World Journal of Microbiology & Biotechnology 10, 178–183

Astaxanthin production by a *Phaffia rhodozyma* mutant on grape juice

P.S. Meyer and J.C. du Preez*

During fermenter cultivation of *Phaffia rhodozyma* on a grape juice medium, the presence of glucose initially delayed fructose utilization, although fructose was consumed before glucose depletion. Total pigment and astaxanthin production were growth associated and reached maximum values of 15.9 μg/ml and 9.8 μg/ml, respectively, after depletion of the carbon source. The total cellular pigment and astaxanthin content increased during the stationary growth phase due to a decrease in biomass, reaching final values of 2120 μg/g and 1350 μg/g, respectively, without the volumetric concentration in the culture changing. The final cell yield was 0.33 g/g sugar utilized. High sugar concentrations in shake-flasks as well as O_2 limitation decreased the astaxanthin content of the cells. Addition of yeast extract to a grape juice minimal medium markedly increased the maximum specific growth rate, total pigment and astaxanthin content of the cells. An excess of ammonia decreased the intracellular astaxanthin content, which reached a maximal value in cultures with no residual glucose or ammonia.

Key words: Ammonia, astaxanthin, carotenoids, fructose, glucose, grape juice, *Phaffia rhodozyma*.

There is a growing commercial interest in astaxanthin as a pigment for salmonid and crustacean aquaculture (Johnson *et al.* 1977, 1980; Gentles & Haard 1991; Chien & Jeng 1992; Storebakken & No 1992). The redness of salmonid flesh which occurs in the wild probably originates from the astaxanthin and related xanthophylls in certain algae, fungi and small crustaceans (Haard 1988; An *et al.* 1989).

The red yeast *Phaffia rhodozyma* is a promising dietary supplement for cultivated fish, shellfish and poultry because it is a natural source of astaxanthin and can also be used as an alternative protein source (Haard 1988). Astaxanthin from broken *P. rhodozyma* cells is readily absorbed by trout and has a high pigmenting efficiency (Johnson *et al.* 1977, 1980; Gentles & Haard 1991). No pigmentation was observed when intact *P. rhodozyma* cells were fed to trout (Johnson *et al.* 1980). Recently, the USA has threatened to raise import tariffs on European white wines by 200% (Sullivan & Levinson 1992), which would in effect close Europe's biggest export market, and this might lead to a grape juice surplus in certain European wine regions. Grape juice might constitute a low cost raw material for commercial astaxanthin production and Longo *et al.* (1992) determined the optimum growth conditions of *P. rhodozyma* on grape juice. In this paper we report on the cultivation of an astaxanthin-overproducing *P. rhodozyma* mutant using grape juice as carbon source, with particular reference to the effect of medium composition on growth and pigment production.

The authors are with the Department of Microbiology and Biochemistry, University of the Orange Free State, P.O. Box 339, Bloemfontein 9300, South Africa; fax: (0)27-51-482004. *Corresponding author.

© 1994 Rapid Communications of Oxford Ltd

Materials and Methods

Microorganism and Culture Conditions

The astaxanthin-overproducing mutant *P. rhodozyma* N9 was obtained by NTG mutation of *P. rhodozyma* CBS 5905T (Meyer *et al.* 1993). A 2-l glass fermenter, equipped with a reflux cooler to minimize evaporation and containing 1 l medium, was operated at 22°C and pH 5. The aeration rate was 1 l/min and the pH was automatically controlled at pH 5 with 1.5 M H_2SO_4 and 3 M NaOH. The dissolved O_2 tension was monitored with a polarographic electrode and the stirrer speed was manually adjusted to maintain it above 40% air saturation. YM medium contained (per l) 5 g peptone, 3 g malt extract, 3 g yeast extract, 1 ml antifoam A (Sigma) and 100 ml white grape juice (Liqui-fruit, Johannesburg). The juice was aseptically added after sterilization of the basal medium. The grape juice contained 100 to 120 g total sugars/l, with the glucose/fructose ratio varying from about 2:1 w/w to 1:1 w/w in different batches.

