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ORIGINAL PAPER

Enhanced production of CoQ_{10} by newly isolated *Sphingomonas* sp. ZUTEO3 with a coupled fermentation–extraction process

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Abstract The use of coenzyme Q_{10} (CoQ_{10}) as a complementary therapy in heart failure will increase in proportion to the growth of the ageing population and the expansion of statins consumption. Economical production of CoQ_{10} by microbes will become more important due to the growing demands of the pharmaceutical industry. Process simplification and integration might be one desirable pathway for economic production of CoQ_{10} by microbial fermentation. In this report, the effect of a coupled fermentation–extraction process on CoQ_{10} production by newly isolated *Sphingomonas* sp. ZUTEO3 was evaluated. It was found that the CoQ_{10} yield of the coupled process was significantly higher than that of the traditional process. As optimal conditions in our experiment, 2% soybean oil was added to the original culture to enhance cell membrane permeability, and 50 mL hexane was added to the 30 h culture as an extracting solvent for the subsequent coupled fermentation–extraction process. The maximal yield of CoQ_{10} reached 43.2 mg/L and 32.5 mg/g dry cell weight after 38 h of total fermentation period. The coupled process represents one potential pathway for CoQ_{10} production with even higher yield and lower cost. This is the first report of CoQ_{10} production by *Sphingomonas* sp. using a coupled fermentation–extraction process.

Keywords Coenzyme Q_{10} · Enzyme · Extraction · Microbial fermentation · Fermentation–extraction coupled process

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Introduction

Coenzyme Q_{10} (also known as ubiquinone-10) has been used successfully as an orally administrated prophylaxis and therapy for various diseases such as cardiovascular disease and mitochondrial respiratory-chain diseases [2], because of its inert toxicity and minimal side effects. It also could be used as an antioxidant in cosmetics and pharmaceuticals, because of its role in protecting membrane phospholipids, lipoproteins and DNA from free radical-induced oxidative damage [22]. In addition, CoQ_{10} is usually recommended as a supplement to 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors (statins), which are common drugs for patients with severe heart failure [2, 3, 17, 19]. The use of coenzyme Q_{10} as a complementary therapy in heart failure will increase in proportion to the growth of the ageing population and the expansion in statins consumption. Economical production of coenzyme Q_{10} using biological processes will become more important due to the growing demands of the pharmaceutical industry.

Coenzyme Q_{10} can be produced by chemical synthesis [18], semi-chemical synthesis [13] and microbial conversion. Wild-type stains and chemical mutants of various microorganisms, including bacteria (e.g. *Agrobacterium*, *Rhodobacter*, *Paracoccus*) and yeasts (e.g. *Candida*, *Rhodotorula*, *Saitoella*) have been reported as coenzyme Q_{10} producers in patent applications [23]. Further strain development, and optimization of fermentation strategies and environmental parameters has resulted in yield improvement of coenzyme Q_{10} in mutant strains [9, 10, 24]. Recombinant *Escherichia coli* containing the *Gluconobacter suboxydans* decaprenyl diphosphate synthase gene [20] and metabolic modification could also improve yields of coenzyme Q_{10} [11, 15, 25].

However, among CoQ_{10}-producing strains in previous reports, *Agrobacterium tumefaciens* exhibited the highest yield of CoQ_{10} production in a fed-batch fermentation process. As reported by Ha et al. [5, 6], the CoQ_{10} yield reached 458 mg/L with high cell density (53.6 g/L) after fed-batch fermentation, and increased to 626.5 mg/L CoQ_{10} at a pilot scale (300 L) in a pH-stat fed-batch system. However, lower cost and higher yield of CoQ_{10} still remains a major research aim of biochemical engineering for the industrial production of CoQ_{10} using biological processes.

