Appl Biochem Biotechnol (2010) 160:1441–1449
DOI 10.1007/s12010-009-8576-1

Cultivation of *Rhodobacter sphaeroides* in the Stirred Bioreactor with Different Feeding Strategies for CoQ_{10} Production

Hong-Wei Yen · Chang-Yu Feng · Jia-Lin Kang

Received: 14 January 2009 / Accepted: 18 February 2009 /
Published online: 10 March 2009
© Humana Press 2009

Abstract The logistic growth model combined with the Luedeking-Piret equation was adopted in this study to model the batch production of CoQ_{10} in the cultivation of *Rhodobacter sphaeroides*. The simulation results indicated that CoQ_{10} production was a primary metabolite. As being a primary metabolite, a longer cell growing stage would tend to accumulate more biomass and lead to a higher CoQ_{10} concentration being produced. In this context, a fed-batch operation by molasses feeding was performed to increase the biomass and subsequent CoQ_{10} production. Three different molasses feeding strategies were operated in this study. Results suggested that the fed-batch operation with molasses controlled at 10±1 g/l could increase the cell mass and CoQ_{10} concentration to reach their maximum values of 18.6 g/l and 83.8 mg/l, respectively, nearly 2.2 times and 1.9 times their respective values obtained in the batch cultivation.

Keywords Kinetic model · CoQ_{10} · Luedeking-Piret · Simulation · Fed-batch

Introduction

Ubiquinone-10 (also called CoQ_{10}) is a common material found in animals, plants, and microorganisms as a coenzyme involved in the respiratory chain reactions. The chemical structure of CoQ_{10} is 2.3-dimethoxy-methyl-benzoquinone with a side chain of 10 monosaturated isoprenoid units [1]. This compound is used as an essential component of ATP generation in the respiratory chains of mitochondria responsible for the electron transfer. The excellent therapeutic effects of CoQ_{10} on heart disease and Parkinson's disease had been reported [2]. It was also recommend as a health supplement to patients who were taking statin drugs for cholesterol control and to decrease the occurrence of heart attack and stroke. The production of CoQ_{10} can be either through chemical synthesis, semi-chemical synthesis, or microbial conversion [3, 4]. Many different microorganisms have been reported

H.-W. Yen (✉) · C.-Y. Feng · J.-L. Kang
Department of Chemical and Materials Engineering, Tunghai University, Taichung, Taiwan, Republic of China
e-mail: hwyen@thu.edu.tw

to be capable of producing CoQ_{10}, such as *Pseudomonas, Agrobacterium*, *Rhodobacter*, and *Paracoccus*. Among that, *Rhodobacter sphaeroides* had been tested the ability of producing CoQ_{10} and identified as one of the talented CoQ_{10} producing strains [5, 6].

In general, a kinetic model was a straight way to describe the biological process, including the product formation, substrate consumption, and biomass growth. A well kinetic model not only could explain the biological process, but also could predict the effects of each parameter on the result. However, the simulation of kinetic model had been always somewhat challenging. The kinetic models could be linear or non-linear, single-phase or multiphase [7]. Linear kinetic models included constant rate and first order kinetics. Non-linear kinetic models comprised the Logistic, Monod, and other defined functional equations [8]. The Logistic growth model combined with the Luedeking–Piret equations to simulate cell growth and the formation rate of the desired metabolic product are widely used in literatures [7].

The fed-batch operation by nutrients feeding was often adopted to avoid substrates limitation in fermentation process. Nutrients feeding could extend the cell growth, which might increase the primary metabolic product production. It is reported that the biomass and CoQ_{10} production was increased to 53.6 g/l and 458 mg/l in a fed-batch cultivation, as compared to 48.4 g/l and 320 mg/l of batch cultivation by *Agrobacterium tumefaciens* [9]. Even for the CoQ_{10} production by a recombinant *Escherichia coli*, the fed-batch operation was also proven able to increase biomass and CoQ_{10} concentration. A high cell density of 103 g/l obtained in the fed-batch fermentation of recombinant *E. coli* increased the CoQ_{10} concentration to 25.5 mg/l, which were nearly 6.9 times higher than the corresponding value for batch fermentation [10].