Shake-flask experiments (500 ml conical flasks each with 50 ml medium) were conducted in YM medium containing either grape

juice, as in the fermenter, or an equal volume of pure glucose/fructose mixture (2:1 w/w), supplemented with 100 mM MES as pH buffer, at 22°C, pH 6 for 5 days on a rotary shaker at 150 rev/min. The minimal medium, used to determine the effect of growth factors, contained (per l) 100 ml white grape juice, 1.84 g NH_4Cl, 2 g KH_2PO_4, 0.5 g $MgSO_4.7H_2O$, 0.1 g $CaCl_2.2H_2O$, trace elements (Du Preez & Van der Walt 1983), 0.1 ml antifoam A and 100 mM MES. The effect of nitrogen concentration was determined in the above minimal medium supplemented with different concentrations of NH_4Cl and 0.5 g yeast extract/l.

Inoculum

The inoculum was prepared from a 48-h-old agar slant of *P. rhodozyma* N9 in a 500 ml Erlenmeyer flask containing 50 ml YM medium at pH 5, incubated for 36 to 40 h on a rotary shaker (150 rev/min) at 22°C. A 1-ml inoculum was used for each shake-flask and a 10-ml inoculum was used for the fermenter.

Analytical Procedures

Growth and dry cell mass were determined as described by Meyer *et al.* (1993). Total intracellular pigments (carotenoids) were determined spectrophotometrically at 480 nm, using a 1% extinction coefficient of 2100, and astaxanthin by HPLC after extraction into methanol (Meyer *et al.* 1993). D-Glucose, D-fructose and glycerol were determined by HPLC and ethanol by GC as described by Van Zyl *et al.* (1988). The contents of O_2 and CO_2 in the fermenter exhaust gas were determined as described by Meyer *et al.* (1992). The indophenol method (Chaney & Marbach 1962) was used to determine the ammonia content of the culture. Individual pigments were analysed by TLC according to An *et al.* (1989). Carotenoids were identified by R_f values and co-chromatography of authentic β-carotene (Sigma) and *trans*-astaxanthin (Roche Products, Isando, South Africa).

Results and Discussion

During fermenter cultivation of *Phaffia rhodozyma* N9 on grape juice, the presence of glucose initially delayed fructose utilization, although fructose assimilation commenced before glucose depletion (Figure 1). The biomass reached a maximum of 9.65 g/l after 34 h, coinciding with depletion of the carbon source, but decreased exponentially thereafter to 5.78 g/l at 120 h (Figure 1). The final cell yield was 0.33 g cells/g sugar consumed. A similar decrease in biomass was observed in glucose-grown cultures (data not shown). Total pigment and astaxanthin accumulation were growth associated and reached a maximum of 15.9 and 9.8 µg/ml, respectively, after approximately 40 h and remained quite constant during the following 50 h. The intracellular total pigment and astaxanthin content increased after depletion of the carbon source (Figure 1), probably due to the decrease in biomass, reaching final values of 2120 and 1350 µg/g, respectively. The total pigment increased at a rate of 28.8 µg/g.h (correlation coefficient, $r = 0.93$) during the exponential growth phase and at a rate of 11.1 µg/g.h ($r = 0.9$) during the stationary phase.

The specific O_2 uptake rate (qO_2) and specific CO_2 production rate (qCO_2) of a 24-h-old *P. rhodozyma* culture

