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EXTRACTION AND PROPERTY STUDIES OF COENZYME Q10 FROM RECOMBINANT *AGROBACTERIUM TUMEFACIENS*

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ABSTRACT

In this report, some results of extraction and characterization of CoQ10 from recombinant *A. tumefaciens* are presented. Four different cell breaking methods (sonication, acidic treatment, ethanol treatment, and enzymatic lysis) in combination with the extracting steps were carried out to extract CoQ10 and the results showed that ethanol treatment was the most efficient method. Appropriate conditions for CoQ10 extraction were 25 ^{o}C, 24 hours incubation and ethanol solvent/biomass ratio of 10:1 (ml/g). Characterization of extracted CoQ10 showed that CoQ10 was sensitive to light, but stable in the temperature ranges of 4 – 60 ^{o}C and the pH range of 6.0 – 9.0. Obtained results in present study should be applied in the large scale for CoQ10 extraction, providing the CoQ10 product for testing production of functional foods.

Keywords: Agrobacterium tumefaciens, coenzyme Q10 (CoQ10), extraction, property.

1. INTRODUCTION

Ubiquinone-10 or Coenzyme Q10 (CoQ10) plays a critical role in the production of energy in nearly every cell of the body. Due to the potent antioxidant properties, CoQ10 has being used much more as a sourse of supplemental food to improve health and be included in the different cosmetics. CoQ10 could be applied in medicine to prevent cancer, treat cardiovascular diseases, diabetes, Parkinson and improve immune system [1]. The current trend is to focus on research in the production of CoQ10 by biological process through bacterial fermentation [2,3,4]. In bacteria, coenzyme Q10 is located in the hydrophobic domain of the phospholipid bilayer of cellular membrane [5], therefore it is neccessary to break the cellular membrane to extract this component. The degradation of the cellular membrane will affect the efficiency of CoQ10 extraction. Several CoQ10 extraction methods were performed with the different efficiencies [6] and mainly suitable for laboratory scale. Some criteria such as simple, low cost and solvent recovery should be considered to set up a method for CoQ10 extraction in the large scale. Furthermore, information on the effects of several factors such as temperature, light and pH to the stability of CoQ10 will helpful to determine appropriate conditions for CoQ10 extraction and

storage of its products. The stability of CoQ10 was maily investigated in complexes [7, 8]. Therefore, the purposes of this study were (1) to establish an effective CoQ10 extraction procedure from *Agrobacterium tumefaciens* suitable for the large scale and (2) to investigate some properties of extracted CoQ10.

2. MATERIALS AND MOTHODS

2.1. Materials

Recombinant *A. tumefaciens* DPXS12 strain was provided from School of Biotechnology and Food Technology, Hanoi University of Science and Technology. Chemicals for culture, fermentation of *A. tumefaciens*, for extraction and property studies of CoQ10 were furchased from Sigma-Aldrich (USA), Merck (Germany), Thermo Scientific (USA), Himedia (India).

2.2. Methods

In this study, four different CoQ10 extraction methods were used in which sonication, hydrocloric acid and enzyme treatment were performed as described by Tian et al. 2010 [6], ethanol extraction method was performed as desbribed by Ranadive et al. [9] with some modifications. For sonication method, the bacterial cell mass was dissolved in 2 mM Tris-HCl, pH 7.5 and sonificated at 60% fulse power for 15 seconds on and 10 seconds off. Total time for sonication was 10 min. For hydrocloric acid treatment method, the bacterial cell mass was dissolved in 3 M HCl and incubated at 85 ^{o}C for 30 min. For enzyme treatment method, a mixture of bacterial cell, proteinase K and lysozyme (0,1 mg/ml) was incubated at 37 ^{o}C for 30 min. Ethanol treatment method was carried out at 60 ^{o}C for 3 hours. Coenzyme Q10 was extracted 3 times from the homogenate with petrolium ether or hexane solvent and dried by speed vacuum concentrator. Coenzyme Q10 was dissolved in ethanol.

