis and metabolic engineering (Okada *et al*., 1998; Yoshida *et al*., 1998; Park *et al*., 2005; Kim *et al*., 2006; Zahiri *et al*., 2006; Jiang and Yu, 2007). It was reported that a green mutant (carotenoid-deficient mutant, Co-22-11) derived from *Rhodopseudomonas sphaeroides* KY-4113 produced 350 mg/l of CoQ_{10} under culturing conditions with a limited supply of air, the CoQ_{10} content being 8.7 mg/g dry cell. In this case, the amount and content were 2.8- and 3.6-times larger than those given by the wild-type strain, respectively (Yoshida *et al*., 1998). Optimization of the media and culture conditions is also one of the most effective strategies to maximize the production of CoQ_{10} by fermentation (Sakato *et al*., 1992;

Yoshida *et al.*, 1998; Wu *et al.*, 2003; Yen and Chiu, 2007; Zhang *et al.*, 2007). For example, the optimal oxidation-reduction potential suggested by Sakato *et al.* (1992) was −150 mV for cell growth and −200 mV for CoQ_{10} accumulation in cells. The cultivation of *R. sphaeroides* under the situation of aerobic-dark at 0% dissolved oxygen (DO) was suggested to be applied in the scale-up CoQ_{10} production (Yen and Chiu, 2007).

In the present study, the effects of the DO concentration and two-stage fermentation process on cell growth and CoQ_{10} production by *Rhodopseudomonas palustris* J001 were investigated. Based on the results obtained, an optimum *R. palustris* J001 fermentation method for the scale-up CoQ_{10} production is suggested.

Materials and Methods

Microorganism and cultivation

Rhodopseudomonas palustris J001 was isolated from mutants of *R. palustris* J (GenBank accession number EU531568 for 16S rRNA gene of the strain) produced by chemical inducers. *R. palustris* J was originally obtained from the environment. The seed medium and fermentation medium contained $NaCH_3COO \cdot 3H_2O$ (4.98 g), NaCl (1.00 g), $(NH_4)_2SO_4$ (0.30 g), KH_2PO_4 (0.50 g), $K_2HPO_4 \cdot 3H_2O$ (0.40 g), $MgSO_4 \cdot 7H_2O$ (0.41 g), $CaCl_2$ (50 mg), $MnSO_4 \cdot H_2O$ (2.8 mg), $FeSO_4 \cdot 7H_2O$ (5 mg), yeast extract (0.5 g), peptone (0.5 g) and deionized H_2O (1000 ml). The pH value was adjusted to 7.0 by addition of NaOH and HCl.

The strain grown on slants at 30 °C and 1500 lux for 48 h was inoculated into an 100-ml Erlenmeyer flask, containing 50 ml of seed medium, and cultivated at 30 °C and 2.196 W/m^2 illumination on a rotary shaker at 100 rev min^{-1} for 36 h. This seed culture was then transferred into a 5-l lab-scale fermenter (Biostat B5, Braun, Melsungen, Germany) containing 3 l of fermentation medium. The culture temperature was maintained at 30 °C and the pH value controlled at 7.0 ± 0.1 by adding 1 M NaOH or 1 M HCl. The dissolved oxygen (DO) content (% of air saturation) was maintained at 5 designed levels (0%, 15%, 30%, 45% and 60%) controlled by adjusting the agitation speed (150–350 rev min^{-1}) and aeration rate (0.1–1.0 v/v/min). The fermenter surface was illuminated using halogen lamps (Moritex LM-50, Japan) positioned at a distance of 15 cm from the surface, and the illumination was in the range of 1.464–2.196 W/m^2. The two-stage fermentation process consisted of one DO stage which favoured the growth of biomass and another DO stage which favoured the production of CoQ_{10}. The switching time from the 1st DO stage to the 2nd DO stage was located at the end of the exponential phase (at the 42nd h after inoculation) and synchronously a proper amount of NaAc was fed.

Analytical methods

The amount of biomass was determined by measuring the optical density at 660 nm with a spectrophotometer (UV-2100, Shimadzu), and the dry cell weight (DCW) was calculated according to the conversion equation: 1 OD_{660} = 0.181 g DCW l^{-1} or g l^{-1}.

