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Notes

Effect of Aeration-Agitation on Coenzyme Q_{10} Production Using *Rhodobacter sphaeroides*

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With the aim of increasing the CoQ_{10} production in mass culture, the effect of aeration-agitation on the CoQ_{10} production using *Rhodobactor sphaeroides* was investigated in a 1-L bioreactor. The maximum CoQ_{10} production was 1.69 mg/g of dry cell weight under conditions of 50 Lux, 30°C, 300 rpm, and 5-vvm aeration. The CoQ_{10} production was improved to produce 2.91 mg/g of dry cell weight under reduced conditions of agitation speed (200 rpm) and aeration rate (0.2 vvm). When *R. sphaeroides* was cultivated under more reduced DO levels during the exponential phase of the cell, the CoQ_{10} production yield of 3.88-mg/g dry cell weight was the maximum obtained. Therefore, poorer conditions of aeration-agitation resulted in higher production of CoQ_{10}, and thus DO content was a crucial factor in the production of CoQ_{10}. Accordingly, it was necessary to control the DO concentration in order to enhance the CoQ_{10} biosynthesis within a large-scale production.

Key words: Aeration, Agitation, *Rhodobacter sphaeroides*, Coenzyme Q_{10}

Introduction

Photosynthetic bacteria can utilize various types of organic matter like carbon and energy substrates, making them common microorganisms in the natural environment (Sasaki et al., 1998). They have been widely applied in the fields of wastewater treatment and bioremediation of sediment mud (Takeno et al., 1999; Nagadomi et al., 2000). Recently, they have also been used in the medical field, since they can produce various types of physiologically active substances such as vitamin B_{12}, ubiquinone (Coenzyme Q_{10}), 5-aminolevulinic acid, porphyrins and RNA (Sasaki et al., 2005; Jeong et al., 2008). Of particular note is the preparation and commercialization of coenzyme Q_{10} (CoQ_{10}) and 5-aminolevulinic acid from these bacteria.

Ubiquinones, which are also referred to as coenzyme Q, are a membrane-bound lipid component. They are common materials found in animals, plants and microorganisms and act as coenzymes involved in various biological reactions. They not only play vital roles as electron carriers in the respiratory chain but also as antioxidants and prooxidants (Ernster and Dallner, 1995; Grant et al., 1997; Wu et al., 2001; James et al., 2004). The number of isoprene units in the prenyl side chain of ubiquinones varies depending on the living organism. CoQ_{10}, 2,3-dimethoxy-5-methyl-benzoquinone with a side chain of 10 monosaturated isoprenoid units, is the only ubiquinone homolog found in human organs (Gale et al., 1961). In humans, CoQ_{10} boosts energy, enhances the immune system, and acts as an antioxidant (Ernster and Dallner, 1995) Recently, CoQ_{10} has been widely used for pharmaceuticals, cosmetics, food supplements, etc. because of its various physiological activities (Takahashi et al., 2003; Sasaki et al., 2005; Zhang et al., 2007).

CoQ_{10} can be produced by chemical (Negishi et al., 2002), semi-chemical (Lipshutz et al., 2002) and biological synthetic methods. The biological synthesis of CoQ_{10} is more widely used than the chemical and semi-chemical syntheses. This is because the starting materials used during chemical synthesis of CoQ_{10} differ from those used in microorganisms and humans (Ha et al., 2007). Therefore, the commercial production of biologically synthesized CoQ_{10} from microorganisms has now attracted increasing attention (Choi et al., 2005), such that a genetically engineered

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microorganism synthesizing CoQ_{10} has been constructed (Lee et al., 2004; Park et al., 2005; Sakai et al., 2005). Until now, however, low yields from microbiological production of CoQ_{10} on an industrial scale have resulted in a high cost of CoQ_{10} production (Ha et al., 2007). Despite recent accomplishments in metabolic engineering of *Escherichia coli* cells for CoQ_{10} production, the production levels are not yet competitive with the levels presently produced by isolation or fermentation (Park et al., 2005). To increase the level of CoQ_{10} production in mass culture, environmental conditions must be optimized. In this study, the effect of aeration-agitation on CoQ_{10} production using *R. sphaeroides* was investigated.

