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Cell lysis methods and coenzyme Q_{10} production of *Methylobacterium* strains

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ABSTRACT

Objective: This research aimed to investigate the effect of cell lysis methods on a quantity of coenzyme Q_{10} (CoQ_{10}) produced by *Methylobacterium* strains. **Methods:** *Methylobacterium organophilum* NBRC 15689^T and *Methylobacterium* strain LRY1-08 were used to produce of CoQ_{10}. The 4 methods of cell lysis treatment were used to disrupt the cell wall of bacteria and to increase the release of CoQ_{10} which was followed by extraction with isopropanol and hexane (3:5). Content of CoQ_{10} was analyzed with HPLC method. **Results:** The results showed that the cell lysis treatment using methanol and 0.3% sodium chloride (10:1) containing 1% Triton X-100 with sonication and then extraction with isopropanol and hexane (3:5) were found to yield the highest CoQ_{10} content for both *Methylobacterium* strains. *Methelobacterium organophilum* NBRC 15689T produced significantly higher CoQ_{10} content than *Methylobacterium* strain LRY1-08 which was consistent with its higher dry cell weight. **Conclusion:** The results indicated that this cell lysis method could be used to extract CoQ_{10} from plasma membrane of the bacteria. Methylobacterium organophilum NBRC 15689^T was a promising strain for CoQ_{10} production source which should be further optimized factors affecting CoQ_{10} production.

Keywords: Coenzyme Q_{10}, cell lysis method, *Methylobacterium* strains, production, content

INTRODUCTION

Coenzyme Q_{10} (CoQ_{10}) or ubiquinone-10 is a vitamin-like lipid-soluble substance. CoQ_{10} can be found in plants, animals, and microorganisms.[1] It is a necessary component of the electron transport system. CoQ_{10} is an important cosmetic ingredient because it has antioxidant properties to slow aging and wrinkle[2,3] and was also used as a nutraceutical supplement.[4] Although CoQ_{10} can be synthesized in the body, the performance of its synthesis is decreased when human get older. Consequently, it leads to the increased commercial production of CoQ_{10} to meet the growing demands.

CoQ_{10} has been produced by three methods including chemical synthesis, extraction from plant or animal tissues, and microbial fermentation.[3,5,6] Chemical synthesis and extraction from plant or animal tissues are environmentally unfavorable because of using chemicals and solvents in the process, high cost,[6,7] and low productivity.[4] On the contrary, microbial fermentation is a method able to choose cheap carbon sources[8] and attractive to the industry due to ease of process control and a relatively low cost of production.[3,6,7] However, there are many factors affecting CoQ_{10} production by microbial fermentation method that needs to be studied to obtain high CoQ_{10} production to meet the needs of the industry.[9] For methods of increasing CoQ_{10} production, there were various methods such as optimization of a fermentation process, mutagenesis of CoQ_{10} producing microorganisms, and gene expression involved in the CoQ_{10} biosynthesis[9] and method development to separate CoQ_{10} from microorganisms.[3]

Many Gram-negative bacteria including *Agrobacterium tumefaciens*, *Paracoccus denitrificans,* and *Rhodobacter sphaeroides* have been reported to be able to produce CoQ_{10}.[5,10] The genus *Methylobacterium* is Gram-negative, facultatively methylotrophic bacteria producing a variety of pink-pigments. The members of this genus were able to produce CoQ_{10},[11] for example, *Methylobacterium extorquens* could yield CoQ_{10} in 94% of all coenzyme Q produced.[12]

CoQ_{10} was located in the plasma membrane of prokaryotes.[13] Therefore, a separation method of CoQ_{10} from bacteria is required. Liquid-liquid extraction was a way to separate a desired substance mixed with others using a mixture of 2-propanol and hexane. This method was the most usual technique for CoQ_{10} extraction from various

