J. Microbiol. Biotechnol. (2003), **13**(2), 175–181

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

High-Level Production of Astaxanthin by Fed-Batch Culture of Mutant Strain *Phaffia rhodozyma* AJ-6-1

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Received: January 14, 2002
Accepted: July 26, 2002

Abstract The production of a carotenoid astaxanthin, a growth-associated principal pigment, is limited in a batch cultivation, because a high glucose concentration severely inhibits the cell growth and also influences the carotenoid production. Therefore, a fermentation strategy including effective chemicals for the high-level production of cells and astaxanthin by a mutant strain *Phaffia rhodozyma* AJ-6-1 was developed in a fed-batch culture. First, a production medium for maximizing the cell and astaxanthin yields was formulated and optimized. Using this optimized medium, the highest cell and astaxanthin concentrations obtained were about 38.25 g/l and 34.77 mg/l, respectively. In addition, an attempt was made to increase the amount of astaxanthin using effective chemicals such as ethanol and acetic acid, which are known as an inducer and/or precursor of carotenoid synthesis. When either 10 g/l ethanol or 5 g/l acetic acid was added to investigate the resulting astaxanthin content, a relatively high astaxanthin concentration of 45.62 mg/l and 43.87 mg/l, respectively, was obtained, and the cell concentrations also increased slightly under these conditions. Therefore, these results imply that a fed-batch culture of the mutant strain *P. rhodozyma* AJ-6-1 could be effectively employed in the commercial production of astaxanthin, although the factors affecting the productivity remain to be elucidated.

Key words: *Phaffia rhodozyma*, carotenoids, astaxanthin, fed-batch culture

As lipid-soluble pigments, carotenoids constitute a wide range of natural pigments that are ubiquitously found in nature. Over 600 natural carotenoid species are currently known as distinct color determinants ranging from yellow to red, and, with such given traits, they are employed in the agrochemical industry as a food and feed additive. Among them, astaxanthin (3,3'-dihydroxy-β,β'-carotene-4,4'-dione), a derivative of β-carotene, is an abundant carotenoid found in marine animals, such as salmonids and crustaceans [11]. The use of astaxanthin for pigmentation in aquaculture, especially as a feed supplement in farmed salmon, trout, and prawns, to obtain a natural red-pink color is necessary, because animals are unable to synthesize it *de novo* [9, 12]. Astaxanthin also has the additional capability of a higher antioxidant activity than β-carotene and α-tocopherol [3, 23]. Therefore, astaxanthin has attracted commercial interest not only as a pigmentation source but also as a potent antioxidative reagent [11] that can delay aging and degenerative diseases in eukaryotic animals, including humans. Furthermore, carotenoids appear to prevent certain cancers [4] and stimulate the immune system [5], and a recent FDA communication permitted the use of astaxanthin as a supplement and listed it for use in salmonid fish feed.

Due to the increasing demand for the commercial production of astaxanthin, it is currently produced by chemical method in an all-trans form by F. Hoffman-La Roche Co. (Switzerland) and is sold for more than $2,000 US per kg [16]. Although synthetic methods have been commercially evaluated as a potential means, biotechnological approaches have gained as much attention due to the strict regulations concerning the safety of chemicals as food additives and the poor adsorption of synthetic carotenoids compounded with a biological source, thereby resulting in a preference for natural carotenoids over synthetic pigments [1].

Various natural biological sources have been identified as astaxanthin producers, including the bacteria *Mycobacterium lacticola* and *Brevibacterium* sp. [22], green algaes *Haematococcus* sp. [16, 19], *Neochloris wimmeri* and *Chlamydomonas nivalis* [11], the basidiomycetous fungus *Peniophora* sp. [7], and the heterobasidiomycetous yeast *Phaffia rhodozyma* [10]. Among them, only two, the green

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alga *H. pluvialis* and yeast *P. rhodozyma*, are currently considered as sources of astaxanthin for industrial production. In *H. pluvialis*, mixotrophic cultivation and suitable irradiance have resulted in a good astaxanthin yield of up to about 60 mg/l [16, 27]. When compared with *Haematococcus*, the astaxanthin yields of *P. rhodozyma* are in the range of 20 to 34 mg/l and thus remains to be improved [15, 25], whereas the *Phaffia* strain is more desirable for use as an industrial pigment source due to its heterotrophic metabolism, rapid growth rate, high cell density achievement, nutritional quality, and safety as a food additive [2]. However, although *P. rhodozyma* is known to produce astaxanthin as a major pigment, its commercial production is restricted by a low content in the wild-type strain. Moreover, it has also been established that the cell yield and astaxanthin production of *P. rhodozyma* are significantly influenced by high glucose concentration [20], therefore, it is difficult to achieve a high volumetric production using a batch culture.

