J Ind Microbiol Biotechnol (2004) 31: 115–121
DOI 10.1007/s10295-004-0122-0

ORIGINAL PAPER

E. D. Simova · G. I. Frengova · D. M. Beshkova

Synthesis of carotenoids by *Rhodotorula rubra* GED8 co-cultured with yogurt starter cultures in whey ultrafiltrate

Received: 6 May 2003 / Accepted: 14 January 2004 / Published online: 3 March 2004

Abstract Two cultures, a yeast (*Rhodorula rubra* GED8) and a yogurt starter (*Lactobacillus bulgaricus* 2–11 + *Streptococcus thermophilus* 15HA), were selected for associated growth in whey ultrafiltrate (WU) and active synthesis of carotenoids. In associated cultivation with the yogurt culture *L bulgaricus* 2–11 + S. *thermophilus* 15HA under intensive aeration (1.3 $l^{-1}min^{-1}$ airflow rate) in WU (45 g lactose l^{-1}), initial pH 5.5, 30 °C, the lactose-negative strain *R. rubra* GED8 synthesized large amounts of carotenoids (13.09 mg l^{-1} culture fluid). The carotenoid yield was approximately two-fold higher in association with a mixed yogurt culture than in association with pure yogurt bacteria. The major carotenoid pigments comprising the total carotenoids were β-carotene (50%), torulene (12.3%) and torularhodin (35.2%). Carotenoids with a high β-carotene content were produced by the microbial association 36 h earlier than by *Rhodotorula* yeast species. No significant differences were notd in the ratio between the pigments synthesized by *R. rubra* GED8 + *L. bulgaricus* 2–11, *R. rubra* GED8 + *S. thermophilus* 15HA, and *R.rubra* GED8 + yogurt culture, despite the fact that the total carotenoid concentrations were lower in the mixed cultures with pure yogurt bacteria.

Keywords Carotenogenesis · *Rhodotorula rubra* · Yogurt starter · Co-cultures · Whey ultrafiltrate

Introduction

The best known function of carotenoids, such as α-carotene, β-carotene, β-cryptoxanthin, torulene and torularhodin, is as a precursor to vitamin A (provitamin A) [16, 29]. Humans and animals convert these derivatives to retinal and further to retinol (vitamin A). The pharmaceutical, chemical, and food industries have shown increased interest in the use of carotenoids, mainly as provitamin A but also as natural food and feed colorants [14, 19, 26, 30]. Compared with extraction from vegetables [12] or chemical synthesis [13], the microbial production of carotenoids is of paramount interest, mainly because of the problems of seasonal and geographic variability in the production and marketing of several of the colorants of plant origin [14], and because of the economic advantages of microbial processes using natural low-cost substrates as carbohydrate sources.

Several bacteria, fungi, and yeasts are good carotenoid producers [19]. Carotenoid biosynthesis is a specific feature of species of *Rhodotorula* [7, 10, 24, 27], *Rhodosporidium* [21], and *Phaffia* [22, 23, 25] genera. The major carotenoid pigments produced by the yeasts *Rhodotorula* and *Rhodosporidium* are β-carotene, torulene, and torularhodin, in various proportions [7, 10, 21, 27], and astaxanthin by *Phaffia rhodozyma* [11, 22, 23, 27]. Yeasts can synthesize carotenoids when cultivated in synthetic media containing various monosaccharides or disaccharides [10, 14, 28]. Studies on carotenogenesis have led to a growing interest in using natural substrates as carbon sources (grape juice, glucose syrup, grape must, soybean flour extract, maize flour extract, peat extract and peat hydrolysate, molasses, corn syrup) [1, 7, 8, 9, 22, 23, 24, 25]. In recent years, raw materials and by-products of agro-industrial origin have been proposed as low-cost alternative carbohydrate sources for microbial metabolite production, with the view also of minimizing environmental and energetic problems related to their disposal [15]. A widespread natural substrate, a residium from cheese manufacture, is milk whey containing lactose as a carbon source. Carotenoid-synthesizing yeasts that are able to assimilate lactose are rarely found in natural conditions [31].

When grown as a monoculture, the carotenoid-synthesizing yeast *Rhodotorula rubra*, used in the present study, does not assimilate lactose but actively synthe-

E. D. Simova (✉) · G. I. Frengova · D. M. Beshkova
Laboratory of Applied Microbiology,
Bulgarian Academy of Sciences, 26 Maritza Blvd.,
Institute of Microbiology, 4002 Plovdiv, Bulgaria
E-mail: lbpmbas@plov.omega.bg

sizes carotenoids in synthetic media with carbon sources such as glucose, galactose, and sucrose. Carotenogenesis by lactose-negative yeasts of milk whey is of interest not only due to its economic and ecological aspects, but also to the possibilities of using lactose as a carbon substrate for synthesis of carotenoids. Carotenoid synthesis by lactose-negative yeasts in lactose-rich substrates can be carried out by creating conditions under which lactose is transformed into carbon sources (glucose, galactose, lactic acid) easily assimilated by the yeasts [17].

The present study reports on carotenogenesis of a selected strain of *Rhodotorula rubra* GED8, in which β-carotene productivity was enhanced through co-cultivation with a yogurt starter culture (*Lactobacillus bulgaricus* 2–11 + *Streptococcus thermopilus* 15HA) on lactose substrate containing cheese whey ultrafiltrate.