samples.[6] Moreover, different cell disruption methods were used to increase CoQ_{10} release from the cells, including chemical, mechanical, and enzymatic methods. The chemical method used chemical interaction with components of the membrane allowing intracellular materials to penetrate through the bacteria cell wall.[13] For the mechanical method, cell membrane is physically broken down with shear force.[14] On the other hand, the enzymatic lysis method is a specifically biological cell lysis.[14] There were many researches that reported the extraction of CoQ_{10} from *A. tumefaciens*, *Rhodospirillum rubrum,* and *Rhodobacter sphaeroides* by cell lysis with enzymes and then extraction with *n*-propanol:hexane (3:5).[3,7,15,16] However, the enzymatic lysis method always provides non-reproducibility due to the instability of enzyme and high processing cost.[3,13] In addition, CoQ_{10} extraction with *n*-propanol:hexane (3:5) or 95% ethanol without a cell lysis process was reported on *R. sphaeroides*.[3] Furthermore, there was a report using a mixture of methanol and 0.3% sodium chloride (10:1) supplemented with 1% Triton X-100 and sonication as a cell lysis method, then extraction with isopropanol and hexane (3:5) for recombinant *Escherichia coli* harboring the *dps* gene.[17]

Although there were many methods for the extraction of CoQ_{10} from numerous bacteria, there was no report on *Methylobacterium organophilum* NBRC 15689^T and *Methylobacterium* strain LRY1-08. *M. organophilum* NBRC 15689^T was recovered from the sediment in the lake,[18] whereas *Methylobacterium* strain LRY1-08 was a new strain isolated from lichen in Rayong. Both *Methylobacterium* strains are Gram-negative bacteria which have similar cell wall structures and cell membranes, but they are different in the 16S rDNA sequences. Therefore, the aim of this study was to investigate the potential of CoQ_{10} production from *M. organophilum* NBRC 15689^T and *Methylobacterium* strain LRY1-08 as well as cell lysis methods for CoQ_{10} extraction from these bacteria cells.

MATERIALS AND METHODS

Bacterial Strains and Culture Conditions

Methylobacterium strain LRY1-08 was gained from the Department of Biochemistry and Microbiology, Faculty of Pharmaceutical Sciences, Chulalongkorn University and *M. organophilum* NBRC 15689^T was obtained from National Institute of Technology and Evaluation. The seed culture was grown in a 250 ml-Erlenmeyer flask containing 100 ml ISP 2 broth (0.4% glucose, 0.4% yeast extract, and 1% malt extract, pH 7.0) at 30°C for 48 h under shaking condition (200 rpm). Then, 10 ml of the seed culture was transferred into a 500 ml-Erlenmeyer flask containing 100 ml ISP 2 broth (pH 7.0) and cultivated at 30°C for 168 h under shaking condition (200 rpm). The cultured broth was further evaluated on the cell mass and CoQ_{10} content produced.

Determination of Cell Mass

The cultured broth (10 ml) was centrifuged at 8000 rpm, 4°C for 15 min. The cell pellets were washed twice with deionized water. The dry cell weight (DCW) of the bacterial strains was evaluated by weighing the cell pellets after dried in an oven at 105°C for 24 h.[3] DCW was calculated as gram per liter of cell suspensions (g/L).

Extraction of CoQ_{10}

The cultured broth (10 ml) was centrifuged at 8000 rpm, 4°C for 15 min. The cell pellets were washed twice with deionized water then treated with the four methods as follows:

1. 5 ml of a mixture of methanol and 0.3% sodium chloride (10:1 v/v) supplemented with 1% Triton X-100 and then sonicated in an ultrasonic bath for 15 times (20 s/ time). The cell debris were extracted with 10 ml of isopropanol and hexane (3:5) under shaking at 200 rpm, 40°C for 30 min (modified from Patt, Cole and Hanson[18])
2. 10 ml of isopropanol and hexane (3:5) and then sonicated with an ultrasonic bath for 15 times (20 s/time) to induce cell lysis and then shaking at 200 rpm, 40°C for 30 min
3. 10 ml of isopropanol and hexane (3:5) under shaking at 200 rpm, 40°C for 30 min (modified from Wu and Tsai[3])
4. 10 ml of 95% ethanol under shaking at 200 rpm, 40°C for 30 min (modified from Wu and Tsai[3]).

Measurement of CoQ_{10} Content

After extraction, 10 ml of each sample was centrifuged at 8000 rpm, 4°C for 15 min. The organic solvent was collected and then evaporated. The 95% ethanol was used to dissolve the pink dried residue. Content of CoQ_{10} was analyzed by high performance liquid chromatography (HPLC) using a Phenomenex 250 mm × 4 mm RP-C18 column. The flow rate of mobile phase containing methanol and hexane (70:30 v/v) was 1 ml/min. Detection was done by a UV/VIS detector at 275 nm.[3] The yields of CoQ_{10} were presented as "CoQ_{10} production" and "specific of CoQ_{10} content" in the units of milligram per liter of cell suspensions (mg/L) and milligram per gram of dry cell weight (mg/g DCW), respectively.[7]

Statistical Analysis

All experiments were done in triplicate. The obtained results were presented as mean ± standard deviation. The data of the cell lysis method in each strain were analyzed by one-way analysis of variance followed by Tukey's multiple comparison test ($P < 0.05$). Independent T-test was used to compare the data between two strains on the same extraction method.

