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Batch cultivation and astaxanthin production by a mutant of the red yeast, *Phaffia rhodozyma* NCHU-FS501

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Phaffia rhodozyma **cells were treated with the mutagenic agent NTG several times and plated on yeast-malt agar containing β-ionone as a selective medium. This mutagenesis of the yeast yielded a mutant (NCHU-FS501) with a total carotenoid content of 1454 μg g^{-1} dry biomass. Temperature and pH had only a slight effect on the volumetric pigment production by the red yeast, however astaxanthin yield and specific growth rate were influenced more significantly by temperature and pH. The optimum inoculum size, temperature and air flow rate for astaxanthin formation by the mutant in a bench-top fermentor were 7.5% (v/v), 22.5°C and 3.6 vvm, respectively. Glucose (1%, w/v) as carbon source yielded the highest volumetric astaxanthin production (6.72 μg ml^{-1}). Peptone (15.8% total nitrogen) was the best nitrogen source for astaxanthin production (6.72 μg ml^{-1}). Pigment formation by the mutant was further improved by increasing the glucose concentration to 3.5%, where the astaxanthin concentration was 16.33 μg ml^{-1}. At 4.5% glucose or above astaxanthin formation was inhibited. Control of the pH of the fermentation broth did not improve pigment production.**

Keywords: astaxanthin; *Phaffia rhodozyma*; batch cultivation; fermentor

Introduction

There is a growing commercial interest in the use of natural sources of feed nutrients. For instance, astaxanthin (3,3′-dihydroxy-β,β-carotene-4,4′-dione) is a commonly encountered keto-carotenoid in certain algae, many invertebrates and fish [11]. The use of astaxanthin as colorant in aquaculture, especially as feed supplement in farmed salmon, trout and prawns to obtain the natural red-pink color is necessary since they are not capable of *de novo* synthesis of carotenoids [8, 9]. The growing aquaculture market, together with the trend towards using natural sources of feed nutrients, expand the need for astaxanthin. In Taiwan the production of kuruma prawn (*Penaeus japonicus*) increased from 100 tons in 1981 to 10707 tons in 1990 [3]. Approximately 4.6×10^5 tons of farmed salmon will be produced worldwide and more than 1×10^5 kg of carotenoid pigments might be required by the end of the decade for inclusion in fish feed [8].

All-trans astaxanthin has been synthesized recently by F Hoffman-La Roche, Switzerland, and is now sold for more than $2000 US per kg, and was approved by the United States Food and Drug Administration (FDA) in May 1995. The use of synthetic pigments in fish feed contributes approximately 10–15% of the total feed cost. Among the microorganisms, such as *Brevibacterium*, *Haematococcus*, *Mycobacterium lacticola*, and *Phaffia rhodozyma*, that can synthesize astaxanthin [6, 18], *Phaffia rhodozyma* is the most promising microbial source for aquacultured animals because of its high content of astaxanthin [1, 10]. For commercial development, red yeast strains with increased astaxanthin are needed. The objective of this study was to obtain astaxanthin-overproducing mutants and to determine the influence of various culture conditions such as carbon and nitrogen sources on astaxanthin production in batch culture.

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Materials and methods

Yeast strains

Phaffia rhodozyma CCRC-21346 was obtained from the Culture Collection and Research Center, Food Industry Research and Development Institute, Taiwan, ROC. Strains of *P. rhodozyma* CBS-6938 and CBS-5098 were obtained from the Centraalbureau voor Schimmelcultures, Netherlands. They were maintained on slants of yeast malt agar (YM agar, Difco, Detroit, MI, USA) at 4°C. Natural isolates and mutants were stored in 40% glycerol/60% YM broth at −70°C.

Mutagenesis of red yeast

Mutagenesis was performed on freshly grown *P. rhodozyma* using *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (NTG, Sigma Chemical Co, St Louis, MO, USA) and in a manner that accords with the method described in Fang and Cheng [4, 5]. After *P. rhodozyma* was treated with various concentrations of NTG for various times, the cells were harvested by centrifugation and washed twice in 1 ml of 0.1 M sodium citrate buffer, pH 5.5. The yeasts were resuspended in the same buffer, and samples were inoculated into YM broth for overnight growth before plating them on a selective medium (YM agar with 3×10^{-4} M β-ionone) [12]. Colonies were visually screened for color changes after 7–10 days of incubation at 20°C.

