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Influence of Oxygen and Glucose on Primary Metabolism and Astaxanthin Production by *Phaffia rhodozyma* in Batch and Fed-Batch Cultures: Kinetic and Stoichiometric Analysis

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The influence of the oxygen and glucose supply on primary metabolism (fermentation, respiration, and anabolism) and astaxanthin production in the yeast *Phaffia rhodozyma* was investigated. When *P. rhodozyma* grew under fermentative conditions with limited oxygen or high concentrations of glucose, the astaxanthin production rate decreased remarkably. On the other hand, when the yeast grew under aerobic conditions, the astaxanthin production rate increased with increasing oxygen uptake. A kinetic analysis showed that the respiration rate correlated positively with the astaxanthin production rate, whereas there was a negative correlation with the ethanol production rate. The influence of glucose concentration at a fixed nitrogen concentration with a high level of oxygen was then investigated. The results showed that astaxanthin production was enhanced by an initial high carbon/nitrogen ratio (C/N ratio) present in the medium, but cell growth was inhibited by a high glucose concentration. A stoichiometric analysis suggested that astaxanthin production was enhanced by decreasing the amount of NADPH required for anabolism, which could be achieved by the repression of protein biosynthesis with a high C/N ratio. Based on these results, we performed a two-stage fed-batch culture, in which cell growth was enhanced by a low C/N ratio in the first stage and astaxanthin production was enhanced by a high C/N ratio in the second stage. In this culture system, the highest astaxanthin production, 16.0 mg per liter, was obtained.

Astaxanthin (3,3′-dihydroxy-β,β′-carotene-4,4′-dione) is an abundant carotenoid pigment in some marine animals such as salmonids and crustaceans. Since these animals cannot synthesize astaxanthin, this carotenoid has been added to the feed of the aquacultivated salmonid, trout, and prawn to improve their color quality (15). Moreover this carotenoid possesses a higher antioxidant activity than β-carotene and α-tocopherol (32). Therefore, astaxanthin has attained a commercial interest not only as a pigmentation source for fish aquaculture but also as a powerful antioxidative reagent (18). Astaxanthin has been produced mainly by the chemical synthetic method on an industrial scale. In recent years, however, the use of chemical synthetic compounds as food additives has been strictly regulated. Since a few microorganisms are capable of synthesizing astaxanthin, the establishment of a natural astaxanthin source by these microorganisms is now required.

An astaxanthin-producing yeast, *Phaffia rhodozyma*, has some advantageous properties: (i) it synthesizes astaxanthin as a principal carotenoid, (ii) it does not require light for its growth and pigmentation, (iii) it can utilize many kinds of saccharides under both aerobic and anaerobic conditions, and (iv) it can grow at a rate of 0.10 to 0.15 h^{-1} (3, 14, 16). Therefore, *P. rhodozyma* is attractive for commercial astaxanthin production, and many studies have reported improvements in astaxanthin production (2, 12, 20, 22, 23, 29).

Recently, Kobayashi et al. (18) have reported that astaxanthin production by *Haematococcus pluvialis*, an astaxanthin-producing microorganism, is much enhanced by oxidative stress, and they have speculated that astaxanthin plays a role as an active oxygen scavenger in the cell. In addition, Schroeder and Johnson (30) have reported that carotenoid biosynthesis is regulated by singlet oxygen and peroxyl radicals in the *P. rhodozyma* cell. Based on these two studies, it could be considered that oxygen might play an important role in astaxanthin biosynthesis. On the other hand, Arnezeder and Hampel (4, 5) have reported that the production of ergosterol, which, like astaxanthin, is synthesized via a mevalonate pathway (10), is enhanced by nitrogen- or phosphate-limited culture of *Saccharomyces cerevisiae*. Therefore, it may be that astaxanthin production could be enhanced under such nutrient-limited conditions, especially with a high carbon/nitrogen ratio (C/N ratio) in the medium. Kakizono et al. (17) have reported that the production of astaxanthin by *H. pluvialis* is enhanced by a high C/N ratio, which in their case was caused by an excess supply of acetate (carbon source). Many workers have reported that primary metabolism, such as anabolism and catabolism (fermentation and respiration), varies with the excess supply of oxygen or carbon source (8, 13, 19, 21, 24, 25, 31). Therefore, a change in primary metabolism might be a trigger for high astaxanthin accumulation.

In the present study, we investigated the influence of the oxygen and glucose supply on primary metabolism and astaxanthin production by *P. rhodozyma*. First, the influences of fermentative conditions and oxygen supply on astaxanthin production were investigated by using a kinetic analysis. Second, the influence of the glucose supply at a fixed nitrogen concentration with high levels of oxygen was investigated. Third, based on the results from the batch culture, we operated a two-stage fed-batch culture with a control C/N ratio in the feed medium. Finally, we analyzed the correlation between primary

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metabolism and astaxanthin production, based on a stoichiometric analysis.

MATERIALS AND METHODS

Microorganism and medium. *P. rhodozyma* ATCC 24202 was used. This yeast was maintained at 4°C on an agar slant containing (per liter) 10 g of glucose, 5.0 g of Bacto Peptone, 3.0 g of malt extract, 3.0 g of yeast extract, and 20 g of agar. YM medium contained (per liter) 10 g of glucose, 5.0 g of Bacto Peptone, 3.0 g of malt extract, and 3.0 g of yeast extract and was adjusted to pH 5.0. The basal medium contained (per liter) 20 g of glucose, 5.0 g of $(NH_4)_2SO_4$, 1.0 g of KH_2PO_4, 0.5 g of $MgSO_4 \cdot 7H_2O$, 0.1 g of $CaCl_2 \cdot 2H_2O$, 1.0 g of yeast extract, 0.1 ml of antifoam reagent (A-nol; Able, Tokyo, Japan), and 20 g of potassium biphthalate (used only for preculture and flask batch culture as a buffer) and was adjusted to pH 4.5. Bacto Peptone, malt extract, yeast extract, and agar were obtained from Difco Laboratories (Detroit, Mich.).

The starter culture broth (4 ml; cultivated with 10 ml of YM medium in a 50-ml test tube for 72 h) was inoculated into a 200-ml Erlenmeyer flask containing 36 ml of the basal medium, and a preculture was carried out for 48 h. Both the starter culture and the preculture were performed with shaking at 140 rpm on a rotary shaker at 20°C.

