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Effect of the carbon source on the carotenoid profiles of *Phaffia rhodozyma* strains

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Phaffia rhodozyma **strains ATCC 24202, ATCC 24203, ATCC 24228, ATCC 24229, ATCC 24261, NRRL Y-10921, NRRL Y-10922 and NRRL Y-17268 were grown on culture media containing glucose, sucrose or xylose as carbon sources. Carotenoids were extracted from biomass and analyzed by HPLC with diode-array detection. The carotenoid profiles depended on both the strain considered and the carbon source employed. Astaxanthin, the main pigment found in *P. rhodozyma*, accounted for 42–91% of total carotenoids. Other carotenoids such as canthaxanthin, echinenone, 3-hydroxyechinenone, lycopene, 4-hydroxy-3′, 4′-didehydro-β-ψ-carotene and phoenicoxanthin were detected. The highest volumetric carotenoid concentration (3.60 mg L^{-1}) was obtained with strain NRRL Y-17268 growing on xylose. In this case, astaxanthin accounted for 82% of total carotenoids.**

Keywords: carotenoid profile; astaxanthin; canthaxanthin; *Phaffia rhodozyma*

Introduction

Carotenoids are lipo-soluble photopigments finding a variety of applications in the food industry.

Astaxanthin (3,3′-dihydroxy-β,β-carotene-4, 4′-dione) is a carotenoid found in nature (especially in marine environments) having a typical orange-pink colour that is responsible for its applications in food products. Since salmonids are unable to synthesize this compound, it must be provided in feed rations to aquacultured fish for obtaining an adequate coloration of the flesh [11,22]. Other beneficial effects derived from the supplementation of salmonid feed with astaxanthin have been reported, including the protection against singlet oxygen, which can induce DNA damage and oxidize proteins [20]. In addition, astaxanthin has been employed as an agent for coloration of egg yolks and poultry flesh [4].

Canthaxanthin (β,β-carotene-4,4′-dione) is an orange-coloured carotenoid extensively employed as a pigment in poultry feed.

Both astaxanthin and canthaxanthin are produced commercially by chemical synthesis. However, food or feed additives from biotechnological processes are preferred by consumers to those produced by chemical technologies, owing to their 'natural' origin and the poor adsorption of synthetic carotenoids [6]. A *Brevibacterium* sp is the most promising source of canthaxanthin [15], whereas astaxanthin has been found in several microorganisms. Bacteria (such as *Mycobacterium lacticola* or *Brevibacterium* sp), algae (such as *Haematococcus* sp, *Neochloris wimmeri* or *Chlamydomonas nivalis*), fungi (such as *Peniophora* sp) and yeast (such as *Phaffia rhodozyma*) have been proposed as astaxanthin producers [15]. Among these microorganisms, the green alga *Haematococcus pluvialis* and the yeast *Phaffia rhodozyma* are being considered for industrial production of astaxanthin [10].

Several authors have reported on the biotechnological production of astaxanthin by *Phaffia rhodozyma*. Recent reviews on this subject are available [8,10,15,18,22].

P. rhodozyma can utilize a variety of carbon sources, including mono- and di-saccharides. We have selected glucose, xylose and sucrose to be tested as carbon sources for proliferating *Phaffia* strains because non-refined solutions of these sugars can be obtained readily on a large scale from starch, lignocellulosic materials, beet or sugarcane.

Glucose and sucrose are typical carbon sources for microorganisms, but information about the cultivation of *Phaffia* on xylose [6,7,9,13,16,19] is scarce and (in some cases) contradictory.

This work deals with the carotenoid profile of wild *Phaffia* strains. In order to provide information useful for assessing the feasibility of the biotechnological production of food pigments, microorganisms were cultured in media containing selected carbon sources, and both the concentration and distribution of carotenoids in microbial biomass were determined.