Process simplification and integration might be one favorable pathway towards economic production of CoQ_{10} by microbial fermentation. For example, the process of simultaneous extraction and fermentation, which has been applied successfully in lactic acid fermentation [16], would be helpful in improving the yield of CoQ_{10} by microbes. It is well known that the accumulation of primary metabolites can result in feedback inhibition in cells [12, 21]. As a kind of primary metabolite within the cell, the gradual accumulation of CoQ_{10} would result in inhibition of the bioconversion of CoQ_{10}. Prompt removal of the CoQ_{10} out of the cell might be helpful in order to maintain continual synthesis of CoQ_{10}. Furthermore, in-situ extraction of CoQ_{10} by a non-aqueous phase contributes to the simplification of downstream processing and cost decrease. However, there has been little in the literature about coupled fermentation–extraction processes for CoQ_{10} production in the past decades.

To develop a more economical process for CoQ_{10} production by microbes, we recently isolated the CoQ_{10}-producing strain *Sphingomonas* sp. ZUTEO3. In this study, the effect of a coupled fermentation–extraction process for CoQ_{10} production by *Sphingomonas* sp. ZUTEO3 was evaluated.

Materials and methods

Chemicals

Coenzyme Q_{10}(purity above 99.9%) was purchased from Wako Pure Chemical Industries (Osaka, Japan). Other chemicals were purchased locally.

Strain and culture conditions

Sphingomonas sp. ZUTE03, which exhibited greater CoQ_{10} production, was isolated from soil from the banks of the Qiangtang River, Zhejiang Province, China. The strain has been deposited with the China Center for Type Culture Collection (CCTCC) at Wuhan University, Wuhan, China, under the accession number CCTCC M207084.

The seed medium contained 20 g/L glucose, 10 g/L peptone, 10 g/L yeast extract, and 5 g/L NaCl. The production medium contained 15 g/L glucose, 10 g/L $(NH_4)_2SO_4$, 1 g/L yeast extract, 0.5 g/L KH_2PO_4, 1.5 g/L Na_2HPO_4, 0.5 g/L $MgSO_4{\cdot}7H_2O$. The initial pH of the above medium was adjusted to 7.0 with 2 M NaOH. The prepared medium was then transferred into 250 or 500 mL Erlenmeyer flasks and autoclaved at 115°C for 20–30 min. All experimental cultures were incubated at 180 rpm and 28°C.

Coenzyme Q_{10} production with traditional methods

In this study, the traditional method for CoQ_{10} production was batch-type cultivation as follows. One loop of strain ZUTE03 from a slant was inoculated into 50 mL of seed medium in a 250 mL flask. After 24 h incubation at 180 rpm and 28°C, 7.5 mL of the broth was inoculated into a 500 mL flask containing 150 mL fermentation culture broth, and then incubated at 28°C and 180 rpm for 24 h. The growth of strain ZUTE03 and CoQ_{10} yield were monitored at regular intervals.

Selection of extraction solvent for coupled fermentation–extraction process

To select a suitable extraction solvent for the coupled fermentation–extraction process, a substance capable of acting as a cell membrane permeability accelerant to allow the coupled process to be achieved is required. Based on a previous report by Benga et al. [1], 1% soybean oil was designated as the cell membrane permeability accelerant in this experiment. Then, hexane, acetone, propanediol, and olive oil were added as the extraction solvent at the beginning of fermentation to evaluate their effect on CoQ_{10} extraction from the broth. In a 500 mL flask, 50 mL extraction solvent was mixed with 100 mL production medium containing 1% soybean oil. After 36 h of fermentation, the extract solvent was collected from the upper layer of the broth as the analytical sample for CoQ_{10} yield. The remaining broth was collected for biomass measurement.

Selection of cell membrane permeability accelerant for coupled fermentation–extraction process

After the optimal extraction solvent was determined by the above test, the optimal cell membrane permeability accelerant needed to be chosen from some potential substances such as plant oil and organic substances. In this experiment, we chose to examine the effect of soybean oil, propanediol and Tween-80 on the coupled process. These substances were added to 100 mL production medium at the beginning of fermentation and mixed with 50 mL extraction solvent. After 36 h of fermentation, the biomass and CoQ_{10} yield were measured in the broth and in the extraction solvent, respectively.

