Journal of Industrial Microbiology & Biotechnology (2001) 26, 327–332

www.nature.com/jim

β-Carotene production in sugarcane molasses by a *Rhodotorula glutinis* mutant

P Bhosale and RV Gadre

Chemical Engineering Division, National Chemical Laboratory, Pune 411 008, India

Several wild strains and mutants of *Rhodotorula* spp. were screened for growth, carotenoid production and the proportion of β-carotene produced in sugarcane molasses. A better producer, *Rhodotorula glutinis* mutant 32, was optimized for carotenoid production with respect to total reducing sugar (TRS) concentration and pH. In shake flasks, when molasses was used as the sole nutrient medium with 40 g l^{-1} TRS, at pH 6, the carotenoid yield was 14 mg l^{-1} and β-carotene accounted for 70% of the total carotenoids. In a 14-l stirred tank fermenter, a 20% increase in torulene content was observed in plain molasses medium. However, by addition of yeast extract, this effect was reversed and a 31% increase in β-carotene content was observed. Dissolved oxygen (DO) stat fed-batch cultivation of mutant 32 in plain molasses medium yielded 71 and 185 mg l^{-1} total carotenoids in double- and triple-strength medium, respectively. When supplemented with yeast extract, the yields were 97 and 183 mg l^{-1} total carotenoid with a 30% increase in β-carotene and a simultaneous 40% decrease in torulene proportion. Higher cell mass was also achieved by double- and triple-strength fed-batch fermentation. *Journal of Industrial Microbiology & Biotechnology* (2001) **26,** 327–332.

Keywords: *Rhodotorula glutinis*; carotenoids; β-carotene; torulene; torularhodin; sugar cane molasses

Introduction

β-Carotene is an important natural pigment found widely in plants and microorganisms. Animals depend on vegetation for β-carotene because they cannot synthesize it *de novo*. Dietary studies showed that β-carotene is useful in combating various types of cancers and other diseases owing to its antioxidant and provitamin A potential [7,13,22]. It can be used as a natural food colorant and is preferred to synthetic colour [19,24].

Among microbial sources, *Rhodotorula glutinis* accumulates minute quantities of β-carotene, in addition to its characteristic carotenoids, *viz.* torulene and torularhodin [9,20,21]. *Rhodotorula* is also rich in lipids, proteins and vitamins, which make it a suitable feed additive [8,15,18].

β-Carotene production by yeast can become industrially feasible if the cost of production can be minimized by strain improvement and use of cheap industrial by-products as nutrient sources. Growth of *Rhodotorula* in sauerkraut brine, sugar cane juice, grape must and whey for carotenoid production has been reported [5,10,16,23]. Sugar manufacture from cane is a very large agro-industry in India. Sugar cane molasses has a high sucrose and mineral content and thus, can be used as a medium for fermentation. Lipid production from *Rhodotorula* and carotenoid production by *Phaffia rhodozyma* in sugar cane molasses have been reported [1,12].

The objective of the present study was to determine the utility of sugar cane molasses as a complete substrate and as carbon source for the production of carotenoid, especially β-carotene, by a yellow mutant of *R. glutinis*.

Correspondence: RV Gadre, Chemical Engineering Division, National Chemical Laboratory, Pune 411 008, India
Received 18 September 2000; accepted 2 March 2001

Materials and methods

Microorganism

R. glutinis NCIM 3168, 3169, 3170, 3353, 3379 and *R. rubra* NCIM 3171, 3172, 3173, 3174 were obtained from the National Collection of Industrial Microorganisms (NCIM), National Chemical Laboratory, Pune, India. All the mutants investigated were developed from *R. glutinis* NCIM 3353 (R-533; Prairie Regional Laboratory, Saskatoon, Canada) by serial UV mutagenesis and selected based on carotenoid production [3]. They were maintained on MGYP medium by monthly transfer.