Materials and Methods

Microorganism and medium

The microorganism, *R. sphaeroides* (GenBank Accession Number: AM69671) used in this study was isolated from silt of Nakdong River by our laboratory (Jeong et al., 2008). The microorganism was maintained on a solid agar plate which contained (per L): 1 g of malic acid; 2 g of casamino acid; 3 g of yeast extract; 1 mL of vitamin solution; 1 mL of mineral solution; and 15 g of agar. The vitamin solution contained (per L): 0.2 g of nicotinic acid; 0.4 g of thiamine-HCl; 0.2 g of nicotinamide; and 0.008 g of biotin. The mineral solution contained (per L): 3 g of $FeSO_4 \cdot 7H_2O$; 0.01 g of H_3BO_3; 0.01 g of $Na_2MoO_4 \cdot 2H_2O$; 0.02 g of $MnSO_4 \cdot H_2O$; 0.01 g of $CuSO_4 \cdot 5H_2O$; 0.01 g of $ZnSO_4$; and 0.5 g of ethylenediamine tetraacetic acid. The pH of the medium was adjusted to 7.2 before autoclaving, and the medium was sterilized at 121°C for 15 min. The microorganism was regularly checked under a microscope in order to eliminate any possibility of contamination. It was stored on the agar plate at 4°C until used and transferred to a fresh agar plate every two weeks.

Batch culture for CoQ_{10} production

A batch type of reaction was carried out in a 1-L bioreactor using 600 mL of working volume (Marubishi, Japan) for CoQ_{10} production. The seed culture was cultivated in a 250-mL flask under 30°C, 180 rpm and 50 Lux, and 60 mL of the broth culture, in which the cells were grown at the end of the exponential growth phase and were used as inoculums. The bioreactor was operated under different conditions of aeration rate (on-off control, 0.2 and 5 vvm) and agitation speed (200 and 300 rpm), respectively. Other culture conditions were 30°C and 50 Lux, with the pH not being controlled during cultivation. Ten-fold diluted anti-foamer, 'Antifoam 204', was occasionally used when severe foaming occurred. Periodic samples from the bioreactor were taken to measure the concentrations of cell and CoQ_{10}. The real-time measurement of pH and the concentration of dissolved oxygen (DO) were accomplished by Labo Controller (Marubishi, Japan). The dry-cell weight (DCW) of the bacteria was determined by weighing the cell pellet after drying in an oven at 100°C for 12 hrs. The cell pellet was prepared by centrifuging a 20 mL sample of broth culture at 5,000 rpm for 10 min and then by decanting the supernatant after washing twice with distilled water.

Extraction and measurement of CoQ_{10}

CoQ_{10} extracted from the isolated photosynthetic bacterium was analyzed using the method described by Matsumura et al. (1983) and Takahashi et al. (2003) with modifications. Ten grams of cells (wet weight), which were grown until the late-logarithmic phase, were suspended in 70-mL methanol, and the slurry was heated at 55°C for 5 min. Chloroform (140 mL) was added, and the suspension was stirred at 30°C for 20 min and filtered through a filter paper (Whatman No. 1). NaCl solution (0.58%, w/v) was added by one-fifth of the filtrate volume. The filtrate and the NaCl solution added were gently mixed, and allowed to separate into two phases. The lower phase was evaporated and resuspended with ethanol. CoQ_{10} was analyzed by HPLC (Agilent 1200, USA) on a Zorbax Eclipse Plus C18 column (100 mm x 4.6 mm, 5 μm) with ethanol as the mobile phase at a flow rate of 1 mL/min. The CoQ_{10} was quantified by an external standard method, based on the peak area, and detected at 275 nm. The intracellular content of CoQ_{10} was estimated by the relationship between dry-cell weight and the amount of CoQ_{10} in the broth.