Batch culture. In the flask batch culture, the preculture broth (2 ml) was inoculated into a 100-ml Erlenmeyer flask containing 18 ml of the basal medium. The flask batch culture was performed with shaking at 140 rpm on a rotary shaker at 20°C. According to the effects of the buffer, the pH was maintained in the range of 4.3 to 4.5.

In the fermentor batch culture, the preculture broth (300 ml) was inoculated into a 5-liter jar fermentor (KMJ-5B; Mitsuwa, Osaka, Japan) containing 2.7 liters of the basal medium. The temperature was controlled at 20°C. The pH was monitored with a pH electrode (Toa Electronics, Tokyo, Japan) and adjusted at 4.4 to 4.5 with a pH controller (MPH-3C; Mitsuwa) by adding a 2 M NaOH solution through a peristaltic pump (NP-3NS; Tokyo Rikakikai, Tokyo, Japan). The dissolved oxygen (DO) concentration was monitored with a DO electrode (Toa Electronics) and controlled with a DO controller (MDO-3C; Mitsuwa) by changing the aeration or agitation speed. In the batch culture, the DO concentration was maintained at 0.0, 2.0, 5.0, and 8.0 mg per liter, which corresponded to 0.0, 23, 57, and 90% of the saturation value (8.84 mg per liter at 20°C in distilled water), respectively. The DO concentration could be maintained at the desired level in the range of ±0.1 mg per liter.

Two-stage fed-batch culture. The preculture broth (200 ml) was inoculated into a 5-liter jar fermentor containing 1.8 liters of the basal medium in which the $(NH_4)_2SO_4$ concentration was reduced to 0.25 g per liter. The first stage of the fed-batch culture was started when the residual glucose concentration in the batch culture fell to about 5.0 g per liter. To prevent precipitate formation, the phosphorous source and minerals were fed separately. Therefore, three feeding solutions were prepared: a glucose solution of 500 g per liter; a mineral solution consisting of 12.5 g of $MgSO_4 \cdot 7H_2O$, 2.5 g of $CaCl_2 \cdot 2H_2O$, and 25 g of yeast extract per liter; and a KH_2PO_4 solution of 25 g per liter. The feedings of these three solutions were set at the same rate (180 ml per h) and were performed simultaneously in response to the pH decrease (26) by using three peristaltic pumps connected to a pH controller. In the batch culture phase and the first stage of the fed-batch culture, pH control was performed with a 2 M ammonium solution to supply a nitrogen source. The feed rate of the ammonium solution was set at 90 ml per h, which was half the rate used for the glucose solution. The DO concentration was controlled at 5.0 mg per liter by changing the agitation speed.

In the second stage of the fed-batch culture, the three pumps for the glucose, mineral, and phosphate solutions were connected to the DO controller. The pump for the ammonium solution was also connected to the DO controller. In this stage, the feedings of these four solutions were performed in response to an abrupt increase in the DO concentration, since this increase could be observed when the glucose in the culture medium was completely consumed (35). Therefore, the feedings of these solutions were performed when the DO concentration exceeded 6.0 mg per liter. The feed rates for the glucose, mineral, and phosphate solutions were set at the same values as in the first stage, whereas the feed rate of the ammonium solution was set at 22.5 ml per h, which was a quarter of that used in the first stage. In order to maintain the DO concentration at 5.0 mg per liter, the agitation speed was increased only when the DO concentration fell below 5.0 mg per liter. pH control was performed with a 2 M NaOH solution.

Throughout the period of the two-stage fed-batch culture, the temperature and pH were controlled at 20°C and 4.4 to 4.5, respectively, and the aeration speed was set at 1.0 volume of air per volume of liquid per min.

Calculation of oxygen uptake rate and carbon dioxide evolution rate. The partial pressures of oxygen and carbon dioxide in the exhaust gas from the fermentor were monitored with an infrared carbon dioxide and thermomagnetic oxygen analyzer (model EX-1562-1; Able, Tokyo, Japan). The flow rate of the exhaust gas was calculated as follows:

$$F_{out} = [(100 - R_{O_2\text{-in}} - R_{CO_2\text{-in}})/(100 - R_{O_2\text{-out}} - R_{CO_2\text{-out}})]F_{in}$$

where F_{in} is the aeration rate into the fermentor (liters per hour), F_{out} is the flow rate of the exhaust gas (liters per hour), $R_{O_2\text{-in}}$ is the percent oxygen in the inlet gas, $R_{O_2\text{-out}}$ is the percent oxygen in the exhaust gas, $R_{CO_2\text{-in}}$ is the percent carbon dioxide in the inlet gas, and $R_{CO_2\text{-out}}$ is the percent carbon dioxide in the exhaust gas. The oxygen uptake and carbon dioxide evolution rates were calculated as follows:

$$dO_2/dt = [F_{in}(R_{O_2\text{-in}}/100) - F_{out}(R_{O_2\text{-out}}/100)]/22.4V$$

$$dCO_2/dt = [F_{in}(R_{CO_2\text{-out}}/100) - F_{out}(R_{CO_2\text{-in}}/100)]/22.4V$$

where dO_2/dt is the oxygen uptake rate (moles per liter per hour), dCO_2/dt is the carbon dioxide evolution rate (moles per liter per hour), and V is the culture volume (liters).

Calculation of carbon recovery. In order to estimate the material balance between (i) glucose consumption and (ii) cell growth and carbon dioxide evolution, carbon recovery was calculated as follows:

$$R_C = (\gamma_X \Delta X + \gamma_{CO_2} \Delta CO_2)/\gamma_S \Delta S \quad (1)$$

where R_c is the carbon recovery (dimensionless), ΔCO_2 is the carbon dioxide evolution (moles per liter), ΔS is the glucose consumption (grams per liter), ΔX is the final cell concentration (grams per liter), γ_{CO_2} is the carbon content in carbon dioxide (12 g per mol), γ_S is the carbon content in glucose (0.40 g per g), and γ_X is the carbon content in the dry cell (grams per gram; defined as 0.50 g per g).

