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Maximum Production of 1,4-Dihydroxy-2-Naphthoic Acid by Fed-Batch and Anaerobic/Aerobic Culture of *Propionibacterium freudenreichii* ET-3

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ABSTRACT
Maximum production strategy of 1,4-dihydroxy-2-naphthoic acid (DHNA) was investigated using a fed-batch and anaerobic/aerobic culture of *Propionibacterium freudenreichii* ET-3. DHNA is a precursor of menaquinone (MK) and the cell produces DHNA and MK in the molar ratio of 3:2 by consuming lactose in skim milk powder medium under normal anaerobic condition. We found that in anaerobic fed-batch culture, which limited lactose concentration, the cell stopped producing MK. As a result, DHNA concentration in fed-batch culture increased as lactose feeding rate decreased, and the maximum DHNA concentration in fed-batch culture was 0.40 mM, which is 3.3-fold that in anaerobic batch culture. However, propionate accumulation inhibited and then stopped DHNA production in anaerobic fed-batch culture. Our previous study showed that the cell could consume propionate under aerobic condition and this knowledge based on metabolic flux distribution for DHNA production was applied here. The cell produced DHNA continuously by switching from anaerobic condition to aerobic condition after lactose feeding. DHNA concentration in the optimal condition was 0.51 mM, which is 4.2-fold that in anaerobic batch culture.

KEYWORDS *Propionibacterium freudenreichii*, DHNA production, menaquinone production, cell growth, fed-batch culture, aerobic culture.

INTRODUCTION

Recently, propionibacteria, particularly *Propionibacterium freudenreichii* ET-3, were found to produce 1,4-dihydroxy-2-naphthoic acid (DHNA), which is a precursor of menaquinone (MK) (Isawa et al., 2002; Furuichi et al., 2006a). DHNA stimulates the growth of bifidobacteria and improves the condition of the human intestine (Satomi et al, 1999; Hojo et al., 2002). Cultures containing DHNA are expected to be applied to developing functional foods by adding the culture broth to dairy products such as yoghurt and milk-based drinks, because propionibacteria have a long history of being used for starter cultures such as for Swiss cheese production in the dairy industry. However, there are some problems to be overcome for using a large amount of the culture broth of *P. freudenreichii* ET-3 for the foods described above, because it has not only a high production cost but also strong bitterness. The bitterness is caused by potassium in K_2CO_3 added as a neutralizer. In our previous study (Furuichi et al., 2006a), the amount of potassium required for unit DHNA production (termed "potassium per DHNA" hereafter) was determined as an index of the quality of the culture. To use cultures containing DHNA for a wide variety of foods, it is necessary to increase DHNA production and decrease "the potassium per DHNA" in the cultures.

Under anaerobic condition, propionibacteria produce propionate and acetate by consuming carbon sources (Piveteau, 1999), and propionate accumulation inhibits DHNA production (Furuichi et al., 2006a). On the other hand, the cell produces DHNA continuously by consuming propionate and producing acetate under aerobic condition. Furthermore, aerobic culture decreases "the potassium per DHNA", because the metabolic change induced by aerobic culture requires no K_2CO_3 to maintain a constant pH of the broth. However, the cell stopped growing after a long exposure to oxygen, and a sharp increase in dissolved oxygen (DO) concentration was observed (Furuichi et al., 2006a; Furuichi et al., 2006b). Moreover, DHNA is eliminated from culture broth after such an increase in DO concentration, because the structure of DHNA is sensitive to oxidation. To decrease the stress caused by oxygen, the celll was cultured by repeating anaerobic and aerobic phases

alternately (Furuichi et al., 2006b). In this study, We investigated the optimal fed-batch method for DHNA production and developed a cultivation method achieving a high DHNA concentration by combining fed-batch and anaerobic/aerobic cultures.

MATERIALS AND METHODS

Microorganism and media

P. freudenreichii ET-3 was used throughout this study. The strain was isolated from Swiss cheese and stocked in the Food Functionality Research Institute of Meiji Dairies, Odawara, Japan. The preculture medium contained 10% (wt/wt) whey powder (Meiji Dairies, Tokyo, Japan) and 0.1% (wt/wt) beer yeast extract (Asahi Food and Health, Tokyo, Japan). The fermentation medium contained 3% (wt/wt) skim milk powder (Meiji Dairies, Tokyo, Japan), 3.4% milk protein concentrate (Murry Goulburn, Melbourne, Australia) and 0.5% (wt/wt) beer yeast extract (Asahi Food and Health). The whey powder was digested by a protease at 47ºC for 3 h. The skim milk powder and milk protein concentrate were dissolved into a proper amount of water after mixing and were digested by protease (Amano A, Amano Pharmaceutical, Tokyo, Japan) at 47ºC for 6 h. During enzyme digestion, pH was controlled at 6.6–7.0 by K_2CO_3. These media were autoclaved at 121ºC for 15 min.