Molasses pretreatment

Sugarcane molasses having an 85% solid content was diluted twofold (w/v) using distilled water. The pH was adjusted to 2.0 with 5 N HCl and it was held in boiling water bath for 40 min for sucrose hydrolysis. After hydrolysis, the solution was cooled to room temperature and its pH was adjusted to 6.0 with 1 N NaOH. The precipitate was removed by centrifugation. Total reducing sugar (TRS) was assayed spectrophotometrically using DNSA [17]. One gram of molasses was found to contain 0.56±0.02 g TRS. The molasses had a C/N ratio of 80 as analyzed using an automated elemental analyzer (Model CHNS-O EA 1108 elemental analyzer; Carlo Erba, Milan, Italy). After addition of 2 g l^{-1} each of K_2HPO_4 and KH_2PO_4, the supernatant was sterilized for 30 min at 121°C and used as nutrient source.

Growth of the yeast

A 5% (v/v) inoculum in logarithmic phase grown in a basal medium containing, per liter: glucose 25, yeast extract 10, K_2HPO_4 2, KH_2PO_4 2, $MgSO_47H_2O$ 0.1 at pH 6.0 was used for all strains.

Inoculum was added to 100 ml medium in a 500-ml Erlenmeyer flask, which was incubated at 28°C±1 on a rotary shaker at 240

Table 1 Carotenoid production by *Rhodotorula* strains in molasses incubated for 72 h

Rhodotorula strains	Biomass ($g\ l^{-1} \pm 0.5$)	Total carotenoids		β-Carotene
		$mg\ l^{-1} \pm 0.5$	$mg\ g^{-1} \pm 0.14$	Proportion (% w/w)±3
3168	9.9	7.4	0.75	6
3169	8.7	7.5	0.86	25
3170	9.9	8.1	0.80	10
3171	6.6	7.6	1.14	13
3172	10.8	6.1	0.56	13
3173	10.8	7.9	0.73	18
3174	7.3	6.0	0.95	13
3353	12.0	6.7	0.56	6
3379	11.0	10.0	1.65	21
Mutant R3	10.2	8.5	1.35	9
Mutant W4	10.8	1.5	0.12	19
Mutant Y	11.3	9.2	1.53	39
Mutant 32	8.3	14.0	1.75	70

rpm for 72 h. Samples were removed periodically for determination of growth and carotenoid content.

Screening of yeasts for carotenoid production

Wild strains of *Rhodotorula* spp. and mutants of *R. glutinis* NCIM 3353 were cultured on hydrolyzed molasses medium containing 20 $g\ l^{-1}$ TRS and samples were analyzed for growth, cellular accumulation ($mg\ g^{-1}$) and volumetric production ($mg\ l^{-1}$) of carotenoids as well as for the proportion of β-carotene. Mutant 32, a mutant producing considerably higher β-carotene, was selected for all further studies.

Optimization studies

Mutant 32 was grown in hydrolyzed molasses medium containing TRS between 10 and 100 $g\ l^{-1}$, achieved by dilution of hydrolyzed molasses in distilled water. The mutant was studied for growth and carotenoid production at different initial pH values between 2 and 10, adjusted by HCl or NaOH in molasses medium having 40 $g\ l^{-1}$ TRS.

Effect of nitrogen supplementation

Effect of molasses supplementation by various inorganic and organic nitrogen sources on carotenoid production by mutant 32 was studied at a C:N ration of 30. All inorganic nitrogen sources were procured from SD Fine Chemical (Boiser, India). Complex organic nitrogen sources were obtained from Hi Media (Mumbai, India). Corn steep liquor (CSL) was obtained from Anil Starch Products (Ahemdabad, India).