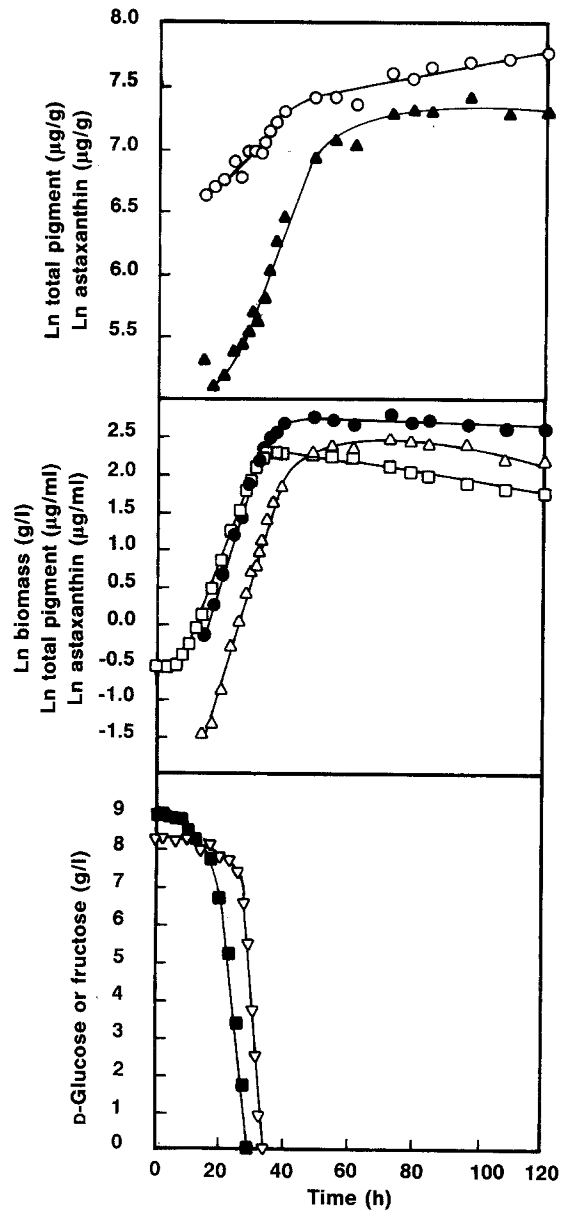

Figure 1. Batch cultivation of *Phaffia rhodozyma* in grape juice medium at pH 5, 22°C for 120 h. ■—Glucose; ▽—fructose; □—biomass; ●—total pigment (µg/ml); ○—total pigment (µg/g); △—astaxanthin (µg/ml); ▲—astaxanthin (µg/g).

were 1.38 and 2.04 mmol/g.h, respectively, giving a respiratory quotient (RQ) of 1.48. The high RQ value was unexpected as the dissolved O_2 tension was maintained at or above 50% saturation and the culture was in the mid-exponential growth phase. No ethanol was detected in the culture, also indicating that the high RQ was not due to a fermentative metabolism. Petrik *et al.* (1983) reported an RQ value of slightly below unity for *Saccharomyces uvarum* in continuous culture at low dilution rates with an increase in RQ to above 2 at higher dilution rates, concomitant with ethanol production. Similarly, Riegler *et*

al. (1983) recorded an RQ value of 1.07 with *S. cerevisiae* at low dilution rates and the value increased to above 3 at higher dilution rates where ethanol production occurred. The formation of mevalonic acid, the first specific precursor of terpenes (carotenoids), requires three molecules of acetyl-CoA (Britton 1983). During conversion of mevalonic acid to isopentyl pyrophosphate, one mol CO_2 is lost for each mol mevalonic acid converted (Goodwin 1971). The high specific rate of CO_2 production observed in *P. rhodozyma* might, therefore, be due to a high activity of this metabolic pathway.