Materials and methods

Microorganisms and cultivation conditions

Five strains of yeast and eight yogurt starter cultures (including strains of the species *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus salivarius* subsp. *thermophilus*) were screened to select a yeast strain and a yogurt starter to be combined in a high-activity association for carotenoid production.

Lactose-negative carotenoid-synthesizing yeasts contaminating commercial yogurt were isolated and identified as *Rhodotorula rubra* according to the method of Kreger van Rij [20]. The strains *R. rubra* GED2, *R. rubra* GED4, *R. rubra* GED5, *R. rubra* GED6, *R. rubra* GED8 were maintained by monthly transfers on 2% malt extract agar slants and stored at 4 °C.

Eight strains of *L. bulgaricus* and eight strains of *S. thermophilus* from our laboratory's collection of yogurt bacteria [4] were associated in yogurt cultures (starters) according to a method described in an earlier publication [4]. *S. thermophillus* strains of relatively high oxytolerance (20–30% dissolved oxygen in milk) were selected [6]. The pure yogurt cultures were maintained in sterile skim cow s milk by transferring a loopful of inoculum every week, and stored at 4 °C.

The yeast and yogurt cultures isolated and identified have been deposited in the collection of the Laboratory of Industrial Microbiology at the Institute of Microbiology of the Bulgarian Academy of Sciences.

Inocula for yeast cultures were grown in 1000-ml Erlenmeyer flasks containing 100-ml culture medium with 2% malt extract, at 29–30 °C, for 48 h, on a rotary shaker at 220 rpm. The inoculum size for all fermentations was 5% (v/v) and the concentration was 1.2–1.4 g dry cells l^{-1}. Inocula for pure yogurt cultures were prepared as follows: skim cow's milk was sterilized at 110 °C for 20 min, cooled down to 43 °C, and inoculated with 2% (v/v) *S. thermophilus* 15HA or with 2% (v/v) *L. bulgaricus* 2–11. The inoculated milk was incubated at 43 °C until the milk had coagulated (pH 4.5–4.6: 5.5 h for *S. thermophilus* 15HA and 4.0 h for *L. bulgaricus* 2–11). Inocula from pure cultures of *L. bulgaricus* 2–11 and *S. thermophilus* 15HA were mixed in a 3:1 ratio immediately before being introduced into the milk for co-cultivation. The inoculated milk was incubated at 43 °C for 2.0 h until the milk had coagulated (pH 4.5-4.6). The amount of the inoculum from the pure and starter yogurt cultures for the fermentation medium was 1% (v/v). Inocula of each yeast and yogurt (pure or mixed) culture were introduced simultaneously into the fermentation medium.

The fermentation medium consisted of whey ultrafitrate (WU), used in its native state (45 g lactose l^{-1}), supplemented with $(NH_4)_2SO_4$ (6.0 g l^{-1}), KH_2PO_4 (5.5 g l^{-1}) Na_2HPO_4 (3.0 g l^{-1}), $MgSO_4{\cdot}7H_2O$ (0.5 g l^{-1}), and yeast extract (5.0 g l^{-1}). The pH was adjusted to 5.5 with lactic acid. The ultrafiltrate was obtained from a whey byproduct (Milk Industry, Plovdiv, Bulgaria) from the manufacture of white brined cheese and deproteinized on a Lab 38 DDS, on GR61PP membranes (Nakskow, Denmark).

Microbial associations (yeast + yogurt cultures) from the preliminary screening were grown on a rotary shaker (220 rpm) for 6 days at 30 °C in 1000-ml Erlenmeyer flasks containing 100 ml fermentation medium.

The three associations [*R. rubra* GED8 + *L. bulgaricus* 2–11; *R. rubra* GED8 + *S. thermophilus* 15HA; *R. rubra* GED8 + (*L. bulgaricus* 2–11 + *S. thermophilus* 15HA)] were batch-grown in a 15-l MBR AG fermentor (Zurich, Switzerland) with a working volume of 7.5 l, at 30 °C and an air-flow rate of 1.3 l $l^{-1}min^{-1}$, agitation 220 rpm, for different cultivation durations (5.5–7.0 days for each separate association). The pH of the fermentation system was not adjusted during the growth period.

Analytical methods

In the mixed culture, viable counts (colony forming units, cfu ml^{-1}) of *R. rubra* GED8 were estimated on plates containing 2% malt extract and 1.2% agar after a 5-day incubation at 29 °C. Viable cells (in cfu ml^{-1}) were determined from the colony counts on streptococcus selective agar for *S. thermopilus* 15HA and LB-agar for *L. bulgaricus* 2–11 after a 6-day incubation at 37 °C. Cell dry weight was determined after heating the cells at 105 °C to a constant weight. Lactose, glucose, galactose, and lactic acid were determined by enzymatic methods as described by Boehringer Mannheim [2]. Extraction of carotenoids from cells and the determination of total carotenoids (spectrophotometrically) and individual carotenoid pigments (by HPLC) were described earlier [17].

Data represent the mean values and standard deviation of three independent experiments.