Media and cultivation

Flask cultures were shaken at 150 rpm in an orbital shaker incubator (S304R, Firstek Scientific, Taiwan, ROC) at 20°C

in 500-ml Hinton flasks. Batch cultivations were conducted in a bench-top fermentor (New Brunswick Scientific, Edison, NJ, USA, Model MF-354) with an initial volume of 2.5 L. YM medium lacking dextrose (basal medium) was used in carbon assimilation experiments while Yeast carbon base (Difco), supplemented with various nitrogen sources, was used to evaluate the influence of different nitrogen sources. The ability of *P. rhodozyma* to use carbon or nitrogen source was tested in a basal medium that was supplemented with carbon sources at 1% (w/v). These carbon sources included glucose, sucrose, fructose, molasses, and D-raffinose. Peptone, beef extract, casein hydrolysate, yeast extract and potassium nitrate were used in studies of nitrogen source supplementation. Additional nitrogen sources, when used, were included at an equimolar level equivalent to 15.8% total nitrogen. The seed culture was prepared by inoculating the yeasts from a fresh slant into a 500-ml Erlenmeyer flask containing 100 ml YM broth, and incubating for 48 h on a rotary shaker (20°C, 150 rpm). The yeasts were then centrifuged, washed twice in distilled water, and resuspended in 100 ml of the basal medium. A 7.5% (v/v) inoculum was used throughout the batch fermentation study except for the effect of inoculum size on the growth and pigment formation. Foam was suppressed, when necessary, by adding an anti-foamer (KM-72, Shinetu Chemicals Co, Tokyo, Japan).

Analytical methods

Residual sugar concentration in the culture medium was determined with 3,5-dinitrosalicylic acid [16]. Sugars in molasses and D-raffinose were determined by phenol-sulfuric acid as described by Brooks and Griffin [2]. Protein concentration was measured with the coomassie blue reagent [21] with bovine serum albumin as standard. Growth was monitored with a Klett-Sumerson colorimeter (Klett, Philadelphia, PA, USA) with a No. 64 red filter. Yeast cell mass was measured by dry weight [19] and expressed as $g\,L^{-1}$. The dimethylsulphoxide (DMSO) method as described by Sedmak *et al* [22] was used to rupture *P. rhodozyma* prior to extracting the carotenoids into hexane : ethyl acetate 50% : 50% (v/v) (HPLC grade). The concentration of astaxanthin was estimated by measuring the absorbance at 480 nm. The extinction coefficient $E^{1\%}_{1\,cm} = 2150$ [22], and the formula provided by An *et al* [1] allowed the calculation of the astaxanthin concentration. In addition, astaxanthin was measured qualitatively by thin-layer chromatography (TLC) and quantified by HPLC [5,17]. Samples for HPLC analysis were diluted in hexanes : ethyl acetate 50% : 50% (v/v) and then made to 0.1% with glacial acetic acid and filtered (0.22 μm pore size) prior to injection. The eluting solvent was ethylacetate : methanol : water (5 : 18 : 2 v/v/v) and the flow rate was 0.5 ml min^{-1}. The eluant was monitored at 474 nm. For calculation of the kinetic parameters, the following formulae were used: the specific growth rate (μ) was determined from the slope of semi-logarithmic plot of cell mass ($g\,L^{-1}$) versus time. The astaxanthin yield ($Y_{P/S}$) is defined as $Y_{P/S} = (P_1 - P_0)/(S_0 - S_1)$, and the yeast cell yield ($Y_{X/S}$) is defined as $Y_{X/S} = (X_1 - X_0)/(S_0 - S_1)$; X_0, S_0 and P_0 are the initial cell, substrate, and total astaxanthin concentrations ($g\,L^{-1}$), respectively; X_1, S_1 and P_1 are the final cell, substrate, and total astaxanthin concentrations ($g\,L^{-1}$), respectively. The specific rate of astaxanthin formation is defined as $q_p = (1/X)(dp/dt)$ [23]. For statistical analyses, the results were analyzed by a statistical analysis system. The results were also analyzed using analysis of variance (ANOVA) and Duncan's test at the 5% significance level [7].