Materials and methods

Microorganisms and culture conditions

Freeze-dried broths of wild *Phaffia rhodozyma* strains were obtained from the American Type Culture Collection (Rockville, MD, USA) or from the Agricultural Research Service Culture Collection (Peoria, IL, USA). Microorganisms were maintained on YM agar plates at 4°C, and transferred monthly. Proliferation experiments were carried out during 7 days at 22°C in orbital shakers (agitation speed, 300 rpm) using 50-ml Erlenmeyer flasks containing 20 ml culture medium. Some fermentation experiments were performed at 22°C in a Braun Biostat B fermentor (2-L jar with 1-L working volume). Air was delivered at

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Table 1 Biomass concentrations, biomass yields, carotenoid concentrations and volumetric carotenoid productivities of *Phaffia rhodozyma* strains

Strain	Carbon source[a]	Biomass conc. (g L^{-1})	$Y_{x/s}$ (g g^{-1})	Total carotenoids (mg L^{-1})	Carotenoid productivity (mg L^{-1} h^{-1})
ATCC 24202	Glucose	5.2	0.26	0.54	0.003
ATCC 24203		5.8	0.29	1.01	0.006
ATCC 24228		5.9	0.30	1.00	0.006
ATCC 24229		4.7	0.23	0.03	0.000
ATCC 24261		5.2	0.26	0.60	0.004
NRRL Y-10921		5.7	0.28	0.64	0.004
NRRL Y-10922		5.6	0.28	0.82	0.005
NRRL Y-17268		5.4	0.27	1.59	0.009
ATCC 24202	Sucrose	6.5	0.33	0.89	0.005
ATCC 24203		8.2	0.41	1.55	0.009
ATCC 24228		6.9	0.35	2.02	0.012
ATCC 24229		4.7	0.24	0.24	0.001
ATCC 24261		6.7	0.34	1.09	0.006
NRRL Y-10921		5.9	0.30	1.06	0.006
NRRL Y-10922		6.3	0.32	0.87	0.005
NRRL Y-17268		6.2	0.31	0.87	0.005
ATCC 24202	Xylose	8.2	0.41	2.17	0.013
ATCC 24203		7.8	0.39	2.16	0.013
ATCC 24228		9.3	0.46	2.57	0.015
ATCC 24229		7.0	0.35	0.88	0.005
ATCC 24261		5.7	0.28	1.49	0.009
NRRL Y-10921		5.7	0.29	1.63	0.010
NRRL Y-10922		9.6	0.48	3.05	0.018
NRRL Y-17268		9.2	0.46	3.60	0.021

[a]Initial substrate concentration = 20 g L^{-1}.

6 L min^{-1}, with the agitation kept at 600 rpm. The percent of O_2 saturation varied in the range 75–90% throughout the fermentations. The composition of the media used for proliferation assays was: yeast extract, 3 g L^{-1}; malt extract, 3 g L^{-1}; peptone, 5 g L^{-1}; carbon source (glucose, sucrose or xylose), 20 g L^{-1}. Duplicate experiments were performed, and the mean results are reported.

Analytical methods

Samples were withdrawn from the fermentation media and centrifuged (4500 × *g*, 10 min). Liquors were analyzed for substrate and metabolic co-products by HPLC with IR detection [17]. Pellets were washed twice with a solution of 9 g sodium chloride L^{-1} in deionized water and centrifuged again. A sample of cells was dried at 102°C for 48 h, in order to allow the calculation of the biomass concentration on a dry weight basis. The remaining fraction of cells was used for carotenoid analysis with sequential DMSO treatments for disrupting cell walls [21] and hexane extraction [3]. Samples from the hexane phase were analysed by HPLC with Diode-Array Detection (DAD) using the following analysis conditions: column, Merck LiChrosorb Si 60; oven temperature, 30°C; gradient elution (flow rate = 1 ml min^{-1}; mobile phase: 100% hexane during 1 min; change up to 50% hexane–50% ethyl acetate in 2 min, this last concentration remaining constant during 6 additional min). Carotenoids were identified by their retention times and by comparison of the spectral features with those of pure compounds or with reported data. All-*trans*-astaxanthin and echinenone standards were kindly provided by Hoffmann-La Roche (Basel, Switzerland). All of the carotenoids were integrated using all-*trans*-astaxanthin as a standard, providing a carotenoid concentration 'equivalent in astaxanthin'.