Results

Eight yogurt cultures and five yeast strains (*R . rubra* GED2, *R. rubra* GED4, *R. rubra* GED5, *R. rubra* GED6, *R. rubra* GED8) were subjected to preliminary large-scale screening to select a yogurt culture and a yeast culture to be co-cultivated as starter cultures for carotenoid synthesis. Of the 40 starter cultures formed (yeast + yogurt cultures), three yogurt cultures (*L. bulgaricus* 2–12 + *S. thermophilus* 18a, *L. bulgaricus* 2–11 + *S. thermophilus* 15HA, *L. bulgaricus* 1–9 + *S. thermophilus* 15a) and five yeast cultures (*R. rubra* GED2, *R. rubra* GED4, *R. rubra* GED5, *R. rubra* GED6, *R. rubra* GED8) were selected on the basis of their carotenoid-synthesizing activity (Table 1). *R. rubra* GED8, cultivated with the yogurt culture (*L. bulgaricus* 2–11 + *S. thermophilus* 15HA), had the highest cell-specific production [0.31 mg carotenoids (g dry cell mass)$^{-1}$] and the greatest cell mass (15.3 g l^{-1}). As a result, the carotenoid titer measured for the association was also the highest [4.72 mg (l culture fluid)$^{-1}$]. The composition of the yogurt culture had less influence on cell-specific production of *R. rubra* GED8 than on cell mass synthesis. In the rest of the yeast strains associated with yogurt cultures, cell-specific carotenoid production [0.16–0.22 mg (g dry cell mass)$^{-1}$] and carotenoid titers [1.56–2.46 mg l culture fluid)$^{-1}$] were lower. The spent culture media of these associations had higher concentrations of residual lactose (10.3–17.5 g l^{-1}) than measured in medium from the association *R. rubra*

Table 1 Carotenoid and cell mass production by *R. rubra* strains co-cultured with yogurt starter cultures in whey ultrafiltrate. *L.b.*, *Lactobacillus bulgaricus*; *S.th.*, *Streptococcus thermophilus*. All assays were carried out after 6 days of growth of microbial associations (yeast + yogurt starter cultures)

Microbial association (yeasts + yogurt starter cultures)	Residual lactose (g l^{-1})	Dry cell mass (g l^{-1})	Total carotenoids (mg l^{-1})	Cell-specific production [mg carotenoids (g dry cell mass)$^{-1}$]
R. rubra GED2 + (*L.b.* 2–12 + *S. th.* 18a)	10.9 ± 0.40	11.0 ± 0.64	2.46 ± 0.15	0.22
R. rubra GED2 + (*L.b.* 2–11 + *S. th.* 15HA)	11.8 ± 0.46	10.3 ± 0.36	1.91 ± 0.24	0.18
R. rubra GED2 + (*L.b.* 1–9 + *S. th.* 15a)	12.5 ± 0.30	9.5 ± 0.26	1.92 ± 0.15	0.20
R. rubra GED4 + (*L.b.* 2–12 + *S. th.* 18a)	13.3 ± 0.53	9.0 ± 0.25	1.80 ± 0.10	0.20
R. rubra GED4 + (*L.b.* 2–11 + *S. th.* 15HA)	14.5 ± 0.62	8.0 ± 0.20	1.64 ± 0.18	0.20
R. rubra GED4 + (*L.b.* 1–9 + *S. th.* 15a)	12.0 ± 0.30	8.3 ± 0.26	1.53 ± 0.20	0.18
R. rubra GED5 + (*L.b.* 2–12 + *S. th.* 18a)	15.3 ± 0.260	7.6 ± 0.40	1.67 ± 0.14	0.22
R. rubra GED5 + (*L.b.* 2–11 + *S. th.* 15HA)	16.0 ± 0.46	10.0 ± 0.50	2.40 ± 0.10	0.24
R. rubra GED5 + (*L.b.* 1–9 + *S. th.* 15a)	17.5 ± 0.62	9.2 ± 0.36	1.91 ± 0.04	0.21
R. rubra GED6 + (*L.b.* 2–12 + *S. th.* 18a)	10.3 ± 0.36	11.4 ± 0.70	1.88 ± 0.31	0.16
R. rubra GED6 + (*L.b.* 2–11 + *S. th.* 15HA)	11.2 ± 0.40	10.6 ± 0.20	2.07 ± 0.18	0.19
R. rubra GED6 + (*L.b.* 1–9 + *S. th.* 15a)	14.0 ± 0.20	8.8 ± 0.35	1.56 ± 0.05	0.18
R. rubra GED8 + (*L.b.* 2–12 + *S. th.* 18a)	9.0 ± 0.25	13.0 ± 0.50	3.56 ± 0.23	0.27
R. rubra GED8 + (*L.b.* 2–11 + *S. th.* 15HA)	8.4 ± 0.53	15.3 ± 0.44	4.72 ± 0.12	0.31
R. rubra GED8 + (*L.b.* 1–9 + *S. th.* 15a)	9.8 ± 0.36	12.2 ± 0.40	3.41 ± 0.13	0.28