The CoQ_{10} content [mg CoQ_{10} (g dried biomass)$^{-1}$ or mg g^{-1}] was assayed by high performance liquid chromatography (HPLC) (Waters 510, USA). To measure the CoQ_{10} content, microbial cells were broken through treatment with acetone, and CoQ_{10} was extracted with hexane, and then the organic phase was passed through 500 mg silica gel with a solid-phase extraction cartridge (pre-activated by hexane). The cartridge was washed with 6 ml hexane; a vacuum was applied on the cartridge. The cartridge was dried in a speed vacuum concentrator and then eluted with 4 ml methanol. The elution solution was passed through a C_{18} solid-phase extraction cartridge (pre-activated by 2 ml methanol and balanced with 2 ml water). The C_{18} solid-phase extraction cartridge was washed with 1 ml methanol and a vacuum was applied on it. The cartridge was eluted with 3 ml isopropanol, and the elution solution was evaporated to dryness in a speed vacuum concentrator. The dry residue (CoQ_{10}) was redissolved with 0.3 ml isopropanol, and the CoQ_{10} content of the isopropanol solution was analyzed by HPLC. The HPLC conditions were: a Diamonsil™ (Dikma, Beijing, China) ODS_2 column (5 μm, 250 mm × 4.6 mm i. d.) as analytical column; isopropanol/methanol (45:55, v/v) at a flow rate of 1 ml min^{-1} as mobile phase; CoQ_{10} as standard and 275 nm as detection wavelength.

The residual CH_3COO^- (Ac^-) concentration (g l^{-1}) in the fermentation broth was assayed by HPLC. The HPLC conditions were: a Diamonsil™ ODS_2 column (5 μm, 250 mm × 4.6 mm i. d.) as analytical column; 2.50% $NH_4H_2PO_4$ (pH 2.50) at a flow rate of 1 ml min^{-1} as mobile phase; acetic acid as standard and 200 nm as detection wavelength.

Results and Discussion

Effect of DO content on cell growth and CoQ_{10} production

The effects of different DO levels on cell growth are shown in Fig. 1. A higher level of DO could be favourable for cell growth when the DO content was controlled at a level lower than 45%. The higher the DO level, the shorter is the lag phase and the higher is the specific growth rate of the exponential phase. A value of 1.02 g l^{-1} biomass was obtained at 45% DO content, which was the highest biomass level among all DO contents.

The effects of different DO levels on CoQ_{10} production (mg CoQ_{10} l^{-1} or mg l^{-1}) are shown in Fig. 2. The higher the DO level, the lower is the CoQ_{10} production when the DO content was changed from 15% to 60%. A yield of 51.1 mg l^{-1} CoQ_{10} was obtained at 15% DO, which was the highest CoQ_{10} production among all DO levels. Furthermore, the formation rate of CoQ_{10} in cells was not synchronous with the rate of cell growth. For example, the time for maximal CoQ_{10} production at 45% DO content was compared 10 h later with the maximal biomass. This result indicated that the effect of the DO level on cell growth was different from that on CoQ_{10} production. Apparently the DO content has to be controlled at different levels and different fermentation periods in order to maximize the production of CoQ_{10}, namely 45% DO during the growth phase with subsequent 15% DO.

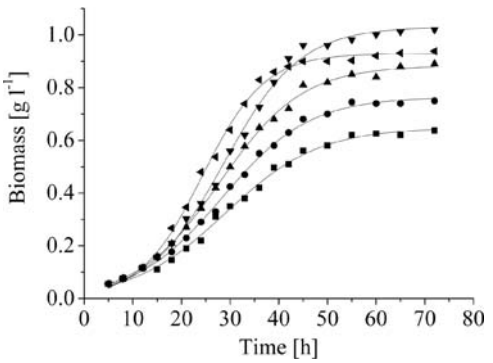

Fig. 1. Effect of the DO content on cell growth of *Rhodopseudomonas palustris* J001. ■, 0% DO; ●, 15% DO; ▲, 30% DO; ▼, 45% DO; ◄, 60% DO.