Increasing the concentration of a glucose/fructose mixture in YM medium from 40 to 112 g/l in shake-flask cultures decreased the intracellular total pigment and astaxanthin contents from 2490 to 1270 µg/g and from 1510 to 820 µg/g, respectively (Table 1). Johnson & Lewis (1979) similarly reported a decrease in astaxanthin content of *P. rhodozyma* with increasing glucose concentration. Residual sugars as well as ethanol and glycerol were detected after 120 h cultivation in cultures grown at approximately 90 g sugars/l (Table 1). The maximum specific growth rate and cell yield of *P. rhodozyma* grown in YM medium containing 10 g glucose/l was 0.12 h^{-1} and 0.46, respectively (Meyer *et al.* 1993). In this investigation, the maximum specific growth rate and cell yield of *P. rhodozyma* decreased markedly in the presence of higher sugar concentrations (Table 1). More than 17 g ethanol/l and 2 g glycerol/l were detected in the cultures with the highest initial sugar concentrations (Table 1) and ethanol might have inhibited growth in these cultures. The decrease in total pigment and astaxanthin content with increasing sugar concentration was more severe in cultures containing grape juice (Table 1), suggesting the presence of an inhibitor. Cultures grown with increasing initial sugar concentration exhibited a gradual increase in yellow hue. The astaxanthin content of *P. rhodozyma* cultivated in red grape juice containing 109 g sugars/l was only approximately 25% of the total pigment content (Table 1). TLC analysis of cultures grown at more than 100 g sugars/l revealed the presence of mainly β-carotene and β-zeacarotene (data not shown).

Increasing the concentrations of the constituents of the YM medium added to undiluted grape juice failed to markedly improve the cell density reached in shake-flasks (Table 2). After 5 days of shake-flask cultivation, sugar utilization was far from complete, with substantial accumulation of ethanol and glycerol in the culture. During fermenter cultivation under similar conditions, however, the culture exhibited a normal orange-red colour and produced significantly higher concentrations of astaxanthin (although still markedly lower than in shake-flasks at a lower sugar concentration) with no ethanol or glycerol production and complete utilization of the carbon source (Table 2). These results indicated O_2 rather than nutrient limitation in shake-flasks. Johnson & Lewis (1979) similarly reported β-carotene and β-zeacarotene accumulation under micro-aerophilic conditions.

The pulse addition of glucose to a *P. rhodozyma* culture in the stationary growth phase resulted in an immediate increase in the volumetric rates of biomass, total pigment and astaxanthin production (Figure 2), although the total

Table 1. Cultivation of *Phaffia rhodozyma* in double-strength YM medium containing grape juice or glucose/fructose mixtures in shake-flasks for 5 days at 22°C and pH 6.*

Total sugars (g/l)	Residual carbon (g/l)				μ_{max}† (h^{-1})	Biomass (g/l)	$Y_{x/s}$‡ (g/g)	Total pigment		Astaxanthin		Final pH
	Glucose	Fructose	Ethanol	Glycerol				(µg/ml)	(µg/g)	(µg/ml)	(µg/g)	
Glucose and fructose mixture												
30.0	0	0	0	0	0.06	10.25	0.26	25.5	2490	15.5	1510	5.87
67.9	0	7.0	19.1	2.4	0.05	12.51	0.21	27.0	2160	15.5	1240	5.74
90.0	22.5	7.9	21.5	3.2	0.04	12.92	0.22	24.5	1900	14.8	1150	5.70
112.2	39.9	18.1	17.3	3.6	ND	12.94	0.24	16.4	1270	10.6	820	5.66
Grape juice (white)												
46.4	0	0	0	0	0.09	12.03	0.26	24.9	2070	14.0	1170	5.78
67.7	0	0	8.7	0	0.08	16.10	0.24	20.6	1280	10.3	640	5.51
92.2	0	0	18.5	1.7	0.08	20.46	0.22	12.7	620	5.2	260	4.58
114.8	23.3	9.4	23.1	2.2	0.04	19.33	0.24	9.6	500	4.2	220	4.29
Grape juice (red)												
40.7	0	0	0	0	0.08	11.09	0.27	24.9	2250	13.8	1240	5.72
64.9	0	0	9.9	0	0.05	15.02	0.23	15.5	1030	8.4	560	5.46
89.8	0	12.3	23.0	0.7	0.04	17.92	0.23	8.6	480	3.4	190	4.40
108.8	23.8	19.6	27.9	2.5	0.02	16.88	0.26	7.9	470	2.0	120	4.18

* Values are means of duplicate determinations for duplicate experiments.
† Maximum specific growth rate.
‡ Cell yield coefficient (g dry cells/g substrate assimilated).