Fermenter

For fermentation studies, 50 ml medium in a 500-ml Erlenmeyer flask was inoculated from a fresh slant culture and incubated for 24 h on a rotary shaker at 28±1°C. For inoculum preparation, the liquid culture was transferred to 450 ml fresh medium in a 2-l flask and incubated for 24 h. Batch fermentation was carried out in a 14-l stirred tank reactor (Labroferm; New Brunswick Scientific, Edison, NJ, USA). All fermentation experiments were carried out at 28°C and 500 rpm stirrer speed. Airflow was maintained at 7 $l\ min^{-1}$. Foam was controlled by Sigma 260 antifoam.

Dissolved oxygen (DO)-based fed-batch fermentation was done with a 6-l initial volume of diluted molasses having 40 $g\ l^{-1}$ TRS. The DO level in the fermenter was controlled between 10% and 40% of saturation by a combination of agitator speed and sugar feed rate. Inoculum size was 5% (v/v) of the initial working volume of the fermenter.

Molasses solutions (4 l) containing 80 $g\ l^{-1}$ TRS (double strength) and 120 $g\ l^{-1}$ TRS (triple strength) were fed to different fermenters continuously throughout the run. In the case of yeast extract-supplemented fed-batch run, a C:N ratio of 30 was maintained in the feed concentrate. Feeding rate varied between 120 and 180 $ml\ h^{-1}$. Samples were drawn from the fermenter aseptically at regular intervals to monitor pH, growth (determined as absorbance at 500 nm after a 100-fold dilution), dry weight and carotenoid content.

Analytical methods

Extraction of carotenoids, their identification by TLC and quantitaion by HPLC are described in our previous work [2].

Results and discussion

Production of carotenoids from biological sources is receiving major attention because of stringent rules and regulations for the use of chemically synthesized carotenoids. Among yeasts, *P. rhodozyma* is widely accepted as a source of astaxanthin for the preparation of feed additives in aquaculture [19]. *Rhodotorula* spp. is a vitamin A source, but its exploitation is limited because wild strains produce a very low quantity of β-carotene.

Selection of strain

All the wild strains studied produced β-carotene, torulene and torularhodin in various proportions when grown in molasses medium. In mutant 32, β-carotene accounted for 70% of the total carotenoids (Table 1) and the quantity was much higher than all *Rhodotorula* strains reported to grow on raw materials of agro-industrial origin [5]. Cellular accumulation of β-carotene (1225 $\mu g\ g^{-1}$) was highest among all the reported mutants of yeasts [11]. Therefore, mutant 32 was chosen for further studies.

The total carotenoid yield, dry cell mass and specific growth rate of mutant 32 achieved in molasses medium were comparable with those in media composed of yeast extract with either glucose, fructose or sucrose. The growth and carotenoid production were much less in a medium composed of non-hydrolyzed molasses (Table 2).

Table 2 Effect of sugars on carotenoid production from mutant 32 grown for 72 h

Sugar ($40\ g\ l^{-1}$)	Biomass ($g\ l^{-1} \pm 0.5$)	μ_{max} ($h^{-1} \pm 0.05$)	Total carotenoids	
			$mg\ l^{-1} \pm 0.9$	$mg\ g^{-1} \pm 0.1$
Glucose	12.2	0.73	22.0	1.8
Fructose	9.3	0.60	14.4	1.5
Sucrose	11.7	0.42	18.3	1.6
Hydrolyzed molasses	10.8	0.36	18.0	1.7
Untreated molasses	6.8	0.29	10.1	1.5

Table 3 Effect of TRS concentration of β-carotene production by mutant 32 grown in shake flasks for 72 h

Initial TRS ($g\,l^{-1}$)	Residual TRS ($g\,l^{-1}$ ±0.2)	μ_{max} (h^{-1} ±0.05)	CDW ($g\,l^{-1}$ ±0.5)	Total carotenoids		β-Carotene proportion (%, w/w)±3
				$mg\,l^{-1}$ ±1.0	$mg\,g^{-1}$ ±0.2	
10	0.3	0.15	4.5	10	2.2	75
20	2.3	0.17	8.0	14	3.1	70
40	2.3	0.36	10.8	18	1.7	69
50	9.1	0.14	8.1	16	1.9	66
60	37	0.15	6.3	18	2.8	72
70	42	0.10	5.3	18	3.4	76
80	43	0.10	5.4	11	2.0	75
90	59	0.07	4.6	12	2.6	77
100	60	0.05	1.3	5	3.8	79

