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Optimal Aerobic Cultivation Method for 1,4-Dihydroxy-2-Naphthoic Acid Production by *Propionibacterium freudenreichii* ET-3

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To investigate the effects of oxygen supply on *Propionibacterium freudenreichii* ET-3 metabolism and 1,4-dihydroxy-2-naphthoic acid (DHNA) production in detail, the strain was cultured by switching from anaerobic condition to aerobic condition at 72 h (termed anaerobic-aerobic switching culture hereafter) employing different oxygen transfer rates (OTRs) in the range of 0.08–0.90 mg/(*l*·h). It was found that a 0.08 mg/(*l*·h) OTR could not change the metabolism or improve the DHNA production of *P. freudenreichii* ET-3. When the OTR was in the range of 0.23–0.66 mg/(*l*·h), propionate, which inhibits DHNA production significantly, was consumed during the aerobic phase. Final DHNA concentration increased to 0.22 mM, irrespective of OTR. When the OTR was 0.90 mg/(*l*·h), a sudden increase in dissolved oxygen (DO) concentration during the aerobic phase resulted in a sudden decrease in DHNA concentration. To attenuate the stresses caused by propionate and oxygen exposure, we designed an optimal cultivation in which the anaerobic and aerobic phases were repeated three times alternately. As a result, propionate concentration was maintained below the level that inhibits DHNA production, and no DO concentration was detected throughout the culture. The final DHNA concentration in this culture was 0.33 mM, which is 2.7-fold that in the anaerobic culture and 1.5-fold that in the anaerobic-aerobic switching culture.

[**Key words:** *Propionibacterium freudenreichii*, 1,4-dihydroxy-2-naphthoic acid production, oxygen transfer rate, propionibacterial metabolism, aerobic culture, propionate oxidation]

The application of propionibacteria to the food industry has a long history, and propionibacteria have wide commercial applications. For example, in the dairy industry, they are essential in the manufacture of Swiss cheese, and in other food industries, they are used in the production of organic acids, biomass, vitamin B_{12} and other metabolites (1, 2).

Under anaerobic condition, propionibacteria consume carbon sources and produce propionate and acetate (3, 4), which are inhibitors of cell growth and metabolites production. Propionate has a stronger inhibitory effect than acetate (5, 6), and its accumulation can be an obstacle to the efficient production of useful metabolites such as vitamin B_{12} on a large scale. Ye *et al*. (5, 7) reported that *Propionibacterium freudenreichii* IFO 12424 starts consuming propionate via the reverse methylmalonyl CoA pathway when anaerobic condition is switched to aerobic condition during its culture. They applied this metabolic change induced by aeration to *P. freudenreichii* IFO 12424 fermentation to improve vitamin B_{12} production by decreasing the inhibitory effect of propionate.

P. freudenreichii ET-3 is a promising strain for producing 1,4-dihydroxy-2-naphthoic acid (DHNA) (8). DHNA stimulates the growth of bifidobacteria and improves the condition of the human intestine (9, 10). Our previous study (11) is the first to characterize *P. freudenreichii* ET-3 in terms of cell growth and DHNA production. In this study, we cultured *P. freudenreichii* ET-3 by switching from anaerobic condition to aerobic condition (termed anaerobic-aerobic switching culture hereafter) to enhance its DHNA production. In this switching culture, DHNA concentration was higher than that in anaerobic culture, because the cells could utilize propionate under aerobic condition. However, in the latter part of the aerobic phase, a continuous oxygen supply reduced cell growth and resulted in a sudden increase in dissolved oxygen (DO) concentration in the culture broth. As a result of the increase in DO concentration, DHNA in the culture broth was broken down drastically, because the structure of DHNA was sensitive to oxidation.

To obtain a high DHNA production by employing aerobic culture in *P. freudenreichii* ET-3 cultivation, it is necessary to design a rational method of aerobic culture. Therefore, in this study, the effects of oxygen supply on *P. freudenreichii* ET-3 metabolism and DHNA production were investigated in anaerobic-aerobic switching culture with different gas flow rates and agitation speeds. Then, we discussed the level of oxygen supply optimum for DHNA production in terms of oxygen transfer rate (OTR). Finally, we proposed

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an optimal cultivation method for *P. freudenreichii* ET-3 with anaerobic-aerobic switching, achieving a high DHNA concentration.

MATERIALS AND METHODS

Microoganism 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). The preculture medium contained 10% (wt/wt) whey powder (Meiji Dairies, Tokyo) and 0.1% (wt/wt) beer yeast extract (Asahi Food and Health, Tokyo). The fermentation medium contained 11% (wt/wt) skim milk powder (Meiji Dairies) and 0.5% (wt/wt) beer yeast extract (Asahi Food and Health). The whey and skim milk powders were digested by the protease Amano A (Amano Pharmaceutical, Tokyo) at 47°C for 3 h. During enzyme digestion, pH was maintained in the range of 6.6–7.0 by K_2CO_3. The media were autoclaved at 121°C for 15 min.

