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Control of Both Foam and Dissolved Oxygen in the Presence of a Surfactant for Production of β-Carotene in *Blakeslea trispora*

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Abstract A production of β-carotene was attempted in a fed-batch culture of *Blakeslea trispora* by controlling both foam and dissolved oxygen in the presence of surfactant, Span 20. Results obtained from the shake flask cultures indicated that a high concentration of dissolved oxygen was needed for both cell growth and β-carotene synthesis, and the optimal concentration of glucose was found to be in the range of 50–100 g/l. In order to maintain the dissolved oxygen concentration level at higher than 50% of air saturation, pure oxygen was automatically sparged into the medium with air. Foam was controlled by bypassing air from the submerged aeration to the headspace in response to the foam that was caused by Span 20. High agitation speed was found to be detrimental to the cell growth due to shear damage, even though it provided sufficient dissolved oxygen. On the other hand, a low aeration speed caused stagnant regions in the fermentor because of improper mixing. Thus, for the fed-batch operation, agitation speed was increased gradually from 300 to 700 rpm to prevent cell damage at the initial stage of fermentation and to give efficient mixing for a viscous culture broth as the culture proceeded. By controlling dissolved oxygen and foam, a high concentration of β-carotene (1,190 mg/l) was obtained in 6 days of the fed-batch culture of *B. trispora* with 2.5% of the dry cell weight, which was approximately 5 times higher than that of the batch cultures.

Key words: β-Carotene, production, *Blakeslea trispora*, dissolved oxygen, foam

Carotenoid β-carotene is a yellow pigment that is abundant in certain plants and microorganisms. It has been used in feed stuffs, food products, and cosmetics since it enhances pigmentation. In addition, it has antioxidant activity and serves as a precursor of vitamin A [6, 13, 18]. Recent reports [4, 15, 20] also confirmed that β-carotene is effective in treating cancer and cardiovascular disease by reducing reactive oxygen species. Consequently, there is an increasing need for obtaining large quantities of β-carotene. Synthesis of β-carotene has been done chemically, by extracting it from naturally occurring plant sources or by microbial fermentations. Among these, production by fermentation has drawn particular interest since natural products are mostly preferred compared to chemically synthesized ones and microbial synthesis is environmentally sound. Moreover, microbial fermentations have an advantage over extraction methods, because those methods make it easier to obtain β-carotene in much higher yields and have a potential for developing overproducing strains by using either mutation or genetic engineering techniques.

Fungus *Blakeslea trispora* has been widely used for the production of β-carotene [2, 3, 7, 10, 11, 12, 14]. Development of mutant strains, medium optimization, light exposure, and addition of stimulators are the factors of particular interest in β-carotene production. In addition, the use of surfactant has been often employed in the fermentation of fungi and mycelial bacteria to improve the yield of fermentation products [1, 5]. Triton X-100, Tweens, and Spans are the most widely used nonionic surfactants. The main function of the surfactants is to enhance mass transfer of substrate and/or product by dispersing cells in the media, otherwise mycelial microorganisms grown in liquid media form aggregates and cluster of cells which hinder mass transfer of nutrients and oxygen [9]. Recently, we reported [7] that Span 20 is the most effective surfactant for dispersed cell growth, increasing β-carotene production to a level 14 times higher than the control. However, surfactants cause severe problems of foaming in a jar fermentation due to submerged aeration. The foam caused by surfactants is so severe that it can not be controlled by the conventional foam control methods such as anti-foam agents or foam breakers. Dissolved oxygen concentration is also a very important factor in carotenoid production

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since carotenoid synthesis is much enhanced by oxidative stress [8, 16].

Therefore, in the present study, we developed a method to control foam and dissolved oxygen in the culture of *B. trispora* in the presence of surfactant. Since foam occurs in linear response to how much air is supplied into the fermentor, both surface aeration and submerged aeration were employed in order to eliminate the problem of severe foaming. Meanwhile, pure oxygen was used to maintain dissolved oxygen concentration at a level higher than 50% air saturation. This article describes the high production of β-carotene (1190 mg/l) by applying a method that precisely controls foam and dissolved oxygen in jar fermentations.