Table 2. Cultivation of *Phaffia rhodozyma* in grape juice medium (containing 50 g glucose/l and 51 g fructose/l) supplemented with various concentrations of YM medium in shake-flasks or fermenters for 5 days at 22°C and pH 6.*

YM medium strength	Residual carbon (g/l)				Biomass (g/l)	$Y_{x/s}$† (g/g)	Total pigment		Astaxanthin	
	Glucose	Fructose	Ethanol	Glycerol			(µg/ml)	(µg/g)	(µg/ml)	(µg/g)
Shake flask cultivation										
2	23.7	8.8	13.6	2.9	16.14	0.24	7.3	450	4.0	250
3	20.0	4.4	20.2	3.0	18.38	0.24	5.1	280	2.8	150
4	14.7	2.7	21.9	4.1	19.87	0.24	4.6	230	2.4	120
5	9.7	2.3	21.6	5.7	20.34	0.23	4.1	200	2.2	110
Fermenter cultivation										
3	0	0	0	0	27.24	0.28	49.8	1830	29.0	1060

* Values are the means of duplicate determinations for duplicate experiments.
† Cell yield coefficient (g dry cells/g substrate assimilated).

pigment and astaxanthin content of the cells initially decreased. This indicates an adaptive phase in which the rate of pigment production lagged behind biomass production. After depletion of the carbon source, the biomass decreased, as in Figure 1.

The maximum specific growth rate, total pigment and astaxanthin content of *P. rhodozyma* in a minimal medium supplemented with biotin or a vitamin solution were lower than when grown in YM medium (Table 3). Residual glucose was only detected when no additions were made to the minimal medium and no growth occurred when glucose was used instead of grape juice (data not shown). The high biomass concentration reached in the minimal medium with grape juice in the absence of added growth factors indicated the presence of growth factors in grape juice (Table 3). Supplementing the minimal medium with yeast extract increased the maximum specific growth rate, total pigment and astaxanthin content (Table 3). The astaxanthin content was approximately two-fold higher in the presence of yeast extract than in its absence, but the highest astaxanthin content was reached when *P. rhodozyma* was cultivated in YM medium (Table 3). This indicated that yeast extract, and especially the YM medium, contained nutrients other than vitamins which enhanced astaxanthin accumulation.

Under N limitation, the astaxanthin content of *P. rhodozyma* decreased slightly (Figure 3). Acetyl-CoA is a precursor for both lipid and astaxanthin synthesis and since a high C:N ratio favours lipid accumulation (Ratledge 1986; Hansson & Dostálek 1988; Bajpai *et al.* 1992; Johnson *et al.* 1992), the decrease in astaxanthin content might be due to accumulation of lipids. The highest astaxanthin content coincided with a low level of residual ammonia and the astaxanthin and total pigment content decreased with increasing residual ammonia concentration (Figure 3). With an initial concentration of 23 g ammonia/l, *P. rhodozyma* contained only 1160 µg total pigment/g and 400 µg

Table 3. The effect of additives on the growth of *Phaffia rhodozyma* at pH 6 and 22°C for 4 days in a minimal medium (containing 7.4 g glucose/l and 6.3 g fructose/l) in shake-flasks.*

Additive	Concentration (%)	μ_{max}† (h^{-1})	Biomass (g/l)	Total pigment		Astaxanthin		Final pH
				(µg/ml)	(µg/g)	(µg/ml)	(µg/g)	
None		0.07	3.99	4.7	1180	2.8	600	5.11
Biotin	0.001	0.09	4.52	6.5	1430	5.0	780	5.18
	0.01	0.09	4.48	5.5	1220	4.2	770	5.20
	0.1	0.07	4.65	5.8	1250	4.9	850	5.05
Vitamin solution‡	0.1	0.10	4.83	8.5	1760	8.8	1030	5.01
Yeast extract	0.1	0.13	5.10	9.1	1780	9.4	1030	5.27
	0.2	0.14	5.31	9.3	1750	10.8	1160	5.42
	0.3	0.14	5.41	9.0	1670	8.9	980	5.53
Complete YM medium		0.14	5.99	10.7	1800	13.3	1240	6.05

* The inoculum was washed twice with sterile distilled water. Values are means of duplicate determinations for duplicate experiments.
† Maximum specific growth rate.
‡ According to Kreger-van Rij (1984).