Table 4 Effect of initial pH of molasses on carotenoid production from mutant 32 grown in shake flasks for 72 h

Initial pH	μ_{max} (h^{-1} ±0.05)	CDW ($g\,l^{-1}$ ±0.5)	Total carotenoids		β-Carotene proportion (%, w/w)±3
			$mg\,l^{-1}$ ±1.1	$mg\,g^{-1}$ ±0.5	
2	0.05	1.6	4	2.5	66
3	0.24	7.9	10	1.27	66
4	0.27	9.1	13	1.43	65
5	0.31	8.9	16	1.79	64
6	0.33	10.8	18	1.67	66
7	0.32	11.3	18	1.59	63
8	0.23	7.6	11	1.44	61
9	0.23	9.4	8	0.85	60
10	0.10	3.5	8	2.28	59

Table 5 Effect of addition of nitrogen sources to molasses on β-carotene production from mutant 32 grown in shake flasks for 72 h

Nitrogen sources	Biomass ($g\,l^{-1}$±0.5)	Total carotenoids		β-Carotene proportion (%, w/w)±6
		$mg\,l^{-1}$ ±1.5	$mg\,g^{-1}$ ±0.3	
Plain molasses control	10.8	18.0	1.7	69
Malt extract	10.1	24.1	2.4	62
Soya bean meal	16.1	18.9	1.2	64
Cotton seed meal	13.6	16.4	1.3	65
Casein acid hydrolysate	12.1	24.5	1.9	70
Peptone	10.5	21.2	2.0	72
Tryptone	11.3	22.0	1.8	75
Soya peptone	12.0	29.3	2.4	75
Yeast extract	12.7	42.6	3.4	79
CSL	13.2	28.3	2.2	88
Ammonium nitrate	10.2	8.8	0.9	56
Ammonium chloride	7.2	17.8	2.4	57
Ammonium sulphate	12.1	14.4	1.3	74
Urea	9.3	17.3	1.9	64

Effect of sugar concentration and initial pH

At concentrations of reducing sugar between 10 and 100 $g\,l^{-1}$ (17.8 and 178.5 $g\,l^{-1}$ molasses, respectively), β-carotene was the major carotenoid produced, comprising 73±6% of the total carotenoids at all sugar concentrations. Although a higher carotenoid content (17.3±1.3 $mg\,l^{-1}$) was observed when the initial TRS concentration was between 40 and 70 $g\,l^{-1}$, higher cell mass and specific growth rate were observed at 40 $g\,l^{-1}$ (Table 3).

At 40 $g\,l^{-1}$ sugar, maximum dry cell mass, specific growth rate (μ_{max}) and volumetric carotenoid production (10.33±1.4 $g\,l^{-1}$,