Calculation of specific rates. The maximum specific growth rate, μ_{max} ($hours^{-1}$), was calculated by a linear regression method from the logarithm of dry cell concentration versus time during the early exponential growth phase. The specific rates of astaxanthin production (q_{Axn}) (micrograms per gram per hour), ethanol production (q_E) (milligrams per gram per hour), and oxygen uptake (q_{O_2}) (millimoles per gram per hour) during the early exponential growth phase were calculated as follows: $q_{Axn} = (\Delta Axn_{exp}/\Delta X_{exp})\mu_{max}$, $q_E = (\Delta E_{exp}/\Delta X_{exp})\mu_{max}$, and $q_{O_2} = (\Delta O_{2exp}/\Delta X_{exp})\mu_{max}$, where ΔAxn_{exp}, ΔE_{exp}, ΔO_{2exp}, and ΔX_{exp} are astaxanthin production (micrograms per liter), ethanol production (milligrams per liter), oxygen uptake (millimoles per liter), and cell growth (grams per liter), respectively, during the early exponential growth phase.

Analyses. The acetone extraction method, as described by Okagbue and Lewis (28), was used to measure the total carotenoid concentration. Because the ratio of astaxanthin to total carotenoids produced was more than 90% in *P. rhodozyma*, this method was employed, with slight modifications, to measure the astaxanthin concentration. The procedures were as follows. The cells in 10 ml of the culture broth were centrifuged (5,000 × *g*, 10 min) and maintained at −80°C for 2 h. After addition of 2 ml of 2.5 M HCl, the cells were heated for 2.5 min in a boiling water bath, quickly cooled, and centrifuged (5,000 × *g*, 10 min). After the cells were washed twice with deionized water, 6 ml of acetone was added to the cells, and the samples were maintained at 4°C for 1.5 h. An excess amount of Na_2SO_4 powder was then added to remove the water, and the cells were maintained at 4°C for 30 min. After centrifugation (5,000 × *g*, 10 min), the absorbance at 478 nm was measured with a spectrophotometer (UV-1600; Shimadzu, Kyoto, Japan). The astaxanthin concentration was calculated by using the absorption coefficient $A_{1\%} = 2{,}100$ (2).

The cell concentration was measured by optical density at 600 nm or by dry cell weight, after filtration on cellulose nitrate (0.45-μm pore size; Advantec Toyo, Tokyo, Japan) and drying at 105°C for 24 h. The ratio of the optical density to dry cell weight (grams per liter) was 2.50 ± 0.10.

The protein content in the cell was measured by a modified biuret method (1, 34) as follows. Ten milliliters of the culture broth was centrifuged (5,000 × *g*, 10 min) and washed twice with deionized water. Then 3 ml of 1 M NaOH was added and boiled for 10 min. After the broth was quickly cooled $CuSO_4$ was added to give a final concentration of 25 mM. After 5 min at room temperature (25°C), the mixture was centrifuged (13,000 × *g*, 2 min). The optical density at 550 nm was then measured by using bovine serum albumin (Sigma Chemical Co., St. Louis, Mo.) as a standard.

Glucose was measured by the glucose oxidase method (Diacolor-GC; Toyobo, Osaka, Japan). Ethanol was measured by gas chromatography (GC-8A; Shimadzu), as described by Nishio et al. (27). Formate, acetate, propionate, and glycerol were measured by high-performance liquid chromatography (TRI ROTAR-V; JASCO, Tokyo, Japan) with a Shodex Ionpak S-801 column (Showa Denco, Tokyo, Japan) and a UV spectrophotometer (UVIDEC-100-V; JASCO), as described by Fukuzaki et al. (9). The ammonium concentration was measured by the indophenol method (11).

All of the analyses were carried out for duplicate or triplicate cultures, and the average values are reported.

RESULTS

Effect of fermentative conditions on growth and astaxanthin production. In general, yeasts ferment a sugar and produce ethanol under oxygen-limiting conditions (Pasteur effect). In addition, yeasts also ferment a sugar at high sugar concentrations, even under aerobic conditions (Crabtree effect). Therefore, in order to examine the effects of the fermentative con-

TABLE 1. Effect of fermentative conditions on growth of and astaxanthin production by *P. rhodozyma* in flask culture[a]

Glucose (g/liter)	Culture vol (ml/100-ml flask)	μ_{max}[b] (h^{-1})	q_{Axn}[c] (μg/g/h)	q_E[d] (mg/g/h)	Δt[e] (h)	ΔX[f] (g/liter)	ΔS[g] (g/liter)	$Y_{X/S}$[h] (g/g)	ΔE[i] (g/liter)	ΔAxn[j] (mg/liter)	ΔAxn/ΔX[k] (mg/g [dry wt] of cells)
20	20	0.14 ± 0.01	39.1 ± 4.3		56	5.93 ± 0.14	20.0 ± 0.0	0.30	ND[l]	1.66 ± 0.15	0.28
	40	0.090 ± 0.010	12.2 ± 1.1	66.0 ± 3.8	48	4.75 ± 0.18	18.2 ± 0.7	0.26	3.89 ± 0.11	0.72 ± 0.05	0.15
	60	0.052 ± 0.004	1.53 ± 0.22	94.2 ± 5.4	80	2.60 ± 0.11	17.9 ± 0.7	0.14	4.90 ± 0.53	0.075 ± 0.005	0.029
40	20	0.10 ± 0.01	21.0 ± 1.6	48.8 ± 2.8	81	7.17 ± 0.30	40.0 ± 0.0	0.18	3.42 ± 0.19	1.48 ± 0.09	0.21
60	20	0.084 ± 0.005	16.0 ± 1.7	44.3 ± 2.7	96	9.90 ± 0.72	58.0 ± 2.4	0.17	5.47 ± 0.49	2.01 ± 0.29	0.20
80	20	0.049 ± 0.006	5.02 ± 0.38	21.1 ± 1.3	168	8.85 ± 0.23	70.0 ± 0.6	0.13	6.21 ± 0.23	1.58 ± 0.10	0.18

[a] All experimental data are expressed as means and standard deviations derived from triplicate experiments. All cultivations were carried out until no more glucose was consumed.
[b] Maximum specific growth rate.
[c] Specific astaxanthin production rate during early exponential phase.
[d] Specific ethanol production rate during early exponential phase.
[e] Culture time.
[f] Final cell concentration.
[g] Glucose consumption.
[h] Growth yield for glucose consumption ($\Delta X/\Delta S$). These values were calculated from the means of the experimental data.
[i] Final ethanol concentration.
[j] Final astaxanthin concentration.
[k] Final specific astaxanthin concentration. These values were calculated from the means of the experimental data.
[l] ND, not detected.