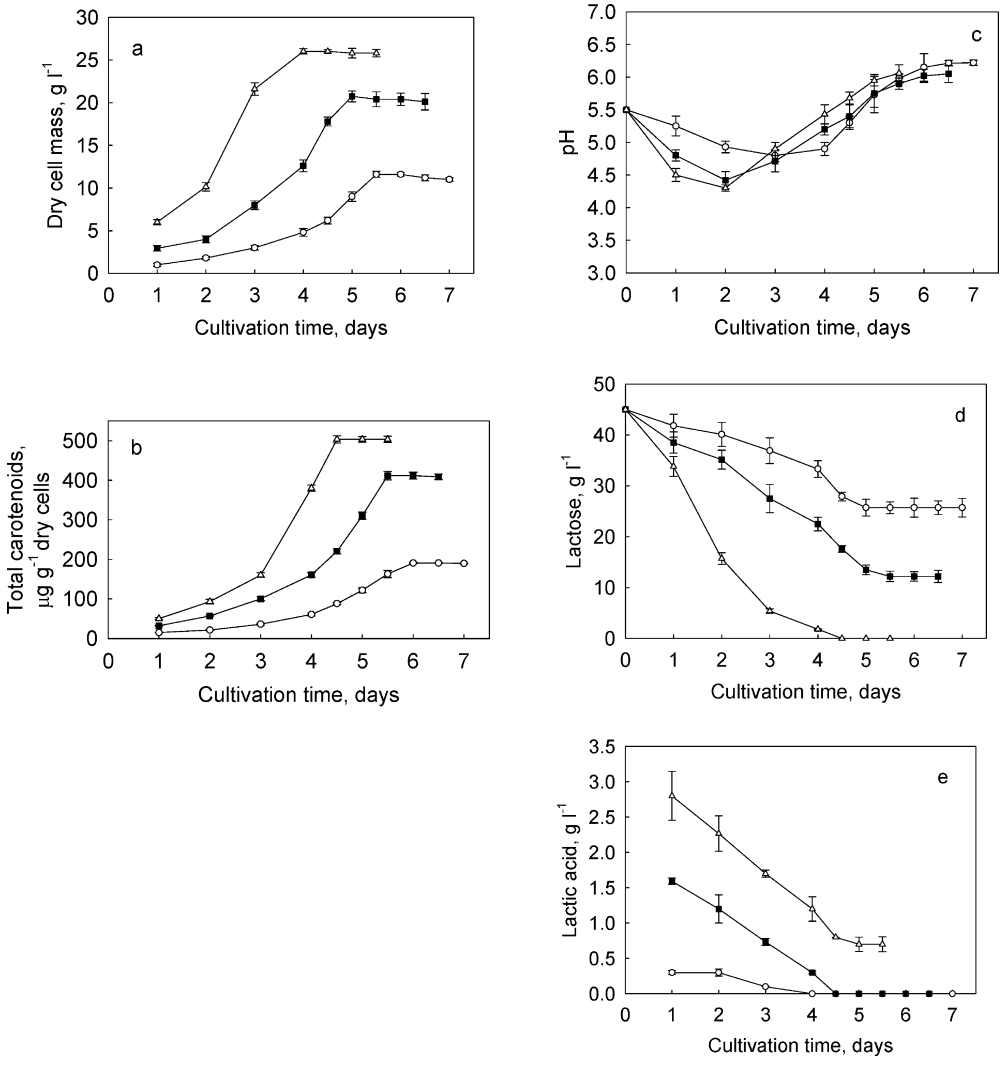

Fig. 1a–c Carotenoids formation and growth of a microbial associations (yeast + yogurt cultures) in whey ultrafiltrate: *Rhodotorula rubra* GED8 + *Lactobacillus bulgaricus* 2–11 (○), *R. rubra* GED8 + *Streptococcus thermophilus* 15HA (■), *R. rubra* GED8 + (*L. bulgaricus* 2–11 + *S. thermophilus* 15HA) (△); dry cell mass (**a**), total carotenoids (**b**), pH (**c**), lactose (**d**), lactic acid (**e**). *Bars* Standard deviation

GED8 + (*L. bulgaricus* 2–11 + *S. thermophilus* 15HA) (8.4 g l^{-1}). Further studies of carotenogenesis by *R. rubra* GED8 were carried out during co-cultivation of the yeast with the yogurt starter culture and with the individual components of the starter, i.e. *L. bulgaricus* 2–11 and *S. thermophilus* 15HA (Figs. 1, 2, and 3).