Cultivation method

Culture temperature was controlled at 33ºC, and the pH of the medium was adjusted to 6.5 using K_2CO_3 during the culture period. Anaerobic culture and aerobic culture were performed using filter-sterilized nitrogen gas and oxygen gas, respectively. The flow rates of nitrogen and oxygen gases were controlled at 0.4 liter min^{-1}. All the fed-batch cultures in this study were started after anaerobic batch culture by supplying a lactose solution at a constant rate because of simplicity of the operation and carried out under anaerobic condition. The concentration of the lactose solution was 1.5 M, and lactose feeding rate was maintained in the range of 0.62–2.54 ml h^{-1}. The working volume of fed-batch cultures was maintained at 2.0 liter by withdrawing samples at regular intervals. Agitation speed was controlled at 100 rpm in all the cultures.

Measurement of DHNA, menaquinone, organic acids

DHNA and MK was quantified in accordance with our previous study (Furuichi et al., 2006a). DHNA and MK concentration was spectrophotometrically measured at 254 nm and 430 nm, respectively using a high-performance liquid chromatography (HPLC) system (Shimadzu 10A HPLC system, Shimadzu, Kyoto, Japan). The amount of organic acid was spectrophotometrically measured at 445 nm using an HPLC system by the postcolumn method (HITACHI L-7000 HPLC system, HITACHI; RS Pack KC-811 column, 8 × 300 mm, SHOWA DENKO, Tokyo, Japan; mobile phase, 0.02% $HClO_4$; reaction solution, 0.125 g of bromthymol blue, 2.129 g of $Na_2HPO_4 \cdot 12 H_2O$ and 20 ml of 0.1 N NaOH, total volume brought to 1000 ml with 0.02% $HClO_4$; temperature, 63ºC; flow rate, 1.0 ml min^{-1}; and injection volume, 20 μ). The relative standard deviations of the experimental data treated in the experiments are all ranged from 5 to 8 % (n=3 to 10).

RESULTS

Effect of lactose feeding rate on *P. freudenreichii* ET-3 metabolism and DHNA production

To investigate the optimal lactose feeding rate for the DHNA production, fed-batch culture with various feeding rates was carried out. At first, anaerobic fed-batch culture, in which lactose solution was fed at 1.90 ml h^{-1}, was planned to continue for 140 h to add totally 0.40 mol of lactose. This fed-batch culture was started after 72 h of anaerobic batch culture. In this culture, DHNA production was stopped when totally 0.24 mol of lactose was added (data not shown). The maximum DHNA concentration in this culture was 0.30 mM. The propionate concentration at the stopping-time of DHNA production was 0.35 M, which was sufficiently high to inhibit DHNA production (Furuichi et al., 2006a). Therefore, finally 0.24 mol of lactose was employed as the total amount of lactose to be added during fed-batch culture for the subsequent fed-batch cultures.

To investigate the effect of lactose feeding rate on DHNA production, the cell was cultured with various lactose feeding rates under anaerobic condition (Fig. 1). All fed-batch cultures were started at 72 h and fed 0.24 mol of lactose. The feeding rates in these fed-batch cultures were maintained at 2.54, 1.90, 1.31, 0.90 and 0.62 ml h^{-1}, and took 63, 86, 122, 178 and 260 h to feed 0.24 mol of lactose, respectively. As shown in Fig. 1, the dry cell weight (DCW) and MK concentrations decreased as feeding rate decreased. However, DHNA concentration increased as feeding rate decreased. The maximum DHNA concentration in fed-batch culture was 0.40 mM, which is about 3.3-fold that in anaerobic culture. Although the production rates of propionate and acetate decreased as feeding rate decreased, the final concentrations of these organic acids were almost the same in all the cultures.