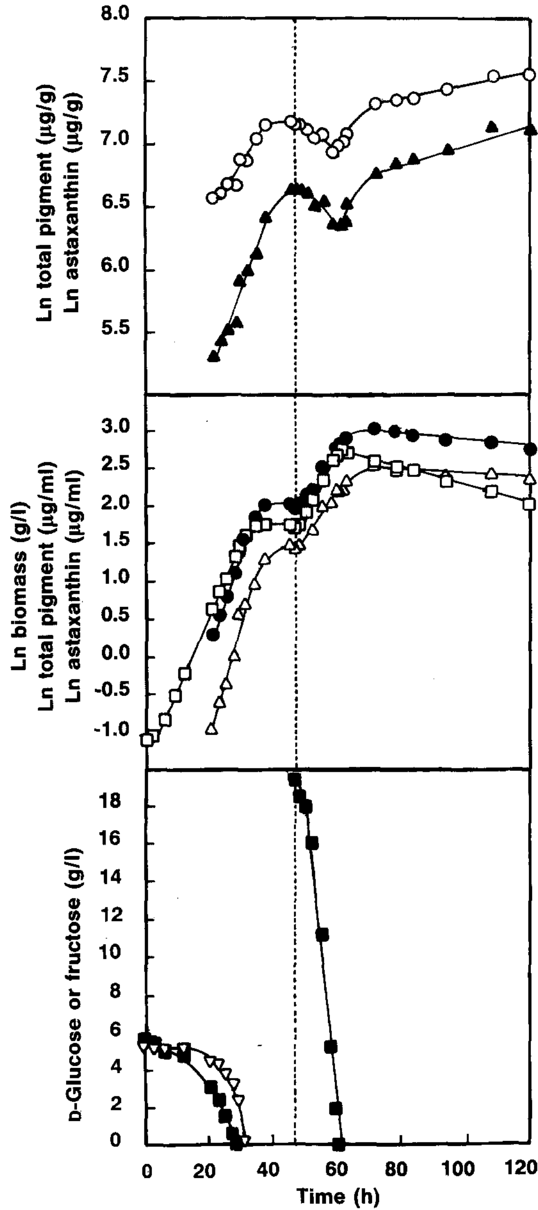

Figure 2. The effect of a glucose pulse (20 g/l), indicated by the broken vertical line, on *Phaffia rhodozyma* in the stationary growth phase during cultivation in grape juice medium at pH 5, 22°C for 120 h. ■—Glucose; ▽—fructose; □—biomass; ●—total pigment (µg/ml); ○—total pigment (µg/g); △—astaxanthin (µg/ml); ▲—astaxanthin (µg/g).