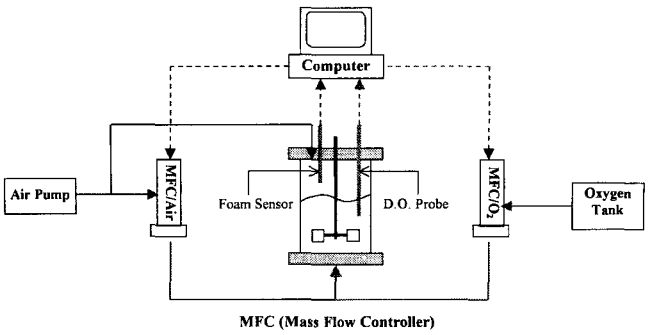

Fig. 1. Schematic diagram of a fermentation system controlling the aeration ratio of surface aeration to submerged aeration by sparger and pure oxygen supplying rate in response to foam and dissolved oxygen concentration.

MATERIALS AND METHODS

Microorganism and Culture Medium

Two strains of *Blakeslea trispora*, ATCC 14271 (NRRL 2456, +mating type) and ATCC 14272 (NRRL 2457, -mating type), were used for all experiments. The two strains were maintained separately on potato-dextrose agar plates at room temperature. These strains were cultivated together (mated culture) for β-carotene production. Fermentation (GAY) medium for the batch culture consisted of 70 g of glucose, 2 g of L-asparagine, 1 g of yeast extract, 1.5 g of KH_2PO_4, 0.5 g of $MgSO_4 \cdot 7H_2O$, and 5 mg of thiamin·HCl in 1 liter of distilled water. The initial pH of the culture was adjusted to 5.5 before autoclaving. Span 20 purchased from Sigma was added to the fermentation medium at a concentration of 1.0% (v/v).

Flask Cultures

Spores of *B. trispora* ATCC 14271 (+) and ATCC 14272 (-) from 2-3-day-old potato-dextrose agar plates were inoculated at concentrations of 5×10^6 and 1×10^7 into 50 ml of the GAY medium in 250-ml Erlenmeyer flasks. Incubations were carried out with shaking at 200 rpm at 27°C on a rotary shaker. To examine the possible effects of dissolved oxygen on β-carotene production, different relative volumes of medium ranging from 10 ml to 150 ml in 250-ml Erlenmeyer flasks were used in shake flask cultures for 2 days. Glucose effect was investigated in the flask culture for 3 d by varying the initial glucose concentration from 50 to 300 g/l. All flask cultures were prepared and analyzed in triplicate. Results are expressed as the mean values of three determinations with a deviation of less than 10%.

Jar Fermentations

Fermentations were done in a 2.5-liter jar fermentor (Korea Fermenter Co., Incheon, Korea) equipped with a six-blade disk-turbine impeller and three baffles. Figure 1 shows the control scheme of both dissolved oxygen and foam using two mass flow controllers (MasTrak, Sierra Instrument Inc., U.S.A.) interfaced to a personal computer (IBM AT). Each mass flow controller for the control of either foam or dissolved oxygen was regulated by using the LABTECH NOTEBOOKpro version 10.02 (Laboratory Technologies Co., U.S.A.) in the feedback on-off control mode. Air was supplied into the fermentor by both surface aeration and submerged aeration at a total flow rate of 1.5 vvm. The ratio of surface aeration to submerged aeration was controlled in response to foam which was created by Span 20. Whenever foam was detected by a foam sensor, submerged aeration was decreased until the foam disappeared. On the other hand, the rest of the air that by-passed the mass flow controller was fed into the headspace of the fermentor for surface aeration. Pure oxygen was automatically supplied through the mass flow controller in order to maintain dissolved oxygen concentration at a level higher than 50% of the air saturation. The pH was not controlled, but it remained between 4.2 and 5.3 throughout the fermentation. Culture temperature was controlled at 27°C. The number of spores inoculated into the fermentor containing 1 liter of the GAY medium was 1×10^8 of *B. trispora* ATCC 14271 (+) and 2×10^8 of ATCC 14272 (-) from the 2-3-day-old potato-dextrose agar plates. For the fed-batch culture, five times higher concentrations of the medium components than those of the batch culture were used, except for glucose. Glucose was initially added at a concentration of 60 g/l and supplemented intermittently into the fermentor to maintain a glucose concentration of between 20 and 70 g/l during the fed-batch culture.