The scope of this study was confined to the investigation of CoQ_{10} production by *R. sphaeroides* in batch and fed-batch operation, and using the Logistic equation combined with the Luedeking–Piret equation to simulate biomass growth, CoQ_{10} production, and carbon source consumption in batch cultivation. In the fed-batch operation, the effects of substrate-molasses feeding were investigated by three different feeding strategies, including the one-time feeding, the control-feeding by maintaining molasses of medium at 5±1 g/l, and the control-feeding by maintaining molasses of medium at 10±1 g/l. The aim of this study is to simulate batch process and to investigate the effects of nutrients feeding strategy on the enhancement of CoQ_{10} production.

Materials and Methods

Microorganism and Cultivation

R. sphaeroides, a photosynthetic bacteria, was purchased from the Bioresource, Collection, and Research Center (BCRC 13100), Taiwan. The freeze-dried *R. sphaeroides* was mixed with sterilized water and inoculated into the medium consisting of (g/l) trypton 15, soyton 15, and NaCl 5 (BCRC suggestion medium). After aerobic incubation at 28 °C for 48 h, a stock of *R. sphaeroides* was prepared by mixing the broth with sterilized glycerol at 10% and was stored in the freezer at 0 °C. This was used as the stock strain (with 10^5 counts/ml) for further inoculation. The inoculum culture medium contained the same ingredients as the medium for the stock preparation. A volume of 50-ml inoculum culture medium was inoculated with a vial containing 1 ml of stock solution. After 48 h of incubation at 30 °C on a rotary shaker (150 r/min), a total 50 ml of culture solution was inoculated into a 5-l lab-scale fermentor (Biotop Ltd, Taiwan) containing 2 l of fermentation medium for the

aerobic cultivation. The fermentation medium used for CoQ_{10} production contained (per liter) 20 g molasses (industrial grade), 10 g yeast extract, 7 g $(NH_4)_2SO_4$, 0.5 g K_2HPO_4, 0.5 g KH_2PO_4, 0.25 g $MgSO_4$ $7H_2O$, 0.008 g vitamin B1 [11], and the fermentation medium was sterilized at 121 °C for 15 min before the inoculation. The pH level was maintained at 7.0 automatically by 1 N NaOH and 1 N HCl solution, if necessary. The aeration and agitation were maintained at 1 vvm and 200 rpm respectively through the whole fermentation process. As observed in the batch process, the cell growth would step into the late exponential phase after 48 h cultivation. Therefore, in the fed-batch operation of one-time feeding, the molasses feeding was performed one time at the 48th hour by feeding 200 ml nutrient solution (containing 200 g molasses/l) into the fermentor. The purpose of one-time molasses feeding was to increase the carbon source concentration back to the initial value of 20 g molasses/l. Due to no on-line molasses measured device for getting a precise molasses concentration control, a manual adjustment of molasses feeding rate was performed every several hours (6 to 12 h interval) to ensure the molasses of fermentation medium within a defined range (at 5±1 g/l and 10±1 g/l, respectively). The value of biomass and molasses measured at each time point would be used for the calculation of biomass yield ($Y_{x/s}$) and specific growth rate (μ), which would be adopted to estimate how much molasses could possibly be consumed in the next several hours. The molasses feeding rate for the batch controlled at 5±1 g/l and 10±1 g/l would be decided upon on the predicting molasses required and the current molasses concentration measured.

Analytical Methods

The cell mass concentration was determined by measuring the optical density at 660 nm with a spectrophotometer (Hitachi) and that on a dry cell weight (DCW) was calculated according to a previously determined conversion equation (1 OD_{660}=0.192 g DCW/l). Total sugar concentrations were analyzed by phenol–sulfuric acid method using molasses as a standard [12]. The CoQ_{10} concentration was assayed by a high-performance liquid chromatography (Hitachi). The volume of 10 ml fermentation broth was first centrifuged and the supernatant was removed. The remaining cell mass was thoroughly mixed with 10 ml of extraction solution consisting of chloroform and methanol at a ratio of 2/1 shaking for 2 h. The supernatant containing CoQ_{10} was analyzed by HPLC. The conditions for HPLC analysis were: column, Vercopack inertsil 10 ODS-3 (250×4.68 mm); temperature, ambient; mobile phase, EtOH/MeOH=2/1; flow rate, 1.0 mL/min; and detector UV detector at 275 nm [11].