Results and discussion

Increase in astaxanthin content by NTG mutagenesis

The total carotenoid content of naturally occurring *P. rhodozyma* strains CBS 5908, CBS 6938 and CCRC 21346 after 120 h of growth in YM broth (20°C) was 235, 286 and 282 $\mu g\,g^{-1}$, respectively. The low total carotenoid content of these strains is typical of the values reported by An *et al* [1] for wild strains. An *et al* [1] indicated that among the mutagenic agents such as EMS, NTG and UV light, NTG was the best mutagen for mutagenesis of *P. rhodozyma*. *P. rhodozyma* CBS 6938 yielded a mutant which produced a total carotenoid content of 958 $\mu g\,g^{-1}$ dry weight. By repeated NTG mutagenesis, the total carotenoid content of a mutant (NCHU-FS501) derived from strain CBS 6938 was increased to 1454 $\mu g\,g^{-1}$ dry weight. The results of HPLC analyses indicated that astaxanthin was the dominant xanthophyll in these strains (Figure 1). NTG generated considerable variation in pigmentation among the colonies screened. β-Ionone, reported by Lewis *et al* [12] for isolation of astaxanthin-overproducing yeast, proved to be a useful agent for the indication of mutants with high astaxanthin content. *P. rhodozyma* NCHU-FS501 was the best strain for astaxanthin production in this investigation. Although further strain improvement was conducted, no strain better than NCHU-FS501 was found, probably because of the lack of a more selective screening procedure.

Growth behaviour of P. rhodozyma NCHU-FS501 in batch culture

During batch cultivation of *P. rhodozyma* NCHU-FS501 in YM broth, the carbon source was utilized within 50 h of incubation (Figure 2). Production of total carotenoid ($mg\,ml^{-1}$) was growth-associated and increased during the exponential growth phase. Similar growth-associated astaxanthin production by *P. rhodozyma* was reported by Johnson and Lewis [10] and Meyer and du Preez [14]. The specific growth of this mutant in batch culture was 0.08 h^{-1}, which was lower than the parent CBS 6938 [4]. A lower growth rate of NTG-treated mutants was also observed by Johnson and An [8].

Parameters influencing astaxanthin production

The effect of air flow rate on the growth and astaxanthin production by *P. rhodozyma* NCHU-FS501 is given in Table 1. A significance difference was found in cell mass when the yeast was grown under different air flow rates. An air flow rate of 3.6 vvm supported the greatest cell growth (6.66 $g\,L^{-1}$). The total carotenoid content ($\mu g\,ml^{-1}$) was fairly independent of oxygen; however, the astaxanthin production of this mutant was significantly reduced by low air flow rate. At the lowest air flow rate (2.4 vvm), the yield