Cultivation One milliliter of *P. freudenreichii* ET-3 frozen culture stored at −80°C was inoculated into 100 ml of preculture medium. After static incubation for 48 h at 37°C, 20 ml of the culture was inoculated into a 3-*l* jar fermentor (BMS 03PI; ABLE, Tokyo) containing 2.0 *l* of the fermentation medium. Culture temperature was maintained at 33°C, and the pH of the medium was adjusted to 6.5 using K_2CO_3 during culture. Anaerobic condition was set using filter-sterilized nitrogen gas, and aerobic culture was set using sterilized air or oxygen gas. Aerobic culture was usually started at 72 h after the start of anaerobic culture, and for aerobic condition, air and oxygen gas flow rates were maintained at 2.0 and 0.4 *l*/min, respectively. Agitation speed was controlled at 150 rpm, unless otherwise specified. When DO control was necessary under aerobic condition, DO concentration was controlled by adjusting agitation speed. For anaerobic-aerobic switching culture, aerobic condition was initially set after 72 h of anaerobic culture.

Oxygen transfer rate OTR was employed to express the level of oxygen supply and was determined from results of the dynamic method of measuring the volumetric coefficient of oxygen transfer (k_La). In this study, the dynamic method was carried out without cell inoculation. For OTR measurement, conditions such as the culture medium, flow rate of air or oxygen, agitation speed, pH and temperature were the same as those for experimental cultures, except for the absence of inoculation. After DO concentration remained constant at 0 mg/*l* with nitrogen gas, DO concentration was increased by supplying air or oxygen, and the time course of DO concentration was recorded. The supply of air or oxygen was continued until DO concentration remained constant to determine saturated oxygen concentration. OTR was determined using k_La and the saturated oxygen concentration obtained from the time course of DO concentration. In this study, OTR was defined as the amount of oxygen transferred per liter per hour.

Quantification of organic acid and CO_2 The concentration of organic acid was spectrophotometrically measured at 445 nm using high-performance liquid chromatography (HPLC) by the post-column method (Hitachi L-7000 HPLC system; Hitachi, Tokyo; RS Pack KC-811 column, 8×300 mm; Showa Denko, Tokyo; mobile phase, 0.02% $HClO_4$; reaction solution, 0.125 g of bromthymol blue, 2.129 g of $Na_2HPO_4 \cdot 12H_2O$, 20 ml of 0.1 N NaOH, and the total volume was brought to 1000 ml with 0.02% $HClO_4$; temperature, 63°C; flow rate, 1.0 ml/min; and injection volume, 20 μl). The amount of CO_2 generated by *P. freudenreichii* ET-3 was calculated from CO_2 percentage in exhaust gas. The CO_2 percentage in exhaust gas was measured with an on-line exhaust gas monitor (off gas Jr. DEX 2562; ABLE). To confirm the accuracy of the measurement of CO_2 percentage in exhaust gas, the mean and standard deviation were calculated from specific CO_2 production rates under the same culture condition. This calculation revealed that the standard deviation was about 10% of the mean.

Quantification of DHNA DHNA was quantified according to our previous study (11). DHNA concentration in the culture broth was spectrophotometrically measured at 254 nm using HPLC (Shimadzu 10A HPLC system; Shimadzu, Kyoto; Cadenza CD-C18 column, 4.6×150 mm; Imtakt, Kyoto; mobile phase, acetonitrile, methanol, water and acetate [15:25:225:0.1, vol/vol/vol/vol, adjusted to pH 5.5 with NH_4OH]; temperature, 45°C; flow rate, 1.0 ml/min; and injection volume, 20 μl)

Molar ratios of CO_2 generated to propionate consumed and acetate produced during aerobic culture Under aerobic condition, propionate is oxidized via the reverse methylmalonyl CoA pathway or the tricarboxylic acid (TCA) cycle (4, 12–14). To evaluate the molar amounts of propionate consumed and acetate produced by this oxygen-inducing metabolic change, we calculated the differences in final propionate and acetate concentrations between anaerobic-aerobic switching culture and anaerobic culture. The molar amount of CO_2 generated induced by aeration was also calculated in the same manner. Then, the molar ratios of CO_2 generated to propionate consumed and acetate produced in the aerobic phase were determined using the molar amounts calculated above.

TABLE 1. Summary of *P. freudenreichii* ET-3 cultures carried out in this study

Culture condition			OTR (mg/[*l*·h])	Final DO concentration (mg/*l*)	Final DHNA concentration (mM)	Yield of DHNA for lactose (mM/M)
Anaerobic culture			ND	0	0.12	1.0
Anaerobic-aerobic switching culture	With various air flow rates in the aerobic phase	1.0 *l*/min	0.08	0	0	0.9
		2.0 *l*/min	0.23	0	0.21	1.4
		4.0 *l*/min	0.35	0	0.22	1.3
	With various agitation speeds in the aerobic phase	100 rpm	0.38	0	0.11	1.4
		150 rpm	0.66	0	0.22	1.3
		200 rpm	0.90	28	0.21	0
	With constant DO concentration in aerobic phase	0.2 mg/*l*	ND	0.2	0	0
	With various aeration start times	72 h	0.23	0	0.22	1.4
		48 h	0.23	0	0.21	1.4
		24 h	0.23	10	0	0
Repeated anaerobic-aerobic switching culture			0.38	0	0.33	1.3

ND, Not determinable.