RESULTS AND DISCUSSION

Determination of Cell Mass

The DCW was determined to study the growth of each strain and used to calculate a quantity of CoQ_{10} production as "specific CoQ_{10} content". The result of DCW is shown in Figure 1. The DCW of *M. organophilum* NBRC 15689^T (4.30 ± 0.11 g/L) was significantly higher than *Methylobacterium* strain LRY1-08 (1.97 ± 0.52 g/L) under cultivation in the ISP2 medium for 7 days ($P < 0.05$). The result indicated that *M. organophilum* NBRC 15689^T grew better than *Methylobacterium* strain LRY1-08 in this culture medium.

Measurement of CoQ_{10} Content

CoQ_{10} was extracted from bacteria cells by liquid-liquid extraction. Before extraction, cell lysis treatment may be needed to improve the release of CoQ_{10} from their plasma

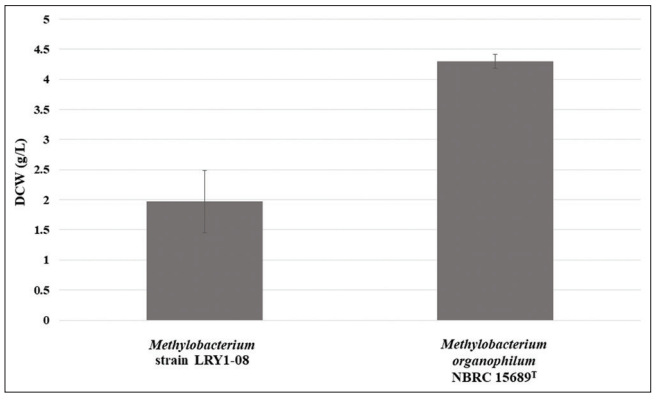

Figure 1: Dry cell weight of *Methylobacterium* strain LRY1-08 and *Methylobacterium organophilum* NBRC 15689^T (mean ± SD, n = 3)

membrane. In this study, the effect of cell lysis methods on the yield of CoQ_{10} was studied and compared on *M. organophilum* NBRC 15689^T and *Methylobacterium* strain LRY1-08, *M. organophilum* NBRC 15689^T. The methods (1) and (2) used different solvent mixtures for cell lysis treatment but the same sonication method. The methods (3) and (4) used different solvents for extraction without cell lysis process.

The CoQ_{10} production and specific CoQ_{10} content are shown in Table 1. HPLC chromatograms of the CoQ_{10} crude extracts in both *Methylobacterium* strains with the CoQ_{10} standard are presented in Figure 2. The result showed that the method (1) using cell lysis treatment by the solvent mixture of methanol, sodium chloride, and Triton-X 100 with sonication was proved to be significantly superior to the other methods because this method yielded the highest CoQ_{10} production and