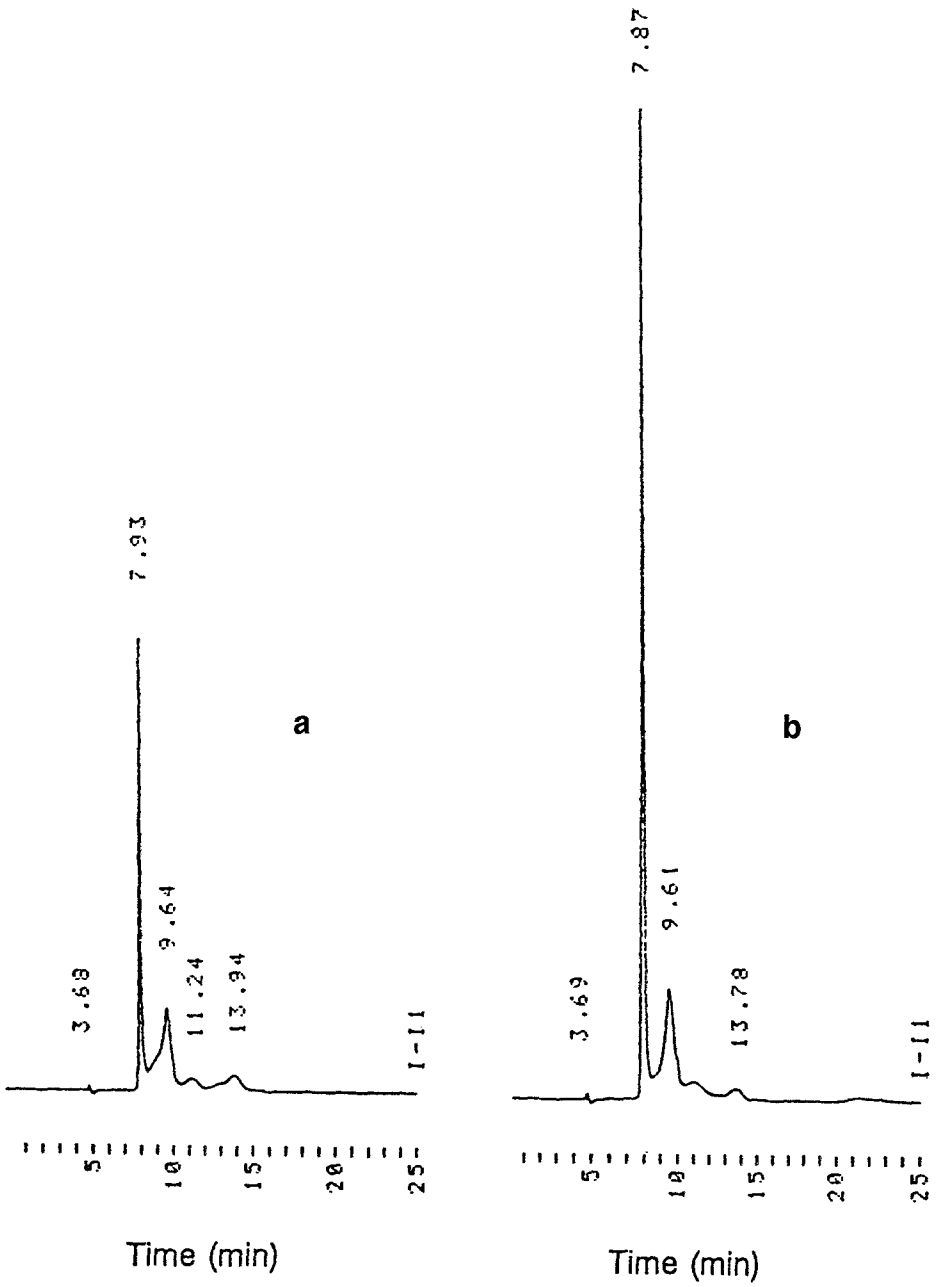

Figure 1 HPLC chromatograms of (a) DMSO-disrupted in hexanes; ethyl acetate-extracted *P. rhodozyma* cells (strain NCHU-FS501) and (b) astaxanthin standard + (a).

of yeast decreased from 0.71 (3.6 vvm) to 0.53 g g^{-1} sugar; and the yield of astaxanthin decreased from 723 (3.6 vvm) to 571 μg g^{-1} sugar (2.4 vvm). Johnson and Lewis [10] reported that at a low oxygen dissolution rate (3.6 mmol L^{-1} h^{-1}), the biomass of *P. rhodozyma* UCD67-210 decreased from the usual value of 4.0 g L^{-1} to 2.0 g L^{-1}.

The optimum temperature for cell growth and total volumetric pigment formation by strain NCHU-FS501 was 22.5°C. The total carotenoid and astaxanthin in terms of μg ml^{-1} and μg g^{-1} cells at 22.5°C were significantly higher than pigment formation at 17.5 and 20°C. The highest maximum specific growth rate (μ_{max}) was also reached at 22.5°C (0.08 h^{-1}) (Figure 3). The specific productivity, cell yield coefficient, yields of total carotenoid and astaxanthin increased slightly with increasing temperatures up to 22.5°C. Johnson and Lewis [10] indicated that the optimum temperature for *P. rhodozyma* UCD67-210 growth and pigment formation was 20 to 22°C, and a similar result was reported by Meyer and du Preez [14] for *P. rhodozyma* strain J4-3. Although the maximum growth temperature of 27.5°C for *P. rhodozyma* was indicated by Johnson and Lewis [10], there was little growth of *P. rhodozyma* strain NCHU-FS501 at 25°C (data not shown).

The effect of different carbon sources on pigment production in batch cultivation by *P. rhodozyma* NCHU-FS501 at a concentration of 10 g L^{-1} is shown in Table 2. Growth of strain NCHU-FS501 on molasses was significantly

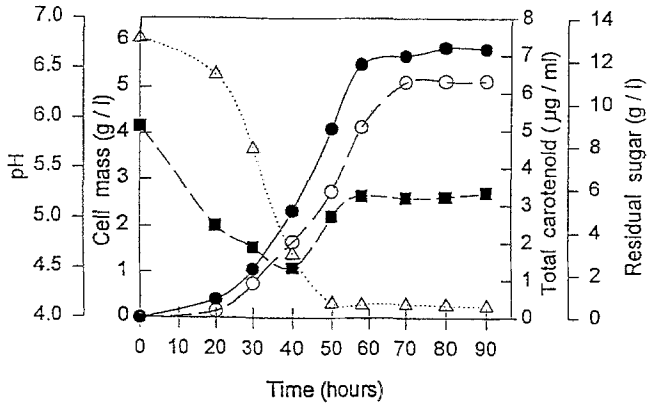

Figure 2 Batch cultivation of *P. rhodozyma* NCHU-FS501 in YM medium containing 10 g glucose L^{-1} at 20°C for 90 h. —●—, cell mass; —○—, total carotenoid; —■—, pH; —△—, residual sugar.