Results and Discussion

In a previous study conducted in our lab (Jeong et al., 2008), the microorganism, *R. sphaeroides,* which was also used in the present study, was reported to have a high content of CoQ_{10} (1.55 mg/g dry cell). Among the photosynthetic bacteria, *Rhodopseudomonas*, *Rhodobacter* and *Rhodospirillum* strains have been known to produce CoQ_{10} (Urakami and Yoshida, 1993), but *Rhodobacter sphaeroides* has been preferentially used as the bacterium for production of CoQ_{10} (Gu et al., 2006). *R. sphaeroides* is a facultative microorganism, which can be cultured under many different growth conditions, including photoheterotrophy, photoautotrophy, chemohetero-

trophy, and fermentation (Kokua et al., 2003). Thus, the effect of aeration-agitation on the CoQ_{10} production using *R. sphaeroides* was interesting in the light of mass culture.

Under the conditions of 50 Lux, 30°C, 300 rpm, and 5 vvm aeration, *R. sphaeroides* was cultivated in a 1-L bioreactor without control of pH in order to observe the effect of aeration-agitation on the CoQ_{10} production. The agitation speed (300 rpm) and aeration rate (5 vvm) were the permissible conditions in this bioreactor without exertion of shear stress on the cells. Under the above conditions, the DO level in the bioreactor was reduced to 0.6 mg/L within 6 hr, with the generation of foam, and this recovered up to 90% afterwards (Fig. 1). This may be attributed to the oxygen requirements of *R. sphaeroides*, a facultative microorganism, at the early growth phase, but its metabolism may have switched to fermentation metabolism afterwards (Saunders and Johnes, 1974; Kokua et al., 2003). The pH was steadily increased up to 9 from the beginning, and maintained at an almost constant level after 10 hr. The cell concentration was increased, but reached a stationary phase (with 2.9 mg/mL) after 28 hr of cultivation. The maximum CoQ_{10} production was 1.69 mg/g of dry cell weight, which was obtained after 24 hr. The CoQ_{10} production was reduced afterwards. The CoQ_{10} production was growth-associated, possibly clarified as primary me-tabolite, correlating with reports on a study of *Rhodobacter* sp. by Yamada et al. (1991) and *R. sphaeroides* by Yen and Chiu (2007). The authors re-ported that CoQ_{10} biosynthesis occurred predominantly during the exponential growth phase.

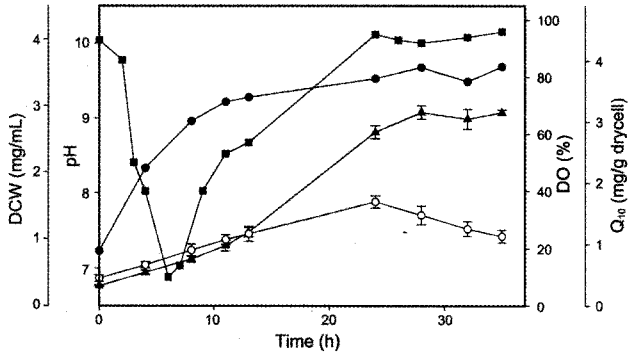

Fig. 1. Profiles of pH (-●-), DO (-■-), dry-cell weight (-▲-) and CoQ_{10} (-○-) in a 1-L bioreactor under 50 Lux, 30°C, 300 rpm, and 5 vvm aeration. Error bars: mean ± S.D. of three replicates.

In Fig. 2, the result of the batch culture under reduced conditions of agitation speed (200 rpm) and aeration rate (0.2 vvm) is shown. The DO level in the bioreactor was reduced to 0.6 mg/L within 3 hr and maintained low for several hours. Then the DO level was steadily increased and recovered up to 90% afterwards. The pH was steadily increased up to 9.28 until the end of the cultivation process. The cellular growth reached a stationary phase after 28 hr of cultivation, with a cell concentration of 3.1 mg/mL. The maximum CoQ_{10} production (2.91 mg/g dry cell) was obtained at 24 hr., which was 1.7 times higher than that obtained at 300 rpm and 5 vvm. Hence, the reduced aeration-agitation therefore resulted in the higher production of CoQ_{10}. This result agreed with those reported in other studies. Kuratu et al. (1984) reported that a limited supply of oxygen was very effective to enhance CoQ_{10} content in the cells of the *Agrobacterium* strain. Urakami and Yoshida (1993) also reported a high yield of CoQ_{10} (2.5 mg/g-cell) under limited supply of oxygen in a culture of *R. sphaeroides*. The CoQ_{10} production was growth-associated, and was reduced after the stationary phase. Therefore, *R. sphaeroides* cells must be harvested at the late-exponential growth phase.