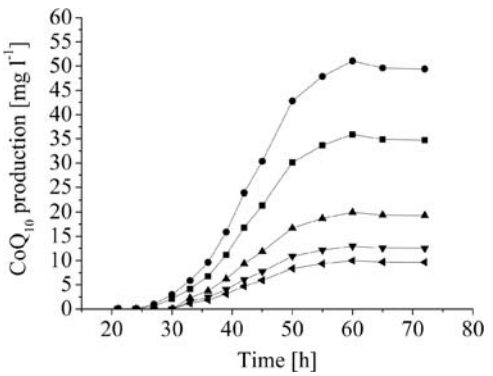

Fig. 2. Effect of the DO content on production of CoQ_{10} by *Rhodopseudomonas palustris* J001. ■, 0% DO; ●, 15% DO; ▲, 30% DO; ▼, 45% DO; ◄, 60% DO.

Effect of DO content on NaAc consumption

As the optimal DO contents for cell growth and CoQ_{10} production were 45% and 15%, respectively, the effects of these DO levels on NaAc consumption by *Rhodopseudomonas palustris* J001 were investigated (Fig. 3). The NaAc consumption matched the cell growth curve very well when Fig. 3 was compared with Fig. 1. The rate of NaAc consumption at 45% DO was higher than that at 15% DO. Both rates of the NaAc consumption became lower when the cultures were in their stable phase. However, the residual Ac^- concentration (g l^{-1}) in the fermentation broth at 45% DO was very low in the stable phase starting 45 h after inoculation, indicating that a low substrate NaAc concentration limited the cell growth at this DO level, and its biomass could be further increased by feeding appropriate amounts of NaAc to the culture on time.

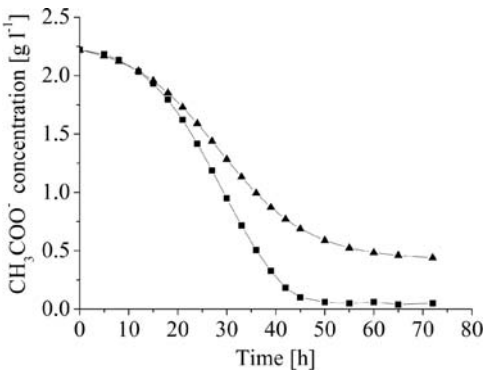

Fig. 3. NaAc consumption by *Rhodopseudomonas palustris* J001 at 15% and 45% DO. ▲, 15% DO; ■, 45% DO.

Effect of two-stage fermentation process on cell growth and CoQ_{10} production

As the optimum DO levels for cell growth and CoQ_{10} production were 45% and 15%, respectively, and low substrate NaAc concentration limited the cell growth at 45% DO, the two-stage fermentation process was investigated in order to maximize the production of CoQ_{10} by the strain. The 1st stage of the process was at 45% DO, which favoured cell growth, and in the 2nd stage the DO content was switched from 45% to 15% and synchronously different amounts of NaAc were fed, which mainly favoured the CoQ_{10} production. The switching time from the 1st DO stage to the 2nd DO stage was founded at the end of the exponential phase (42 h after inoculation). The effects of the two-stage fermentation process on cell growth and CoQ_{10} biosynthesis are documented by in Table I. The result showed that the optimal feeding amounts of NaAc for cell growth and CoQ_{10} production were the same (2.0 g l^{-1}). The maximum biomass was 1.31 g l^{-1} or 28% higher than that of the one-stage batch process with 45% DO, the maximum CoQ_{10} production was 89.1 mg l^{-1} or 585% higher than that of the one-stage batch process with 45% DO, and the CoQ_{10} overproduction rate was 1.142 mg l^{-1} h^{-1} or 426% higher than that of the one-stage batch process with 45% DO. Both the CoQ_{10} production and the CoQ_{10} overproduction rate of the two-stage process without feeding NaAc were increased by 365% but the biomass amount was similar compared to the one-stage batch process with 45% DO. These results indicated that a proper DO level is the major factor to increase the CoQ_{10} production by the two-stage process. A comparison of cell growth and CoQ_{10} production between the two-stage process with feeding of 2.0% NaAc and the one-stage batch process is shown in Fig. 4. The starting time of the stable phase of cell growth and CoQ_{10} production by the two-stage process was delayed by 15 h and 18 h, respectively, as compared to the one-stage batch process but the CoQ_{10} production rate of the two-stage process did not decrease.