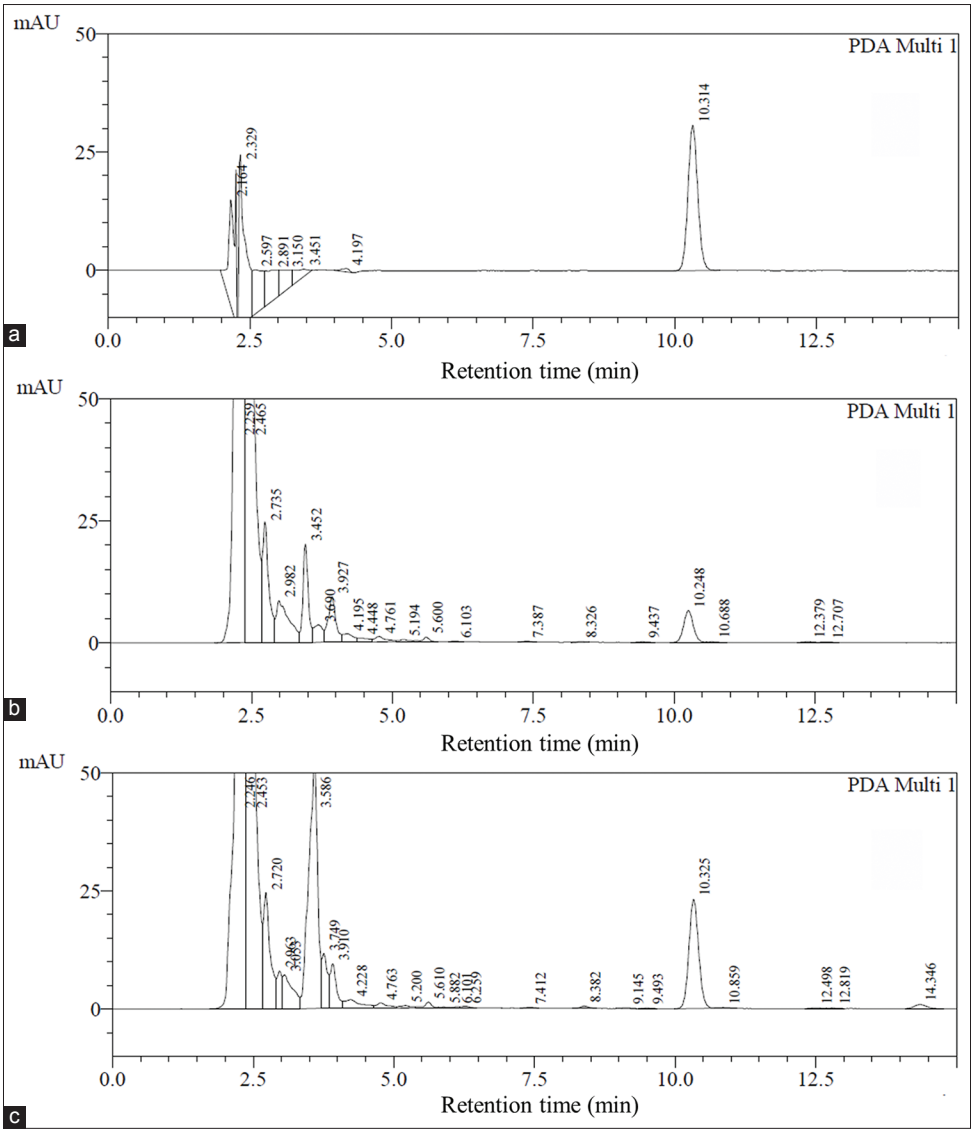

Figure 2: HPLC chromatograms of CoQ_{10} standard (a), CoQ_{10} crude extracts from fermentation broth of *Methylobacterium* strain LRY1-08 (b) and *Methylobacterium organophilum* NBRC 15689_T (c) under treating with the method (1), presenting the CoQ10 peaks at the retention time of 10.314, 10.248, and 10.325 min, respectively

Table 1: Effect of cell lysis methods on CoQ_{10} content of *Methylobacterium* strain LRY1-08 and *Methylobacterium organophilum* NBRC 15689^T (mean±SD, n=3)

Method	*Methylobacterium* strain LRY1-08		*Methylobacterium organophilum* NBRC 15689^T	
	CoQ_{10} production (mg/L)*	Specific CoQ_{10} content (mg/g DCW)*	CoQ_{10} production (mg/L)*	Specific CoQ_{10} content (mg/g DCW)*
1	0.9173 ± 0.05^{aA}	$0.4656\pm0.03^{a\bullet}$	3.0881 ± 0.17^{aB}	$0.7182\pm0.04^{a\#}$
2	0.2320 ± 0.04^{bA}	$0.1178\pm0.02^{b\bullet}$	2.6825 ± 0.10^{bB}	$0.6238\pm0.02^{b\#}$
3	0.2818 ± 0.06^{bA}	$0.1431\pm0.03^{b\bullet}$	1.9133 ± 0.10^{cB}	$0.4450\pm0.02^{c\#}$
4	0.0000^{cA}	$0.0000^{c\bullet}$	0.0000^{dA}	$0.0000^{d\bullet}$