Table 2 Xylitol concentrations determined for samples obtained at the end of fermentations

Strain	Xylitol conc. (g L^{-1})
ATCC 24202	2.28
ATCC 24203	1.69
ATCC 24228	1.05
ATCC 24229	4.16
ATCC 24261	4.64
NRRL Y-10921	7.28
NRRL Y-10922	1.4
NRRL Y-17268	0.78

Results and discussion

Phaffia rhodozyma strains ATCC 24202, ATCC 24203, ATCC 24228, ATCC 24229, ATCC 24261, NRRL Y-10921, NRRL Y-10922 and NRRL Y-17268 are potential candidates for the biotechnological production of commercially valuable carotenoids. A systematic study was carried out in order to establish the concentration and the profile of carotenoids of the above strains cultured on media containing selected carbon sources. Three sugars were considered for this purpose: sucrose (which can be extracted at large scale from beet or sugarcane), glucose (that can be produced by hydrolysis of starchy or cellulosic substrates) and xylose (that can be obtained from mild acid hydrolysis of the hemicellulosic fraction of some lignocellulosics such as hardwoods, corncobs or bagasse).

Biomass yield and total carotenoid concentration

Literature data show that the carotenoid profile of *Phaffia* depends on the duration of fermentations, and that prolonged fermentations result in increased astaxanthin concentrations. We have carried out fermentations lasting 7 days, a time at which a plateau in astaxanthin concentration was observed. In the last stages of fermentation, cell lysis was not significant, and the carbon source was completely depleted in all the cases considered.

Table 1 shows the biomass concentrations and the biomass yields ($Y_{x/s}$) obtained in experiments performed in shaked flasks. The values of $Y_{x/s}$ are within the wide variation ranges reported in the literature [6,9,13,14].

Table 1 shows the volumetric carotenoid concentrations determined in the same assays. The experimental values (0.03–3.60 mg L^{-1}, corresponding to 6–391 mg carotenoid kg^{-1} dry biomass) are in the range reported for natural isolates of *Phaffia*. An adequate evaluation of results should consider the following aspects: (i) wild-type strains were employed in assays, their carotenoid contents being significantly lower than the ones corresponding to mutant strains. For example, astaxanthin concentrations below 330 mg kg^{-1} are typical for optimized cultures of wild *Phaffia* strains [13]. The stability of wild strains is an important advantage for industrial purposes. (ii) A significant number of studies reported on this subject employed spectrophotometric analysis for measuring carotenoids, but the results are reported as astaxanthin concentrations. This methodology can lead to remarkable differences in results and to overestimation of astaxanthin. (iii) The carotenoid content of biomass found in our study presumably could be increased by further modifications in culture conditions,