For comparison with batch culture, the data of the anaerobic culture of the cell is described in the next section. It is considered that limiting the specific lactose consumption rate did not affect lactose metabolism and energy yield of lactose, and that the

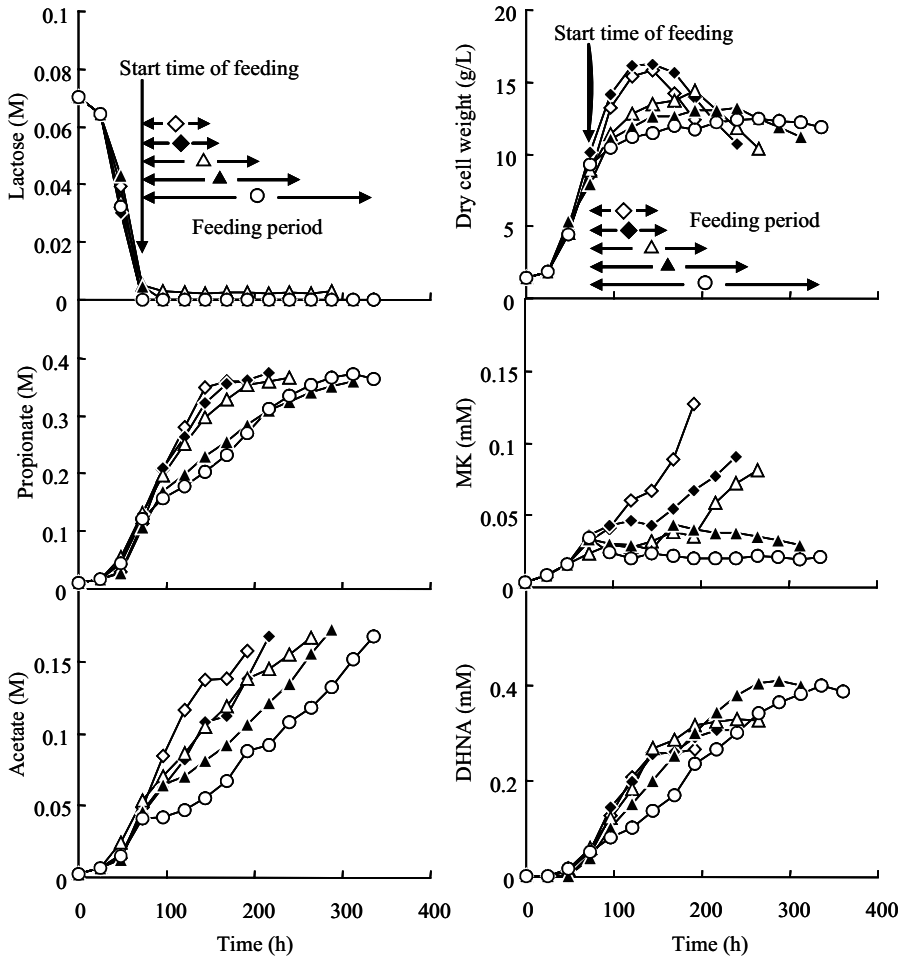

FIG 1. Effect of feeding rate on cell growth and productions of organic acids, MK and DHNA. In these cultures, 0.24 mol of lactose was fed, and lactose feeding rates were maintained at 2.54, 1.90, 1.31, 0.90 and 0.62 ml h^{-1}. The concentration of the lactose solution fed was 1.5 M, and the working volumes of these cultures were maintained at 2.0 liter. The vertical and horizontal arrows represent the start time and the periods of lactose feeding, respectively. The symbols within the horizontal arrows correspond to those in the plots: open diamonds, 2.54 ml h^{-1}; filled diamonds, 1.90 ml h^{-1}; open triangles, 1.31 ml h^{-1}; filled triangles, 0.90 ml h^{-1}; open circles, 0.62 ml h^{-1}.

increase in DHNA production was induced by increasing the ratio of energy utilization for DHNA production to cell growth and MK production. DHNA yields for lactose in fed-batch cultures were higher than that in batch culture, and yields increased as specific lactose consumption rate decreased. On the other hand, cell and MK yields for lactose decreased as specific lactose consumption rate decreased, because cell growth and MK production were depressed corresponding to the limitation of lactose concentration. As a result, DHNA production might be increased.