Analytical Methods

Mycelial cells were filtered through muslin in a Buchner funnel, washed thoroughly with distilled water, and then lyophilized. The lyophilized cells were weighed to determine cell growth and used for β-carotene analysis. To extract β-carotene, the lyophilized cells were cut into small pieces, homogenized in hexane: methanol (1:1, v/v), and shaken at

30°C until they became colorless. The hexane phase containing β-carotene was obtained by adding distilled water followed by centrifugation, and used to determine the concentration of β-carotene by the high-performance liquid chromatography (HPLC) method. HPLC was performed with a Waters chromatograph (Milford, MA, U.S.A.) equipped with Waters Model 501 pump, a Waters 740 data module, and Waters Model 486 detector. Separations were performed on a Waters Nova-Pak C18 column (150×3.9 mm, 4 μm particle size). The mobile phase of acetonitrile-tetrahydrofuran-water (50:40:10, v/v/v) was eluted at a flow rate of 0.7 ml/min. Sample detection was achieved at 450 nm, and chromatographic peaks were determined by comparing with an authentic sample purchased from Sigma. Under the conditions used, β-carotene was eluted at the retention time of 9.3 min. The glucose concentrations in the culture media were analyzed by using the Glucose/Lactate Analyzer 2300STAT (Yellow Spring Instruments Co., U.S.A.).

RESULTS AND DISCUSSION

Effect of Aeration on Cell Growth and β-Carotene Production in Flask Cultures

High cell density culture was required for high production of β-carotene, because β-carotene accumulated inside of cells. In the case of high cell density culture of mycelial fungi, the viscosity of the culture broth increases as cell concentration goes up, reducing mass transfer rates and consequently causing dissolved oxygen limitation. To examine the effect of dissolved oxygen on cell growth and β-carotene production, mated cultures of *B. trispora* were performed in 250-ml flasks containing various volumes of the GAY medium. As shown in Fig. 2, both concentrations of cells (dry weight of 13.5 g/l) and β-carotene (202 mg/l) were the highest in flasks containing the smallest volume (10 ml) of the medium. As the culture volume increased from 10 to 150 ml, the cell growth decreased and the β-carotene production decreased at an even greater rate, resulting in a decrease of cellular β-carotene content from 1.6% to less than 0.5%. These results suggested that sufficient supply of oxygen is a prerequisite for producing β-carotene. Some reports [8, 16] described that oxidative stresses enhanced the synthesis of carotenoids since antioxidant compounds seemed to be stimulated under high oxygen pressure. Results of these flask cultures support the fact that oxygen plays an important role in the synthesis of carotenoids.

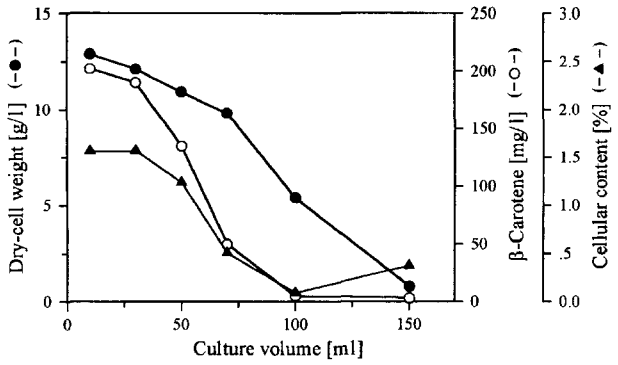

Fig. 2. Effect of culture volume in 250-ml flasks on cell growth and β-carotene production by *B. trispora*.
Cells were cultivated with shaking at 200 rpm for 2 days at 27°C in the GAY medium containing 1% (v/v) Span 20.