Table 1 The influence of air flow rate on growth, total carotenoid and astaxanthin content of *P. rhodozyma* NCHU-FS501 during batch fermentation at 22.5°C[a]

Parameters	Air flow rate (vvm)		
	2.4	3.2	3.6
Cell growth			
Biomass (g L^{-1})	$_z$4.88[b]	$_y$5.40	$_x$6.66
Yield (g cells g^{-1} sugar)	0.53	0.56	0.71
Total carotenoid			
Volumetric (μg ml^{-1})	$_x$7.28	$_x$7.30	$_x$7.53
Cellular (μg g^{-1})	$_x$1492	$_y$1352	$_z$1131
Yield (μg g^{-1} sugar)	790	762	802
Astaxanthin			
Volumetric (μg ml^{-1})	$_y$5.30	$_x$6.03	$_x$6.72
Cellular (μg g^{-1})	$_y$1086	$_x$1117	$_z$1009
Yield (μg g^{-1} sugar)	571	632	723
Specific growth rate (h^{-1})	0.05	0.08	0.07
Specific productivity (μg g^{-1} h^{-1})	43.9	80.9	78.2
Final pH	6.76	5.50	5.29

[a]Growth was measured in a bench top fermentor containing YM medium after 90 h of incubation at inoculum size of 7.5% and stirring rate of 400 rpm.
[b]Data are means of triplicate determinations. Values in the same row with the same subscript letter were not significantly different at the 5% confidence level.

higher (7.20 g L^{-1}) than on any other carbon source tested. However, the highest total volumetric carotenoid and cellular astaxanthin content (μg g^{-1}) was found when the batch culture was grown in fructose as the sole carbon source. Although fructose supported more pigment formation by the mutant, it did not support the highest growth of the yeast, resulting in a lower total volumetric carotenoid concentration than in a glucose medium (Table 2). Johnson and Lewis [10] reported that higher astaxanthin contents were found when *P. rhodozyma* was grown on D-cellobiose, maltose, mannitol and sucrose. It has been shown that D-cellobiose can only be utilized by *P. rhodozyma* aerobically [20]. Meyer *et al* [15] found that the high astaxanthin contents of 1973 μg g^{-1} and 1926 μg g^{-1} were obtained in *P.*

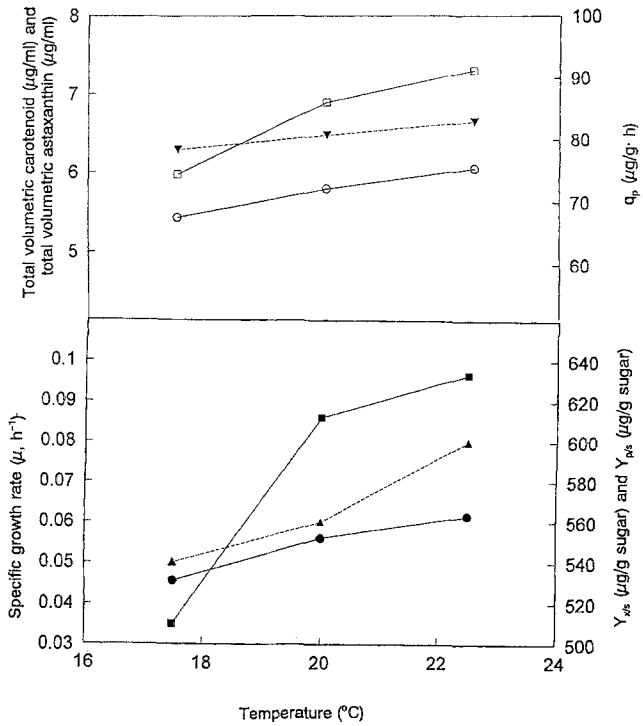

Figure 3 Effect of temperature on *P. rhodozyma* NCHU-FS501 during batch cultivation for 90 h. Growth was measured in YM medium at inoculum size of 7.5% (v/v), stirring rate of 400 rpm and air flow rate of 3.2 vvm. —□—, total carotenoid; —○—, total astaxanthin; —▼—, q_p (specific rate of astaxanthin formation); —▲—, (specific growth rate); —●—, $Y_{x/s}$ (cell mass yield coefficient); —■—, $Y_{p/s}$ (astaxanthin yield coefficient).

rhodozyma J4-3 with mannitol and succinate as sole carbon sources, respectively. The highest specific growth rate and specific productivity were found when strain NCHU-FS501 was grown in glucose medium. Although the maximum specific growth rate of *P. rhodozyma* J4-3 was high when glucose was used as the sole carbon source, Meyer *et al* [15] reported that mannose supported the highest maximum specific growth rate of strain J4-3. Fang and Cheng [5] found that the pentoses L-arabinose and D-xylose can be utilized by *P. rhodozyma* NCHU-FS301, however low cell mass and pigment production by strain FS301 was observed.