Effects of oxygen transfer rates on *P. freudenreichii* ET-3 metabolism and DHNA production

The anaerobic culture of the cell was first carried out as a control experiment. As shown in Fig. 2, the productions of propionate and acetate stopped after lactose depletion, and the concentrations of these organic acids remained constant afterwards. DHNA production also stopped after lactose depletion, and the final DHNA concentration in this culture was 0.12 mM.

To investigate the effects of oxygen supply on DHNA production, the cell was cultured at various airflow rates. In anaerobic-aerobic switching culture, aerobic culture was started after 72 h of anaerobic culture and airflow rate was controlled at 1.0, 2.0 and 4.0 *l*/min during the aerobic phase. The oxygen transfer rates (OTRs) were 0.08, 0.23 and 0.35 mg/(*l*·h) for the cultures with airflow rates of 1.0, 2.0 and 4.0 *l*/min, respectively. As shown in Fig. 2, for the culture with the airflow rate of 1.0 *l*/min, the formation patterns of propionate and acetate were similar to those for the anaerobic culture. The DHNA concentration in the culture with the airflow rate of 1.0 *l*/min was 0.11 mM, which was the same as that in the anaerobic culture. However, in the cultures with the airflow rates of 2.0 and 4.0 *l*/min, the cell consumed propionate after lactose depletion, and DHNA

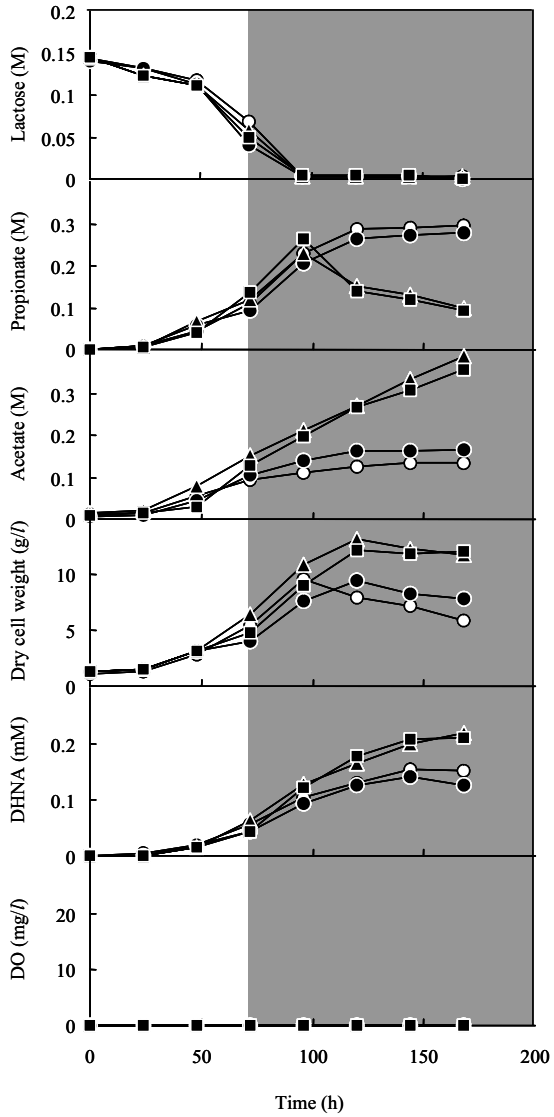

FIG. 2. Time courses of anaerobic-aerobic switching culture with different air flow rates. Aeration was started at 72 h (grey background represents aerobic phase), and agitation speed was set at 150 rpm. Symbols: open circles, anaerobic culture (control profile); closed circles, air flow rate at 1.0 *l*/min; closed triangles, air flow rate at 2.0 *l*/min; closed squares, air flow rate at 4.0 *l*/min.