CoQ10 was quantified by Craven reaction as described by Shimada et al. [10]. Briefly, a volume of 500 µl of 100 mM ethylcyanoacetate was added to 500 µl of CoQ10 solution, mixed well and incubated for 6 min, followed by adding 100 µl 0.4 M KOH. The absorbance intensity of reaction mixture at 625 nm was recorded by spectrophotometer and used to determine CoQ10 amount. Determination of CoQ10 was also validated by high performed liquid chromatography (HPLC). The CoQ10 extract was analyzed by HPLC (Agilent 1200 Series, USA) using C18 column (250 mm x 4,6 mm), UV detector at 35 ^{o}C. After loading sample, the column was washed by methanol for 19 min and eluted by ethanol gradient for 4 min, followed by isocratic run up to 45 min at 0.5 ml/min. The CoQ10 amount was determined on the basis of area of absorbent peak at 275 nm wavelength and standard curve of CoQ10.

Stabilities of CoQ10 against pH, light, temperature were determined by quantifying the remaining CoQ10 amount after exposuring to the different conditions for different times. Antioxidant property of CoQ10 was determined as described by Wang et al. [11].

3. RESULTS AND DISCUSSION

3.1. The result of cell lysis by different methods

Coenyzme Q10 (CoQ10) is a hydrophobic compound, insoluble in water. In living cells, CoQ10 is located on the plasma membrane of the bacterial cell and the inner membrane of

mitochondria of eukaryotic cells [5]. Therefore, CoQ10 will be directly extracted from bacterial cell mass. In this study, the cellular membrane of *A. tumefaciens* was lysed by four different methods including sonication, hydrocloric acid, ethanol and enzyme treatment. The result indicated that the highest amount of CoQ10 was obtained by the ethanol extraction method compared with other methods (Fig. 1). *A. tumefaciens* contains a thin inner cell wall and outer membrane and therefore the hydrophobic linkage in this membrane will be weaken when interacting with ethanol, leading to cellular lysis [12] and CoQ10 release. Similarity, hydrocloric acid also plays role in the lysis of cellular membrane [6]. However, CoQ10 is hydrophobic compound therefore its releasing efficiency is higher by ethanol than hydrocloric acid. In addition to chemical usage, some enzymes such as proteinase K, lysozyme were also used to break the cell membrane in several researches. However, the efficiency of enzymatic lysis is lower than chemical lysis by ethanol and hydrocloric acid. Sonication method is known to generate microscopic bubbles leading to incomplete cell lysis [6], therefore the CoQ10 recovery was the lowest. On the basis of obtained results, the ethanol treatment method should be further investigated and applied for CoQ10 extraction.

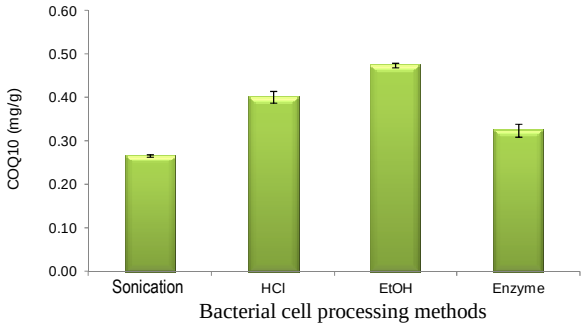

Figure 1. Different cell lysis methods for CoQ10 extraction.