Effect of Agitation Speed in Jar Fermentors

In one of our previous studies [7], we reported that a surfactant, Span 20, could disperse mycelial cells in the medium, thereby enhancing β-carotene production in flask cultures by increasing mass transfer rates. However, operations with jar fermentors using surfactants causes severe foam problems when the conventional submerged aeration is applied to them. An excessive foaming increases contamination through leakage of culture broth from fermentors and also decreases productivity of desired products by the loss of cell mass from the culture broth. It is of importance to mention that reduced aeration and agitation can control foam. However, the results shown in Fig. 2 demonstrate that a sufficient supply of oxygen is necessary for high production of β-carotene. Thus, a unique aeration strategy combining surface aeration and submerged aeration was designed in order to prevent foaming while supplying pure oxygen to maintain the dissolved oxygen concentration at a desired level.

In addition, since agitation speed is one of the key factors influencing dissolved oxygen concentration, mixing property, and cell morphology, we examined its effect on cell growth and β-carotene production during the jar fermentation. Pure oxygen together with air was used to prevent the dissolved oxygen limitation that can be caused by low agitation speed. At the agitation speed of 350 rpm, the concentrations of the cells and β-carotene were 16 g/l and 304 mg/l, respectively, which were significantly higher than those at 250 rpm and at 500 rpm (Table 1). At the agitation speed of 250 rpm, stagnant regions caused by poor mixing were observed in the fermentor. It seems that low cell growth and low β-carotene production can be ascribed to the dissolved oxygen limitation occurring in those regions of poor mixing. On the other hand, at 500 rpm, many fragmented mycelia were observed in the early stage of fermentation. It implies that there was a serious mechanical damage to the mycelia, resulting in the decrease of cell growth and β-carotene production. Therefore, we suggest that agitation speed in *B. trispora* culture for β-carotene production should be carefully controlled to satisfy the requirements of both minimizing cell damage and maximizing the effect of mixing.

Table 1. Effects of agitation speed on cell growth and β-carotene production during batch fermentations of *B. trispora* in a jar fermentor[a].

Agitation speed (rpm)	Cell concentration[b] (g/l)	β-Carotene production (mg/l)	β-Carotene content[c] (mg-carotene/g-cell)
250	9	36	4
350	16	304	19
500	13	104	8

[a]Dissolved oxygen concentration and foam were controlled with the aeration mode and pure oxygen as described in Materials and Methods. Total aeration rate was 1.5 vvm. Cultures were carried out for 4 d.
[b]Cell concentration is described as dry weight of cells.
[c]β-Carotene content is defined as the ratio of β-carotene accumulated to cell weight.

Effect of Glucose Concentration on β-Carotene Production

A high level of β-carotene production can be achieved by obtaining a high concentration of *B. trispora* with high cellular content. For this, the substrate concentration should be high enough to support a high density of cells in batch fermentation. However, microbial metabolism is often inhibited in excessively high concentrations of nutrients. Thus, we investigated the effect of glucose as a main source of carbon and energy on cell growth and β-carotene production. As shown in Fig. 3, β-carotene production drastically decreased from 200 mg/l to a trace amount as glucose concentration increased from 50 to 300 g/l, although cell growth was not significantly affected by the glucose concentration, consequently resulting in a remarkable decrease of the cellular β-carotene content. Therefore, it was concluded that the glucose concentration in fed-batch cultures should be maintained below 100 g/l because β-carotene production was greatly inhibited by high glucose concentrations. On the other hand, no inhibition of cell growth or β-carotene production was observed with 5-times higher concentrations of the other nutrients in the GAY medium (data not shown).