Kinetic Models

The logistic (Eq. 1) models would be used to simulate the specific biomass growth rate (μ) in this study. The Luedeking–Piret equation combined with the Logistic growth model were used to simulate CoQ_{10} (expressed as P) production (Eq. 2) and molasses (expressed as S) consumption (Eq. 3) [13]. This Luedeking–Piret model was originally developed for the lactic acid fermentation process by *Lactobacillus delbrucckii*. According to this model, the product formation rate depended linearly upon both the instantaneous biomass concentration, x, and the biomass growth rate, dx/dt. A carbon substrate (molasses) was assimilated for cell growth, for metabolites production as well as for cells maintenance energy required. Therefore, the molasses consumption could be reasonable regarded as partially linked to cell growth rate and cell concentration as following the description in a Luedeking–Piret

type model (Eq. 3); where $\gamma=1/Y_{x/s}$ and δ=ms. The value of $Y_{x/s}$ and ms stood for the biomass yield and the specific substrate consumption rate. In the simulation of metabolic product by the Luedeking–Piret type equation, a high α value incorporated with a low β value would represent a primary metabolic product production model. All kinetic parameters shown in the models would be estimated through the iteration calculation of genetic algorithm (GA). The optimal values of the parameters were approximated by minimizing the discrepancy between the model predictions and corresponding experimental data.

$$\mu = \mu_m\left(1 - \frac{x}{m}\right) \quad \text{(logistic)} \tag{1}$$

$$\frac{dP}{dt} = \alpha\frac{dx}{dt} + \beta x \quad \text{(Luedeking} - \text{Piret for product)} \tag{2}$$

$$-\frac{dS}{dt} = \gamma\frac{dx}{dt} + \delta x \quad \text{(Luedeking} - \text{Piret for substrate)} \tag{3}$$

x, the biomass concentration; x_m, the maximum biomass concentration

Results and Discussion

Simulation of Batch Cultivation Process

As seen in Fig. 1, the logistic growth model combined with the Luedeking–Piret equation was applicable for the simulation of CoQ_{10} production, molasses consumption, and cell

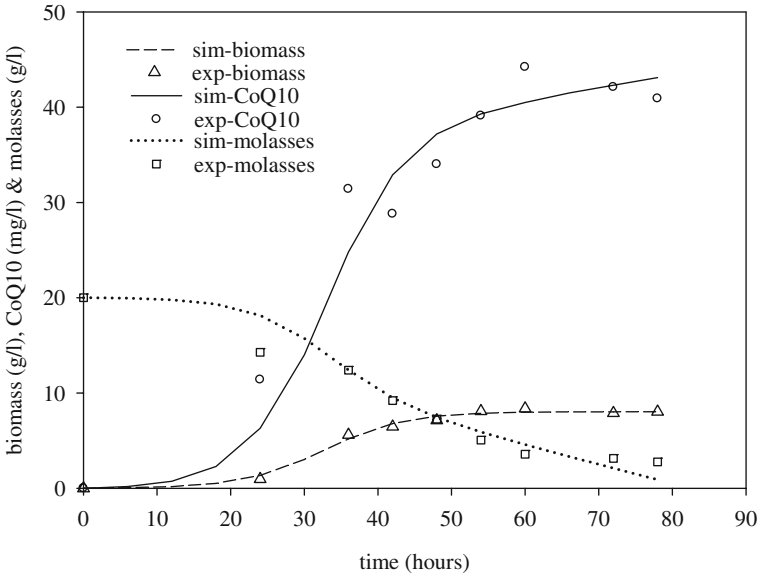

Fig. 1 Experimental data and simulation results using the logistic equation and the Luedeking-Piret type equations in batch cultivation

growth in the batch cultivation of *R. sphaeroides* (R^2 would be 0.936, 0.924, and 0.888, respectively). The kinetic parameters for the biomass simulation would be 0.183 (1/h) of μ_m and 8.03 (g/l) of x_m. The batch culture of *R. sphaeroides* using chemically defined medium showed a classical growth trend. As shown in the figure, the cells entered the exponential growth phase and the following stationary phase after a short lag phase. The initial carbon source (20 g molasses/l) would be rapidly consumed during the exponential growth phase. After 60 h cultivation, the molasses concentration would decline to nearly 3 g/l and no significant molasses consumption was observed after then. If the consumption of carbon source was stop, it reflected that the cell growth would step into the stationary phase, in which only the maintenance energy was required for the cells.