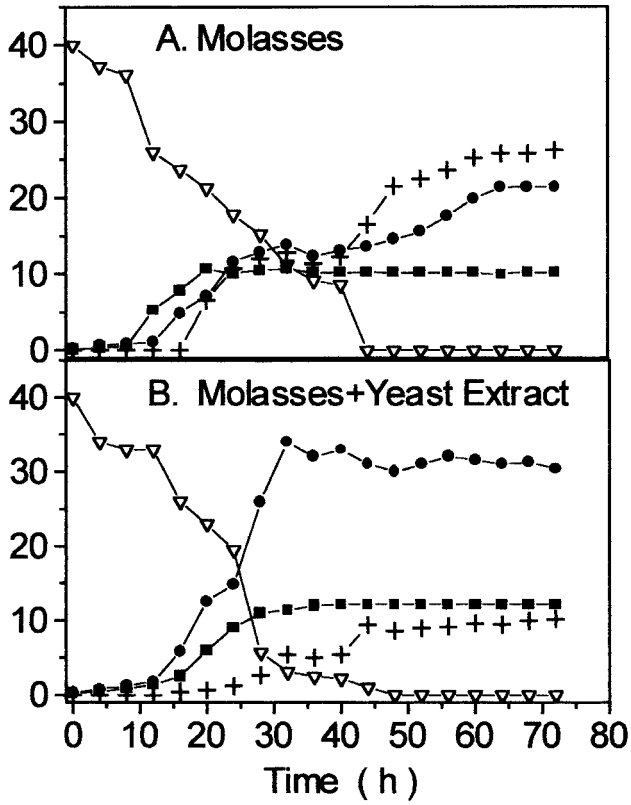

Figure 1 Carotenoid production during batch fermentation in (A) plain molasses medium, (B) yeast-extract-supplemented molasses. Dry cell weight (■, g l^{-1}), glucose (▽, g l^{-1}), β-carotene (●, mg l^{-1}) and torulene (+, mg l^{-1}).

0.32 ± 0.1 h^{-1} and 17.3 ± 1.3 mg l^{-1}, respectively) were observed at pH range of 5–7 (Table 4). At extreme pH values, poor growth and low carotenoid yields were observed as expected, although the proportion of the principal carotenoids remained unaltered.

Effect of addition of nitrogen sources

When various inorganic and organic nitrogen sources were added to the molasses to have a C:N ratio of 30, yeast extract and CSL showed 2.4- and 1.6-fold increases in carotenoid production with 10% and 19% increases in β-carotene proportion, respectively. Yeast extract was preferred over CSL for further studies because of better yield (Table 5).

Fermentation

Batch fermentation: The development of biomass, carotenoid production profiles and consumption of glucose are shown in Figure 1. In plain molasses medium, the maximum specific growth rate was 0.93 h^{-1}. Carotenoid accumulation was observed during the exponential and early stationary phases. Cultures became stationary after approximately 24 h. The maximum volumetric carotenoid concentration was 49.3 mg l^{-1}. After 72 h, β-carotene content, which was 69±5% of total carotenoids in shake flasks, was only 44±2% in the fermenter. Specific carotenoid productivity increased with a simultaneous decrease in μ until 28 h, after which the specific growth rate (0.04 h^{-1}) and carotenoid production rate (2.3 mg l^{-1} h^{-1}) were constant and 53% of the total carotenoid accumulation was seen beyond the exponential growth phase.

When molasses medium was supplemented with yeast extract, a 30% increase in β-carotene content was observed with a simultaneous 40% decrease in torulene. Maximum carotenoid content was observed at 32 h with a carotenoid production rate of 1.79 ± 0.2 mg l^{-1} h^{-1}. No major effect on specific growth rate (μ_{max} 0.87 h^{-1}) or final dry cell mass (12.2 g l^{-1}) was observed. The cellular accumulation (3.5 mg g^{-1}) was less, however, compared to plain molasses medium used as nutrient source (4.8 mg g^{-1}).

The total pigment and β-carotene production were mainly growth-associated and increased during the exponential growth phase. It also increased during stationary phase. The continued increase in the pigment content of mutant 32 in the stationary phase was presumably due to a shift from growth to pigment biosynthesis [6].

In plain molasses medium, torulene proportion increased in the fermenter compared to shake flasks, perhaps due to higher oxygen transfer in the fermenter compared to shake flasks [14]. However, this effect was reversed by addition of yeast extract with an increase in β-carotene.

In a batch fermentation, pigment production was mainly growth-associated and the increase in cell mass could lead to an increase in volumetric carotenoid production.