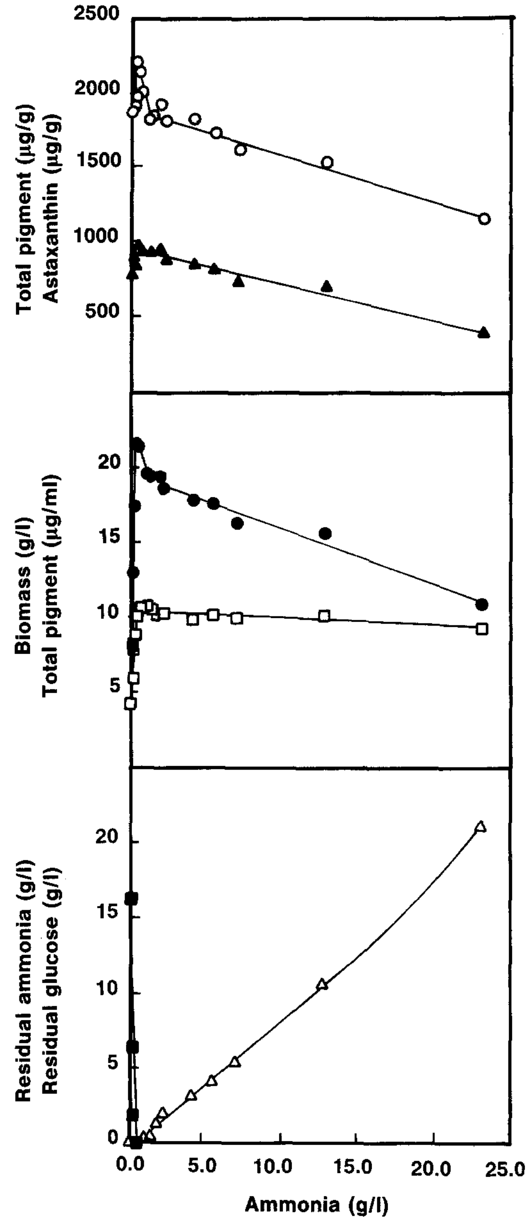

Figure 3. The effect of initial ammonia concentration on *Phaffia rhodozyma* in minimal medium during shake-flask cultivation at pH 6, 22°C for 4 days. ■—Residual glucose; △—residual ammonia; □—biomass; ●—total pigment (µg/ml); ○—total pigment (µg/g); ▲—astaxanthin (µg/g).

astaxanthin/g compared with 2140 µg/g and 980 µg/g, respectively, with an initial ammonia concentration of 0.5 g/l and no residual ammonia or glucose. The decrease in astaxanthin content at high ammonia concentrations might be due to ammonia toxicity.

From a plot of biomass versus ammonia concentration, the cell yield was 19.62 g cells/g nitrogen utilized (data not shown). This yield coefficient was comparable with the value of 16.78 reported for *Candida blankii* (Meyer *et al.* 1992) and was within the range of 8.3 to 20 reported for other yeasts by Egli & Fiechter (1981).

In conclusion, our results show that *P. rhodozyma* could be cultivated in grape juice, assimilating both glucose and fructose as carbon sources, although supplementation with vitamins or yeast extract was essential for the production of high levels of biomass and astaxanthin. If present in excess, ammonia inhibited astaxanthin production markedly and the highest astaxanthin content was obtained when no residual glucose or ammonia were detected. A high sugar

concentration and O_2 limitation suppressed astaxanthin accumulation.

Acknowledgements

The authors gratefully acknowledge a grant from the Foundation of Research and Development. We thank P.J. Botes for technical assistance with the chromatographic analysis.

References

An, G.-H., Schuman, D.B. & Johnson, E.A. 1989 Isolation of *Phaffia rhodozyma* mutants with increased astaxanthin content. *Applied and Environmental Microbiology* **55**, 116–124.

Bajpai, P.K., Bajpai, P. & Ward, O.P. 1992 Optimization of culture conditions for production of eicosapentaenoic acid by *Mortierella elongata* NRRL 5513. *Journal of Industrial Microbiology* **9**, 11–18.

Britton, G. 1983 Carotenoids. In *The Biochemistry of Natural Pigments*, eds Benkovic, S.J., Elmore, D.T., Lewis, J., Muetterties, E.L., Schofield, K., Thomas, J.M. & Thrush, B.A. pp. 23–73. Cambridge: Cambridge University Press.

Chaney, A.L. & Marbach, E.P. 1962 Modified reagents for determination of urea and ammonia. *Clinical Chemistry* **8**, 130–132.

Chien, Y.-H. & Jeng, S.-C. 1992 Pigmentation of kuruma prawn, *Penaeus japonicus* Bate, by various pigment sources and levels of feeding regimes. *Aquaculture* **102**, 333–346.

Du Preez, J.C. & Van Der Walt, J.P. 1983 Fermentation of D-xylose to ethanol by a strain of *Candida shehatae. Biotechnology Letters* **5**, 357–362.