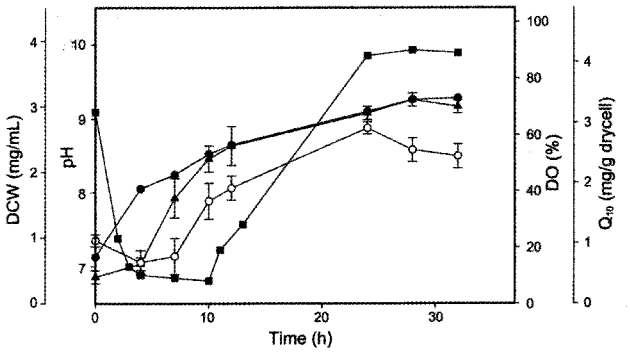

Fig. 2. Profiles of pH (-●-), DO (-■-), dry-cell weight (-▲-) and CoQ_{10} (-○-) in a 1-L bioreactor under 50 Lux, 30°C, 200 rpm, and 0.2 vvm aeration. Error bars: mean ± S.D. of three replicates.

In order to observe the effect of a further reduced aeration rate on the CoQ_{10} production, a batch culture was carried out under the on-off control of DO during the exponential phase of cell, i.e., air was supplied at 0.2 vvm from the beginning of experiment, and DO was controlled at a low level (less than 5%) after the cellular growth entered the exponential growth phase. As shown in Fig. 3, the level of DO in the bioreactor was reduced down to 0.5 mg/L within 3.5 hr, and then DO was maintained at a low level until 24 hr of cultivation. The pH was increased up to 8.8 within 11 hr, and then almost maintained until the end of the cultivation. The maximum cell density (3 mg/mL) was obtained at 24 hr of cultivation, with a maximum

CoQ_{10} production of 3.88-mg/g dry cell weight. Hence, poorer conditions of aeration-agitation resulted in higher production of CoQ_{10}, with no significant reduction of the cell mass. This was in agreement with other studies: Sasaki et al. (1998) reported that CoQ_{10} production was enhanced under microaerobic dark cultivation, which was the aerobic culture with an almost nil level of DO in the culture broth; Wu et al. (2003) found that a maximal CoQ_{10} (1.91 mg/g dry cell weight) was obtained at low DO concentration; Urakami and Yoshida (1993) obtained a high yield of CoQ_{10} (2.5 mg/g-cell) under limited supply of oxygen in the culture of *R. sphaeroides*; Yen and Chiu (2007) suggested that the cultivation of *R. sphaeroides* under the situation of aerobic-dark at 0% DO could be applied in the scale-up CoQ_{10} production.

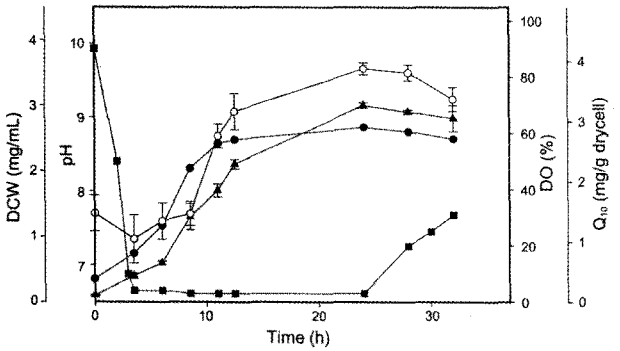

Fig. 3. Profiles of pH (-●-), DO (-■-), dry-cell weight (-▲-) and CoQ_{10} (-○-) in a 1-L bioreactor under 50 Lux, 30°C, 200 rpm, and on-off control. Error bars: mean ± S.D. of three replicates.

Due to the ability of *R. sphaeroides* to grow under aerobic-dark and anaerobic-light conditions, the DO level in the culture medium might be detrimental to the cell growth and CoQ_{10} production (Yen and Chiu, 2007). Therefore, DO content was a crucial factor in the production of CoQ_{10}. Accordingly, it was necessary to control the DO concentration in order to enhance the CoQ_{10} biosynthesis (Wu et al., 2003; Ha et al., 2007).

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