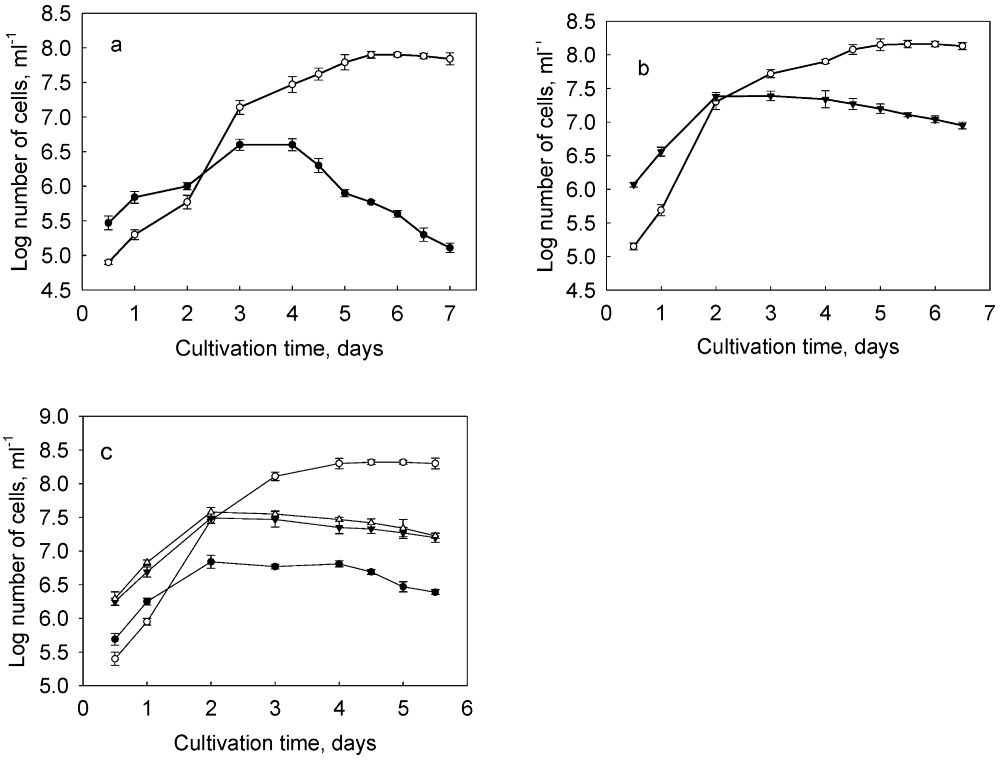

Fig. 2a–c Growth of mixed cultures of yeast and yogurt cultures in whey ultrafiltrate. *R. rubra* GED8 + *L. bulgaricus* 2–11 (**a**), *R. rubra* GED8 + *S. thermophilus* 15HA (**b**), *R. rubra* GED8 + (*L. bulgaricus* 2–11 + *S. thermophilus* 15HA) (**c**); *R. rubra* GED8 (○), *L. bulgaricus* 2–11 (●), *S. thermophilus* 15HA (▼), *L. bulgaricus* 2–11 + *S. thermophilus* 15HA (▽). *Bars* Standard deviation

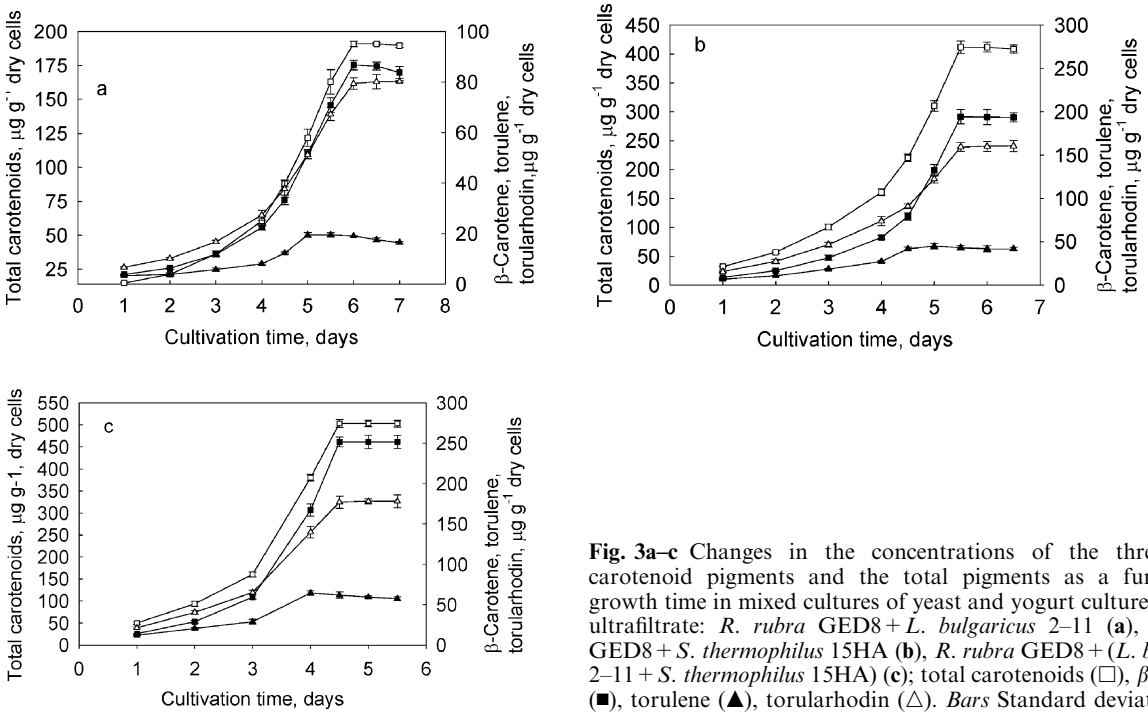

Fig. 3a–c Changes in the concentrations of the three major carotenoid pigments and the total pigments as a function of growth time in mixed cultures of yeast and yogurt cultures in whey ultrafiltrate: *R. rubra* GED8 + *L. bulgaricus* 2–11 (**a**), *R. rubra* GED8 + *S. thermophilus* 15HA (**b**), *R. rubra* GED8 + (*L. bulgaricus* 2–11 + *S. thermophilus* 15HA) (**c**); total carotenoids (□), *β*-carotene (■), torulene (▲), torularhodin (△). *Bars* Standard deviation