Nelis and De Leenheer [18] reported that changing the nitrogen source had no significant effect on β-carotene production. However, Fang and Cheng [5] indicated that peptone, beef extract, casein hydrolysate and nutrient broth supported high pigment formation of *P. rhodozyma* NCHU-FS301 although poor growth and astaxanthin production were found when urea was used as the nitrogen source. Similar results were observed when *P. rhodozyma* J4-3 was grown on yeast carbon base with MES buffer and peptone as nitrogen source [15]. In this investigation, *P. rhodozyma* NCHU-FS501 produced significantly more astaxanthin (1289 μg g^{-1}) when it was grown on casein hydrolysate as nitrogen source in batch culture (Table 3). However, peptone supported the highest specific growth rate (0.07 h^{-1}), astaxanthin yield (713 μg g^{-1} sugar) and specific productivity (78.2 μg g^{-1} h^{-1}). The cell yield coefficients on carbon substrate (g cells g^{-1} sugar) in the presence of differ-

Table 2 The influence of carbon sources on growth, total carotenoid and astaxanthin content of *P. rhodozyma* NCHU-FS501 during batch fermentation at 22.5°C[a]

Parameters	Carbon source (at 10 g L^{-1})				
	Fructose	Glucose	Molasses	Raffinose	Sucrose
Cell growth					
Biomass (g L^{-1})	$_{y}$5.06[b]	$_{w}$6.43	$_{v}$7.20	$_{z}$3.04	$_{x}$5.72
Yield (g cells g^{-1} sugar)	0.54	0.68	0.92	0.32	0.60
Total carotenoid					
Volumetric (μg ml^{-1})	$_{w}$6.94	$_{v}$7.14	$_{y}$4.69	$_{z}$2.95	$_{x}$6.08
Cellular (μg g^{-1})	$_{v}$1371	$_{w}$1110	$_{z}$651	$_{y}$970	$_{x}$1063
Yield (μg g^{-1} sugar)	751	761	604	313	640
Astaxanthin					
Volumetric (μg ml^{-1})	$_{w}$5.91	$_{v}$6.72	$_{y}$3.52	$_{z}$2.48	$_{x}$5.59
Cellular (μg g^{-1})	$_{v}$1168	$_{w}$1045	$_{z}$489	$_{y}$816	$_{x}$977
Yield (μg g^{-1} sugar)	644	713	641	262	591
Specific growth rate (h^{-1})	0.06	0.07	0.05	0.05	0.04
Specific productivity (μg g^{-1} h^{-1})	44.9	78.2	21.9	20.7	32.3
Final pH	6.01	5.29	6.99	7.83	6.10

[a]Growth was measured in a bench top fermentor containing YM-based medium after 70 h of incubation at inoculum size of 7.5%, air flow rate of 3.6 vvm and stirring rate of 400 rpm.
[b]Data are means of triplicate determinations. Values in the same row with the same subscript letter were not significantly different at the 5% confidence level.

Table 3 The influence of different nitrogen sources on growth, total carotenoid and astaxanthin content of *P. rhodozyma* NCHU-FS501 during batch fermentation at 22.5°C[a]

Parameters	Nitrogen source (at 15.8% total nitrogen)[b]				
	BE	CH	PP	PN	YE
Cell growth					
Biomass (g L^{-1})	$_{v}$7.09[c]	$_{y}$5.09	$_{w}$6.43	$_{x}$6.07	$_{x}$6.05
Yield (g cells g^{-1} sugar)	0.85	0.54	0.68	0.63	0.66
Total carotenoid					
Volumetric (μg ml^{-1})	$_{y}$5.66	$_{w}$6.89	$_{v}$7.14	$_{x}$5.96	$_{w}$6.87
Cellular (μg g^{-1})	$_{z}$789	$_{v}$1354	$_{x}$1110	$_{y}$982	$_{w}$1136
Yield (μg g^{-1} sugar)	681	742	763	621	751
Astaxanthin					
Volumetric (μg ml^{-1})	$_{z}$4.79	$_{x}$6.56	$_{v}$6.72	$_{y}$5.58	$_{w}$6.17
Cellular (μg g^{-1})	$_{z}$676	$_{v}$1289	$_{w}$1045	$_{y}$919	$_{x}$1020
Yield (μg g^{-1} sugar)	583	705	713	582	681
Specific growth rate (h^{-1})	0.03	0.03	0.07	0.04	0.05
Specific productivity (μg g^{-1} h^{-1})	12.5	41.6	78.2	48.4	52.3
Final pH	7.81	7.71	5.29	6.88	7.87