The current study describes astaxanthin production by a mutant of *P. rhodozyma* in fed-batch fermentation. To achieve a high-level production of astaxanthin, the nutrient sources were optimized using glucose and yeast extract in the feed medium, and then an effective substrate, ethanol or acetic acid, was added to increase the accumulation of astaxanthin in the grown cells.

MATERIALS AND METHODS

Microorganisms and Media

The yeast strain derivative *P. rhodozyma* AJ-6 used in the present work was generated from the parent strain *P. rhodozyma* ATCC24202 by conventional mutagenesis in the author's laboratory [26]. In brief, NTG mutagenesis was performed by washing the grown yeasts with distilled water in 5 ml phosphate buffer (pH 7.0), and then the cells were suspended in the same buffer. Mutation was induced by addition of 5 ml NTG solution (0.3%) for 30 min [13], and an aliquot of sample was spread on the selective plate containing 1 mM β-ionone. Acriflavine mutagenesis was also done with grown cells (5 ml), followed by further incubation at 24°C for 2 days after the addition of 0.4% (w/v) acriflavine solution. UV mutagenesis was carried out according to the method previously described by An *et al.* [1]. The parent strain was obtained from KRIBB (Korea Research Institute of Bioscience and Biotechnology). Unless otherwise described, the strain was routinely maintained on YM agar plates at 4°C and transferred monthly. The initial culture medium was formulated on the basis of the components and concentrations of the most common culture medium for *P. rhodozyma*. The seed culture was grown in medium I (YPD; glucose 10 g/l, yeast extract 5 g/l, bacto peptone 5 g/l), while the fed-batch culture was initially grown in medium II (10 g/l of glucose and yeast extract).

Inoculation and Culture Conditions

A loopful of yeast was inoculated into a 250-ml baffled flask containing 25 ml of medium I and then incubated at 24°C for 48 h. An aliquot (5 ml) of the culture broth was used to re-inoculate a 500-ml baffled flask containing medium I (45 ml) and further incubated for 36 h. For the main cultures, flasks and a fermentor were inoculated with an appropriate volume of seed broth to a volumetric ratio of 10% (v/v).

To select an appropriate nitrogen source from common sources, flask cultures were carried out in 250-ml baffled flask containing 40 ml of a glucose medium (10 g/l). The fed-batch cultures were grown at 24°C in a 2.5-l stirred tank fermentor (KoBioTech, Incheon, Korea) with an initial 1 l of medium II. The final working volume and total glucose concentration were 1.4 l and 100 g/l, respectively. The aeration rate and initial agitation speed were set at 1.5 vvm and 300 rpm, respectively. Under these conditions, the dissolved oxygen concentration was controlled at above 40% by increasing the agitation speed. In an experiment to determine the optimal C/N ratio for the feed medium, the initial and final working volumes were 0.8 and 1.5 l, respectively, while the other conditions were identical to those described above.

Analyses

The cell growth was monitored by measuring the optical density at 660 nm of suspended cells, which were collected from the culture broth and then washed with distilled water, based on a correlation factor defined as the gram dry cell weight per liter of culture broth that corresponded to 1.77 of $OD_{660\,nm}$. For the dry cell weight determination, an aliquot of the culture broth was filtrated through a membrane filter (0.45 μm) and then washed twice with distilled water. The resulting pellet was dried to a constant weight at 60°C under vacuum. The glucose and ethanol concentrations were measured using a glucose analyzer (YSI, Yellow Springs, U.S.A.) and gas chromatography (Hitachi, Tokyo, Japan), respectively.