ditions on growth and astaxanthin production, we performed two series of experiments. First, to investigate the Pasteur effect, batch cultures were performed with 20 g of glucose per liter in a 100-ml Erlenmeyer flask with culture volumes of 20, 40, and 60 ml. The results are summarized in Table 1. Because no ethanol was produced with a 20-ml culture volume, this culture was considered to be grown under aerobic conditions. With 40- and 60-ml culture volumes, however, ethanol was produced. The final ethanol concentration (ΔE) (grams per liter) increased, and the maximum specific growth rate (μ_{max}) ($hours^{-1}$), the final astaxanthin concentration (ΔAxn) (milligrams per liter), and the specific astaxanthin concentration (ΔAxn/ΔX) (milligrams per gram) decreased with increasing culture volume.

Next, to investigate the Crabtree effect, the initial glucose concentrations in the batch cultures were increased to 40, 60, and 80 g per liter with a culture volume of 20 ml, since aerobic conditions were maintained with a culture volume of 20 ml in the 100-ml Erlenmeyer flask, as mentioned above. These results are also summarized in Table 1. In all of the cultures, ethanol production was observed. In addition, with an increase in the initial glucose concentration, ΔE increased and μ_{max}, ΔAxn, and ΔAxn/ΔX decreased.

Based on the data in Table 1, a relationship between fermentation and astaxanthin production during the early exponential phase was analyzed by a kinetic procedure. The values for the specific astaxanthin production rate, q_{Axn}, were plotted against the values for the specific ethanol production rate, q_E. As shown in Fig. 1, except for one data point, a linear relationship was observed between q_{Axn} and q_E:

$$q_{Axn} = -K_{Axn/E} q_E + 37.8 \quad (2)$$

where $K_{Axn/E}$ is a kinetic constant which shows the ratio of astaxanthin production to ethanol production and is estimated from the slope of the straight line in Fig. 1 to be 0.39 μg of astaxanthin per mg of ethanol. Equation 2 clearly shows that astaxanthin production was repressed by the fermentation of *P. rhodozyma*. Although a remarkable deviation from equation 2 was observed at 80 g of glucose per liter (Fig. 1), this could be due to physiological changes in cell metabolism due to a considerable decrease in μ_{max} and the remarkably long culture time.

Effect of oxygen supply on growth and astaxanthin production. In order to avoid the decrease in astaxanthin production due to the Pasteur effect, the DO concentration must be maintained at an appropriate level. To investigate the effects of varying the oxygen supply, batch cultures were performed in the fermentor with the basal medium under DO control at 0.0, 2.0, 5.0, and 8.0 mg per liter. As shown in Table 2, μ_{max} was maintained at a high level (>0.18 h^{-1}) at 5.0 and 8.0 mg of DO per liter but decreased at less than 2.0 mg of DO per liter. ΔAxn was enhanced remarkably at 2.0 mg per liter, whereas there was no significant difference at a DO concentration higher than 2.0 mg per liter. From these results, the most suitable DO concentration for growth and astaxanthin production was determined to be 5.0 mg per liter.

Effect of glucose supply on growth and astaxanthin production at high levels of oxygen. To investigate the effects of varying the glucose supply at a fixed nitrogen concentration, batch cultures were performed under DO control at 5.0 mg per liter in the fermentor. The initial glucose concentrations ranged from 10 to 120 g per liter, whereas the concentrations

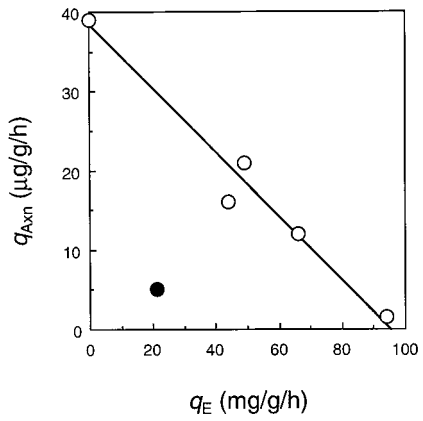

FIG. 1. Relationship between the initial specific astaxanthin production rate (q_{Axn}) and the specific ethanol production rate (q_E) during the early exponential phase. The mean values from Table 1 are shown. The line was calculated by the least-squares regression method (r = 0.983), except for the data (●) obtained for 80 g of glucose per liter.

TABLE 2. Effect of oxygen supply on growth of and astaxanthin production by *P. rhodozyma* in a fermentor under DO control[a]

DO (mg/liter)	μ_{max} (h^{-1})	q_{Axn} (μg/g/h)	q_{O_2}[b] (mmol/g/h)	Δt (h)	ΔX (g/liter)	$Y_{X/S}$[c] (g/g)	ΔAxn (mg/liter)	ΔAxn/ΔX[c] (mg/g [dry wt] of cells)
0.0	0.10 ± 0.01	16.0 ± 2.1	1.50 ± 0.12	48	4.80 ± 0.21	0.24	0.75 ± 0.01	0.16
2.0	0.13 ± 0.01	43.2 ± 5.2	4.16 ± 0.23	42	9.40 ± 0.05	0.47	3.10 ± 0.10	0.33
5.0	0.18 ± 0.01	54.0 ± 4.3	5.22 ± 0.30	36	9.27 ± 0.20	0.46	2.79 ± 0.01	0.30
8.0	0.19 ± 0.01	63.0 ± 5.3	5.89 ± 0.29	30	10.2 ± 0.08	0.51	3.39 ± 0.01	0.33

[a] All experimental data are expressed as means and variances derived from duplicate experiments. Nomenclature is the same as in Table 1. All cultivations were carried out in a fermentor with the basal medium containing 20 g of glucose per liter and were continued until glucose was consumed completely.
[b] Specific oxygen uptake rate during early exponential phase.
[c] Calculated from the means of the experimental data.

of the other medium components were not changed from those in the basal medium. The results are summarized in Table 3. Ethanol production was not detected or was at trace levels (<0.3 g per liter) in all cultures, suggesting that the Crabtree effect was repressed by a high oxygen supply. Extracellular products such as formate, acetate, propionate, and glycerol were not detected in any of the cultures (except for the trace amount of ethanol). The maximum specific growth rate decreased with increasing initial glucose concentration. This might be due to an inhibitory effect of high concentrations of glucose. The final cell concentration (ΔX), ΔAxn, and ΔAxn/ΔX increased with increasing initial glucose concentration, but they decreased at 120 g of glucose per liter. At 80 g of glucose per liter, the highest ΔAxn (14.8 mg per liter) was obtained in all batch cultures. ΔAxn/ΔX reached 0.51 mg per g, which was 1.8-fold higher than that at 10 g of glucose per liter. The residual ammonium concentration decreased with increasing the initial glucose concentration and was not detected at 80 g of glucose per liter, indicating the presence of an ammonium deficit. These results suggest that at high DO concentrations, astaxanthin production is enhanced by an initial high C/N ratio in the medium.