R. rubra GED8 displayed the highest carotenoid-synthesizing activity (503.5 µg total carotenoids per g dry cells) in associated growth with the yogurt starter culture. The mixed culture *R. rubra* GED8 + (*L. bulgaricus* 2–11 + *S. thermophilus* 15HA) also had the highest cell mass (26 g l^{-1}). The association of the yeast strain with the monocultures *L. bulgaricus* 2–11 and *S. thermophilus* 15HA resulted in lower carotenoid-synthesizing activity—2.6-fold less for the association with the lactobacillus and 1.2-fold less for

the association with the streptococcus—as well asa lower cell mass (11.6 and 20.4 g l^{-1}, respectively). Furthermore, carotenogenesis was delayed by 36 and 24 h, respectively. The maximum carotenoid titers for the three associations were: *R. rubra* GED8 + (*L. bulgaricus* 2–11 + *S. thermophilus* 15HA), 13.09 mg (l culture fluid)$^{-1}$ on day 4.5; *R. rubra* GED8 + *S. thermophilus* 15HA, 8.39 mg (l culture fluid)$^{-1}$ on day 5.5; and *R. rubra* GED8 + *L. bulgaricus* 2–11, 2.21 mg (l culture fluid) on day 6.

The major carotenoid pigments comprising the total carotenoids synthesized by *R. rubra* GED8 during its cocultivation with both the pure yogurt bacteria and with the yogurt cultures were β-carotene, torulene, and torularhodin (Fig. 3). The dynamics of carotenoid pigment synthesis were similar for all three fermentation systems. During carotenoid formation, the time required to reach a maximum concentration of total carotenoids was 4.5 days[503.5 μg (g dry cells)$^{-1}$] for the association *R. rubra* GED8 + (*L. bulgaricus* 2–11 + *S. thermophilus* 15HA), 5.5 days [411.7 μg (g dry cells)$^{-1}$] for the association *R. rubra* GED8 + *S. thermophilus* 15HA, and 6.0 days [190.0 μg (g dry cells)$^{-1}$] for the association *R. rubra* GED8 + *L. bulgaricus* 2–11). These times coincided with the time required for maximum accumulation of β-carotene and torularhodin. Torulene was formed earlier during the growth cycle of the yeast, and the maximum concentration [19.9 μg (g dry cells)$^{-1}$] was obtained 24 h earlier in the cultivation of the association yeast + lactobacillus. Maximum torulene concentrations [45.0 and 64.6 μg (g dry cells)$^{-1}$] for the associations yeast + streptococcus and yeast + yogurt starter were obtained 12 h earlier. No significant differences were registered in the relative share of individual pigments in the total carotenoids for the three associations, i.e. 45.4% β-carotene, 10.0% torulene, and 41.6% torularhodin for *R. rubra* GED8 + *L. bulgaricus* 2–11; 47.2% β-carotene, 10.5% torulene, 38.7% torularhodin for *R. rubra* GED8 + *S. thermophilus* 15HA; and 50.0% β-carotene, 12.3% torulene, and 35.2% torularhodin for *R. rubra* GED8 + (*L. bulgaricus* 2–11 + *S. thermophilus* 15HA).

Discussion

The maxima for cell mass accumulation and carotenoid formation did not coincide (Fig. 1a, b) since the carotenoid content of the cells reached a maximum after the cultures had completed growth, i.e. during the stationary growth phase of the yeast. Of the individual carotenoid pigments making up total carotenoids, torulene was formed earlier in the growth cycle of the yeast cells and on day 4 had reached a relative level of 100% compared to 66 and 78% for β-carotene and torularhodin, respectively, for the association *R. rubra* GED8 + (*L. bulgaricus* 2–11 + *S. thermophilus* 15HA). The results for associations of yeast with lactobacillus and streptococcus were similar. Carotenoid production followed the change in the pH of the fermentation medium for the three associations (Fig. 1c). The pH was the lowest in the mixed culture with yogurt starter (pH 4.3 on day 2), rose during active carotenogenesis, and then remained around 6.0, indicating that fermentation had concluded. The yogurt pure cultures and the starter culture actively transformed lactose to lactic acid under intensive aeration during associated cultivation with the yeast strain. In the course of this process, no glucose was found, and the concentration of galactose was minimal. In the mixed culture *R. rubra* GED8 + yogurt starter, lactic acid was present from day 1 to day 5 in concentrations ranging from 2.8 to 0.7 g l^{-1} but was absent from the mixed culture of yeast + pure yogurt bacteria after the day 4 (Fig. 1c). The two monosaccharides and the lactic acid were easily assimilated by *R. rubra* GED8 thereby allowing the yeast culture to grow exponentially in medium in which the carbon source was directly inassimilable (Fig. 2a, b, c). Lactose was assimilated at different rates in the three fermentation systems (Fig. 1d). In the culture fluids of yeast + pure yogurt cultures, lactose assimilation was delayed and higher concentrations of residual lactose were recorded. In the mixed yogurt culture + yeast, 96% of the lactose was assimilated by day 4 compared to 26 and 50% in the mixed cultures *R. rubra* GED8 + *L. bulgaricus* 2–11 and *R. rubra* GED8 + *S. thermophilus* 15HA. The mixed culture yeast + yogurt starter had utilized lactose by the end of the process (day 4.5). In the mixed cultures consisting of *L. bulgaricus* 2–11 and *S. thermophilus* 15HA, 43 and 73% of the lactose was assimilated. The lower results for cell mass and carotenoid synthesis by microbial associations of the yeast with lactobacillus or streptococcus (Fig. 1a, b) are of particular significance in that respect.