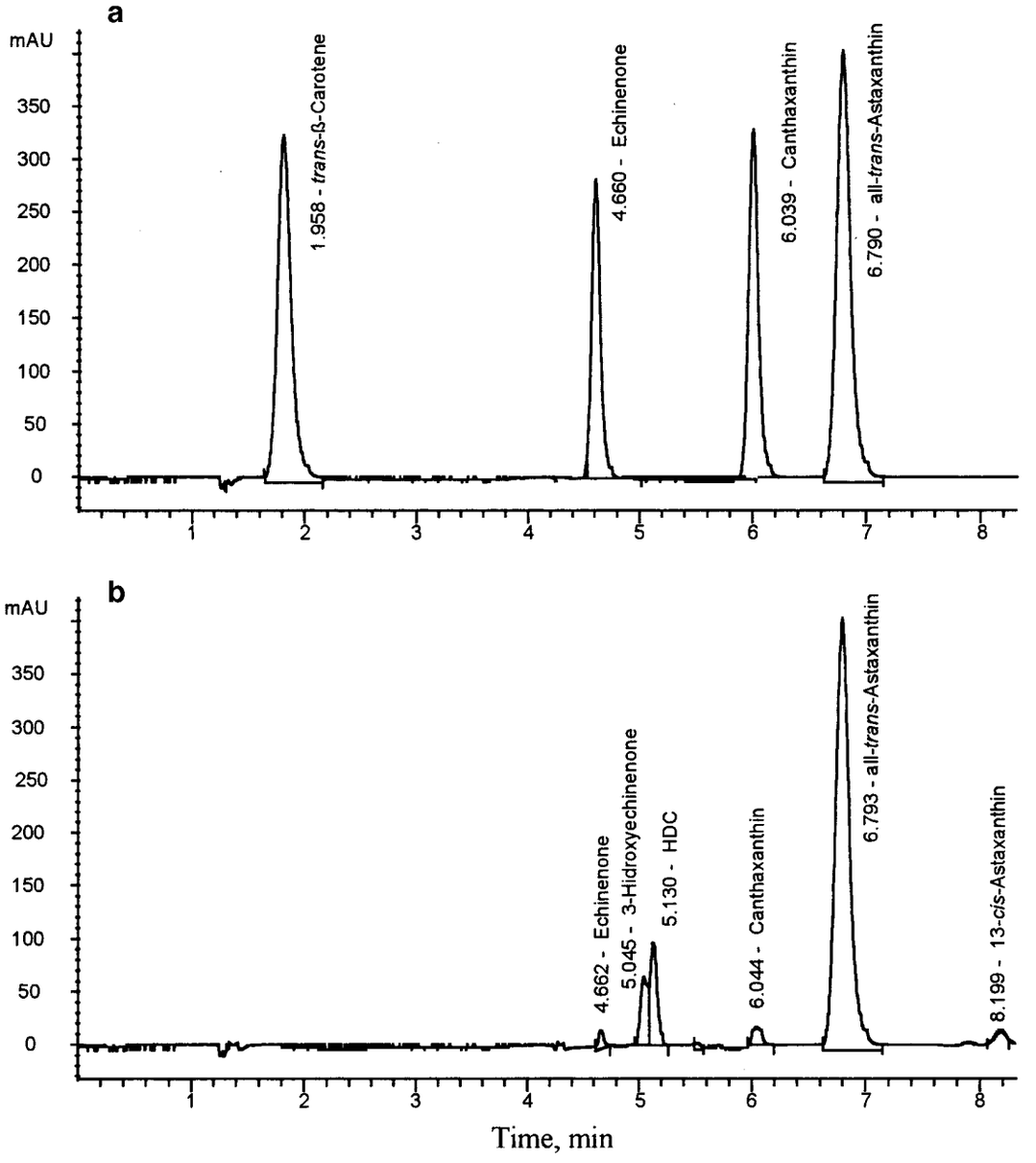

Figure 1 Chromatograms of: (a) standard carotenoids; (b) carotenoids extracted from *Phaffia rhodozyma* NRRL Y-17268 cells grown on xylose.

Table 3 Carotenoid distribution (%) of strains cultured on glucose-, sucrose- or xylose-containing media

		Ec	3-HE	HDC	Cx	T-Ax			
A) Glucose	ATCC 24202	7	22	0	11	59			
	ATCC 24203	3	10	8	6	73			
	ATCC 24228	3	11	5	8	73			
	ATCC 24229	0	0	0	0	100			
	ATCC 24261	5	13	7	12	63			
	NRRL-Y-10921	3	11	3	11	72			
	NRRL Y-10922	7	13	11	34	34			
	NRRL Y-17268	13	11	11	11	54			
		Ly	Ec	3-HE	HDC	Pho	Cx	C-Ax	T-Ax
B) Sucrose	ATCC 24202	1	16	24	0	0	11	0	48
	ATCC 24203	3	6	10	3	3	8	6	60
	ATCC 24228	1	7	13	6	4	10	6	52
	ATCC 24229	8	21	29	0	0	0	0	42
	ATCC 24261	3	12	18	6	6	12	0	42
	NRRL Y-10921	3	8	15	6	0	10	0	58
	NRRL Y-10922	3	9	13	7	6	13	0	49
	NRRL Y-17268	2	0	8	7	3	15	0	64
		Ly	Ec	3-HE	HDC	Cx	C-Ax	T-Ax	
C) Xylose	ATCC 24202	0	0	5	11	2	0	78	
	ATCC 24203	0	0	5	10	1	0	81	
	ATCC 24228	0	0	4	9	1	0	83	
	ATCC 24229	0	2	7	2	9	0	80	
	ATCC 24261	0	1	4	11	3	0	79	
	NRRL Y-10921	0	0	2	4	2	8	85	
	NRRL Y-10922	0	0	6	10	2	0	82	
	NRRL Y-17268	1	1	4	9	3	4	78	

Nomenclature: Ly = Lycopene; Ec = Echinenone; 3-HE = 3-hydroxyechinenone; HDC = 4-hydroxy-3′,4′-didehydro-β-ψ-carotene; Pho = Phoenicoxanthin; Cx = canthaxanthin; C-Ax = *cis*-astaxanthin; T-Ax = all-*trans*-astaxanthin.

but this kind of work (based on optimization of both composition of culture media and initial substrate concentration) can be more reasonably accomplished in future studies based on the best strains and carbon sources selected from the information provided in this work. Therefore, we have focused our attention on the assessment of the comparative ability of the selected strains for producing commercially valuable carotenoids from carbon sources available at a large scale.

