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การผลิต Coenzyme Q10 จากเชื้อสายพันธุ์กลาย *Rhodopseudomonas* sp. S12-13

The Coenzyme Q10 Production from *Rhodopseudomonas* sp. S12-13 Mutant

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Rhodopseudomonas sp. S12-13 เป็นเชื้อสายพันธุ์กลายจากสายพันธุ์พ่อแม่ *Rhodopseudomonas* S12 ที่ได้รับการฉายแสงอัลตร้าไวโอเลตเพื่อชักนำการกลายพันธุ์ สายพันธุ์กลาย S12-13 มีความสามารถในการย่อยสลายแป้งมันสำปะหลัง และให้การสร้าง coenzyme Q10 ปริมาณสูงถึง 148.12±21.74 µg/L เมื่อนำมาเลี้ยงเชื้อในอาหาร AM medium ที่มีแหล่งคาร์บอน และแหล่งไนโตรเจน เป็น 2% แป้งมันสำปะหลัง และ 1% กลูตาเมต ตามลำดับ แต่สายพันธุ์พ่อแม่ S12 ให้การสร้าง coenzyme Q10 เพียง 12.51±1.34 µg/L เมื่อเลี้ยงเชื้อในอาหาร AM medium เชื้อสายพันธุ์กลาย S12-13 ให้การสร้าง coenzyme Q10 สูงสุด เพิ่มขึ้นเป็น 186.91±28.97 µg/L เมื่อนำมาเลี้ยงเชื้อในขวดทรงสี่เหลี่ยม (rectangle bottle) ซึ่งมีพื้นที่รับแสง 200 ตารางเซ็นติเมตร ขณะที่การเลี้ยงเชื้อในขวดทรงกระบอก (cylindrical bottle) ซึ่งมีพื้นที่รับแสงเพียง 143 ตารางเซ็นติเมตร จะให้การสร้าง coenzyme Q10 เพียง 151.70±21.64 µg/L

คำสำคัญ : การย่อยแป้งมันสำปะหลัง ; coenzyme Q10 ; *Rhodopseudomonas* sp.

Abstract

Rhodopseudomonas sp. S12-13 was retrieved from the mutation of the wild type, *Rhodopseudomonas* S12, by UV-irradiation. The mutant, S12-13 contained the cassava starch digestibility resulting in the high amount of coenzyme Q10 production at 148.12±21.74 µg/L when cultivating in AM medium containing of 2% cassava starch and 1% glutamate as carbon and nitrogen sources, respectively. In case of wild type, S12, the coenzyme Q10 production was only 12.51±1.34 µg/L when cultivating in AM medium. Mutant S12-13 produced maximal coenzyme Q10 (186.91±28.97 µg/L), when it was cultivated in the rectangle bottle, that contained 200 cm^2 of light exposure surface area. For the cultivation in cylindrical bottle, which contained 143 cm^2 of light exposure surface area, one would produce 151.70±21.64 µg/L of coenzyme Q10.

Keywords : cassava starch digestion ; coenzyme Q10 ; *Rhodopseudomonas* sp.

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Introduction

Rhodopseudomonas sp., a purple non-sulfur photosynthetic bacteria, contains carotenoids and photosynthetic pigments, bacteriochlorophylls that converts light energy into chemical energy by the process of anoxygenic photosynthesis. Moreover, it can grow autotrophically with CO_2 as the sole carbon source (Pfennig, 1969). Bacteriochlorophylls and various carotenoids are active components of light harvesting complexes in purple non-sulfur photosynthetic bacteria. Both compounds play different functional roles and absorb light in different wavelengths ranging from 700 to 1000 nm. Incandescent light (from a tungsten lamp) and halogen lamp are often used for illumination when cultivating purple non-sulfur photosynthetic bacteria, as they emit wide spectra absorbed by carotenoids, namely 450–550 nm, and by bacteriochlorophylls, namely 715–1050 nm (Kuo *et al*., 2012).