On the simulation of CoQ_{10} production by the Luedeking–Piret type equation, a relatively lower β value (1.1×10^{-5} g/g/h) as compared to the α value (0.0048 g/g) was found. A high ratio of α value related to β value (α/β=436) indicated that CoQ_{10} should be classified as a primary metabolite in the cultivation of *R. sphaeroides*, which implied that the CoQ_{10} production would strongly depend on the biomass growth rate. This statement was also supportive from the biochemical function of CoQ_{10} in cells, which CoQ_{10} was an essential component on the respiratory chain. Therefore, the increase of biomass concentration would be naturally leading to a high CoQ_{10} concentration found. On the substrate simulation by the Luedeking–Piret equation, the estimated values of γ and δ would be 2 g molasses/g cell and 0.025 g molasses/g cell/h, respectively. These values corresponded to 0.5 g cell/g molasses of the biomass yield ($Y_{x/s}$) and 0.025 g molasses/g cell/h of energy maintenance coefficient (m_s).

The Fed-batch Operation by Molasses Feeding

As suggested previously, CoQ_{10} was regarded as a primary metabolite. Therefore, the increase of cell growth stage could increase the CoQ_{10} production. To extend the growing phase, a fed-batch operation was performed at the 48th hour of the late exponential phase. Three different feeding strategies were investigated, including the one-time feeding, the nutrients control-feeding by maintaining medium molasses at 5±1 g/l and at 10±1 g/l, respectively. The time course data would be presented in Figs. 2, 3, and 4, respectively.

In Fig. 2 of the one-time molasses feeding, a total 40 g of molasses was fed into a 2-l working medium at the 48th hour to raise the carbon source back to the initial concentration. After feeding, molasses would rapidly be consumed from the concentration of 19.3 g/l to 9.8 g/l within 6 h. About 32 h after feeding, the molasses concentration would go down to about 3 g/l, almost the same level as the molasses concentration before feeding. However, the cell growth stage in the fed-batch of one-time feeding was apparently longer than the period observed in the batch. The maximum cell and CoQ_{10} concentration were 12.1 g/l and 56.9 mg/l at the 78th hour, which were slightly higher than 8.4 g/l and 44.2 mg/l obtained in the batch.

The one-time feeding was succeeding to extend the growth stage and led to an increase of both biomass and CoQ_{10} concentration. However, the one-time feeding would result in the severely vibration of molasses concentration. It would not be a well process control strategy. Therefore, two control-feeding strategies by adjusting feeding rate to achieve 5±1 and 10±1 g/l of medium molasses were initiated after 48 h cultivation. As shown in Figs. 3 and 4 of medium molasses controlled at 5±1 and 10±1 g/l, both control-feeding strategies would result in the increase of biomass and CoQ_{10} production. The maximum biomass and CoQ_{10} were 11.6 g/l and 53.7 mg/l in the 5±1 g molasses/l controlled batch and 18.6 g/l and 83.8 mg/l in the 10±1 g molasses/l controlled batch. The control-feeding batch at 5±