The concentration of the carbon sources was fixed at 20 g L^{-1} in order to increase the volumetric concentration of carotenoids. From a practical point of view, the volumetric concentration of carotenoids is more important than the corresponding carotenoid concentration expressed on dry biomass basis, because this latter variable is increased by low substrate concentrations.

A comparison of the carbon sources shows that glucose led to lower pigment concentrations, whereas the best results on carotenoid concentration, astaxanthin proportion and carotenoid productivity were achieved in media containing xylose. The fact that glucose supported a high rate of growth but only a moderate carotenoid yield is in agreement with reported results [9,12].

Table 1 also includes data on the carotenoid productivity. The highest value (0.021 mg L^{-1} h^{-1}) was obtained by strain NRRL Y-17268 grown on xylose, this value being similar to that reported in the literature for the mutant strain NCHU-FS301 cultured on the same carbon source [6].

Xylitol production

Xylitol, an intermediate of xylose metabolism, was detected in all the xylose fermentations. Xylitol concentrations in the range 0.78–7.28 g L^{-1} were found at the end of the experiments (Table 2). The coproduction of xylitol and astaxanthin by *Phaffia* on xylose-containing media has been reported recently [19].

Carotenoid profiles

DAD-HPLC is a very useful tool for carotenoid analysis. With previous steps of cell disruption and liquid–liquid extraction, this methodology provides a fast, reliable and accurate procedure for establishing the composition of cell extracts. Figure 1 shows chromatograms of standard carotenoids and cell extracts.

Table 3 shows the relative proportion of carotenoids obtained from the various strains grown on the carbon sources tested. The results show an interesting dependence of the carotenoid profile on both the type of strain and the carbon source utilized.

Astaxanthin was the most abundant carotenoid. Andrewes *et al* [2] identified a *cis*-isomer from *Phaffia*, but its complete formula was not established. Little information

exists on the *cis-trans* isomerism in astaxanthin from *Phaffia*. Isomers all-*trans*-, 9-*cis*- and 13-*cis*- were identified in this work, the all-*trans*-isomer being the most prominent (90–100% of total astaxanthin).

A wide variation (34–100%) was observed in the relative astaxanthin content of cells grown on glucose. Narrow ranges were observed for astaxanthin content in cells grown on sucrose (42–66%) or xylose (80–91%). The highest volumetric concentration of astaxanthin (2.96 mg L^{-1}) was reached using strain NRRL Y-17268 grown on xylose, whereas the most complex carotenoid profiles were obtained using sucrose as a carbon source.

Several authors [2,3] reported the presence of β-carotene in *Phaffia*. This compound appeared in the earlier stages of all the fermentations performed in the present work but was absent at the end of the fermentations. This is in agreement with the mechanism proposed for carotenoid biosynthesis [2] considering the prolonged fermentation times employed in our study.

Lycopene was identified by Andrewes *et al* [2] in extracts from *P. rhodozyma*. Under the conditions used in the present work, no lycopene was detected when using glucose as the carbon source. Strain NRRL Y-17268 produced minimal amounts of lycopene (1% of total carotenoids) in experiments carried out with xylose as substrate, whereas lycopene accounted for 1–8% of total carotenoids in *Phaffia* strains grown on sucrose.

Echinenone and 3-hydroxyechinenone were detected in *Phaffia* and proposed as intermediates in astaxanthin biosynthesis [2]. Limited amounts of echinenone (0–2% of total carotenoids) were observed when using xylose as a carbon souce, but increased amounts of this compound were determined for microorganisms grown on the other sugars tested (up to 13% on glucose and up to 21% on sucrose). A similar pattern was observed for 3-hydroxyechinenone, with low concentrations (2–7%) in cells cultured on xylose and higher amounts in cells cultured on other sugars (up to 22–29% of total carotenoids for microorganisms grown on glucose or sucrose, respectively).