Coenzyme Q10, which is known as ubiquinone 10, has a quinone skeleton and a long hydrophobic isoprenoid side-chain, as shown in Figure 1. The species of coenzyme Q vary among organisms due to the difference in the length of the isoprenoid side chain, which is determined by polyprenyl diphosphate synthase (Cluis *et al*., 2007; Jeya *et al*., 2010). Coenzyme Q10 occurs widely in animals, plants, and the cells of microorganisms. It plays a crucial role in participation with ATP production of the mitochondrial electron transport chain. Due to its antioxidant activity, coenzyme Q10 is used not only as a medicines and cosmetics but also as a food supplement due to its various physiological activities. The production of coenzyme Q10 by microbes is a successful approach for generating large amounts of this natural product (Kuo *et al*., 2012). However, coenzyme Q10 has some drawbacks such as its instability against heat and light and its poor oral absorption due to the high hydrophobicity (Stocker *et al*., 1991; Thomas *et al*., 2001; Fuller *et al*., 2006).

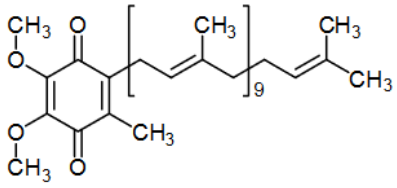

Figure 1 Chemical structures of coenzyme Q10 (Miyamoto *et al*., 2009)

It has been found that photosynthetic bacteria contain the highest coenzyme Q10 concentrations in nature (Carr & Exell, 1965). Recently, some studies on the production of coenzyme Q10 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; Jiang & Yu, 2007). To date, production of coenzyme Q10 is produced by one of three methods: extraction from biological tissues (Laplante *et al.*, 2009), chemical synthesis (Ehud & Doron, 1988), and microbial fermentation (Yoshida *et al.*, 1998). Currently, microbial fermentation is the most viable method for coenzyme Q10 production (Lu *et al.*, 2013; Yuting *et al.*, 2010) because of the ability to produce biologically potent coenzyme Q10 without optical isomers and at reduced costs (Tian *et al.*, 2010). Margaritis and Vogrinetz (1983) and Ndikubwimana and Lee (2014) found that types of C-source and N-source and pH level presented the strong effect on cell growth and coenzyme Q10 production from *Rhodopseudomonas* sp. Light intensity is also a very important factor in photosynthetic bacteria growth (Zhou *et al.,* 2014).

This study aimed to maximize the coenzyme Q10 production of *Rhodopseudomonas* sp. S12 by UV mutation and select the mutant contained the cassava starch digestibility. The optimal carbon and nitrogen sources for coenzyme Q10 production by the mutant were studied.

Methods

Materials

Microorganism and cultivation

Rhodopseudomonas sp. S12 was cultivated in AM medium, contained Na-acetate (5.0 g), glutamate (10.0 g), KH_2PO_4 (10.0 g) and yeast extract (1.0 g) and deionized H_2O (1000 ml) at pH 6.8-7.2, for 7 days in N_2 atmosphere at 35-40 ^{o}C with 1500 lux (Manjean *et al.*, 2012).

Strain improvement by UV mutation

The mutation of *Rhodopseudomonas* sp. S12 was carried by UV-irradiation exposure (253 nm, TUV30Wt8, Phillips). The cell suspension of *Rhodopseudomonas* sp. S12, (1.0 of OD 600 nm), was treated with UV-irradiation at 5, 10, 15, 20 and 25 min, respectively, at the distance of 30 cm. One-ml of treated sample was spread on AM agar with cassava starch as C-source and then incubation in N_2 atmosphere at 35-40 ^{o}C under 1500 lux of halogen lamp (Osram, Germany) for 7-10 days. The cassava starch utilized mutant was selected by iodine test. The mutant presented the clear zone was selected and maintained in deep tube of AM agar.

Inoculum preparation

The selected mutant that showed the maximal clear zone of cassava starch digestibility was stab in deep tube AM agar and then incubated under 1500 lux at 35-40 ^{o}C for 7 days. One-loop of selected mutant transferred to 10 ml of AM broth, contained in 15-ml test tube, and then incubated in the same condition. 1.0% of this culture was used as an inoculum for the further experimentations.

Effect of carbon sources on coenzyme Q10 production

1.0 % inoculum of selected mutant was transferred to 200 ml of AM medium in rectangle bottle. The C-source in AM medium was varied by using 0.5, 1.0 and 1.5% of Na-acetate, glucose and cassava starch concentration, respectively. The cultivation was carried under anaerobic condition with 1500 lux at 35-40 ^{o}C for 7 day. Triplicate samples were examined for the cell growth and coenzyme Q10 production.