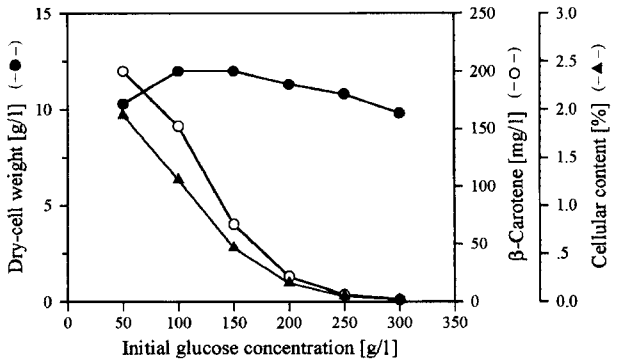

Fig. 3. Effect of glucose concentration on cell growth and β-carotene production in 50 ml of GAY medium in 250-ml flasks.
Cells were cultivated with shaking at 200 rpm for 3 days at 27°C in the GAY medium containing 1% (v/v) Span 20.

Controlled Fed-Batch Culture for High Production of β-Carotene

Based on the above results, we attempted to produce β-carotene at a high concentration in a fed-batch fermentation. For this, we employed a new fermentation system, which can suppress foam while maintaining higher levels of dissolved oxygen (see Materials and Methods). Glucose was initially added at a concentration of 60 g/l and intermittently supplemented into the fermentor to maintain glucose concentrations ranging between 20 and 70 g/l. Concentrations of the other nutrients were 5 times higher than those of the batch culture medium. It should also be noted that the agitation speed was increased from 300 to 700 rpm in 50 rpm steps, taking time-dependent features of mycelial culture into account [17]. Indeed, it was necessary to increase the agitation speed for providing proper mixing with the rising viscosity of the culture.

Figure 4 clearly shows how the dissolved oxygen concentration was maintained at around 50% of the air saturation while successfully controlling foam by regulating the ratio of submerged to surface aeration. Only a little submerged aeration was found to be sufficient in the early growth phase because of low oxygen demand due to low cell concentrations. Foam trouble by submerged aeration was mitigated as the cell concentration increased. Thus, after two and a half days, submerged aeration governed the aeration. Dissolved oxygen concentration was designed to be maintained at above 50% of the air saturation by supplying pure oxygen. However, it should be noted that during the 2 days from the onset of pure oxygen supply (18 h), the dissolved oxygen concentration fluctuated in the range of 20–120% air saturation from the desired 50%, since supply of pure oxygen was too high for the requirement of low cell concentrations. After the cell concentration reached 15 g/l at 2.5 day, the dissolved oxygen concentration began to be fairly well controlled with pure oxygen and the maximum supply (1.5 vvm) of air by submerged aeration at 700 rpm. The results shown in Fig. 5 show the time courses of cell growth, β-carotene production, culture pH, and cellular carotene content during the fed-batch