Carotenoid-synthesizing yeasts are aerobes

Lactic acid bacteria are facultative anaerobes with a preference for anoxic conditions, and their growth and metabolism in natural media (milk, whey) do not require oxygen. Previous work showed that the selected oxytolerant strain *S. thermophilus* 15HA plays a leading role in lactic acid metabolism in a batch aerated yogurt culture [6]. This is supported by the behavior of the yogurt culture during carotenogenesis of the yeast *R. rubra* GED8 in associated cultivation. Under intensive aeration during carotenoid formation, active cell growth was observed in the mixed yogurt culture and pure cultures of *S. thermophilus* 15HA. The oxytolerant *S. thermophilus* 15HA manifested comparatively more intensive growth and acidification in the mixed yogurt culture, which reflected carotenoid formation (Fig. 2c, 1b). Oxygen influenced the growth curve of the pure culture *L. bulgaricus* 2–11 (Fig. 2a), suppressing growth of the pure culture during carotenogenesis but stimulating growth of the mixed yogurt culture during carotenoid formation (Fig. 2c). The latter was probably due to oxygen being

involved in the mechanism of assimilating the carbon carrier by the oxytolerant *S. thermophilus* 15HA, which in turn created anoxic conditions in the mixed culture, thus enhancing *L. bulgaricus* 2–11 growth. However, the highest viability of the yogurt bacteria during carotenogenesis occurred in the mixed culture. Microscopy of the microorganisms showed domination of the bacteria over the yeast on days 1 and 2 in the three fermentation systems. The bacterial cells were young and ranged in size from 4.0–10.0×0.8–1.0 μm for the lactobacillus, situated singly, in pairs or short chains, to 0.8–1.0 μm for the streptococcus, which formed diplococci and short chains. Yeast growth during the first two days was characterized by a few non-budding but well-shaped oval cells (5.0–10.0 μm). Intensive growth of the yeast with young budding cells prevailed after the day 2, and the yeast population was further along in the growth cycle than the bacterial population. By day 5, the latter consisted of cells that continued to divide and which predominated over the aging cells. After days 4–5, the bacteria diminished significantly and consisted mainly of aging cells in long chains. Growth curves indicated enhanced cell viability of streptococci and lactobacilli up to days 4–5 (Fig. 2). Thus, even under extreme conditions (intensive aeration) during carotene formation, the metabolism of the mixed yogurt culture was stimulated by a positive interaction between the two species of yogurt bacteria. In earlier studies, mixed yogurt cultures stimulated the synthesis of metabolites important for lactic acid transformation and for the organoleptic properties of the product [3, 4, 5]. The present studies show that mutual support of the two species of yogurt bacteria stimulated not only their metabolic activities but also the metabolism of the microorganisms cultivated with them. Furthermore, the B-complex vitamins and amino acids produced by yeast seem to stimulate the growth of yogurt bacteria [18]. Evidence of positive reciprocal metabolism between lactobacteria and yeast in a carotenoid-synthesizing fermentation system is provided by the high level of carotenoid formation by the yeast culture, and active growth and acidification of the yogurt culture (*L. bulgaricus* 2–11 + *S. thermophilus* 15HA) under intensive aeration.

β-carotene, torulene and torularhodin are typical carotenoids of the *Rhodotorula* genus [7, 8, 10, 27]. Carotenogenesis of *R. rubra* GED8 in association with a yogurt culture showed that the amount and ratio among the pigments depends on the species specificity of the producing strain [17]. The ability of *R. rubra* GED8 to produce carotenoids with a high *β*-carotene content (50% of the total amount) in associated cultivation with the yogurt culture is of particular interest. In the mixed culture with *S. thermophilus* 15HA, *β*-carotene made up 47.2% of total carotenoids, while with *L. bulgaricus* 2–11 the amount was 45.4%. *β*-Carotene produced by *R. rubra* GED8 cultivated with a yogurt culture was about six times higher than that of the strain R. *glutinis* 22P [18] and about ten times higher than the amount reported for *R. lactosa* BKM 1264 [31].

Conclusion

The microbial association *R. rubra* GED8 + yogurt culture (*L. bulgaricus* 2–11 + *S. thermophilus* 15HA) grown in WU under intensive aeration synthesized higher titers of carotenoids and *β*-carotene (50% of the total) than reported so far for *Rhodotorula* species. The process of carotenoid formation was shortened by 36 h compared to other species of *Rhodotorula*. Carotenoid and *β*-carotene synthesis by *R. rubra* GED8 cultivated in a directly unassimilable substrate (lactose) suggests the possibilities of mixed cultivation for effective carotenoid synthesis.

Acknowledgements The authors gratefully acknowledge the financial support for this work from the National Science Fund of Bulgaria.