Effect of concentration and time of addition of membrane-permeability accelerant on CoQ_{10} production in the coupled fermentation–extraction process

After the optimal membrane-permeability accelerant was selected, the effect of concentration and addition time was determined. In this experiment, the time intervals for solvent addition and sampling were determined according to the results of the above traditional fermentation.

Effect of extraction solvent addition time and extraction time on CoQ_{10} production by the coupled fermentation–extraction process

After the optimal extraction solvent was selected, the effect of addition time and extraction time was determined. In this experiment, the time intervals for solvent addition and sampling were also determined according to the results of the above traditional fermentation.

Extraction and measurement of CoQ_{10}

For the broth resulting from conventional fermentation, cells were harvested by centrifugation at 15,000 rpm and 4°C for 15 min with a centrifuge (Sanyo, Japan). After washing the cell pellet twice with distilled water, 2 g cell pellet was transferred into a fresh round-bottom flask (150 mL) and mixed with 0.35 g pyrogallic acid, 1.25 g KOH, 9.5 mL methanol and 3.5 mL distilled water. The mixture was kept in reflux state at a 90°C water bath for 30 min before cooling rapidly with tap water, and then transferred to a separating funnel where it was mixed with 40 mL hexane. After vortexing vigorously for 5 min, the upper solution of the organic phase was collected in a fresh tube. The extraction procedure was repeated twice and the extracted solvent was collected together followed by a condensing process in a rotary vacuum evaporator. After overnight storage at 4°C, impurities such as cholesterol would be precipitated. After removal of impurities by filtration, the solvent quantity was adjusted accurately to 50 mL with hexane before CoQ_{10} assay.

For the broth resulting from the coupled fermentation–extraction process, to which soybean oil and hexane had been added at different times, the upper layer of the non-aqueous phase could be recovered by a separating funnel. The non-aqueous phase was then condensed in the rotary vacuum evaporator. After overnight storage at 4°C, impurities such as cholesterol would be precipitated. After removal of impurities by filtration, the solvent quantity was adjusted accurately to 50 mL with hexane before CoQ_{10} assay.

Coenzyme Q_{10} concentrations in all the liquid samples were analyzed by high-performance liquid chromatography (HPLC) (SPD-10AVP, SHIMADZU, Japan) equipped with Agilent SB-C18 (4.6 $\times$ 150 mm^2). A mixture of methanol and hexane (83:17 by volume) was used as the mobile phase at a flow rate of 0.6 ml/min, UV detector wavelength 275 nm, and a sampling quantity of 20 μL.

Analysis of cell mass

Biomass could be calculated according to a standard curve of the relationship between optical density of cells and dry cell weight (DCW) of *Sphingomonas* sp. ZUTE03. Cells were harvested by centrifugation (12,000 rpm, 10 min, 4°C) from 5 mL liquid culture each time. The optical density of cells was determined at 550 nm using a 752 spectrophotometer (Shanghai, China) after the cells were washed three times with 5 mL 50 mM potassium phosphate buffer (pH 7.0).

Statistical analysis methodology

Every sample in the experiment was in triplicate. Software Origin 6.0 was used to draw the figures with error bars.

Results and discussion

Coenzyme Q_{10} production by *Sphingomonas* sp. ZUTEO3 using the traditional process

Sphingomonas sp. ZUTE03 was isolated using solanesol as precursor, and showed greatest CoQ_{10} production among strains isolated from the banks of the Qianjiang Qiver, PR China. Under optimal conditions of batch-type cultivation, as determined in our previous experiments [14], *Sphingomonas* sp. ZUTEO3 could produce about 1.14 mg/L and 0.48 mg/g-DCW CoQ_{10} as maximal yield (Fig. 1) at 30 and 24 h, respectively. It was suggested that an improvement of CoQ_{10} yield by *Sphingomonas* sp. ZUTEO3 was required. Further strain development by mutation or metabolic modification would be one method to achieve this. However, process simplification and integration might be a more favorable pathway towards economic production of CoQ_{10} by microbes. Thus, the focus of this paper is to evaluate the effect of a coupled process on CoQ_{10} production by *Sphingomonas* sp. ZUTEO3.