The study of DO effects on CoQ_{10} production in *Paracoccus denitrificans* led to the conclusion that a lower content of oxygen in the inlet gas (as

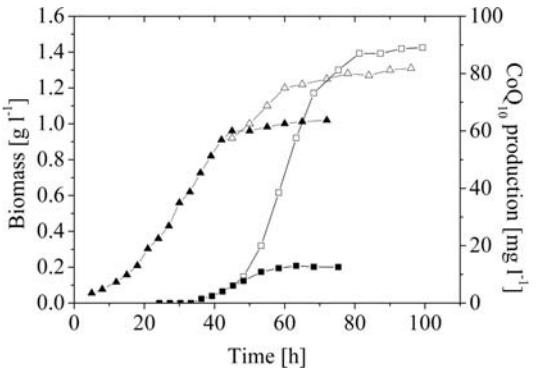

Fig. 4. Comparison of cell growth and CoQ_{10} production by *Rhodopseudomonas palustris* J001 between the two-stage fermentation process and the one-stage batch fermentation process. Biomass: ▲, one-stage batch process (45% DO content); △, two-stage process (feeding of 2.0% NaAc and synchronously switching the DO content from 45% to 15% at the end of the exponential phase). CoQ_{10} production: ■, one-stage batch process; □, two-stage process.

Table I. Effects of the two-stage fermentation process on cell growth and CoQ_{10} production by *Rhodopseudomonas palustris* J001.

Run	CK_1	CK_2	0	1	2	3	4	5
DO content (%) in 1st stage	45	15	45	45	45	45	45	45
DO content (%) in 2nd stage	45	15	15	15	15	15	15	15
Feeding amounts of NaAc [g l^{-1}]	0	0	0	0.5	1.0	1.5	2.0	2.5
Maximum biomass [g l^{-1}]	1.02	0.75	1.01	1.11	1.19	1.25	1.31	1.29
(%)	100	73.5	99.0	109	117	123	128	126
Maximum CoQ_{10} production [mg l^{-1}]	13.0	51.1	60.5	73.1	80.9	84.5	89.1	88.1
(%)	100	393	465	562	622	650	685	678
CoQ_{10} content [mg g^{-1}]	12.7	69.0	59.9	65.9	68.0	67.6	68.0	68.3
(%)	100	543	472	519	535	532	535	538
CoQ_{10} overproduction rate [mg l^{-1} h^{-1}]	0.217	0.852	1.008	1.015	1.037	1.083	1.142	1.129
(%)	100	393	465	468	478	499	526	520

CK_1, in control 1, one-stage batch process with 45% DO; CK_2, in control 2, one-stage batch process with 15% DO; 0–5, two-stage fermentation process, feeding different amounts of NaAc and synchronously switching DO content from 45% to 15% at the end of the exponential phase.

low as 2.5%) would yield a higher CoQ_{10} production (Kaplan *et al*., 1993). Sakato *et al.* (1992) suggested that the CoQ_{10} production was enhanced by microaerobic-dark cultivation of *Rhodopseudomonas sphaeroides,* which was the anaerobic culture with an almost nil level of dissolved oxygen in the culture broth. Wu *et al*. (2003) investigated the DO effect (in the range of 20–50% of saturated DO level) on the CoQ_{10} concentration in a 7-l fermenter, and found a maximum CoQ_{10} concentration of 32.1 mg l^{-1} obtained at 40% DO. Yen and Chiu (2007) suggested that the cultivation of *R. sphaeroides* under the aerobic-dark condition at 0% DO could be applied in the scale-up CoQ_{10} production. These differences for an optimal DO level suggested in the literature for CoQ_{10} production by microbes (from a value close to 0% DO to a 40% DO level) probably resulted from the different microorganisms used. However, we can conclude that a proper DO level is important to increase the CoQ_{10} production by microbes. The two-stage process with *Rhodopseudomonas palustris* J001, which consisted of 45% DO for the 1st stage and 15% DO for the 2nd stage together with a synchronous feeding of NaAc, is suggested to be applied in the scale-up CoQ_{10} production.