The effect of the glucose supply on astaxanthin production was then analyzed kinetically. The values of q_{Axn} were plotted against the initial glucose concentration (Fig. 2). The values of q_{Axn} at various glucose levels in the flask cultures (Table 1) were also plotted (Fig. 2) to compare them with those values at high levels of oxygen. In the flask culture, in which it was difficult to maintain DO at a high level throughout the entire cultivation period, q_{Axn} decreased remarkably as the initial glucose concentration was increased. On the other hand, q_{Axn} could be successfully maintained at almost 0.54 μg per g per h at 10 to 80 g of glucose per liter under DO control at high levels. These results suggest that to maintain high astaxanthin productivity, high levels of oxygen are necessary to repress the Crabtree effect and other inhibitory effects derived from the high concentrations of glucose. At 120 g of glucose per liter, however, q_{Axn} decreased remarkably, even if the DO concentration was maintained at a high level. Since a considerable decrease in μ_{max} was observed and a remarkably long culture time was required at 120 g of glucose per liter (Table 3), this was considered to be due to the physiological changes in cell metabolism stemming from the inhibitory effects of high glucose concentrations.

The results shown in Fig. 2 clearly show that oxygen uptake plays an important role in astaxanthin production. Therefore, the role of oxygen uptake, or respiration, on astaxanthin production was then analyzed by a kinetic procedure. The values of q_{Axn} in Tables 2 and 3 were plotted against the values of q_{O_2}. As shown in Fig. 3, a linear relationship between q_{Axn} and q_{O_2}, except for one data point, was obtained:

$$q_{Axn} = K_{Axn/O_2} q_{O_2} + 0.23 \quad (3)$$

where K_{Axn/O_2} is a kinetic constant representing the ratio of the rate of astaxanthin production to the rate of oxygen uptake, which is estimated from the slope of the straight line in Fig. 3 to be 10.3 μg of astaxanthin per mmol of oxygen. Equation 3 clearly shows that astaxanthin production was enhanced by an increase in the respiratory activity of *P. rhodozyma*. A remarkable deviation from equation 3 was observed at 120 g of glucose per liter (Fig. 3), which may be due to the reasons mentioned in the discussion of Fig. 2 above.

In order to analyze their influences on anabolism, the growth yield for glucose consumption ($Y_{X/S}$), the ratio of cell growth to nitrogen consumption ($\Delta X/\Delta N$), the ratio of cell growth to oxygen uptake ($\Delta X/\Delta O_2$), the ratio of cell growth to carbon dioxide evolution ($\Delta X/\Delta CO_2$), and the ratio of astaxanthin production to glucose consumption (ΔAxn/ΔS) were calculated from the data obtained in Table 3 (with the omission of the

TABLE 3. Results for DO-controlled batch cultures with various initial glucose concentrations[a]

Glucose (g/liter)	μ_{max} (h^{-1})	q_{Axn} (μg/g/h)	q_{O_2} (mmol/g/h)	Δt (h)	ΔX (g/liter)	ΔS (g/liter)	Residual N[b] (g/liter)	ΔAxn (mg/liter)	ΔAxn/ΔX[c] (mg/g [dry wt] of cells)
10	0.19 ± 0.01	55.0 ± 0.3	5.50 ± 0.20	26	4.56 ± 0.34	10 ± 0	1.03 ± 0.08	1.34 ± 0.17	0.29
20	0.18 ± 0.00	54.0 ± 0.2	5.22 ± 0.10	36	9.27 ± 0.20	20 ± 0	0.69 ± 0.04	2.79 ± 0.01	0.30
40	0.13 ± 0.00	54.6 ± 2.1	5.20 ± 0.25	46	16.1 ± 0.6	40 ± 0	0.24 ± 0.02	6.74 ± 0.35	0.42
80	0.10 ± 0.01	51.0 ± 3.2	5.00 ± 0.30	96	28.8 ± 1.7	80 ± 0	ND[d]	14.8 ± 0.8	0.51
120	0.078 ± 0.005	27.3 ± 2.8	6.28 ± 0.40	146	22.6 ± 1.6	110 ± 8	ND	7.80 ± 0.80	0.35

[a] All values are expressed as means and variances derived from duplicate experiments. All cultivations were performed under DO control at 5.0 mg per liter. Nomenclature is the same as in Tables 1 and 2.
[b] Residual ammonium concentration.
[c] Calculated from the means of the experimental data.
[d] ND, not detected.

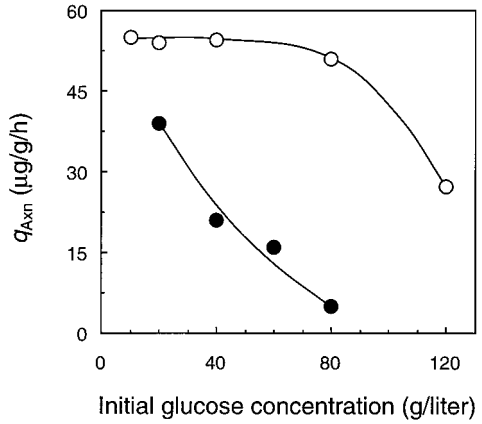

FIG. 2. Influence of the initial glucose concentration on the specific astaxanthin production rate (q_{Axn}) during the early exponential phase. Symbols: ○, mean values obtained in batch cultures under DO control at 5.0 mg per liter (Table 3); ●, mean values obtained in flask batch cultures (Table 1).