For the pigment content analyses, the cells were harvested by centrifugation at 4,000 rpm for 15 min, washed with distilled water, and then dried. The dried cells were resuspended in 2 ml of preheated dimethylsulfoxide (DMSO) and then vortexed. Next, 1 ml of phosphate buffer (pH 7.0) and 5 ml of hexane-ethylacetate (50:50) were added, mixed by vortexing, and then the resulting solution was centrifuged (4,000 rpm) and analyzed [21]. The total carotenoid concentration was determined using a spectrophotometer at 474 nm, based on a previously reported extinction coefficient [1]. The astaxanthin was quantified by HPLC (Waters, Miliford, U.S.A.), using a UV detector at 474 nm

and Nova-Pak C18 column (3.9×300 mm, Waters), with an isocratic solvent of methanol-acetonitrile (75:25) as the mobile phase at a flow rate of 1.0 ml/min.

RESULTS

Generation and Evaluation of an Astaxanthin-Overproducing Mutant Strain

As a preliminary experiment, the wild-type yeast *P. rhodozyma* ATCC24202 was mutated to generate a hyper-strain for the production of the carotenoid, astaxanthin. The over-producing mutant strains generated by mutagenesis and protoplast fusion were then evaluated for their potential in astaxanthin production [1, 14]. The mutant *P. rhodozyma* AJ-6 from serial mutagenesis, consecutively treated with conventional mutagenic agents, such as NTG, UV, acriflavine, and γ-ray [26], produced the highest astaxanthin concentration, at about 4.49 mg/g-DCW, which was about 15-fold higher than that from the wild-type strain. In general, there is a trend toward a decreasing specific growth rate during the progression to a hyper-strain, especially in the production of a growth-related compound, due to metabolic imbalance, thereby resulting in physiological stress. Although the strain *P. rhodozyma* AJ-6 exhibited higher productivity than that from the wild-type or other mutated strains, the serial mutagenesis resulted in defected cell growth and reduced final cell mass, and also frequently reverted after several successive transfers in a solid plate or solution broth. Therefore, to overcome this barrier to practical application, an attempt was made to further screen the mutant from revertant pools. Consequently, a stable clone which retained a high content of astaxanthin (2.0 mg/g-DCW) and showed a growth rate comparable to the wild-type strain, was finally isolated. The resulting clone was renamed as *P. rhodozyma* AJ-6-1.

Using the isolated strain, the characteristics of its cell growth and astaxanthin production by batch culture were examined under various conditions. The maximum cell and carotenoid concentrations were about 7.44 g/l and 14.46 mg/l, respectively, thereby resulting in the values close to those of a hyper- but unstable mutant *P. rhodozyma* AJ-6, when the mutant strain (AJ-6-1) was cultivated in a predetermined medium [26] supplemented with 20 g/l of glucose, 20 g/l of corn steep liquor, 0.5 g/l of $MgSO_4 \cdot 7H_2O$, and 0.16 g/l of $MnCl_2 \cdot 4H_2O$.

Media Formulation for Astaxanthin Production by Fed-Batch Culture

Since astaxanthin production by *P. rhodozyma* is known to depend on the cell mass, much efforts have previously been devoted to increasing the cell mass using a commercially available glucose. In spite of various experiments conducted, the levels of cell mass and productivity were similar to those obtained presently in the batch culture [24, 25]. This is mainly due to the fact that the cell growth and astaxanthin production are severely affected at a high glucose concentration in a typical batch culture. Therefore, the use of an alternative cultivation strategy, such as a fed-batch culture [13], for a high cell mass and density could be a possible strategy for further progress.