References

1. An G, Jang B, Cho M (2001) Cultivation of the carotenoid-hyperproducing mutant 2A2 N of the red yeast *Xanthophyllomyces dendrorhous* (*Phaffia rhodozyma*) with molasses. J Biosci Bioeng 92:121–125
2. Anonymous (1983) In: Methods of enzymatic food analysis using test combinations. Boehringer Mannheim GmbH Biochemica, Mannheim, pp 21, 33, 35
3. Beal C, Corrieu G (1994) Viability and acidification activity of pure and mixed starters of *Streptococcus salivarius* spp. *thermophilus* 404 and *Lactobacillus delbrueckii* ssp. *bulgaricus* 398 at the different steps of their production. Lebensm Wiss Technol 27:86–92
4. Beshkova D, Simova E, Frengova G, Simov Z (1998) Production of flavour compounds by yogurt starter cultures. J Ind Microbiol Biotechnol 20:180–186
5. Beshkova D, Simova E, Frengova G, Simov Z, Adilov E (1998) Production of amino acids by yogurt bacteria. Biotechnol Prog 14:963–965
6. Beshkova D, Simova E, Frengova G, Simov Z, Spasov Z (2002) Effect of oxygen on batch yogurt cultures. World J Microbiol Biotechnol 18:361–365
7. Buzzini P, Martini A (1999) Production of carotenoids by strains of *Rhodotorula glutinis* cultured in raw materials of agro-industrial origin. Bioresource Technol 71:41–44
8. Buzzini P (2001) Batch and fed-batch carotenoid production by *Rhodotorula glutinis-Debaryomyces castellii* co-cultures in corn syrup. J Appl Microbiol 90:843–847
9. Buzzini P, Rubinstein L, Martini A (2001) Production of yeast carotenoids by using agro-industrial by-products. Agro Food Ind Hi-tech 12:7-10
10. Bhosale P, Gadre R (2001) Production of *β*-carotene by a *Rhodotorula glutinis* mutant in sea water medium. Bioresource Technol 76:53–55
11. Bon J, Leathers T, Jayaswal R (1997) Isolation of astaxanthin-overproducing mutants of *Phaffia rhodozyma*. Biotechnol Lett 19:109–112
12. Coulson J (1980) Miscellaneous naturally occurring colouring materials for food stuff. In: Walford J (ed) Developments in food colour. Applied Science, London, pp 189–218
13. Counsell J (1980) Some synthetic carotenoids as food colours. In: Walford J (ed) Developments in food colour. Applied Science, London, pp 151–187
14. De Haan A, Burke R, Bont J (1991) Microbial production of food colorants. Med Fac Landbouww Rijisuniv Gent 56:1655–1660
15. Demain A, Phaff H, Kurtzman C (1998) The industrial importance of yeasts. In: Kurtzmann C, Fell J (eds) The yeasts. A taxonomic study. Elsevier, Amsterdam, pp 13–19

16. Ershov Y, Dmitrovsky A, Polulyakh O, Podoprigova O, Bykhovsky V (1992) Enzymatic conversion of torulene and torularhodin to retinal. Prikl Biochim Microbiol 28:680–684
17. Frengova G, Simova E, Pavlova K, Beshkova D, Grigorova D (1994) Formation of carotenoids by *Rhodotorula glutinis* in whey ultrafitrate. Biotechnol Bioeng 44:888–894
18. Frengova G, Simova E, Beshkova D (1997) Caroteno-protein and exopolysaccaride production by co-cultures of *Rhodotorula glutinis* and *Lactobacillus helveticus*. J Ind Microbiol 18:272–275
19. Johnson E, Schroeder W (1996) Microbial carotenoids. In: Fiechter A (ed) Advances in biochemical engineering and biotechnology. Springer, Berlin, pp 119–178
20. Kreger van Rij N (1984) In: The yeast: a taxonomic study. Elsevier, Amsterdam, pp 902–903
21. Kvasnikov E, Grinberg T, Vaskivnjuk V, Nagornaja S, Sudenko V, Stelokova I (1978) Yeasts synthesizing carotenoids. Izv ANSSR Seria Biologicheskaja 4:565–575
22. Longo E, Siero C, Velazguez J, Calo P, Cansado J, Villa T (1992) Astaxanthin production from *Phaffia rhodozyma*. Biotech Forum Europe 9:565–567
23. Martin A, Acheampong E, Patel T, Chornet E (1993) Study of growth parameters for *Phaffia rhodozyma* cultivated in peat hydrolysates. Appl Biochem Biotechnol 37:235–341
24. Martin A, Lu C, Patel T (1993) Growth parameters for the yeast *Rhodotorula rubra* grown in peat extracts. J Ferm Bioeng 76:321–325
25. Meyer P, Du Preez J (1994) Astaxanthin production by a *Phaffia rhodozyma* mutant on grape juice. World J Microbiol Biotechnol 10:178–183
26. Nelis A, De Leenheer A (1991) Microbial sources of carotenoids pigments used in foods and feeds. J Appl Bacteriol 70:181–191
27. Perrier V, Dubreucg E, Gaizy E (1995) Fatty acid carotenoid composition of *Rhodotorula* strains. Arch Microbiol 164:173–179
28. Polulyakh O, Podoprigova O, Eliseev S, Ershov Y (1991) Biosynthesis of torulene and torularhodin by the yeast *Phaffia rhodozyma*. Appl Biochem Microbiol 27:411–419
29. Simpson K (1983) Relative value of carotenoids as precursors of vitamin A. Proc Nutr Soc 42:7-17
30. Vandamme E (1992) Production of vitamins, coenzymes and related biochemicals by biotechnological processes. J Chem Tech Biotechnol 53:313–327
31. Zalashko M (1990) In: Solokova E (ed) Biotechnology of milk whey processing. Science Press, Moscow, pp 161–163