*Different lowercase letters in the same column (a-d) indicate significant differences among cell lysis methods on CoQ_{10} production and specific CoQ_{10} content for each strain. Different uppercase letters in the same line (A and B) indicate significant differences on CoQ_{10} production between *Methylobacterium* strain LRY1-08 and *Methylobacterium organophilum* NBRC 15689^T. Different symbols in the same line ($^{\bullet, \#}$) indicate significant differences on specific CoQ_{10} content between *Methylobacterium* strain LRY1-08 and *Methylobacterium organophilum* NBRC 15689^T

specific CoQ_{10} content for both *M. organophilum* NBRC 15689^T (3.0881 ± 0.17 mg/L, and 0.7182 ± 0.04 mg/g DCW) and *Methylobacterium* strain LRY1-08 (0.9173 ± 0.05 mg/L, and 0.4656 ± 0.03 mg/g DCW). The reason was due to the cells breaking first with chemicals and mechanicals. Chemicals can permeate cell wall of bacteria and disturb the normal physiology of bacteria leading to a rapid release of CoQ_{10}. Sonication is a method for cell disintegration because it generates microscopic air bubbles. These transient cavities may cause high-shear gradients by microstreaming and the effects of cavitation were mostly found only in areas adjacent to the vibrating surface.[13]

The method (2) using isopropanol and hexane (3:5) with sonication yielded less CoQ_{10} production and specific CoQ_{10} content than the method (1) [Table 1]. This result indicated that the chemicals had more influence than the mechanical method used in this study. In addition, the method (2) with sonication gave significantly more yield of CoQ_{10} than the method (3) without sonication ($P < 0.05$) for *M. organophilum* NBRC 15689^T but there was insignificant difference for *Methylobacterium* strain LRY1-08. The result may demonstrate the importance of sonication. However, an ultrasonic bath used in this sonication process may provide not enough energy due to less frequency wave as compared to an ultrasonic probe.

As compared between the methods (3) and (4) using different extraction solvents without cell lysis process [Table 1], the solvent mixture of isopropanol and hexane (3:5) showed more ability of CoQ_{10} extraction from plasma membrane of both *M. organophilum* NBRC 15689^T (1.9133 ± 0.10 mg/L, and 0.4450 ± 0.02 mg/g DCW) and *Methylobacterium* strain LRY1-08 (0.2818 ± 0.06 mg/L, and 0.1431 ± 0.03 mg/g DCW), whereas 95% ethanol could not extract CoQ_{10}. The result of 95% ethanol disagreed with the previous study[3] reporting that 95% ethanol could be used to well extract CoQ_{10} from *R. sphaeroides* without a cell lysis process. Both *R. sphaeroides* and *Methylobacterium* are Gram-negative bacteria that have similar cell wall structures. The 95% ethanol can disturb the normal physiology of bacteria, but less extraction efficiency than the mixture of isopropanol and hexane (3:5). Because CoQ_{10} is a lipid-soluble and less polar substance, the mixture of isopropanol and hexane (3:5) was more suitable solvent than 95% ethanol for the extraction of CoQ_{10} according to the like dissolves like principle.

For the influence of *Methylobacterium* strains on CoQ_{10} production and specific CoQ_{10} content, Table 1 shows that *M. organophilum* NBRC 15689^T produced significantly higher yield of CoQ_{10} than *Methylobacterium* strain LRY1-08 ($P < 0.05$) for all extraction methods. The higher yield of CoQ_{10} from *M. organophilum* NBRC 15689^T was consistent with its higher DCW [Figure 1]. This result might indicate that growth of *Methylobacterium* strains affected their production of CoQ_{10} on which influence of cultivation condition should be further studied. However, *Methylobacterium* strains still provided low yields of CoQ_{10} as compared with other bacteria strains that have been reported to able to produce CoQ_{10} in the range of 25.5–770 mg/L.[19]

CONCLUSION

Cell lysis treatment was used to disrupt the cell wall of bacteria and to increase the release of CoQ_{10} which was followed by extraction with isopropanol and hexane (3:5). The methods used in the cell lysis process affected CoQ_{10} production and specific CoQ_{10} content of both *Methylobacterium* strains. Cell lysis treatment using a mixture of methanol and 0.3% sodium chloride (10:1 v/v) supplemented with 1% Triton X-100 and sonication, then extraction with isopropanol and hexane (3:5) was found to yield the highest CoQ_{10} content. *M. organophilum* NBRC 15689^T produced significantly higher CoQ_{10} content than *Methylobacterium* strain LRY1-08. *M. organophilum* NBRC 15689^T should be further studied for the effect of culture media on CoQ_{10} production to provide higher yield of CoQ_{10}.

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