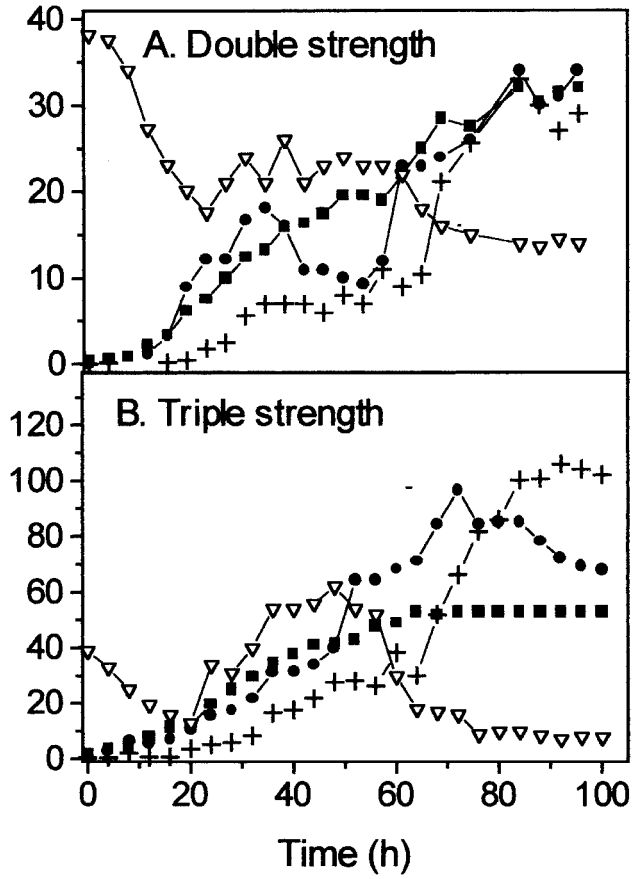

Figure 2 Carotenoid production during fed-batch fermentation in plain molasses medium. Dry cell weight (■, g l^{-1}), glucose (▽, g l^{-1}), β-carotene (●, mg l^{-1}) and torulene (+, mg l^{-1}).

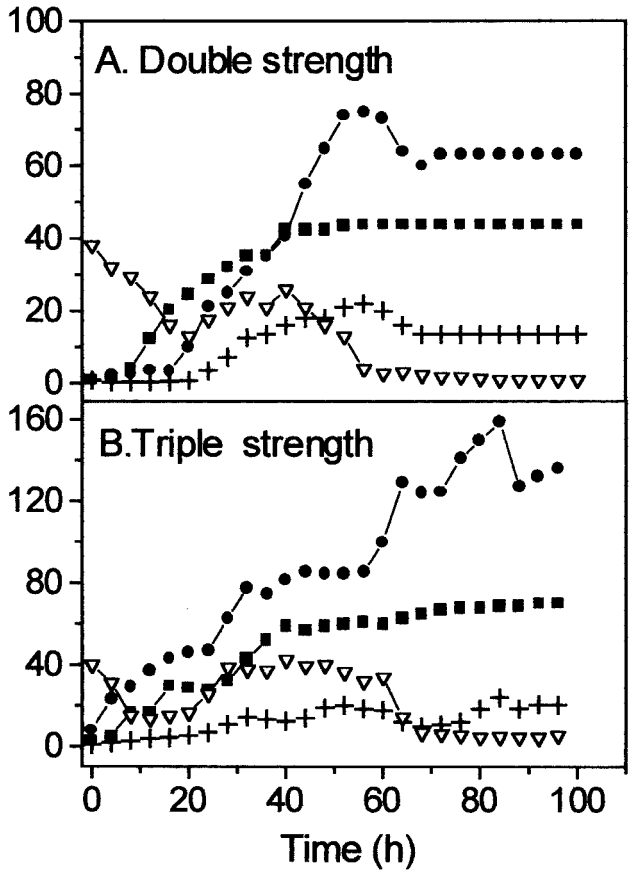

Figure 3 Carotenoid production during fed-batch fermentation in molasses medium supplemented with yeast extract. Dry cell weight (■, g l^{-1}), glucose (▽, g l^{-1}), β-carotene (●, mg l^{-1}) and torulene (+, mg l^{-1}).