In order to maximize the cell productivity of astaxanthin production in a fed-batch culture, it is first necessary to formulate an effective medium for cell growth, since astaxanthin is produced as a growth-associated secondary metabolite, thereby requiring a high cell mass. In the current study, we selected glucose as the carbon source, since it is generally known to be the most effective carbon source for cell growth. To identify a suitable nitrogen source for cell production, the characteristics of cell growth and pigment production were investigated using flask cultures of the mutant strain *P. rhodozyma* AJ-6-1. As a commercially available nitrogen source, corn steep liquor (CSL) was used first for the production of astaxanthin in a fed-batch culture, as CSL has already been successfully used as the nitrogen source in a batch culture [26]. However, poor solubility of the suspended insoluble particles limited the use of CSL as the nitrogen source in fed-batch cultivation. The reason for this was because making a concentrated feed medium was difficult and the suspended particles were not fully utilized by *P. rhodozyma* AJ-6-1, resulting in an accumulation of suspended particles during the cultivation. Therefore, an attempt was made to select an effective source from among the components of a YM medium, which typically contains 3 g/l of yeast extract, 3 g/l of malt extract, and 5 g/l of bacto peptone, and supports the full growth of *P. rhodozyma*. The characteristics of cell growth were investigated using flask cultures in a medium containing 20 g/l of glucose, with a total of 10 g/l of nitrogen source(s). The glucose concentration was tentatively selected from a preliminary experiment by considering total cell mass and pigment content. As a result, the cell growth was more favorable in a medium containing yeast extract as the nitrogen source, compared with any of the others tested.

The effect of nitrogen sources in combination with yeast extract on cell growth was also investigated. Thus, a total of four sets, including yeast extract only, yeast extract-bacto peptone (1:1), yeast extract-bacto peptone-malt extract (3:4:3), and yeast extract-malt extract (1:1), were used. As shown in Table 1, the dry cell weight obtained from each culture broth was 5.88, 5.12, 3.85, and 1.42 g-cell/l, respectively. Among the nitrogen sources tested, yeast extract exhibited the best result for the biomass production of *P. rhodozyma* AJ-6-1, while a comparable yield was also obtained with the yeast extract-bacto peptone (1:1). In the cultures supplemented with malt extract, either singulary or in combination, the cell growth was relatively poor compared to others tested.

Table 1. Effect of organic nitrogen sources on cell growth.

	X_m[a] (g/l)	Productivity (g-cell/l·h)	$Y_{X/S}$[b] (g-cell/g-glucose)
YE	5.88	0.08	0.30
YE+BP	5.12	0.07	0.27
YE+BP+ME	3.85	0.04	0.19
YE+ME	1.46	<0.01	0.14

The cells were cultivated in a baffled flask containing 40 ml of a medium supplemented with 20 g/l of glucose and a nitrogen source (total 10 g/l): YE, yeast extract; BP, bacto peptone; ME, malt extract. Each culture was conducted in duplicate.

[a]X_m; maximum cell concentration.

[b]$Y_{X/S}$; yield of biomass defined as the dry cell weight per gram of glucose utilized.

Because of its importance in achieving a high cell density in a fed-batch culture, a second experiment was carried out to establish the effect of the initial glucose concentration on the cell growth. The cell growth was examined in a fermentor containing 10 g/l of yeast extract and different initial glucose concentrations ranging from 5 to 12.5 g/l. As expected, the cell growth and resulting cell mass were significantly influenced by the initial glucose concentration. A higher cell concentration and productivity were obtained with an increasing glucose concentration up to 10 g/l, whereas only a minor difference in the carbon yield relative to cell growth was observed with an increasing glucose concentration (Table 2). A further increase in the glucose concentration (12.5 g/l) resulted in a drastic decrease in the cell concentration, a particularly low yield with 0.42 g yeast per gram of glucose utilized, as previously reported by Johnson and Lewis [10] in which a reduced cell yield was observed when the glucose concentration exceeded 15 g/l. Based on the results obtained, 10 g/l of glucose was considered to be the optimal concentration for further experiments, because of the highest carbon yield (0.58 g/g-glucose). Increasing the glucose concentration resulted also in an increased lag period (data not shown).

To confirm the above result that yeast extract or yeast extract-bacto peptone was the best nitrogen sources at 20 g/l glucose concentration, we further analyzed the cell growth and pigment content for dependency of nitrogen source in the optimized glucose concentration (10 g/l). However, there were no significant differences in a preference to nitrogen sources, although the final cell mass and astaxanthin content varied somewhat.

Table 2. Effect of initial glucose concentration on cell growth.