Effect of nitrogen sources on coenzyme Q10 production

1.0 % inoculum of selected mutant was transferred to 200 ml of AM medium in rectangle bottle. The N-source in AM medium was varied by using 0.5, 1.0 and 1.5% of glutamate, $(NH_4)_2SO_4$ and $NaNO_3$ concentration, respectively. The cultivation was carried under anaerobic condition with 1500 lux at 35-40 ^{o}C for 7 day. Triplicate samples were examined for the cell growth and coenzyme Q10 production.

Effect of light exposure surface area on coenzyme Q10 production

The cultivation of selected mutant was cultivated with 200 ml of optimal medium formula in 250-ml cylindrical bottle and rectangle bottle, which indicated the light exposure area of 143 cm^2 and 200 cm^2, respectively. The cultivation was carried under anaerobic condition with 1500 lux at 35-40 ^{o}C for 7 day. Triplicate samples were examined for the cell growth and coenzyme Q10 production.

Analytical methods

The cell growth was determined by dry cell weight. About 5-ml of culture was centrifuged with 8000x g for 15 min and then dried at 70 ^{o}C overnight to a constant weight. The extracted coenzyme Q10 from the selected mutant of *Rhodopseudomonas* sp. was analyzed by the method of Matsumura *et al*. (1983) and Jeong *et al*. (2008) with modifications. The cell pellet, which retrieved from the centrifugation, was used to extract by mixing with a solvent mixture of ethanol and hexane (1:2, v/v) for 1 hr. The heavy phase was collected and applied for the coenzyme Q10 analysis by HPLC (LC-10AD, Shimadzu, Columbia, MD) with a μBondapak C18 (3.9 mm × 300 mm, Waters, Milford, MA) coupled to a UV detector (SPD-10A, Shimadzu, Columbia, MD). The column was eluted with ethanol and methanol (9:1, v/v) at a flow rate of 1.0 ml/min and a chromatogram was obtained by monitoring the absorbance at 275 nm identified and quantified by known concentrations of coenzyme Q10 standard (Sigma–Aldrich, St. Louis, MO).

Results

Strain improvement by UV mutation

The retrieved mutant was pointed inoculation technique on the AM agar with cassava starch as C-source and cultivated under N_2 atmosphere at 35-40 ^{o}C and 1500 lux of halogen lamp for 7-10 days. The mutant with

cassava starch digestive zone was observed by iodine detection. Only 8 selected mutants showed the cassava starch digestive zone (Table 1).

Table 1 The cassava starch digestibility of 8 selected mutants from UV mutation on *Rhodopseudomonas* sp. S12

Isolate	Growth zone (Gz) (cm)	Clear zone (Cz) (cm)	Digestibility (Cz/Gz)
S12 (wild type)	0.25	0.25	1.00
12-13	1.10	4.25	3.86
12-14	0.95	2.35	0.25
12R1	1.00	1.50	1.50
12R2	0.75	1.62	2.16
12R3	0.45	0.65	1.44
12R4	0.65	0.95	1.46
12C1	0.55	0.75	1.36
12C2	0.35	0.45	1.28

The *Rhodopseudomonas* sp. mutant, isolate S12-13 showed the largest cassava starch digestibility of 3.86 times than the others. Figure 2 shows the cassava starch digestibility of S12-13. Therefore, S12-13 would be used for the further experiments.

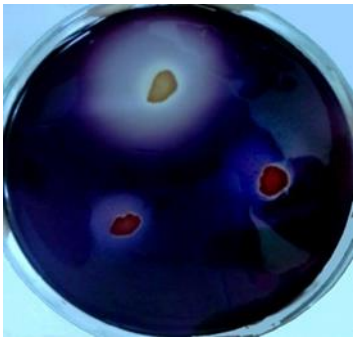

Figure 2 The cassava starch digestibility of some mutants shows the growth zone and clear zone.

Effect of carbon source on coenzyme Q10 production

The *Rhodopseudomonas* sp. S12-13 was cultivated in AM medium by using 0.5, 1.0, 2.0 and 3.0% of Na-acetate, glucose and cassava starch as C-source, respectively. The cultivation was carried on 200 ml medium in cylindrical bottle and incubated anaerobic condition under 1500 lux at 35-40 °C for 7 days. Figure 3 shows the coenzyme Q10 production in various types and concentrations of C-source. The result showed that the cultivation in AM medium with 2% of cassava starch as C-source presented the maximum coenzyme Q10 production at 151.70±10.23 µg/L. In case of 3% cassava starch, the high viscosity might cause some effect on cell dispersion during the first state of cultivation. However, 2% and 3% glucose concentration indicated the inhibition effect on cell growth. The 2% of cassava starch would be used for the further experiment.