Selection of extraction solvent for coupled fermentation–extraction process

Having selected 1% soybean oil as the accelerant of cell membrane permeability, the effect of hexane, acetone, propanediol, and olive oil on CoQ_{10} production was determined. It was found that acetone and propanediol were impossible

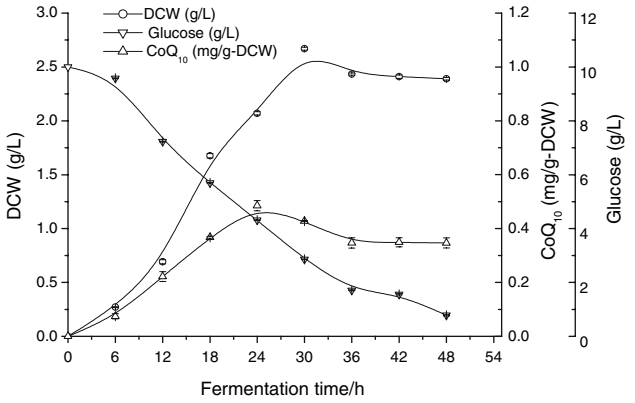

Fig. 1 Time course of coenzyme Q_{10} (CoQ_{10}) production and growth of *Sphingomonas* sp. ZUTEO3 using the traditional process under optimal conditions

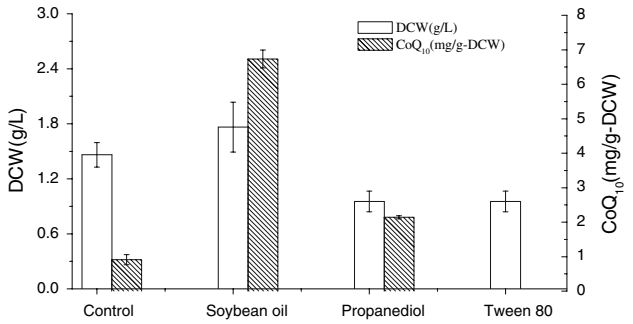

Fig. 2 Effect of different accelerants of cell membrane permeability on CoQ_{10} production by *Sphingomonas* sp. ZUTEO3 in the coupled fermentation–extraction process

to separate from the mixture because of their preferential solubility in water. Olive oil was also impossible to separate from the mixture, and significantly increased the viscosity of the final broth. Hexane could be easily separated from the final broth. Therefore, hexane was one favorable solvent for the extraction of CoQ_{10} directly from the broth. However, hexane is easy to volatilize and may be harmful to cell growth. Thus, it was first necessary to determine the optimal amount of hexane to be added to the medium.

To determine the effect of different amounts of hexane on CoQ_{10} production, cultures were prepared in 500 mL flasks with 20, 30, 40, 50, and 60 mL hexane mixed with 100 mL medium, respectively, and incubated for 36 h at 180 rpm. The results (data not shown) showed that the yield of CoQ_{10} in 20, 30, and 60 mL hexane was lower than that in 40 mL hexane. The yield of CoQ_{10} in 50 mL hexane was almost the same as that in 40 mL hexane. Moreover, when 50 mL hexane was added, stratification formed more easily in the final broth and a higher amount of hexane (85%) could be recycled than with the other hexane amounts. Therefore, 50 mL hexane was selected as the extraction solvent in the following experiments. The effect of addition time of hexane is shown in Fig. 5.