Glucose (g/l)	X_m (g/l)	Productivity (g-cell/l·h)	$Y_{X/S}$ (g-cell/g-glucose)
5.0	3.20	0.10	0.53
7.5	3.84	0.12	0.50
10.0	5.81	0.15	0.58
12.5	4.20	0.09	0.33

The cell growth was examined in a fermentor containing 10 g/l of yeast extract and various glucose concentrations. All experiments were carried out in duplicate.

Optimal C/N Ratio of Feed Medium

In the next experiment, the C/N ratio for the feed medium was tested and determined for cell growth and pigment production. For the optimization experiments, the yeast cells were cultivated in a medium containing a predetermined concentration of glucose, while varying the concentration of yeast extract and maintaining a set working volume. A feeding strategy was employed to keep the glucose concentration above a certain lower limit, below which cell growth was inhibited. When the residual glucose concentration decreased below 5 g/l, the flow rate was manually adjusted to feed the glucose solution at a faster rate. Under these conditions, the cell cultivation was continued until the glucose was exhausted.

As shown in Table 3, although a gradual increase of the astaxanthin concentration was observed in the feed medium with an increasing C/N ratio, the productivity was decreased, when the ratio became higher than 2:1. Therefore, the maximum cell and astaxanthin concentrations of about 33.08 g/l and 22.17 mg/l, respectively, were produced, when a feed medium with C/N ratio of 2:1 was used. A comparative analysis of the present results with a report by Yamane *et al.* [24] clearly revealed that the astaxanthin production was significantly influenced by the C/N ratio, and that cell growth was inhibited with a high C/N ratio in the feed medium.

Effect of Ethanol and Acetic Acid Addition on Astaxanthin Production

Based on a fed-batch culture with an optimized C/N ratio in the feed medium, the final set of experiments to increase the astaxanthin content was performed using two favorable substances, ethanol and acetic acid, both of which are known to have a stimulating effect on astaxanthin accumulation in growing and/or grown cells.

Table 3. Effect of C/N ratio of feed medium on cell growth and astaxanthin production.

C/N ratio (Glucose:Yeast extract)	Cell conc. (g/l)	Astaxanthin (mg/l)	Productivity (g-cell/l·h)
1:1	25.14	17.03	0.32
2:1	33.08	22.17	0.35
3:1	29.46	24.87	0.34
4:1	27.00	26.58	0.28

In this experiment, the initial and final working volumes were 0.8 and 1.5 l, respectively, and all other conditions were identical to those described in Materials and Methods. The fed-batch cultivation was performed until the glucose was depleted in the feeding solution.

Table 4. Cell growth and astaxanthin production by ethanol and acetic acid feeding in fed-batch cultures.

Feeding conditions		Cell mass (g/l)	Astaxanthin (mg/l)	Total carotenoids (mg/l)
Control	0 g/l	38.25	34.77	40.90
Ethanol	5 g/l	34.63	39.96	45.98
	10 g/l	37.15	45.62	50.02
	15 g/l	30.90	38.27	44.86
Acetic acid	5 g/l	40.59	43.87	48.14
	10 g/l	36.27	37.06	46.26
	15 g/l	35.88	28.81	40.07

In this experiment, the initial and final working volumes were 1.0 and 1.4 l, respectively, and all other conditions were identical to those described in Materials and Methods. The fed-batch cultivation was performed until the glucose was depleted in the feeding solution, then each effective substance (ethanol and acetic acid) was fed. For a clear comparison, the control cells were also incubated after the residual glucose was depleted.

To investigate the effect of ethanol induction, the cells were first grown in the optimized medium with a final glucose concentration of 100 g/l until the glucose was depleted. Then, ethanol, ranging from 0 to 15 g/l, was added to the culture broth. As shown in Table 4, the astaxanthin content was further increased in the presence of ethanol, compared to the control without any supplement. Although the trend varied, the final cell concentration decreased slightly with an increased ethanol concentration. This was probably due to the toxic effect of ethanol on the yeast cells. The maximum cell and astaxanthin concentrations obtained were about 37.15 g/l and 45. 62 mg/l, respectively, in a 24 h culture after the addition of 10 g/l ethanol. At this concentration, ethanol showed a negligible effect on the cell growth (Fig. 1).