Canthaxanthin was detected in microorganisms cultured in all the carbon sources tested, its relative proportions being dependent on both the strain and sugar used (0–34% for microorganisms cultured on glucose, 0–15% for microorganisms cultured on sucrose and 1–9% for microorganisms cultured on xylose). Canthaxantin has been identified in carotenoid-producing microorganisms such as the alga *Haematococcus lacustris*, which converts β-carotene into astaxantin via the intermediate canthaxanthin [5].

HDC (4-hydroxy-3′,4′-didehydro-β-ψ-carotene) and phoenicoxanthin were also detected. HDC was found in most strains grown on glucose or sucrose and in all strains grown on xylose, and accounted for up to 11% of total carotenoids in several strains. To our knowledge, only one reference [1] has cited this compound. Phoenicoxanthin, a carotenoid that has been reported as a pigment of *Phaffia* [2], was found in several strains grown on sucrose, with a maximum of 6% of total carotenoids in strain ATCC 24261, but it was not detected at the end of the fermentation in strains grown on glucose or xylose.

Figure 2 shows the dynamics of carotenoid concentrations during the fermentation of a xylose-containing medium with *P. rhodozyma* ATCC 24228 in a batch fermentor. The most important feature observed was the increased astaxanthin concentration obtained at prolonged fermentation times.

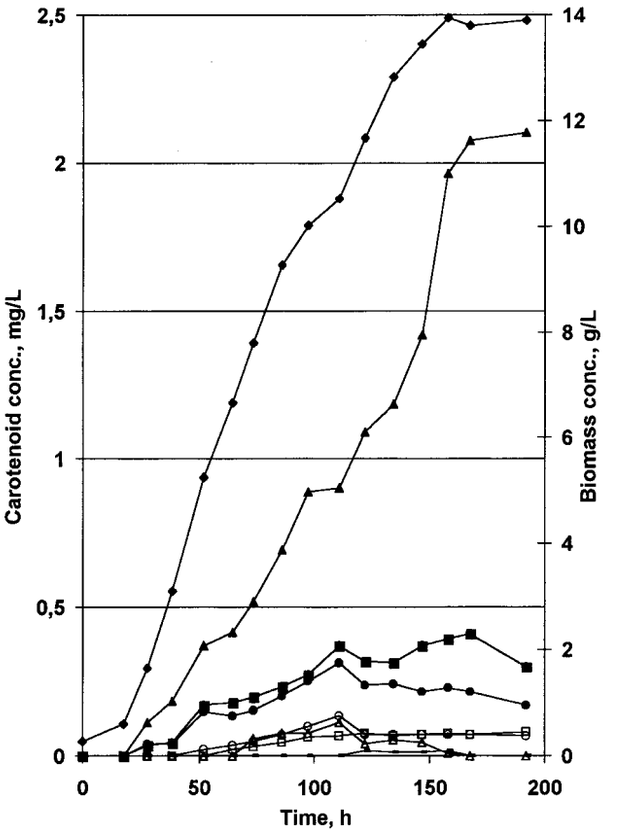

Figure 2 Carotenoid production by *Phaffia rhodozyma* ATCC-24228 cultured on xylose. —▲—, Astaxanthin; —△—, β-carotene; —○—, Echinenone; —●—, 3-hydroxyechinenone; —■—, 4-hydoxy-3′,4′-didehydro-β-ψ-carotene; —▬—, Phoenicoxanthin; —□—, canthaxanthin; —◆—, biomass.

In conclusion, the carotenoid profile of wild *Phaffia rhodozyma* strains depended on both the strain and the carbon source. Taking into account both the total pigment concentration and the carotenoid profile, xylose was identified as the carbon source leading to the highest volumetric concentrations of astaxanthin. The information reported in this work on the comparative ability of *Phaffia* strains for producing carotenoids should be useful for assessing the biotechnological production of feed pigments.

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References

1 An G, DB Schuman and EA Johnson. 1989. Isolation of *Phaffia rhodozyma* mutant with increased astaxanthin content. Appl Environ Microbiol 55: 116–124.