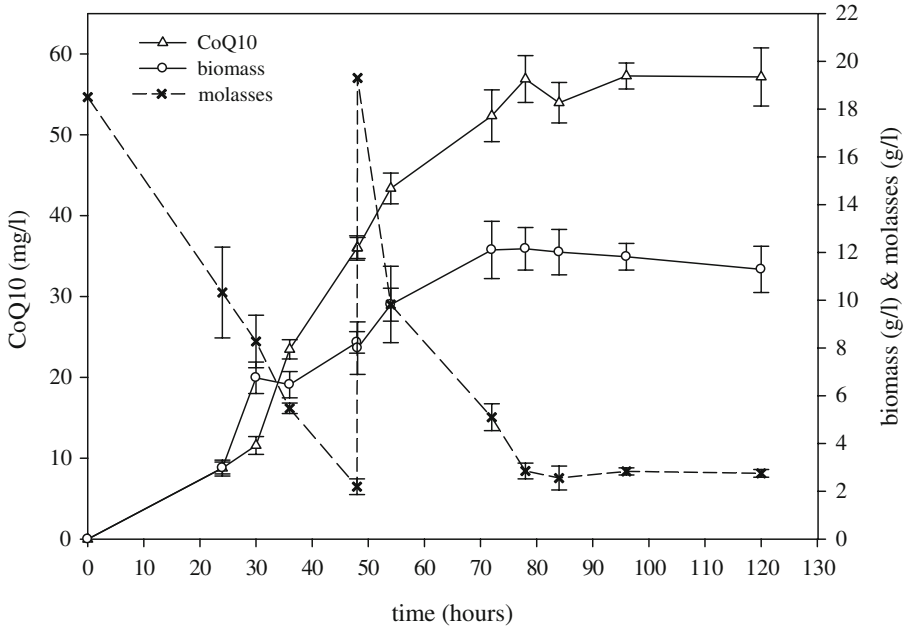

Fig. 2 Time course data of CoQ_{10}, biomass, and molasses concentration in a one-time feeding batch (molasses feeding at the 48th hour)

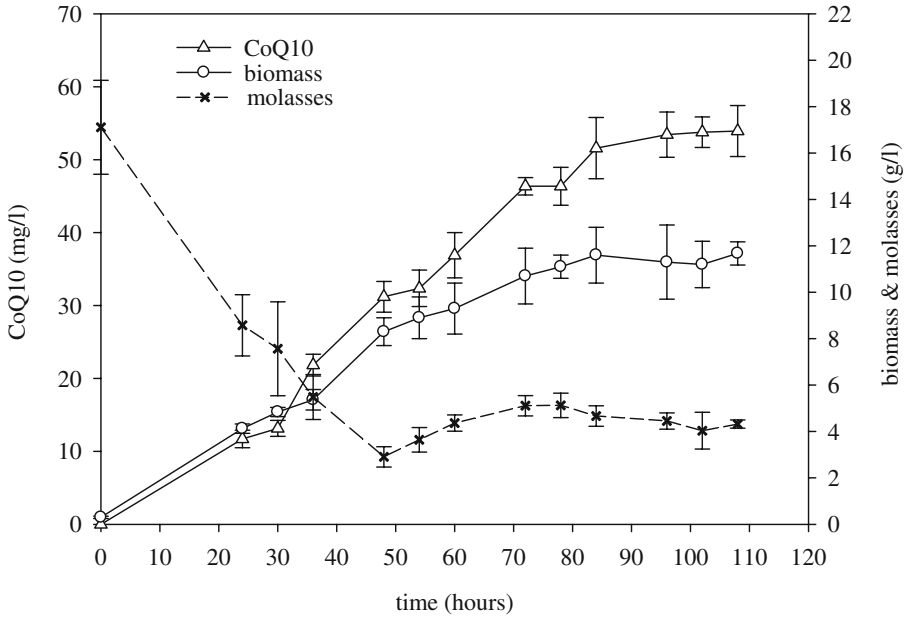

Fig. 3 Time course data of CoQ_{10}, biomass, and molasses concentration in a fed-batch fermentation (molasses concentration was controlled at 5±1 g/l by adjusting feeding rate after 48 h cultivation)