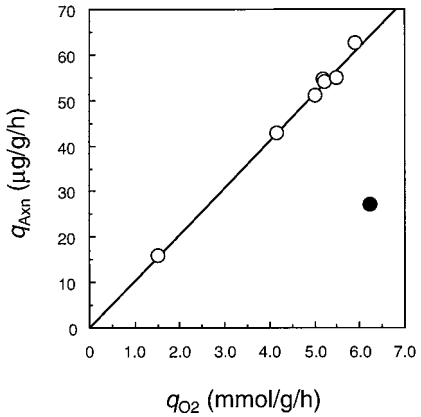

FIG. 3. Relationship between the specific astaxanthin production rate (q_{Axn}) and the initial specific oxygen uptake rate (q_{O_2}) during the early exponential phase. The mean values from Tables 2 and 3 are shown. The line was calculated by the least-squares regression method (r = 0.999), except for the data (●) obtained for 120 g of glucose per liter.

data point at 120 g of glucose per liter). As shown in Table 4, $Y_{X/S}$, $\Delta X/\Delta O_2$, and $\Delta X/\Delta CO_2$ decreased with increasing initial glucose concentration, whereas $\Delta X/\Delta N$ increased. The variations in $Y_{X/S}$, $\Delta X/\Delta O_2$, $\Delta X/\Delta CO_2$, and $\Delta X/\Delta N$ suggest that changes in anabolism occur as a result of variations in the C/N ratio due to the varying glucose supply. On the other hand, $\Delta Axn/\Delta S$ increased with increasing initial glucose concentration. Thus, it was considered that an increase in astaxanthin production might be accompanied by a change in anabolism.

To examine the material balance between (i) glucose consumption and (ii) cell growth and carbon dioxide evolution, the carbon recovery, R_C was calculated. Larsson et al. (19) have reported that the carbon content in the *S. cerevisiae* cell was almost constant at 0.50 g of carbon per g (dry weight) of cells at each dilution rate in chemostat cultures under carbon-, nitrogen-, or carbon- and nitrogen-limiting conditions. In the present study, therefore, it was speculated that the carbon content in the cell, γ_X in equation 1 should be 0.50 g per g. As R_C could be regarded as almost 1.00 (Table 4), the glucose consumed would primarily be converted to cell materials and carbon dioxide in all cultures.

Two-stage fed-batch culture. As shown in Table 3, cell growth was inhibited by high concentrations of glucose. To maintain the glucose concentration at a low level, therefore, a fed-batch culture is suitable for *P. rhodozyma* cultivation. As shown in Tables 3 and 4, the astaxanthin production was enhanced by a high initial C/N ratio in the medium, whereas a lower C/N ratio was suitable for cell growth. Thus, to simultaneously obtain high cell mass and high astaxanthin production, the cultivation period should be separated into two stages: the growth stage (first stage), in which the medium feed maintains a low C/N ratio, and the astaxanthin production stage (second stage), in which the medium feed maintains a high C/N ratio to enhance astaxanthin production.

In the first stage of the fed-batch culture, efficient cell growth must be attained. As shown in Table 3, the highest μ_{max} was obtained in the batch culture at 10 g of glucose per liter. In this culture, the ratio of consumption of glucose to consumption of ammonium during exponential growth was calculated as 2.80 mol of glucose per mol of ammonium. In order to perform the medium feed with the C/N ratio at 2.80 mol of glucose per mol of ammonium, the feed rate of the glucose solution (500 g per liter, which is almost equal to 2.80 mol per liter) was set at a value twice that of the ammonium solution (2 M). Since no pH-altering extracellular products were detected in the aerobic culture of *P. rhodozyma*, as mentioned above, the decrease in pH was considered to be primarily due to the ammonium consumption. In such a case, exponential cell growth could easily be maintained by the simultaneous feeding of a glucose solution and an ammonium solution (for pH control), which would respond to decreases in pH (26). In the first stage, therefore, the glucose solution was fed simultaneously with an ammonium solution, for pH control.

In the second stage of the fed-batch culture, it was necessary to enhance astaxanthin production by a medium feed with a high C/N ratio. Therefore, the feed rate for the ammonium solution was set at 12.5% of that for the glucose solution, and the medium feed was performed with the C/N ratio at 11.2 mol of glucose per mol of ammonium. It is well known that an abrupt increase in DO concentration is observed in an aerobic culture when the carbon source has been consumed (35). In the second stage, therefore, the glucose and ammonium solutions were fed simultaneously in response to an increase in DO concentration.

Typical time profiles for the two-stage fed-batch culture are shown in Fig. 4, and the results are summarized in Table 5. As shown in Table 5, a high cell concentration, 20.4 g per liter, was obtained in a relatively short period of time (40 h), giving μ_{max} = 0.12 h^{-1} in the first stage. In the second stage, a relatively high level of astaxanthin production, 10.2 mg per

TABLE 4. Calculated results from the experimental data in the DO-controlled batch cultures[a]

Glucose (g/liter)	$Y_{X/S}$ (g/g)	$\Delta X/\Delta N$[b] (g/g)	$\Delta X/\Delta O_2$[c] (g/mol)	$\Delta X/\Delta CO_2$[d] (g/mol)	$\Delta Axn/\Delta S$[e] (mg/g)	R_C[f]
10	0.46	13.8	35.1	30.4	0.13	1.09
20	0.46	13.8	34.3	32.0	0.14	1.01
40	0.40	14.4	25.2	23.7	0.17	0.93
80	0.36	21.2	19.9	19.3	0.19	1.01

[a] All values were calculated from the means of the experimental data shown in Table 3.
[b] Ratio of final cell concentration to ammonium consumption.
[c] Ratio of final cell concentration to oxygen uptake.
[d] Ratio of final cell concentration to carbon dioxide evolution.
[e] Ratio of final astaxanthin concentration to glucose consumption.
[f] Carbon recovery for glucose consumption against final cell concentration and carbon dioxide evolution.