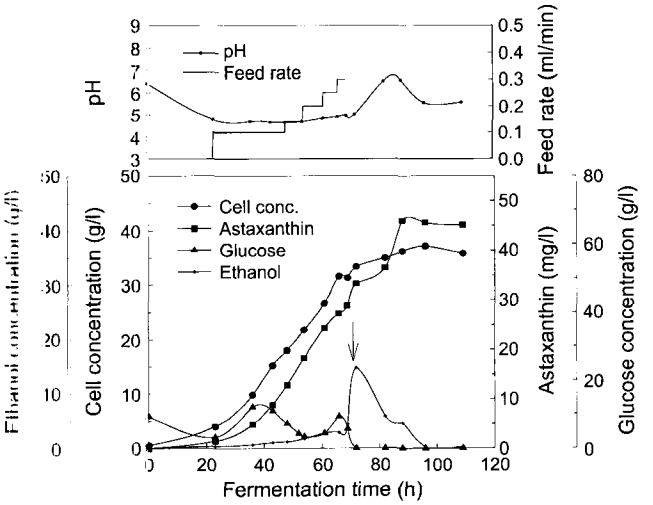

Fig. 1. Enhancement of astaxanthin production by *Phaffia rhodozyma* AJ-6-1 with ethanol feeding.
Glucose and yeast extract (2:1) were fed by manual adjustment of the feeding solution, and 10 g/l of ethanol was fed at the time indicated by an arrow. The dissolved oxygen concentration was maintained at above 40%.

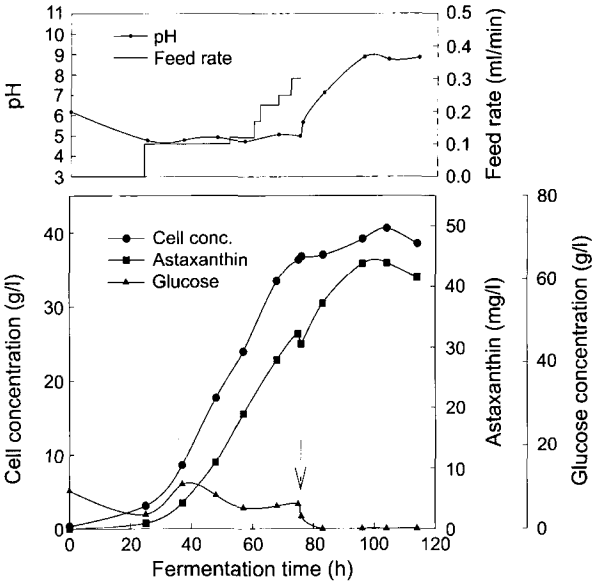

Fig. 2. Enhancement of astaxanthin production by *Phaffia rhodozyma* AJ-6-1 with acetic acid feeding.
Glucose and yeast extract (2:1) were fed by manual adjustment of the feeding solution, and 5 g/l of acetic acid was fed at the indicated time. The dissolved oxygen concentration was maintained at above 40%.

The effect of acetic acid on astaxanthin accumulation was also tested. When 5 g/l of acetic acid was added, the maximum cell and astaxanthin concentration obtained were about 40.59 g/l and 43.87 mg/l, respectively (Fig. 2). The values were slightly lower than those obtained with ethanol feeding. The addition of more than 5 g/l of acetic acid also led to a gradual decrease in the cell and astaxanthin concentrations, thereby suggesting that a higher concentration of acetic acid also inhibited cell growth, similar to ethanol feeding. In a earlier study, Meyer and du Preez [17] described the effect of various chemicals on cell growth and astaxanthin production, and reported that acetate acts as a pH titrant for pigment production by *P. rhodozyma* and has no significant effect on the cell growth and astaxanthin content.

DISCUSSION

The yeast strain *Phaffia rhodozyma* appears to be the best candidate for producing a natural carotenoid astaxanthin among all the strains reported so far, because it is non-toxic to humans and easier to process development. Therefore, extensive efforts have been made to improve pigment production by strain development and high-cell density cultures, however, only a limited number of cases on the production of astaxanthin by fed-batch cultivation have been reported [22, 27]. In the present study, we

Table 5. Comparison of cell and astaxanthin concentrations reported in related experiments.