3.2. Determination of appropriate conditions for the cell lysis by ethanol

Determination of ethanol/cell mass ratio

In order to evaluate the effect of solubility of the cell mass in ethanol to CoQ10 recovery from *A. tumefaciens*, different ratios of ethanol/cell mass were investigated including 5, 10, 15 ml/g and the cell lysis was taken at 37 ^{o}C for 3 hours. The obtained results showed that the amount of extracted CoQ10 was proportional to the increase of ethanol/cell mass ratio (Fig. 2A). The comparison of obtained CoQ10 amounts from different ethanol/cell mass ratios showed that the extracted CoQ10 amount from 10:1 ratio was 1.32 fold higher (equivalently 24.3 %) than 5:1 ratio; whereas the CoQ10 recovery from 15:1 ratio was only 1.08 fold higher than 10:1 ratio (equivalently 7.7 %). On the basis of obtained results, the ethanol/cell mass ratio (10 ml/g) was selected for the CoQ10 extraction in further works.

Determination of assimilating time

In order to improve solubility in ethanol, the cell mass was homogenized by sonication method at the low energy level (approximately 30 – 40 %) for different times including 1, 2, 3, 5, 10, and 15 min. The result showed that the highest level of CoQ10 was obtained after 3 min assimilation. However, the more increase of assimilating time was leading to the decrease of

CoQ10 recovery (Fig. 2B). This decrease might be due to the inverse effects of ultrasound in a long time to the structure of CoQ10.

Determination of incubation temperature and time

The effect of incubation temperature to the CoQ10 recovery from *A. tumefaciens* was determinized by incubating the homogenized mixture at different temperatures (25, 37 và 60 ^{o}C) for 3 hours. The result showed that the increase of incubation temperature enhanced the recovery of CoQ10 from the lysed cell (the increase was 1.15 and 1.17 fold at 37 ^{o}C and 60^{o}C respectively in comparison with 25 ^{o}C) (Fig. 2C). The obtained results indicates that the temperature of 37^{o}C is suitable for incubation of homogenized mixture. Similarity, the obtained CoQ10 amount was proprotional to the incubation time. The obtained CoQ10 amount after 24 hours incubation was increased 1.27 fold in comparison with that after 1 hour incubation (Fig. 2D).

From the obtained results, a procedure for CoQ10 extraction was established, which includes: the steps of mixing ethanol with cell mass (10:1, v/w), assimilation by sonication for 3 min at low energy, incubation at 37^{o}C for 24 hours, centrifugation for collecting the upper phase, three times extraction of CoQ10 with hexane (1:1), concentration of CoQ10 to dryness and redissolved in 100 % ethanol. The obtained CoQ10 was analyzed by HPLC and the result showed the appearance of a CoQ10 peak on the chromatographic pattern (Fig. 2F) in comparison with the standard CoQ10 pattern (Fig. 2E). This result confirmed that CoQ10 was successfully extracted from the cell mass of recombinant *A. tumefaciens*.