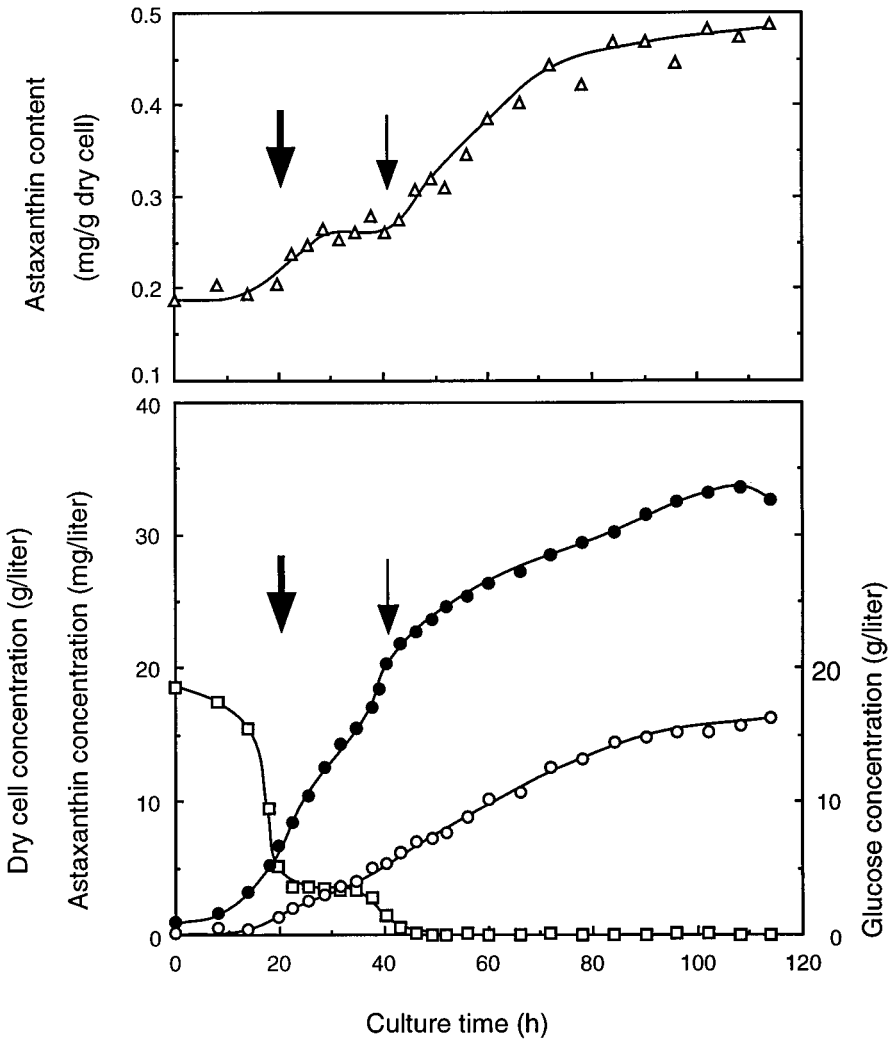

FIG. 4. Results for two-stage fed-batch culture. The thick and thin arrows show the starts of medium feeding with pH and DO control, respectively. Symbols: ●, cell concentration; □, glucose concentration; ○, astaxanthin concentration; △, astaxanthin content.

liter, was obtained. ΔAxn/Δ*X* in the second stage was ca. 3-fold higher than that in the first stage and 1.5-fold higher than that in the DO-controlled batch culture at 80 g of glucose per liter (Table 3), indicating that the astaxanthin production was enhanced much more in the second stage than in the first stage. As a result of the two-stage fed-batch culture, Δ*X* and ΔAxn reached 33.6 g per liter and 16.0 mg per liter, respectively, which were the highest values for all of the cultivations performed in the present study. These results suggest that the two-stage fed-batch cultures used in the present study are very effective for maximizing *P. rhodozyma*'s production of astaxanthin.

DISCUSSION

The results in the present study indicate that astaxanthin production by *P. rhodozyma* is affected by fermentation, respiration, and anabolism. Two questions then arise: (i) why astaxanthin production is enhanced by respiration and repressed by

TABLE 5. Results for two-stage fed-batch culture[a]

Stage	μ_{max} (h^{-1})	Δ*t* (h)	Δ*X* (g/liter)	ΔX_t[b] (g)	ΔS_t[c] (g)	ΔAxn (mg/liter)	ΔAxn_t[d] (mg)	ΔAxn/Δ*X* (mg/g [dry wt] of cells)
First (growth)	0.12	40	20.4	43.2	104.3	5.80	12.3	0.28
Second (astaxanthin production)	0.03	74	13.2	29.9	166.9	10.2	23.3	0.78
First + second		114	33.6	73.1	271.2	16.0	35.6	0.48

[a] Results are from a single experiment. Nomenclature is the same as in Tables 1 and 2.
[b] Total cell production.
[c] Total glucose consumption.
[d] Total astaxanthin production.

fermentation and (ii) why astaxanthin production is enhanced by a higher initial C/N ratio at high levels of oxygen. To answer these two questions, we analyzed the experimental data by a stoichiometric procedure.

To answer the first question, we will discuss the NADH balance. It is known that under oxygen-limiting conditions, NADH accumulates due to a decrease in the efficiency of oxidative phosphorylation (33). Therefore, to maintain the NADH balance in cell, it is necessary that the excess NADH be reoxidized, and it would be decreased by repressing a biosynthetic pathway which produces a large amount of NADH.

Furthermore, Johnson and Schroeder (16) have described a reaction model for astaxanthin biosynthesis from glucose by *P. rhodozyma*:

$$12\text{Glucose} + 4O_2 + 14\text{NADPH} + 48\text{NAD}^+ =$$
$$1\text{Astaxanthin} + 32CO_2 + 14\text{NADP}^+ +$$
$$48\text{NADH} + 12H_2O + 34H^+ \quad (4)$$

Equation 4 indicates that a large amount of NADH is produced as a side product of the astaxanthin production, indicating that astaxanthin production might also be repressed with an increase in NADH accumulation due to the oxygen-limiting conditions. This agrees with equation 2. On the other hand, when a sufficient amount of oxygen is supplied to the cell, the accumulated NADH can be reoxidized to NAD^+, using 1 mol of oxygen for 2 mol of NADH. In addition, since an oxygen molecule is introduced into the astaxanthin molecule (6), oxygen is also material requirement for astaxanthin biosynthesis. Therefore, it is considered that a sufficient supply of oxygen can enhance astaxanthin production by preventing the accumulation of NADH and by supplying oxygen molecules as a starting material, which agrees with equation 3. We therefore considered that this was why the astaxanthin production was enhanced with increasing oxygen uptake (respiration) and repressed with increasing ethanol production (fermentation).