Strain	Culture	Cell (g/l)	Astaxanthin (mg/l)	Productivity (mg/l·h)	Reference
Haematococcus pluvialis	Batch	-	49.50	(14 days)[a]	Fábregas *et al.* [6]
Haematococcus pluvialis	Batch FBC	2.65 2.74	53.43 64.36	(20 days)[a] (20 days)[a]	Zhang *et al.* [27]
Phaffia rhodozyma (ATCC 24202)	FBC[b]	33.60	16.00	0.13	Yamane *et al.* [24]
Phaffia rhodozyma (ATCC 24202)	FBC[c]	30.00	21.60	0.31	Yamane *et al.* [25]
Phaffia rhodozyma (NRRL Y-17268)	Batch[d]	9.10	5.20	-	Parajó *et al.* [18]
Phaffia rhodozyma PR-190	Batch[e]	24.90	33.70	0.20	Kusdiyantini *et al.* [15]
Phaffia rhodozyma AJ-6-1	FBC	37.15	45.62	0.47	This work

[a]As a freshwater green alga, *Haematococcus pluvialis* was grown photoautotrophically, yet the astaxanthin productivity was not described.
[b]Two-stage fed-batch culture relative to pH and DO control.
[c]Two-stage fed-batch culture with glucose and ethanol feeding.
[d]Culture grown in a flask using xylose as the carbon source.
[e]Culture conducted using glycerol as the carbon source.

demonstrated that high-level production of cells and astaxanthin by a fed-batch culture could be successfully achieved, when an optimized medium was used. This was mainly accomplished by using a hyper-strain together with a fed-batch strategy. For the media formulation, glucose was used in the fed-batch culture, because of its cheap price and high biomass yield. However, as reported elsewhere, the growth of *P. rhodozyma* was inhibited, when the glucose concentration was above 15 g/l [10, 20]. Moreover, the lag time and astaxanthin production were also influenced by a high glucose concentration. Thus, when glucose is used as the carbon source, fed-batch cultivation is more desirable for the process development, as shown in this work. As an alternative strategy, a different carbon source, that could produce a comparable result for cell growth and astaxanthin production, could be employed.

As shown in the present study, which is in accordance with previous reports, two chemicals, ethanol and acetic acid, were found to have a positive effect on the astaxanthin content and productivity. This change in the astaxanthin productivity in the yeast cells indicated that the strain is amenable to engineering for further increased pigment production. This suggestion is also strongly supported by earlier results that ethanol stimulated the induction of alcohol dehydrogenase and hydroxy-methyl-glutaryl-CoA (HMG-Co-A) reductase activity [8] and also serves, along with acetic acid, as a suitable precursor of acetyl-CoA for carotenoid synthesis, although these compounds were not readily utilized as carbon sources by strain *P. rhodozyma*. Our present results also demonstrated the possibility of establishing a two-stage culture system in which cells grown in the first stage continuously produce the astaxanthin of *P. rhodozyma*. In this system, the second stage mainly stimulates the further accumulation of pigments through the use of effective chemicals.

It was reported previously that the highest concentration of yeast cells and astaxanthin content were 24.90 g/l and 33.70 mg/l, respectively, in a batch culture [15], whereas Yamane *et al.* [24, 25] reported cell and astaxanthin concentrations of 30.0 g/l and 21.60 mg/l, respectively, with a fed-batch culture (Table 5). The results obtained in the current work showed that the highest astaxanthin content and cell concentration, when an optimized medium was simply used, were obtained with a fed-batch culture of the mutant strain *P. rhodozyma* AJ-6-1. Although an exact comparison of productivity and production costs was not conducted, due to different media formulations and cultivation strategies, the current result shows promise for further consideration. Accordingly, a fed-batch culture using the current method, along with an effective substance, appears to be a promising route for the high-level production of the pigment astaxanthin, although more effective and cheaper media formulations remain to be explored further.

Acknowledgment

This work was supported by a grant from the Korea Health R&D Project (HMP-97-F-5-0027), Ministry of Health & Welfare.

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