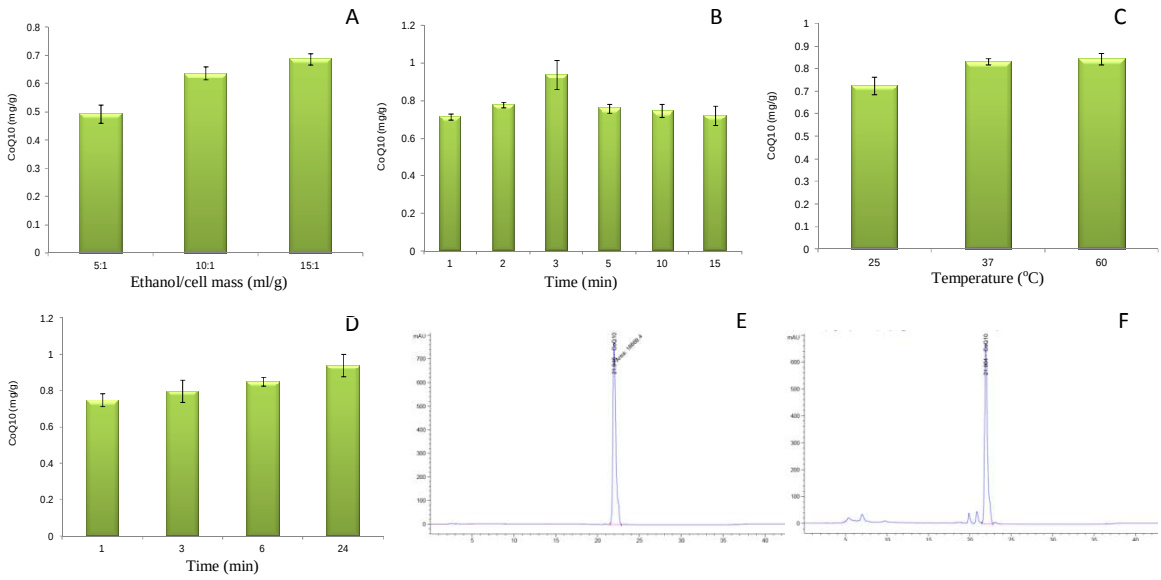

Figure 2. Effect of ethanol/cell mass ratio (A), assimilating time (B), ethanol incubation temperature (C), ethanol incubation time (D) on the CoQ10 recovery from recombinant *A. tumefaciens*. The HPLC chromatography of standard CoQ10 (E) and the extract of recombinant *A. tumefaciens* (F).

3.3. Property studies of extracted CoQ10

3.3.1. The effect of temperature on the stability of CoQ10

CoQ10 solution was incubated at different temperatures (4 ^{o}C, 25 ^{o}C, 37 ^{o}C, 60 ^{o}C) for 144 h and the remaining CoQ10 amount was determined after each 24 hours. The results showed that the CoQ10 amount was almost no change at all incubation temparatures after 144 hours (Fig. 4A). The obtained result coincided with the observation by Fir et al. [7]. This result indicated that CoQ10 was thermostable and this result is valuable for extraction, processing, storage and application of CoQ10.

3.3.2. The effect of pH on the stability of CoQ10

The study result showed that CoQ10 was relatively stable in the pH range of 6-9. Relative stability of CoQ10 reached 95 % after 120 h (Fig. 3B). However, the stability was decreased in the acidic condition. The CoQ10 amount was decreased approximately by 18 % after 24 h and 32 % after 120 h.

3.3.3. Effect of light on the stability of CoQ10

To determine the stability of CoQ10 under the light, CoQ10 solution was exposed to the fluorescent light for 144 hours and the day-night light for 72 hours. The results showed that the CoQ10 amount was decreased 32 %, 56 %, and 72% after 24 h, 48 h and 144 h, respectively, under the fluorescent light (Fig. 4C). The CoQ10 amount was significantly decreased (41 % after 24 h and 70 % after 48 h) when exposed to the day-night light. In the same conditions, the CoQ10 amount was no change when it was completely darkened. The significant decrease of CoQ10 under the day-night light may be due to direct effect of UV light to CoQ10. According to [7] CoQ10 was destroyed by 27 % under the UV light after 120 min. Previous study also showed that CoQ10 was destroyed by 80 % after 15 days exposed to the day-night light [8].