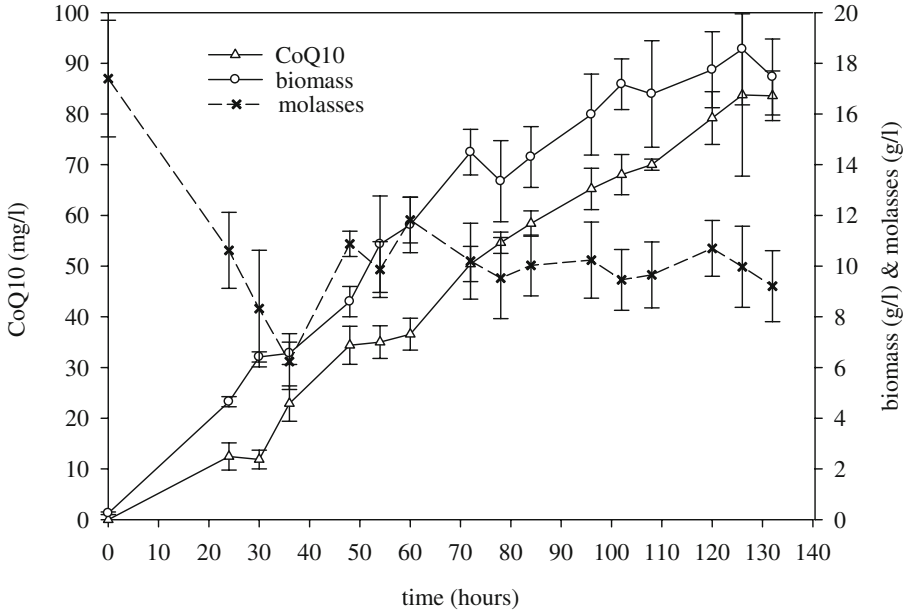

Fig. 4 Time course data of CoQ_{10}, biomass, and molasses concentration in a fed-batch fermentation (molasses concentration was controlled at 10±1 g/l by adjusting feeding rate after 48 h cultivation)

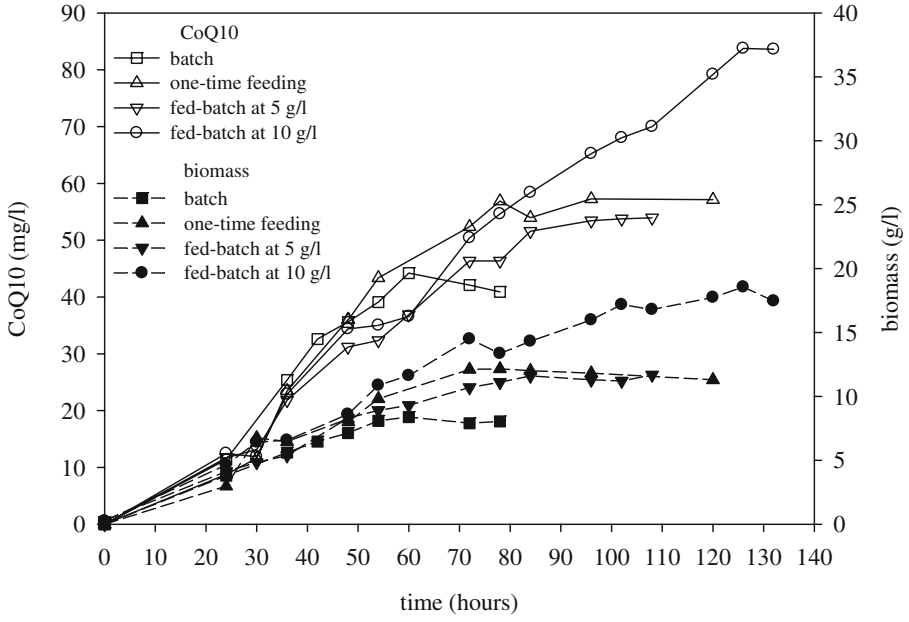

Fig. 5 The comparison of CoQ_{10} and biomass obtained in the batch, the one-time feeding batch, and the fed-batch with molasses controlled at 5±1 and 10±1 g/l

1 g molasses/l seemed not to have much different of biomass and CoQ_{10} concentration to the values obtained in the one-time feeding. These results suggested that the medium molasses controlled at 5±1 g/l might not be high enough to enhance cell growing. Conversely, the fed-batch with the molasses concentration controlled at 10±1 g/l would have obvious enhancement on biomass and CoQ_{10} production. The comparison of CoQ_{10} and biomass production between the batch, the one-time feeding batch, the control-feeding batch at 5±1 g molasses/l and at 10±1 g molasses/l was shown in Fig. 5. As seen in the figure, the residual molasses concentration was a key parameter for keeping cell growing and for the production of CoQ_{10} by *R. sphaeroides*. The maximum CoQ_{10} concentration obtained from the fed-batch of molasses controlled at10±1 g/l would be 83.8 mg/l, which was almost 1.9 times the value of batch operation (44 mg/l). However, as seen in Table 1, the molasses content in the fed-batch operation was slightly lower than the value obtained in the batch. Such results suggested that the nutrients feeding would direct metabolic flux toward biomass production more than CoQ_{10} production. Many studies in literatures already revealed that a high CoQ_{10} content would be obtained under a limited oxygen condition during the batch process [11]. To create a severe oxygen-limited environment by turning down the agitation or decreasing aeration rate after the carbon source feeding might be worth to investigate in the future trials.