[a]Growth was measured in a bench top fermentor containing YM-based medium after 70 h of incubation at inoculum size of 7.5%, air flow rate of 3.6 vvm and stirring rate of 400 rpm.
[b]BE, CH, PP, PN and YE indicate beef extract, casein hydrolysate, peptone, potassium nitrate and yeast extract, respectively.
[c]Data are the means of triplicate determinations. Values in the same row with the same subscript letter were not significantly different at the 5% confidence level.

ent nitrogen sources (15.8% total nitrogen) were similar except for the higher value on beef extract. The pH profile of the batch fermentation on different nitrogen sources varied. Usually, the pH dropped during the first stage of growth and then increased again (data not shown). In this study, the final pH of 7.87 occurred after 70 h of incubation when yeast extract was used as a nitrogen source. Lin and Demain [13] indicated that different patterns of pH change might be an important factor during the cultivation of microorganisms on various nitrogen sources. The effect of pH control on the growth and pigmented formation will be discussed later.

There was no significant difference on the cellular content of astaxanthin and total carotenoid content between pH 5 and 6 (Table 4). On the other hand, pH affected the specific growth rate and cell yield coefficient markedly. The highest specific growth rate (0.06 h^{-1}) was found at pH 6.0, however the highest cell yield (0.63 g g^{-1} sugar) and biomass (20.02 g L^{-1}) were found at an initial pH of 7.0. An optimum value of pH 4.5 was reported by Johnson and Lewis [10] for *P. rhodozyma* UCD67-210 in terms of growth rate, cell yield and astaxanthin formation. Lin and Demain [13] found that the biomass production of a *Monascus* sp decreased with increasing pH, whereas the pig-

Table 4 The influence of pH on growth and astaxanthin formation of *P. rhodozyma* NCHU-FS501 during batch fermentation at 22.5°C[a]

Parameters	pH		
	5.0	6.0	7.0
Cell growth			
Biomass (g L^{-1})	$_{y}$8.48[b]	$_{y}$8.30	$_{x}$20.02
Yield (g cells g^{-1} sugar)	0.25	0.36	0.63
Total carotenoid			
Volumetric (μg ml^{-1})	$_{x}$9.56	$_{x}$9.54	$_{y}$7.74
Cellular (μg g^{-1})	$_{x}$1127	$_{x}$1149	$_{y}$387
Yield (μg g^{-1} sugar)	291	413	240
Astaxanthin			
Volumetric (μg ml^{-1})	$_{x}$9.15	$_{y}$8.81	$_{z}$6.65
Cellular (μg g^{-1})	$_{x}$1079	$_{x}$1061	$_{y}$332
Yield (μg g^{-1} sugar)	272	383	212
Specific growth rate (h^{-1})	0.02	0.06	0.02
Specific productivity (μg g^{-1} h^{-1})	34.7	12.5	15.1

[a]Cells were cultured in a bench top fermentor containing YM-based medium with 35 g glucose L^{-1} after 144 h of incubation at inoculum size of 7.5%, stirring rate of 400 rpm and air flow rate of 3.6 vvm.
[b]Data are means of triplicate determinations. Values in the same row with the same subscript letter were not significantly different at the 5% confidence level.

ment content increased with increasing pH. Meyer and du Preeze [14] reported that a pH decrease was observed during the exponential growth phase of *P. rhodozyma* in batch culture. The possibility of acid production by the red yeast on a chemically defined medium might cause a decrease of cell yield in the chemostat culture [14]. In this investigation, the cellular astaxanthin content of *P. rhodozyma* NCHU-FS501 and the final pH in a batch culture containing YM-base medium with 35 g L^{-1} glucose and initial pH of 6.1 was 1180 μg g^{-1} and 4.43, respectively. Under the same conditions with pH control at 6, the astaxanthin content was found to be 1061 μg g^{-1} after 144 h of incubation. This result indicates that control of the pH of the fermentation broth did not benefit the astaxanthin production.