Fed-batch fermentation: A fed-batch process was designed to improve culture homogeneity and to increase productivity because an increase in molasses concentration led to inhibition of cell growth and carotenoid yield. Initial fed-batch studies in shake flasks revealed that maximum carotenoid accumulation was achieved when feeding was started at early stationary phase. Feeding molasses at later stages had no significant effect on carotenoid production.

In the fermenter, during fed-batch runs, mutant 32 produced carotenoid in three phases. Initially, during the growth phase, carotenoids were synthesized. During early stationary phase, when feeding was started and the DO level was maintained between 10% and 40% saturation, a slight decrease in volumetric production (mg l^{-1}) was noticed, which may be because of dilution of the broth by continuous addition. However, during this period, cellular accumulation was constant. A significant increase in β-carotene and torulene content was observed after feeding was over.

In plain molasses medium, maximum volumetric carotenoid concentration for double- and triple-strength feed supplement was observed at 84 h (71 mg l^{-1}) and 88 h (185 mg l^{-1}), respectively, and β-carotene and torulene were produced in equal proportion. Mutant 32 also produced a very low concentration of torularhodin (3±1%), which remained unchanged throughout the fermentation period (Figures 2 and 3). Yeast extract supplementation showed no major increase in total carotenoid production in double-strength (97 mg l^{-1}) and triple-strength feed supplement (183 mg l^{-1}), but showed better accumulation of β-carotene (77% and 87% respectively). Yeast extract supplementation thus favored β-carotene production that reached 63 and 130 mg l^{-1} in fermenters fed with double- and triple-strength molasses medium.

The fed-batch technique maximized the specific growth rate, resulted in higher biomass and minimized substrate inhibition of pigment formation. Feeding molasses in the fed-batch mode led to increased biomass by 3- and 4.4-fold in double- and triple-strength feed, respectively, whereas this increase was 4.4- and 7-fold, respectively, in yeast-extract-supplemented molasses feed.

Both batch and fed-batch studies suggest that mutant 32 should be maintained at stationary phase in order to increase cellular and volumetric carotenoid level, as 67% of the final concentration of carotenoid was synthesized after stationary phase was achieved.

Optimization studies for carotenoid production in grape juice reported recently [4] stress the need for economic production of higher β-carotene production from *R. glutinis* and the present study is a step in this direction.

Acknowledgements

The authors are thankful to the Council of Scientific and Industrial Research (CSIR), India, for award of a senior research fellowship to Prakash Bhosale.