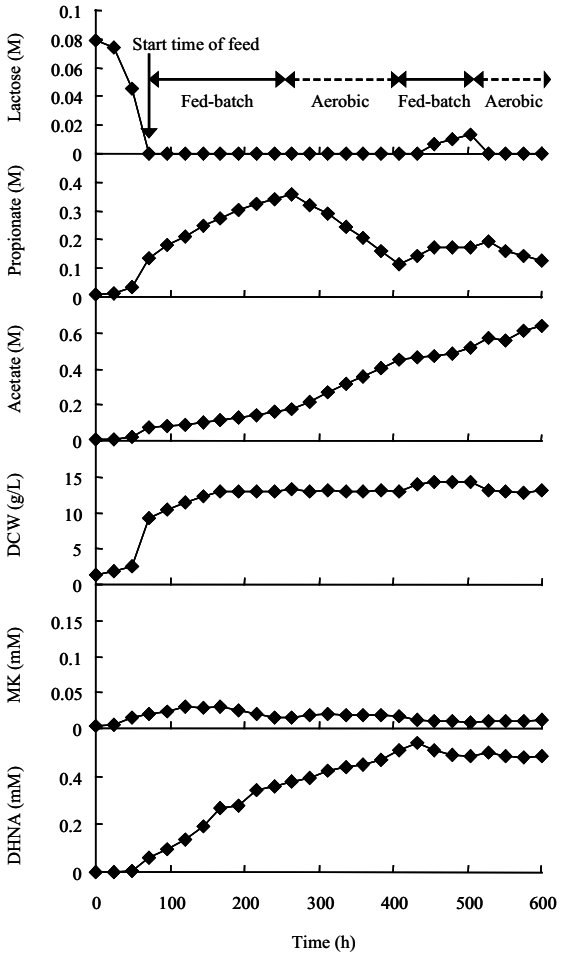

FIG 3. Profiles of cultivation repeating fed-batch and aerobic cultures alternately. During fed-batch culture, feeding rate was maintained at 0.90 ml h^{-1}, and in the first and the second feeding periods, 0.24 and 0.12 mol of lactose were fed, respectively. The concentration of the lactose solution fed was 1.5 M, and the working volume of the culture was maintained at 2.0 liter. The vertical arrow represents the start time of lactose feeding. The solid and dotted horizontal arrows represent the periods of lactose feeding and aerobic culture, respectively.

concentration reached 0.22 and 0.21 mM, respectively. DO concentration remained constant at about 0 mg/*l* irrespective of airflow rate. However, excess supply of oxygen gave negative result such that a sudden DO concentration increase was observed in the aerobic phase, and DHNA concentration decreased sharply at the same time (data not shown).

DHNA production by repeating fed-batch and aerobic cultures alternately

Considering the effect of limitation of lactose supply and aerobic condition on DHNA production, an optimal operation by combination of fed-batch and anaerobic/aerobic cultures was investigated. DHNA production by repeating anaerobic fed-batch and aerobic batch cultures alternately was carried out (Fig. 3). In this cultivation, aerobic batch culture was switched to anaerobic fed-batch culture before an increase in DO concentration occurred. As shown in Fig. 3, fed-batch and aerobic batch cultures were repeated two times alternately in this experiment. In the first and second anaerobic fed-batch cultures, 0.24 and 0.12 mol of lactose were fed at 0.90 ml h^{-1}, and lactose feeding was continued for 178 and 90 h, respectively. The cell stopped DHNA production immediately after the start of the second anaerobic fed-batch culture. DO concentration remained constant at about 0 mg $liter^{-1}$ throughout this cultivation, and at the stopping-time of DHNA production, the propionate and acetate concentrations were 0.14 and 0.45 M, respectively. The maximum

TABLE 1. Amount of potassium required for unit DHNA production in fed-batch culture and the cultivation combining fed-batch and aerobic cultures[a]

Culture condition	Lactose feeding rate (ml h^{-1})	Amount of potassium required for unit DHNA production (g mM^{-1})
Batch		0.21 (100)
Fed-batch	2.54	0.16 (74)
	1.90	0.14 (65)
	1.31	0.13 (61)
	0.90	0.10 (48)
	0.62	0.11 (50)
Cultivation combining fed-batch and aerobic cultures	0.90	0.08 (38)

[a] The concentration of lactose solution fed was 1.5 M, and the working volume of these cultures was maintained at 2.0 liter.

The values in parenthesis are the percentages of corresponding anaerobic batch culture values.

DHNA concentration in this cultivation was 0.52 mM, which was almost the same as that of the cultivation combining fed-batch and aerobic cultures described above.