Selection of cell membrane permeability accelerant for the coupled fermentation–extraction process

CoQ_{10} is one kind of fermentation product within the cell. Improved cell membrane permeability is necessary for the application of the combined processes of fermentation and extraction. Two grams of either soybean oil, propanediol, or Tween-80—all common solvents used to improve cell membrane permeability—was added to 1 L medium at the beginning of fermentation to evaluate their effects on CoQ_{10} production (Fig. 2).

It was found that both cell mass and CoQ_{10} yield were highest when soybean oil was added. Propanediol produced higher specific CoQ_{10} content and lower cell mass than that of the control. The addition of Tween-80 also resulted in a lower cell mass than that of the control. In addition, Tween-80 made the final broth too viscous to separate the organic solvent directly from the broth for CoQ_{10} measurement. Thus, Tween-80 was not suitable for the coupled process. As reported by Benga et al. [1], the addition of soybean oil could increase the permeability of the cell and depress the resistance of the membrane to penetration. In addition, it could improve cell growth. Propanediol is also one kind of organic compound that could increase the permeability of the cell [8] and improve the specific CoQ_{10} content. However, it inhibited cell growth. Therefore, soybean oil was finally chosen as the accelerant of cell membrane permeability of *Sphingomonas* sp. ZUTE03.

Effect of increased concentration of membrane permeability accelerant on CoQ_{10} production

To further test the effect of lower or higher soybean oil concentration, 1%, 2%, 3% and 4% soybean oil was added to the medium at the beginning of cultivation. After 36 h of culture, biomass and CoQ_{10} yield were determined. The results (Fig. 3) showed that the addition of soybean oil at different concentrations had no significant effect on cell growth of *Sphingomonas* sp. ZUTE03. However, the CoQ_{10} yield varied with the concentration of soybean oil. Among the concentrations tested, 2% soybean oil led to the highest CoQ_{10} yield. Lower or higher concentrations of soybean oil resulted in lower CoQ_{10} yield. Therefore, 2% soybean oil was selected for subsequent experiments.

Effect of addition time of cell membrane permeability accelerant on CoQ_{10} production

To further detect the effect of addition time on CoQ_{10} production, 2% soybean oil was added at the beginning, and at 6, 12, and 18 h of fermentation, respectively. The results

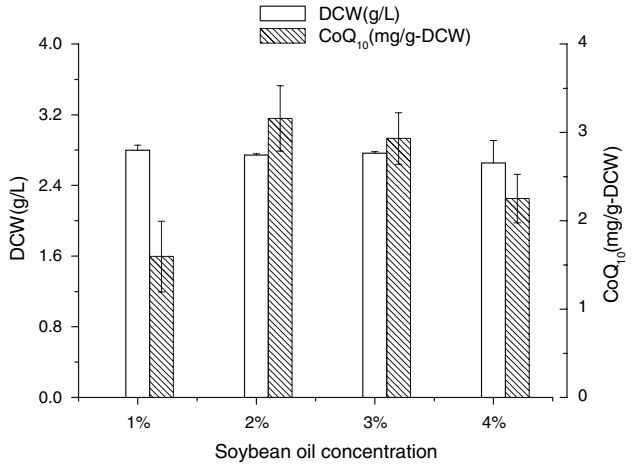

Fig. 3 Effect of soybean oil concentration on CoQ_{10} production by *Sphingomonas* sp. ZUTEO3 in the coupled fermentation–extraction process

are shown in Fig. 4. It was found that different addition times had no significant effect on cell growth. However, the CoQ_{10} yield decreased as addition times extended. Thus, adding soybean oil at the beginning of fermentation was most favorable for CoQ_{10} yield, and should be helpful in improving extraction of CoQ_{10} from the cell at the appropriate time, consequentially improving production of CoQ_{10} within the cell without product feedback inhibition.