2 Andrewes AG, HJ Phaff and MP Starr. 1976. Carotenoids of *Phaffia rhodozyma*, a red-pigmented fermentating yeast. Phytochemistry 15: 1003–1007.
3 Calo P, JB Velazquez, C Sieiro, P Blanco, E Longo and TG Villa. 1995. Analysis of astaxanthin and other carotenoids from several *Phaffia rhodozyma* mutants. J Agric Food Chem 43: 1396–1399.
4 Dike AO, F Lettner and W Zollitsch. 1992. The supplementation of layers' feed with the yeast *Phaffia rhodozyma* as pigment carrier. Arch Geflugelk 56: 205–209.
5 Donkin P. 1976. Ketocarotenoid biosynthesis by *Haematococcus lacustris*. Phytochemistry 15: 711–715.
6 Fang TJ and Y Cheng. 1993. Improvement of astaxanthin production by *Phaffia rhodozyma* through mutation and optimization of culture conditions. J Ferment Bioeng 75: 466–469.
7 Hayman GT, BM Mannarelli and TD Leathers. 1995. Production of carotenoids by *Phaffia rhodozyma* grown on media composed of corn wet-milling co-products. J Ind Microbiol 14: 389–395.
8 Johnson EA and G An. 1991. Astaxanthin from microbial sources. Crit Rev Biotechnol 11: 297–326.
9 Johnson EA and MJ Lewis. 1979. Astaxanthin formation by the yeast *Phaffia rhodozyma*. J Gen Microbiol 115: 173–183.
10 Johnson EA and WA Schroeder. 1995. Microbial carotenoids. Adv Biochem Eng Biotechnol 53: 119–178.
11 Johnson EA, T Villa and M Lewis. 1980. *Phaffia rhodozyma* as an astaxanthin source in salmonid diets. Aquaculture 20: 123–134.
12 Meyer PS and JC du Preez. 1994. Effect of culture conditions on astaxanthin production by a mutant of *Phaffia rhodozyma* in batch and chemostat culture. Appl Microbiol Biotechnol 40: 780–785.
13 Meyer PS, JC du Preez and SG Kilian. 1993. Selection and evaluation of astaxanthin-overproducing mutants of *Phaffia rhodozyma*. World J Microbiol Biotechnol 9: 514–520.
14 Meyer PS, JC du Preez and MS van Dyk. 1994. The effect of monoterpenes on astaxanthin production by a mutant of *Phaffia rhodozyma*. Biotechnol Lett 16: 125–128.
15 Nelis HJ and AP DeLeenher. 1991. Microbial sources of carotenoid pigments used in foods and feeds. J Appl Bacteriol 70: 181–191.
16 Okagbue RN and MJ Lewis. 1983. Mixed cultures of *Bacillus circulans* wl-12 and *Phaffia rhodozyma* on different carbon sources: yeast-wall lytic enzyme production and extractability of astaxanthin. Biotechnol Lett 5: 731–736.
17 Parajo JC, H Domınguez and JM Domınguez. 1995. Production of xylitol from raw wood hydrolysates by *Debaryomyces hansenii* NRRL Y-7426. Bioproc Enging 13: 125–131.
18 Parajo JC, V Santos and M Vazquez. 1996. Produccion biotechnologica de astaxantina por *Phaffia rhodozyma*. Aliment Equip Tecnol 15: 153–160.
19 Parajo JC, V Santos and M Vazquez. 1997. Co-production of carotenoids and xylitol by *Xanthophyllomyces dendrorhous* (*Phaffia rhodozyma*). Biotechnol Lett 19: 139–141.
20 Schroeder WA and EA Johnson. 1995. Carotenoids protect *Phaffia rhodozyma* against singlet oxygen damage. J Ind Microbiol 14: 502–507.
21 Sedmak JJ, DK Weerasinghe and SO Jolly. 1990. Extraction and quantitation of astaxanthin from *Phaffia rhodozyma*. Biotechnol Techniq 4: 107–112.
22 Tangerås A and E Slinde. 1994. Coloring of salmonids in aquaculture: the yeast *Phaffia rhodozyma* as a source of astaxanthin. In: Fisheries Processing: Biotechnological Applications (Martin AM, ed), pp 391–431, Chapman & Hall, London