Carr N. G. and Exell G. (1965), Ubiquinone concentrations in Athiorhodaceae grown under various environmental conditions. Biochem. J. **96**, 688–692.

Crane F. L. (2001), Biochemical functions of coenzyme Q_{10}. J. Am. Coll. Nutr. **20**, 591–598.

Eern D. and Keinan E. (1988), Total of linear polyprenoids. 3. Synthesis of ubiquinones via palladium-catalyzed oligomerization of monoterpene monomers. J. Am. Chem. Soc. **110**, 4356–4362.

Jiang S.-y. and Yu L.-j. (2007), The pathway for CoQ biosynthesis in microorganisms and the recent progress in the genetic improvement of microbial strains for CoQ_{10} production with the aid of molecular biological methods. China Biotechnol. **27**, 103–112.

Kaplan P., Kucera I., and Dadak V. (1993), Effect of oxygen on ubiquinone-10 production by *Paracoccus denitrificans.* Biotechnol. Lett. **15**, 1001–1002.

Kim S. J., Kim M. D., Choi J. H., Kim S. Y., Ryu Y. W., and Seo J. H. (2006), Amplification of 1-deoxy-D-xylulose 5-phosphate (DXP) synthase level increases coenzyme Q_{10} production in recombinant *Escherichia coli*. Appl. Microbiol. Biotechnol. **72**, 982–985.

Mikael T., Jerker O., and Gustav D. (2004), Metabolism and function of coenzyme Q. Biochim. Biophys. Acta **1660**, 171–199.

Okada K., Kainou T., Tanaka K., Nakagawa T., Matsuda H., and Kawamukai M. (1998), Molecular cloning and mutational analysis of the ddsA gene encoding decaprenyl diphosphate synthase from *Gluconobacter suboxydans.* Eur. J. Biochem. **255**, 52–59.

Park Y. C., Kim S. J., Choi J. H., Lee W. H., Park K. M., Kawamukai M., Ryu Y. W., and Seo J. H. (2005), Batch and fed-batch production of coenzyme Q_{10} in recombinant *Escherichia coli* containing the decaprenyl diphosphate synthase gene from *Gluconobacter suboxydans.* Appl. Microbiol. Biotechnol. **67**, 192–196.

Sakato K., Tanaka H., Shibata S., and Kuratsu Y. (1992), Agitation-aeration studies on coenzyme Q_{10} production using *Rhodopseudomonas sphaeroides.* Biotechnol. Appl. Biochem. **16**, 19–28.

Sasaki K., Watanabe M., Sude Y., Ishizuka A., and Noparatnaraporn N. (2005), Applications of photosynthetic bacteria for medical fields. J. Biosci. Bioeng. (Japan) **100**, 481–488.

Wu Z. F., Du G. C., and Chen J. (2003), Effects of dissolved oxygen concentration and DO-stat feeding strategy on CoQ_{10} production with *Rhizobium radiobacter*. World J. Microbiol. Biotechnol. **19**, 925–928.

Yen H. and Chiu C. (2007), The influences of aerobic-dark and anaerobic-light cultivation on CoQ_{10} production by *Rhodobacter sphaeroides* in the submerged fermenter. Enzyme Microbiol. Technol. **41**, 600–604.

Yoshida H., Kotani Y., Ochiai K., and Araki K. (1998), Production of ubiquinone-10 using bacteria. J. Gen. Appl. Microbiol. **44**, 19–26.

Zahiri H. S., Yoon S. H., Keasling J. D., Lee S. H., Kim S. W., Yoon S. C., and Shin Y. C. (2006), Coenzyme Q_{10} production in recombinant *Escherichia coli* strains engineered with a heterologous decaprenyl diphosphate synthase gene and foreign mevalonate pathway. Metab. Eng. **8**, 406–416.

Zhang D., Shrestha B., Li Z., and Tan T. (2007), Ubiquinone-10 production using *Agrobacterium tumefaciens dps* gene in *Escherichia coli* by coexpression system. Mol. Biotechnol. **35**, 1–14.