References

1 Alvarez RM, B Rodriguez, JM Romano, AO Diaz, E Gomez, D Miro, L Navarro, G Saura and JL Garcia. 1992. Lipid accumulation in *Rhodotorula glutinis* on sugarcane molasses in single-stage continuous culture. *World J Microbiol Biotechnol* 8: 214–215.
2 Bhosale P and RV Gadre. 2001a. Production of β-carotene from *Rhodotorula glutinis* mutant in sea water medium. *Biores Technol* 76: 53–55.
3 Bhosale P and RV Gadre. 2001b. Production of β-carotene by a mutant of *Rhodotorula glutinis*. *Appl Microbiol Biotechnol*, in press.
4 Buzzini P. 2000. An optimization study of carotenoid production by *Rhodotorula glutinis* DBVPG 3853 from substrates containing concentrated rectified grape must as the sole carbohydrate source. *J Ind Microbiol Biotechnol* 24: 41–45.
5 Buzzini P and A Martini. 1999. Production of carotenoids by strains of *Rhodotorula glutinis* cultured in raw materials of agro-industrial origin. *Biores Technol* 71: 41–44.
6 Costa I, HL Martelli, IM Dasilva and D Pomeroy. 1987. Production of β-carotene by a *Rhodotorula* strain. *Biotechnol Lett* 9: 373–375.
7 Edge R, DJ McGarvey and TG Truscott. 1997. The carotenoids as antioxidants — a review. *J Photochem Photobiol B: Biol* 41: 189–200.
8 Ershov YU, AA Dmitrovsky, OV Poloyokh, OI Podoprigora and VY Bykhovsky. 1992. Enzymic conversion of torulene and torularhodin to retinal. *Prikl Biokhim Mikrobiol* 28: 680–684.
9 Frengova G, E Simova, K Pavlov, D Beshkova and D Grigorova. 1994. Formation of carotenoids by *Rhodotorula glutinis* in whey ultrafiltrate. *Biotechnol Bioeng* 44: 888–894.
10 Frengova G, E Simova and D Beshkova. 1997. Caroteno-protein and exopolysaccharide production by coculture of *Rhodotorula glutinis* and *Lactobacillus helveticus*. *J Ind Microbiol Biotechnol* 18: 272–277.
11 Girard P, B Falconnier, J Bricout and B Vladesco. 1994. β-Carotene-producing mutants of *Phafia rhodozyma*. *Appl Microbiol Biotechnol* 41: 183–191.
12 Haard NF. 1988. Astaxanthin formation by the yeast *Phafia rhodozyma* on molasses. *Biotechnol Lett* 10: 609–614.
13 Hennekens CH. 1997. β-Carotene supplementation and cancer prevention. *Nutrition* 13: 697–699.
14 Hideyuki S, N Hidesato, N Tatsuya, M Wararu, F Tokio and K Sadao. 1999. Effect of culture condition on biosynthesis of carotenoids in *Rhodotorula glutinis* No. 21. *Seibutsu Kogaku Kaishi* 77: 55–59.

15 Jacek L. 1997. Oleaginous microorganisms: an assessment of the potential. In: Advances in Applied Microbiology, vol. 43. Academic Press, London, pp. 195–243.
16 Martelli HL, DIM Silva, NO Souza and D Pomeroy. 1990. Production of β-carotene by a *Rhodotorula* strain grown on sugar cane juice. *Biotechnol Lett* 12: 207–208.
17 Miller GL. 1959. Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Anal Chem* 31: 426–428.
18 Mocanu E, D Talos, M Panaitescu, A Contrea, A Trif, R Caprita, GH Bogdan, C Gravila, C Manu, R Driha, M Coman and AV Marinovici. 1997. Studies on metabolic role of *Rhodotorula rubra* 120 r carotenoids pigments, used as a fodder additive concentrate, in laying hens nutrition. *Roum Biotechnol Lett* 2: 55–60.
19 Nelis HJ and AP De Leenheer. 1991. Microbial sources of carotenoid pigments used in food and feeds. *J Appl Bacteriol* 70: 181–191.
20 Perrier V, E Dubreucq and P Galzy. 1995. Fatty acid and carotenoid composition of *Rhodotorula* strains. *Arch Microbiol* 164: 173–179.
21 Peterson WJ, WR Evans, E Leecce, TA Bell and JL Etchells. 1958. Quantitative determination of the carotenoids in yeast of the genus *Rhodotorula*. *J Bacteriol* 75: 586–589.
22 Rock CL. 1997. Carotenoids: biology and treatment. *Pharmacol Ther* 75: 185–197.
23 Shih CT and YD Han. 1996. Production of carotenoids by *Rhodotorula rubra* from sauerkraut brine. *Food Sci Technol* 29: 570–572.
24 Yamaguchi K. 1997. Recent advances in microalgal bioscience in Japan, with special reference to utilization of biomass: a review. *J Appl Phycol* 8: 487–502.