Effect of addition time of extraction solvent on CoQ_{10} production

Although CoQ_{10} yield increased when soybean oil and hexane were added at the beginning of fermentation, the time point for hexane addition need optimization for further improvement of CoQ_{10} yield. The optimal time point might be the time at which the CoQ_{10} concentration reaches the threshold value that inhibits its own synthesis. As shown in

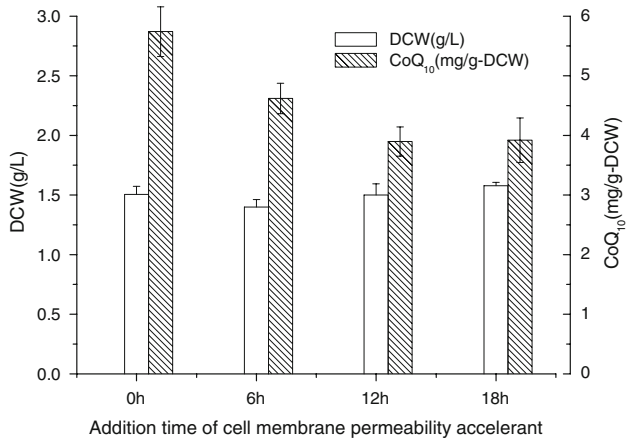

Fig. 4 Effect of soybean oil addition time on CoQ_{10} production by *Sphingomonas* sp. ZUTEO3 in the coupled fermentation–extraction process

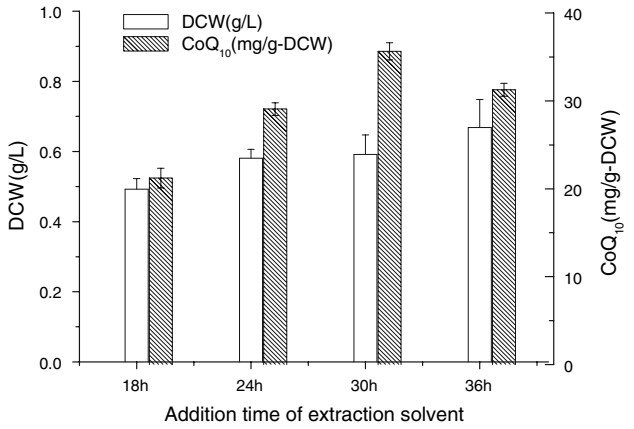

Fig. 5 Effect of time of addition of extraction solvent on CoQ_{10} production by *Sphingomonas* sp. ZUTEO3 in the coupled fermentation–extraction process

Fig. 1, the yield of CoQ_{10} reached a maximal value at 24 h, suggesting that the CoQ_{10} concentration might reach this threshold value at 24 h of traditional batch fermentation. However, the addition of hexane might inhibit cell growth. Therefore, hexane should be added in the middle or late exponential phase to avoid significant inhibition of cell growth. Based on the cell growth curve in Fig. 1, hexane was added at 18, 24, 30, or 36 h in order to evaluate the optimal time of hexane addition. The results shown in Fig. 5 indicated that 30 h was the optimal time point for the highest CoQ_{10} yield while 24 h was not. Therefore, 30 h was selected as the time point for hexane addition in subsequent experiments.

Effect of extraction time after addition of extraction solvent on CoQ_{10} production

After hexane addition at 30 h, the subsequent period was designated as 2, 4, 6, 8, and 12 h respectively to further detect the effect of extraction time on CoQ_{10} production. The final measurement results of biomass and CoQ_{10} yield are shown in Fig. 6. CoQ_{10} yield was found to

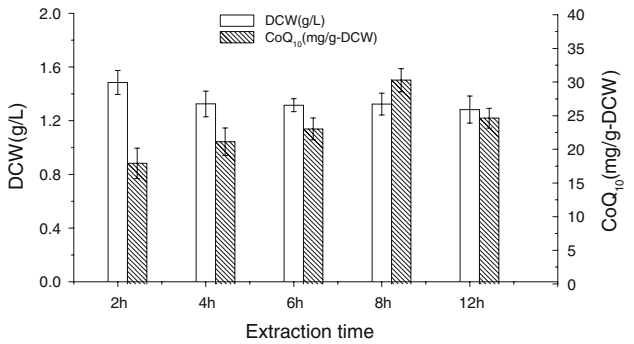

Fig. 6 Effect of extraction time on CoQ_{10} production by *Sphingomonas* sp. ZUTEO3 in the coupled fermentation–extraction process