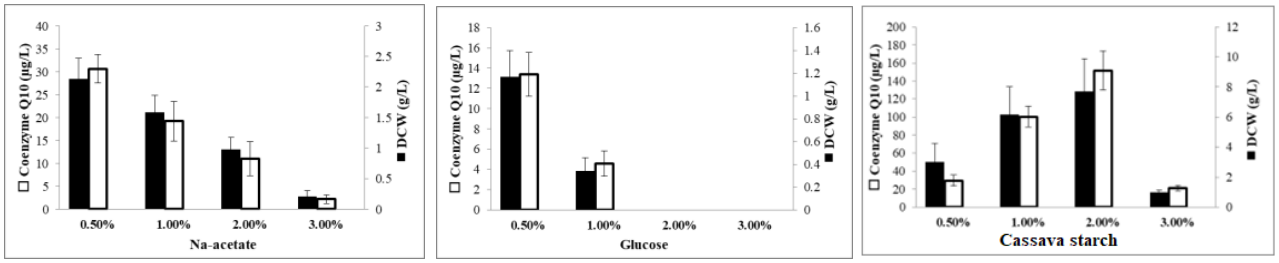

Figure 3 The cell growth and coenzyme Q10 production of S12-13 that was cultivated in AM medium containing 0.5%, 1.0%, 2.0% and 3.0% of Na-acetate, glucose and cassava starch, respectively. The incubation was carried on anaerobic condition with 1500 lux at 35-40 °C for 7 days.

Effect of nitrogen source on coenzyme Q10 production

2.0% of cassava starch would be used as the C-source in AM medium. 0.5, 1.0, 2.0 and 3.0% of glutamate, $(NH_4)_2SO_4$ and $NaNO_3$ were tested for the optimal N-source for coenzyme Q10 production by S12-13. The cultivation was carried on 200 ml medium in cylindrical bottle and incubated anaerobic condition under 1500 lux at 35-40 °C for 7 days. Figure 4 shows the coenzyme Q10 production in various types and concentrations of N-source. The result showed that the cultivation in AM medium with 1% of glutamate as N-source gave the maximum coenzyme Q10 production at 148.12±15.72 µg/L. The coenzyme Q10 decreased as glutamate concentration was increased to 2.0% and 3.0%. In case of $(NH_4)_2SO_4$ and $NaNO_3$, the cell growth and coenzyme Q10 production were lower than glutamate. It was found the final pH of the cultures in glutamate, $(NH_4)_2SO_4$ and $NaNO_3$ were about 7.2, 5.6 and about 8.8, respectively.

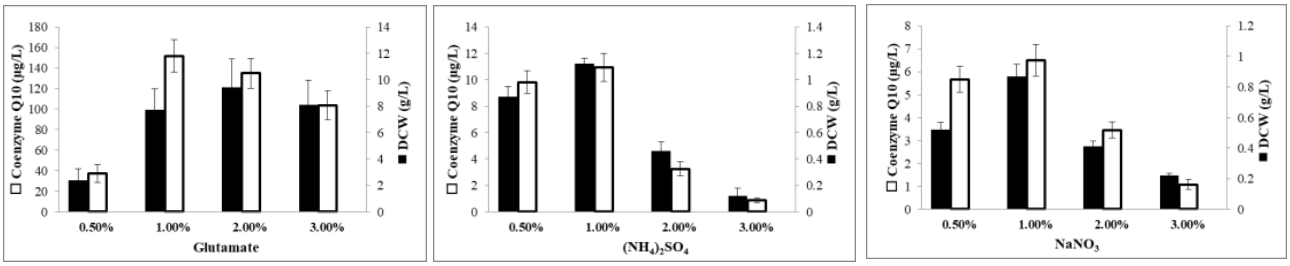

Figure 4 The cell growth and coenzyme Q10 production of S12-13 that was cultivated in AM medium containing 0.5%, 1.0%, 2.0% and 3.0% of glutamate, $(NH_4)_2SO_4$ and $NaNO_3$, respectively. The incubation was carried on anaerobic condition with 1500 lux at 35-40 °C for 7 days.