The quinone skeleton of MK is derived from DHNA, and MK is synthesized by combining DHNA and isoprene. Therefore, inhibiting MK production by stopping isoprene synthesis is considered to enhance the release of DHNA out of the cell. In 1993, Rohmer et al. reported that isoprene is synthesized from pyruvate and glyceraldehyde 3-phosphate in several bacteria. Although, to our knowledge, there have been no studies investigating the pathway of isoprene synthesis in propionibacteria, propionibacteria are also considered to synthesize isoprene from pyruvate and glyceraldehyde 3-phosphate, because many types of bacterium have recently been shown to utilize this pathway to synthesize isoprene (Rohmer et al., 1996; Schwender et al., 1996). Under anaerobic condition, propionibacteria consume carbon sources via the Embden-Meyerhof-Parnas (EMP) pathway, which involves glyceraldehyde 3-phosphate (Piveteau, 1999), but, under aerobic condition, consume propionate via the reverse methylmalonyl CoA pathway, which does not involve glyceraldehyde 3-phosphate (Furuichi et al., 2006b; Ye et al., 1999). Our previous study (Furuichi et al., 2006a) showed that the cell stopped MK production when utilizing the reverse methylmalonyl CoA pathway. It is considered that the metabolism under aerobic condition, which stopped the supply of isoprene precursors inhibited MK production. Fed-batch culture, which decreased lactose concentration, could decrease specific lactose consumption rate. This indicates that fed-batch culture decreased the flux of the EMP pathway. Fed-batch culture might have decreased the amounts of glyceraldehyde 3-phosphate and pyruvate to be utilized for isoprene production by decreasing the flux of the EMP pathway. For efficient DHNA production, specific lactose consumption rate should be controlled at the minimum value in the range that maintains specific DHNA production rate constant.

Propionate accumulation is considered to stop DHNA production in the end of fed-batch culture, because at the stopping-time of DHNA production, the propionate concentration was 0.35 M, which was sufficiently high to inhibit DHNA production (Furuichi et al., 2006a). Therefore, aerobic culture was started at the end of lactose feeding. Under aerobic condition, the cell consumes propionate and produces acetate via the reverse methylmalonyl CoA pathway, and produces DHNA continuously while inhibiting MK synthesis (Furuichi et al., 2006a; Furuich et al., 2006b). As shown in Fig. 3, this cultivation revealed that the cell could also consume propionate in the aerobic phase after fed-batch culture, and that the decrease in propionate concentration enabled the cell to produce DHNA continuously.

Acetate accumulation might have stopped DHNA production, because acetate concentration increased continuously throughout the cultivation. At the stopping-time of DHNA production, the acetate concentration was 0.45 M. To investigate the cause of the stop of DHNA production, 0.84 mol of acetate

was added after 48 h of anaerobic batch culture with a 2.0 liter working volume. In this culture, the cell stopped DHNA production immediately after the addition of acetate (data not shown), although DHNA was produced continuously in batch culture without acetate addition. Hence, it is concluded that the acetate accumulation stopped DHNA production completely in the cultivation repeating fed-batch and aerobic cultures alternately.

Amount of potassium required for unit DHNA production in each culture

"Potassium per DHNA" in fed-batch cultures and the cultivation combining fed-batch and aerobic cultures was calculated (Table 1). As shown in Table 1, "the potassium per DHNA" in fed-batch cultures decreased as feeding rate decreased. The minimum value of "the potassium per DHNA" in fed-batch cultures was obtained by maintaining feeding rate at 0.90 ml h^{-1}, and "the potassium per DHNA" in fed-batch culture was one-half that in batch culture. Furthermore, "the potassium per DHNA" in the cultivation combining fed-batch and aerobic cultures was about one-third that in batch culture.

CONCLUSION

This study showed that fed-batch culture, which limited lactose concentration, could increases DHNA production by inhibiting cell growth and MK production (Fig. 1). This indicates that fed-batch culture has a great advantage for DHNA production. Fed-batch culture has been applied to many fermentation processes. However, there are, few studies of fed-batch culture using anaerobic bacteria. Considering the effect of limitation of lactose supply and aerobic condition based on the metabolic flux distribution on DHNA production, an optimal operation by combination of fed-batch and anaerobic/aerobic cultures was investigated. DHNA production by repeating anaerobic fed-batch and aerobic batch cultures alternately was carried out. *P. freudenreichii* ET-3 produced DHNA continuously by switching from anaerobic condition to aerobic condition after lactose feeding. DHNA concentration in the optimal condition was 0.51 mM, which is 4.2-fold that in anaerobic batch culture.

In this study, "the potassium per DHNA" was also determined as an index of the quality of the culture. The minimum value of "the potassium per DHNA" in the cultivation combining fed-batch and aerobic cultures was about one-third that in batch culture. This means that the taste of *P. freudenreichii* ET-3 culture was also drastically improved by the optimal cultivation.

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