Conclusions

The logistic growing model incorporated with the Luedeking–Piret equation presented a very good description on the biomass formation, CoQ_{10} production, and substrate consumption. The resulting biomass yield ($Y_{x/s}$) of 0.5 and the energy maintenance coefficient (m_s) of 0.025 obtained in the batch would be useful for the design of a continuous CoQ_{10} production process by *R. sphaeroides*. The high value of α/β ratio obtained in the Luedeking–Piret simulation model indicated that CoQ_{10} was regarded as a primary metabolite, and a fed-batch operation could successfully lead to a longer growth stage with a higher biomass concentration and a higher CoQ_{10} concentration. The results of three different feeding strategies in the fed-batch operation suggested that the molasses concentration controlled at 10±1 g/l during the fermentation process could have the maximum CoQ_{10} concentration of 83.8 mg/l obtained, nearly 2.2 times the values obtained in the batch process.

Table 1 The comparison between batch and fed-batch with different feeding strategies.

	Batch	One-time feeding	Fed-batch at 5±1 g/l	Fed-batch at 10±1 g/l
Maximum biomass (g/l)	8.4	12.1	11.6	18.6
Maximum CoQ_{10} concentration (mg/l)	44	56.9	53.7	83.8
CoQ_{10} product rate (mg/l/h)[a]	0.733	0.729	0.525	0.664
CoQ_{10} content (mg CoQ_{10}/g biomass)[a]	5.25	4.70	4.62	4.5

[a] Based on the maximum CoQ_{10} concentration obtained

Acknowledgements The authors wish to thank the National Science Council of the R.O.C. for the financial supports (NSC 95-2221-E-029-007) in this study.

References

1. Kawamukai, M. (2002). *Journal of Bioscience & Bioengineering*, *94*, 51–57.
2. Lieberman, A., Lyons, K., Levine, J., & Myerburg, R. (2005). *Parkinsonism & Related Disorders*, *11*, 81–84. doi:10.1016/j.parkreldis.2004.07.012.
3. Choi, J.H., Ryu, Y.W., & Seo, J.H. (2005). *Applied Microbiology & Biotechnology*, *68*, 9–15. doi:10.1007/s00253-005-1946-x.
4. Cluis, C.P., Burja, A.M., & Martin, V.J.J. (2007). *Trends in Biotechnology*, *25*, 514–521. doi:10.1016/j.tibtech.2007.08.008.
5. Sasaki, K., Watanabe, M., Suda, Y., Ishizuka, A., & Noparatnaraporn, N. (2005). *Journal of Bioscience & Bioengineering*, *100*, 481–488.l. doi:10.1263/jbb.100.481.
6. Yoshisa, H., Kotani, Y., Ochiai, K., & Araki, K. (1998). *The Journal of General & Applied Microbiology*, *44*, 19–26. doi:10.2323/jgam.44.19.
7. Benkortbi, O., Hanini, S., & Bentahar, F. (2007). *Biochemical Engineering Journal*, *36*, 14–18. doi:10.1016/j.bej.2006.06.015.
8. Songa, H., Jang, S.H., Park, J.M., & Lee, S.Y. (2007). *Biochemical Engineering Journal*, *40*, 107–115. doi:10.1016/j.bej.2007.11.021.
9. Ha, S.J., Kim, S.Y., Seo, J.H., Oh, D.K., & Lee, J.K. (2007). *Applied Microbiology & Biotechnology*, *74*, 974–980. doi:10.1007/s00253-006-0744-4.
10. Park, Y.C., Kim, S.J., Choi, J.H., Lee, W.H., Park, K.M., Kawamukai, M., et al. (2005). *Applied Microbiology & Biotechnology*, *67*, 192–196. doi:10.1007/s00253-004-1743-y.
11. Yen, H.W., & Chiu, C.H. (2007). *Enzyme & Microbial Technology*, *4*, 1600–1604.
12. Wee, Y.J., Kima, J.N., Yunb, J.S., & Ryua, H.W. (2004). *Enzyme & Microbial Technology*, *34*, 568–573. doi:10.1016/j.enzmictec.2004.08.008.
13. Luedeking, R., & Piret, E.L. (1959). *Journal of Biochemical & Microbiological Technology & Engineering*, *1*, 393–412. doi:10.1002/jbmte.390010406.