Effect of light exposure surface area on coenzyme Q10 production

The optimal medium for coenzyme Q10 production was composed of 2.0% cassava starch and 1.0% glutamate as C-source and N-source, respectively. The cultivation of S12-13 was conducted in cylindrical bottle and rectangle bottle which had the light expose area at 143 cm^2 and 200 cm^2, respectively. The cultivation was incubated anaerobic condition at 35-40 °C under 1500 lux for 7 days. The result is shown in Figure 5. The coenzyme Q10 production by cultivated in rectangle bottle gave the maximal value at 186.91±28.97 µg/L that higher than one with cylindrical bottle (151.70±21.64 µg/L). It was indicated that the higher light expose area the lower light shading. Light is one of the essential factors for the growth and coenzyme Q10 from *Rhodopseudomonas* sp. S12-13.

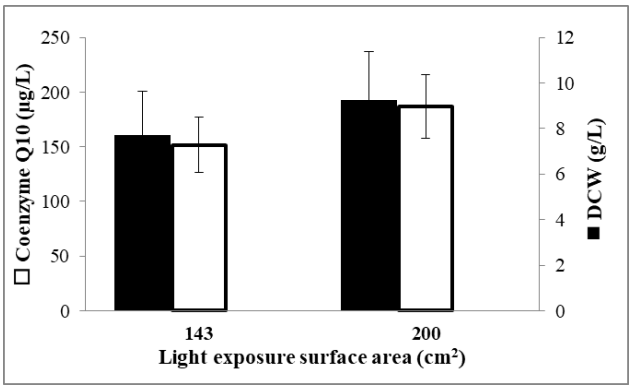

Figure 5 The cell growth and coenzyme Q10 production of S12-13 that was cultivated in 200 ml of optimal medium with surface area of 143 cm^2 (cylindrical bottle) and 200 cm^2 (rectangle bottle), respectively. The incubation was carried on anaerobic condition with 1500 lux at 35-40 °C for 7 days.

Discussion

Rhodopseudomonas sp. S12 produced 12.51±1.34 µg/L of coenzyme Q10 when cultivated in AM medium. While the mutant S12-13 gave 19.21±4.91 µg/L in the same medium. After medium optimization, the production was increased to 148.12±21.74 µg/L. The mutant S12-13 showed the cassava starch digestibility resulting in high growth and coenzyme Q10 production, but the wild type did not assimilate cassava starch as C-source. The Na-acetate and glucose concentrations were increased resulting in the more substrate inhibition. Meanwhile, glucose showed the serious inhibition effect on cell growth of the mutant S12-13. For using $(NH_4)_2SO_4$ as N-source, it was found that the final pH of all experiments was in acidic value (about 5.6). In case of $NaNO_3$, the final pH of all experiments was in alkaline value (about 8.9). The pH changing during the cultivation might relate to the coenzyme Q10 production. The high glucose concentration and pH level showed the strong inhibition effect on cell growth and product formation of *Rhodopseudomonas sphaeroides* VM 81 (Margaritis & Vogrinetz, 1983). Ndikubwimana and Lee (2014) found that the pH might be the key factors affecting coenzyme Q10 production. When the surface area of light exposure was increased from 148 cm^2 to 200 cm^2, the cell growth and coenzyme Q10 were improved due to the light shading was minimized. Light is also a very important factor in photosynthetic bacteria growth since photosynthetic bacteria convert light energy into chemical energy via anaerobic photosynthesis (Zhou *et al*., 2014).

Conclusions

Rhodopseudomonas sp. S12-13 was retrieved from the mutation of the wild type, *Rhodopseudomonas* S12, by UV-irradiation. The mutant, S12-13 contained the cassava starch digestibility resulting in the high amount of coenzyme Q10 production (148.12±21.74 µg/L) when cultivating in 2% cassava starch and 1% glutamate as carbon and nitrogen sources, respectively. In case of wild type, S12, the coenzyme Q10 production was only 12.51±1.34 µg/L when cultivating in AM medium. The high glucose concentration and pH level showed the strong inhibition effect on cell growth and coenzyme Q10 formation of *Rhodopseudomonas* sp. S12-13. For mutant S12-13, the cassava starch digestion might release the suitable glucose residue to compromise the glucose effect. Mutant S12-13 produced maximal coenzyme Q10 (186.91±28.97 µg/L), when it was cultivated in the rectangle bottle, that contained 200 cm^2 of light exposure surface area. For the cultivation in cylindrical bottle, which contained 143 cm^2 of light exposure surface area, one would produce 151.70±21.64 µg/L of coenzyme Q10. Light is also the other factors that affected cell growth and coenzyme Q10 production of S12-13.

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