Second, we discuss the reason why the astaxanthin production is enhanced by a high glucose supply at a fixed nitrogen concentration, that is, a high initial C/N ratio in the medium with high levels of oxygen. An increase in astaxanthin production might be accompanied by variations in anabolism, such as $Y_{X/S}$ and $\Delta X/\Delta N$, as shown in Table 4. To answer this question, therefore, the differences in anabolism between *P. rhodozyma* cells with a low astaxanthin content and those with a high astaxanthin content were analyzed by a stoichiometric procedure. First, in order to estimate the difference in the growth characteristics, the growth stoichiometric equations were established. To establish these equations, we assumed that (i) glucose was the sole carbon source and was converted primarily to cell materials and carbon dioxide, (ii) ammonium was the sole nitrogen source, and (iii) 5% of the cell mass is composed of ash. The basic stoichiometric equation can be given as follows:

$$C_6H_{12}O_6 + aNH_3 + bO_2 = 6Y_C CH_xO_yN_z + cH_2O + dCO_2$$

where Y_c is the ratio for carbon conversion from glucose to cells (dimensionless) and a, b, c, and d are the stoichiometric constants. Based on the calculated values of $Y_{X/S}$, $\Delta X/\Delta N$, $\Delta X/\Delta O_2$, and $\Delta X/\Delta CO_2$ (Table 4), a, b, and d can be calculated. Y_C can be rewritten by using $Y_{X/S}$, x, y, and z. Therefore, one can estimate c, x, y, and z by taking into account the balances of C, O, H, and N. For example, the growth stoichiometric equation for the batch culture with 80 g of glucose per liter can be estimated as follows:

TABLE 6. Estimated results for the primary metabolism of *P. rhodozyma* in batch culture[a]

Glucose (g/liter)	Formula	Mol wt[b]	N/C[c] (g/g)	ΔNADPH[d] (mmol/g)
10	$CH_{1.74}O_{0.52}N_{0.10}$	24.7	0.057	8.1
20	$CH_{1.71}O_{0.53}N_{0.10}$	24.5	0.057	7.3
40	$CH_{1.44}O_{0.49}N_{0.09}$	23.7	0.053	4.0
80	$CH_{1.44}O_{0.56}N_{0.06}$	24.5	0.034	2.9

[a] All values were estimated from the values shown in Table 4.
[b] Estimated molecular weight of cell.
[c] Estimated nitrogen content in cell.
[d] Estimated NADPH consumption per gram (dry weight) of cells formed (see equation 5).

$$C_6H_{12}O_6 + 0.17NH_3 + 3.26O_2 = 2.65CH_{1.44}O_{0.56}N_{0.06} +$$
$$4.35H_2O + 3.35CO_2$$

The results are summarized in Table 6.

Cell assimilation equations with cosubstrates such as ATP and NADPH were then established by using the cell formula. For this, we made two assumptions: (i) Y_{ATP} is 10.5 g per mol, and (ii) a reductant for the synthesis of cell materials is NADPH. The basic equation can be expressed as follows:

$$C_6H_{12}O_6 + 6zNH_3 + e\text{NADPH} + eH^+ + f\text{ATP} =$$
$$6CH_xO_yN_z + e\text{NADP}^+ + f\text{ADP} + gH_2O$$

where e, f, and g are the stoichiometric constants. These constants can be calculated based on the formula estimated above and the Y_{ATP} concept. For example, the cell assimilation equation for the batch culture with 80 g of glucose per liter can be given as follows:

$$C_6H_{12}O_6 + 0.36NH_3 + 0.42\text{NADPH} + 0.42H^+ +$$
$$13.98\text{ATP} = 6CH_{1.44}O_{0.56}N_{0.06} +$$
$$0.42\text{NADP}^+ + 13.98\text{ADP} + 2.76H_2O \quad (5)$$

These results are also summarized in Table 6.

As shown in Table 6, it was estimated that the nitrogen content per gram (dry weight) of cells decreased with an increase in the glucose supply. The decrease in nitrogen content might be due to a decrease in the protein content of the cell. Actually, the protein content decreased with increasing glucose supply. For example, the protein content was 0.62 ± 0.02 g of protein per g (dry weight) of cells at 10 g of glucose per liter, but it decreased to 0.53 ± 0.02 g per g at 80 g of glucose per liter. As shown in Table 6, it was also estimated that the NADPH consumption for anabolism per gram (dry weight) of cell decreased with an excess glucose supply. Albers et al. (1) and Bruinenberg et al. (7) have reported that when yeast is grown in a minimal medium containing glucose as a carbon source and ammonium as a nitrogen source, a large amount of NADPH is consumed in amino acid synthesis. Therefore, it was considered that a decrease in NADPH consumption might be due to a decrease in protein synthesis. The astaxanthin synthesis requires a large amount of NADPH, as shown in equation 4. The decrease in NADPH consumption for primary metabolism such as anabolism leads to an increase in the amount of NADPH which can be utilized for astaxanthin production. We therefore believe that this is why astaxanthin production was enhanced by the high initial C/N ratio at high levels of oxygen.

As mentioned above, astaxanthin production might be in-

creased by the repression of primary metabolism such as protein synthesis, whereas cell growth might be decreased by the repression of primary metabolism. Therefore, a two-stage fed-batch culture, in which the astaxanthin production stage is separated from the cell growth stage, should be effective. This hypothesis was supported by the results of the two-stage fed-batch culture in the present study.

In conclusion, it appears that astaxanthin production in *P. rhodozyma* is very much affected by cell metabolism, i.e., anabolism, respiration, and fermentation. This indicates that in order to improve astaxanthin production, it is necessary not only to optimize the synthetic pathway of astaxanthin but also to optimize the whole metabolic pathway in the cell, especially the anabolic and respiratory pathways. An et al. (2) have isolated astaxanthin-overproducing mutants of *P. rhodozyma* by using antimycin A, which is an uncoupling reagent for oxidative phosphorylation, as a selection marker. Because this selection method was developed based on the relationship between respiration and astaxanthin production, it can be considered that this method is very reasonable and supports our conclusions.

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