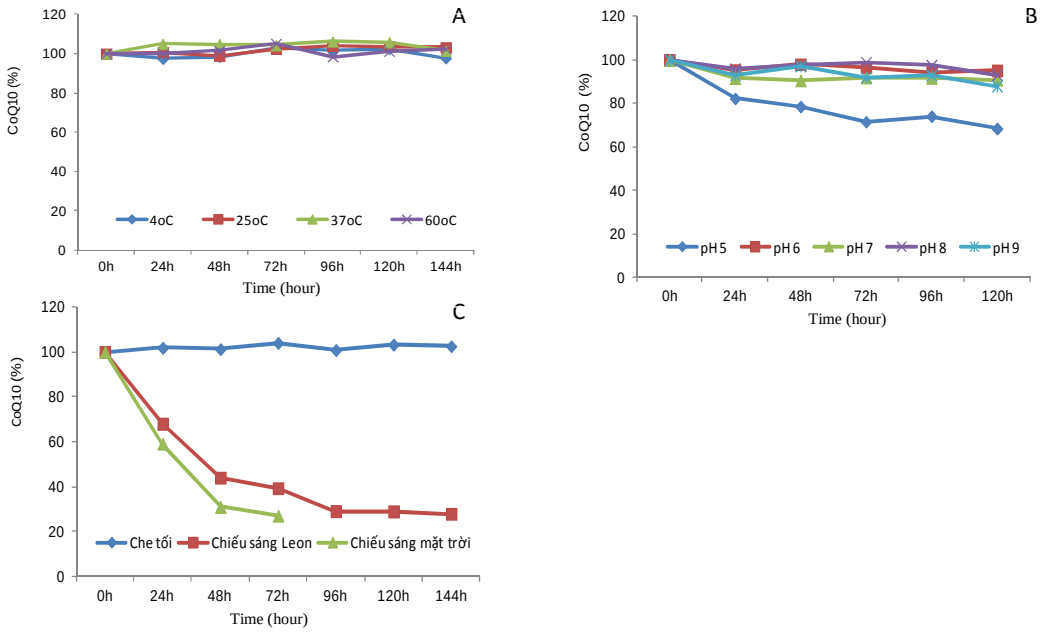

Figure 3. The effect of temperature (A), pH (B), light (C) on the stability of CoQ10 extracted from recombinant *A. tumefaciens.*

4. CONCLUSIONS

Four different methods including sonication, hydrocloric acid, enzyme and ethanol treatment were used to break the cell membrane for recovering the CoQ10. The result indicated that ethanol treatment method was the best method for the extraction of CoQ10. Appropriate conditions for ethanol treatment were determined, including: ethanol/cell mass (10:1), 3 min assimilation by sonication, incubation temperature 37 ^{o}C for 24 h. Property study indicated that CoQ10 was sensitive to light, and decreased by 32 and 56 % after 1 and 2 days, respectively. The CoQ10 amount was no change under 4 – 60 ^{o}C after 144 h. CoQ10 was stable under the pH range of 6.0 – 9.0.

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TÓM TẮT

NGHIÊN CỨU QUY TRÌNH TÁCH CHIẾT VÀ MỘT SỐ ĐẶC TÍNH CỦA COENZYME Q10 TỪ CHỦNG *AGROBACTERIUM TUMEFACIENS* TÁI TỔ HỢP

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Bài báo này trình bày một số kết quả về tách chiết và đặc tính của CoQ10, một sản phẩm từ *A. tumefaciens* tái tổ hợp. Bốn phương pháp gồm phá vỡ tế bào bằng siêu âm, xử lí bằng axit HCl, xử lí bằng cồn và xử lí enzyme kết hợp với các bước tách chiết đã được thực hiện và kết quả cho thấy phương pháp xử lí bằng cồn cho hiệu quả cao nhất. Với phương pháp này, một số điều kiện tách chiết thích hợp được xác định là nhiệt độ 37 ^{o}C và thời gian 24 giờ, tỉ lệ cồn/sinh khối là 10:1 (ml/g). Kết quả nghiên cứu đặc tính cho thấy CoQ10 nhạy cảm với ánh sáng, hàm lượng CoQ10 giảm đi 32 và 56 % sau 1 và 2 ngày tương ứng. Hàm lượng CoQ10 không bị giảm trong dải nhiệt độ 4 – 60 ^{o}C sau 144 giờ. CoQ10 bền trong dải pH 6.0 – 9.0. Những kết quả thu được sẽ được áp dụng để tiến hành tách chiết CoQ10 ở quy mô lớn, phục vụ nghiên cứu tạo chế phẩm CoQ10 để sản xuất thử nghiệm thực phẩm chức năng.

Từ khóa: Agrobacterium tumefaciens, coenzyme Q10 (CoQ10), tách chiết, đặc tính.