Influence of glucose concentration

Growth and pigment formation of *P. rhodozyma* NCHU-FS501 were investigated in glucose concentration ranging from 10 g L^{-1} to 45 g L^{-1} in a bench top fermentor. At glucose concentrations below 35 g L^{-1}, the cell mass yield (g g^{-1} sugar) decreased with increasing glucose concentration (Figure 4). However, a high yield of yeast per gram glucose utilized was observed (0.81 g g^{-1} sugar) in YM medium containing 45 g L^{-1} of glucose. The total volumetric carotenoid (μg ml^{-1}), total volumetric astaxanthin (μg ml^{-1}), and biomass (g L^{-1}) increased accordingly at concentrations of glucose below 35 g L^{-1} (Figure 4). For instance, the astaxanthin concentration was 16.33 μg ml^{-1} in 35 g L^{-1} glucose compared to 6.72 μg ml^{-1} in YM medium containing 10 g L^{-1} of glucose. Above 45 g L^{-1} concentration of glucose, the growth of cells and pigment formation decreased significantly. The specific growth rate of *P. rhodozyma* NCHU-FS501 in YM medium containing 45 g L^{-1} glucose was 0.01 compared to 0.05 at 35 g L^{-1} glucose concentration (data not shown), indicating a substrate inhibition effect. Similar results were observed by

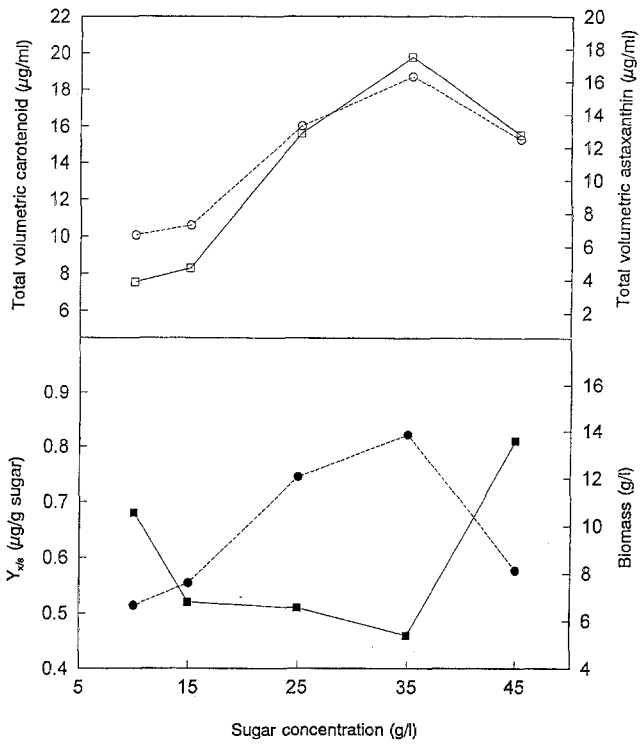

Figure 4 Effect of glucose concentration on growth and pigment formation of *P. rhodozyma* NCHU-FS501. —□—, total volumetric carotenoid; —○—, total volumetric astaxanthin; —●—, biomass; —■—, $Y_{x/s}$ (cell mass yield coefficient). The values were measured in YM-based medium at inoculum size of 7.5% (v/v), stirring rate of 400 rpm, air flow rate of 3.6 vvm and 22.5°C fermentation temperature. The incubation time for the medium containing 10, 15, 25, 35 and 45 g glucose L^{-1} were 90, 90, 100, 140 and 180 h, respectively.

Johnson and Lewis [10] for a *P. rhodozyma* strain in medium containing 1.5% (w/v) glucose. They claimed that when *P. rhodozyma* was cultured with increasing concentrations of glucose, fermentative growth was indicated by decreased yields (biomass of yeast g^{-1} sugar utilized) and this was accompanied by a decreased astaxanthin concentration. In this investigation, however, the cell yield at 45 g L^{-1} glucose was higher than the cell yield at 35 g L^{-1} glucose (Figure 4), indicating that fermentative metabolism might not play an important role at high glucose concentrations.

In conclusion, the total carotenoid content (μg g^{-1} cells) of *P. rhodozyma* CBS 6938 was increased approximately five times by repeated NTG mutagenesis. Batch fermentation studies of the NTG mutant strain NCHU-FS501 indicated that the optimum inoculum size, temperature and air flow rate for astaxanthin production in the bench-top fermentor were 7.5% (v/v), 22.5% and 3.6 vvm, respectively. Under optimum conditions in a batch culture with 3.5% glucose concentration, an additional three-fold increase of volumetric astaxanthin concentration was obtained (16.33 μg ml^{-1}), compared to the initial cultivation method (5.30 μg ml^{-1}). Because of the substrate effect of high sugar concentrations on astaxanthin accumulation in batch culture, the use of a fed-batch cultivation for high cell density fermentation might be a better alternative, and investigations in this regard are currently underway in our laboratory.

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