Egli, T. & Fiechter, A. 1981 Theoretical analysis of media used in the growth of yeasts on methanol. *Journal of General Microbiology* **123**, 365–369.

Gentles, A. & Haard, N.F. 1991 Pigmentation of rainbow trout with enzyme-treated and spray-dried *Phaffia rhodozyma. The Progressive Fish-Culturist* **53**, 1–6.

Goodwin, T.W. 1971 Biosynthesis. In *Carotenoids*, ed Isler, O. pp. 577–636. Basel: Birkhäuser Verlag.

Haard, N.F. 1988 Astraxanthin formation by the yeast *Phaffia rhodozyma* on molasses. *Biotechnology Letters* **10**, 609–614.

Hansson, L. & Dostálek, M. 1988 Effect of culture conditions on mycelial growth and production of *gamma*-linolenic acid by the fungus *Mortierella ramanniana. Applied Microbiology and Biotechnology* **28**, 240–246.

Johnson, E.A., Conklin, D.E. & Lewis, M.J. 1977 The yeast *Phaffia rhodozyma* as a dietary pigment source for salmonids and crustaceans. *Journal of the Fish Research Board of Canada* **34**, 2417–2421.

Johnson, E.A. & Lewis, M.J. 1979 Astaxanthin formation by the yeast *Phaffia rhodozyma. Journal of General Microbiology* **115**, 173–183.

Johnson, E.A., Villa, T.G. & Lewis, M.J. 1980 *Phaffia rhodozyma* as an astaxanthin source in salmonid diets. *Aquaculture* **20**, 123–134.

Johnson, V., Singh, M., Saini, V.S., Adhikari, D.K., Sista, V. & Yadav, N.K. 1992 Bioemulsifier production by an oleaginous yeast *Rhodotorula glutinis* IIP-30. *Biotechnology Letters* **14**, 487–490.

Kreger-van Rij, H.J.W. 1984 *The Yeasts: a Taxonomic Study*, 3rd edn. Amsterdam: Elsevier Science.

Longo, E., Sieiro, C., Velázquez, J.B., Calo, P., Cansado, J. & Villa, T.G. 1992 Astaxanthin production from *Phaffia rhodozyma. Biotech Forum Europe* **9**, 565–567.

Meyer, P.S., Du Preez, J.C. & Kilian, S.G. 1992 Chemostat cultivation of *Candida blankii* on sugar cane bagasse hemicellulose hydrolysate. *Biotechnology and Bioengineering* **40**, 353–358.

Meyer, P.S., Du Preez, J.C. & Kilian, S.G. 1993 Selection and evaluation of astaxanthin-overproducing mutants of *Phaffia rhodozyma. World Journal of Microbiology & Biotechnology* **9**, 514–520.

Petrik, M., Käppeli, O. & Fiechter, A. 1983 An expanded concept for the glucose effect in the yeast *Saccharomyces uvarum*: involvement of short- and long-term regulation. *Journal of General Microbiology* **129**, 43–49.

Ratledge, C. 1986 Lipids. In *Biotechnology*, Vol. 4, eds Pape, H. & Rehm, H.-J. pp. 185–213. Weinheim: Verlag Chemie.

Riegler, M., Käppeli, O. & Fiechter, A. 1983 The role of limited respiration in the incomplete oxidation of glucose by *Saccharomyces cerevisiae. Journal of General Microbiology* **129**, 653–661.

Storebakken, T. & No, K.H. 1992 Pigmentation of rainbow trout. *Aquaculture* **100**, 209–229.

Sullivan, S. & Levinson, M. 1992 GATT: the wrath of grapes. Washington threatens a trade war with Europe. *Newsweek* **November 16**, 42.

Van Zyl, C., Prior, B.A. & Du Preez, J.C. 1988 Production of ethanol from sugar cane bagasse hemicellulose hydrolyzate by *Pichia stipitis. Applied Biochemistry and Biotechnology* **17**, 357–369.

(Received in revised form 28 July 1993; accepted 8 August 1993)