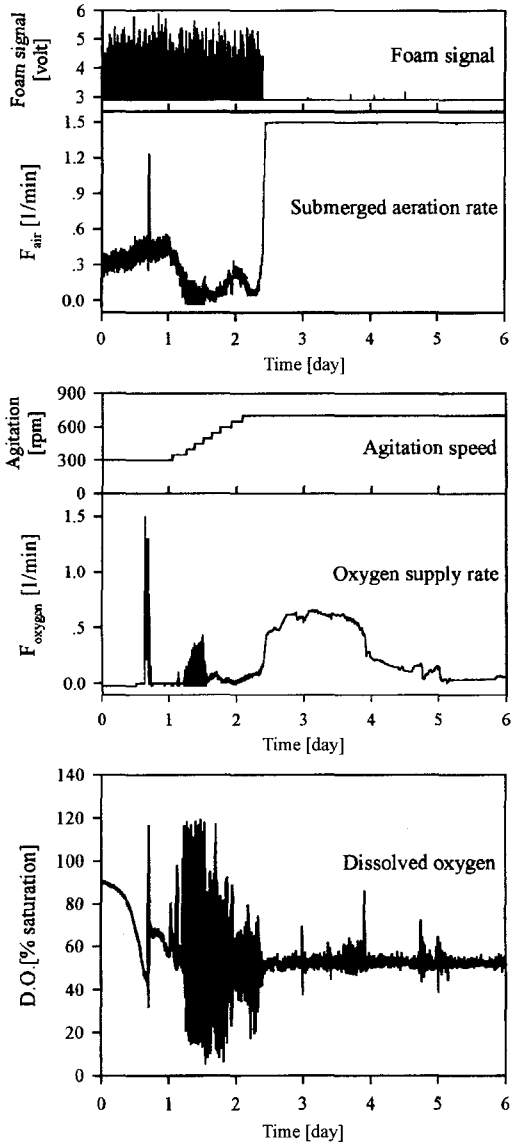

Fig. 4. Changes of submerged aeration rate, agitation speed, pure oxygen supply, and dissolved oxygen concentration during the cultivation of *B. trispora* in the fed-batch fermentation.
Surface aeration rate is the total aeration rate (1.5 vvm) minus submerged aeration rate.

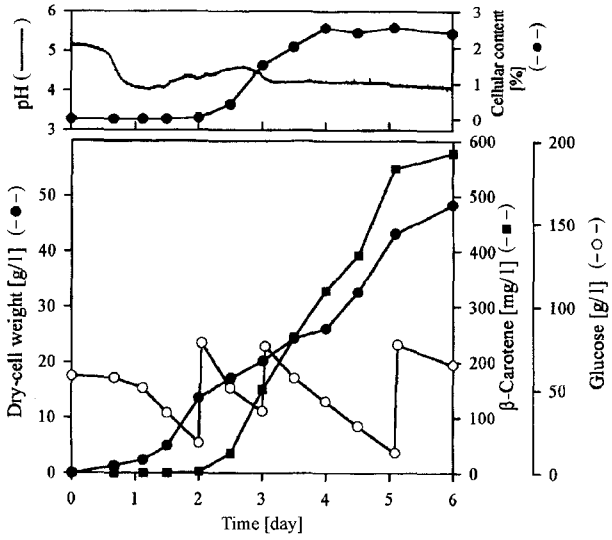

Fig. 5. Time courses of cell growth, β-carotene production, culture pH, and cellular β-carotene content during the fed-batch culture of *B. trispora*.
Control of fermentation was described in Fig. 4.

cultivation of *B. trispora*. Cell concentration increased over the whole fermentation period, and reached the maximum concentration of 48 g/l in 6 days, which was approximately 5 times higher than that in the batch cultures. β-Carotene began to accumulate after the second day when the cell concentration was about 15 g/l. The maximum production (1,190 mg/l) of β-carotene was obtained at day 6 of the fed-batch culture. Cellular β-carotene content increased from a trace amount to 2.5% during the 2-4 days of culture, and remained at its highest value to the end of the fermentation. Culture pH was initially set at 5.2 and remained relatively constant throughout the fermentation.

In the batch fermentation, we obtained 304 mg/l of β-carotene in 6 days when the fermentor was operated at 350 rpm. For the fed-batch fermentation, we used 5 times higher concentrations of medium than batch fermentation medium while supplying glucose intermittently to maintain its concentration in the range of 20-70 g/l. In addition, we increased the agitation speed gradually from 300 rpm to 700 rpm to compensate for rising cell concentration and viscosity. By controlling foam and dissolved oxygen in the fed-batch fermentation, we obtained a high β-carotene concentration of 1,190 mg/l in 6 days with the cellular content of 2.5%, showing a significant improvement in carotene production compared to that in the batch culture. Since we have made a great improvement in β-carotene production by computer-aided control of foam and dissolved oxygen, we believe that this process can be used in many mycelial microorganism fermentations where surfactants play important roles.

Acknowledgments

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