Table 1 Coenzyme Q_{10} (CoQ_{10}) production by various microorganisms with different process

Process	Microorganism	CoQ_{10} concentration, mg/L	Specific CoQ_{10} content, mg/g-DCW	Working volume and time	Reference
Fed-batch	*Agrobacterium tumefaciens*	71.5	2.1	2 L, 96 h	Gu et al. [4]
Batch	*Agrobacterium tumefaciens*	320	6.61	2.8 L, 96 h	Ha et al. [5]
Fed-batch	*Agrobacterium tumefaciens*	458	8.54	2.8 L, 96 h	
		446	8.24	250 L, 96 h	
		441	8.05	2,800 L, 96 h	
Fed-batch	*Agrobacterium tumefaciens*	626.5	9.25	160 L, 120 h	Ha et al. [6]
Fed-batch	*Agrobacterium tumefaciens*	562.3	9.1	2.8 L, 96 h	Ha et al. [7]
Fed-batch	Recombinant *Escherichia coli*	25.5	0.247	1 L, 38 h	Park et al. [18]
Batch	*Sphingomonas* sp. ZUTEO3	1.14	0.48	0.15 L, 30 h	This study
Coupled process	*Sphingomonas* sp. ZUTEO3	43.2	32.5	0.15 L, 38 h	This study

DCW Dry cell weight

reach a maximal value (43.2 mg/L, 32.5 mg/g-DCW) after an 8 h period of simultaneous extraction and fermentation, i.e., after total of 38 h of fermentation. In comparison to the traditional process illustrated in Fig. 1, the coupled process with *Sphingomonas* sp. ZUTEO3 produced a specific CoQ_{10} content more than 60 times higher than that achieved with traditional fermentation.

Although the CoQ_{10} concentration of *Sphingomonas* sp. ZUTEO3 after 38 h of the coupled fermentation–extraction process was lower than that found in fed-batch process in previous reports [4–8], this study achieved the highest specific CoQ_{10} content (Table 1). *Agrobacterium tumefaciens* exhibited the highest CoQ_{10} concentration among all strains for CoQ_{10} production. However, the specific CoQ_{10} content of *Agrobacterium tumefaciens* was lower than that of our strain, suggesting that the coupled process presented here might potentially be used in combination with fed-batch process to further improve CoQ_{10} production by *Agrobacterium tumefaciens*. In the same way, we need to increase the cell mass in the coupled process to the same high level as that obtained in fed-batch process to achieve higher CoQ_{10} production by *Sphingomonas* sp. ZUTEO3.

In addition, in-situ extraction of CoQ_{10} by non-aqueous phase in a coupled process contributes to the simplification of downstream processing. In particular, the extraction process after fermentation, which contains a series of steps such as cell lysis and centrifugation, can be omitted, which certainly could result in decreased production costs.

Therefore, further optimization of conditions for the coupled process, along with the selection of strains or mutants that show increased productivity, should allow even higher levels of CoQ_{10} production at lower cost and improve industrial production by microbes.

Conclusion

Soybean oil and hexane were found to be key substances to improve the production of CoQ_{10} by *Sphingomonas* sp. ZUTEO3 with a coupled fermentation–extraction process. Soybean oil might improve the release of CoQ_{10} from the cells, and hexane was a suitable solvent to extract the product (mainly CoQ_{10}) from the broth without any significant detrimental effect on cell growth.

By coupling the processes of fermentation and extraction, more CoQ_{10} could be extracted directly from the broth of *Sphingomonas* sp. ZUTEO3. The coupled process led to maximal CoQ_{10} yields of 43.2 mg/L and 32.5 mg/g-DCW. Furthermore, the coupled process contributes to the simplification of downstream processing and might result in decreased production costs.

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