

# Carotenoid Production by Lactoso-Negative Yeasts Co-Cultivated with Lactic Acid Bacteria in Whey Ultrafiltrate

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Two strains were selected – the lactoso-negative yeast *Rhodotorula rubra* GED2 and the homofermentative *Lactobacillus casei* subsp. *casei* Ha1 for co-cultivation in cheese whey ultrafiltrate (WU) and active synthesis of carotenoids. Under conditions of intensive aeration (1.0 l/l min, 220 rpm), a temperature of 30 °C, WU with 55.0 g lactose/l, initial pH = 5.5, the carotenoid content in the cells reached a maximum, when the growth of the cultures had come to an end, *i.e.* in the stationary phase of the yeast. The maxima for dry cell accumulation (27.0 g/l) and carotenoid formation (12.1 mg/l culture medium) did not coincide on the 5<sup>th</sup> and 6<sup>th</sup> day, respectively. A peculiarity of the carotenoid-synthesizing *Rh. rubra* GED2 strain, co-cultivated with *L. casei* Ha1, was the production of carotenoids with high  $\beta$ -carotene content (46.6% of total carotenoids) and 10.7% and 36.9% for torulene and torularhodin, respectively.

**Key words:** Carotenogenesis, Co-Cultivation, Whey

## Introduction

Several bacteria, fungi and yeasts are good carotenoid producers (Johnson and Schroeder, 1996). Of the yeasts, those species belonging to the genera *Rhodotorula*, *Rhodospiridium* and *Phaffia* are known to form carotenoids from both synthetic and raw substrates for a long time (Martin *et al.*, 1993a, b; Meyer and Du Preez, 1994; Buzzini and Martini, 1999; Bhosale and Gadre, 2001, Bon *et al.*, 1997; Vijayalakshmi *et al.*, 2001). The major carotenoid pigments produced by the yeasts *Rhodotorula* and *Rhodospiridium* are  $\beta$ -carotene, torulene and torularhodin in various proportions (Buzzini and Martini, 1999; Bhosale and Gadre, 2001; Perrier *et al.*, 1995; Buzzini, 2001; Buzzini *et al.*, 2001; Kvasnikov *et al.*, 1978) and astaxanthin by *Phaffia rhodozyma* (Martin *et al.*, 1993a; Meyer and Du Preez, 1994). The studies on carotenogenesis reveal a growing interest in using natural substrates as a carbon source (grape juice, glucose syrup, peat extract and peat hydrolysate, molasses, corn syrup) (Martin *et al.*, 1993 a, b; Meyer and Du Preez, 1994; Buzzini and Martini, 1999; Buzzini, 2001; Buzzini *et al.*, 2001). In recent years, raw material and by-products of agro-industrial origin have been proposed as low cost alternative carbo-

hydrate sources for microbial metabolite production, with the view also of minimizing environmental and energetic problems related to their disposal (Demain *et al.*, 1998). A widespread natural substrate, a residue from cheese manufacture, is milk whey containing lactose as a carbon source. Carotenoid-synthesizing yeasts able to assimilate lactose are rarely found in natural conditions (Zalashko, 1990).

When grown as monocultures, the carotenoid-synthesizing yeast *Rhodotorula rubra* used in the present study do not assimilate lactose but actively synthesize carotenoids in synthetic media with carbon carriers such as glucose, galactose and sucrose. The studies on carotenogenesis by lactoso-negative yeasts of milk whey are certainly interesting not only for their economic and ecological aspects, but also for the possibilities of using lactose as a carbon substrate for synthesis of carotenoids. Carotenoid synthesis by lactoso-negative yeasts in lactose-rich substrates can be carried out under conditions for lactose transformation into carbon sources (glucose, galactose, lactic acid) easily assimilated by the yeasts (Frengova *et al.*, 1994).

The present work reports on production of carotenoids with high  $\beta$ -carotene content by a selected high-activity strain-producer *Rhodotorula rubra*

GED2 in association with *Lactobacillus casei* subsp. *casei* Ha1 strain in cheese whey ultrafiltrate.

## Materials and Methods

### Microorganisms and cultivation conditions

Three yeast strain and 40 strains of lactic acid cultures were screened to select yeast strains and lactic acid bacteria to be associated in a high-activity association for carotenoid production. The lactose-negative carotenoid-synthesizing yeast contaminating commercial yogurt were isolated and identified as *Rhodotorula rubra* using the Kreger van Rij determiner (1984). The strains *Rhodotorula rubra* GED2, *Rhodotorula rubra* GED4 and *Rhodotorula rubra* GED5 were maintained by monthly transfers on 2% malt extract agar slants and stored at 4 °C. The strains of lactic acid bacteria of the species *Lactococcus lactis* subsp. *lactis*, *Lactobacillus plantarum*, *Lactobacillus helveticus* and *Lactobacillus casei* subsp. *casei* were supplied by the Department of Milk Technology at the Higher Institute of Food and Flavour Industries. The cultures were maintained in sterile skim cow's milk and MRS broth, according to De Man, Rogosa and Sharpe (Fluka, Buchs, Switzerland) by transferring a loopful of inoculum every week, and stored at 4 °C.

The inocula of the yeast cultures were grown in 1000-ml Erlenmeyer flasks containing 100-ml culture medium with 2% malt extract, at 29–30 °C, in the course of 48 h, on a rotary shaker with 220 rpm. The inoculum size for all fermentation was 5% (v/v) and its cell concentration was 1.3–1.4 g dry cells/l. The inocula of the lactic acid cultures were grown statically in skim cow's milk and incubated at temperatures as follows: *L. lactis* – at 30 °C for 16 h; *L. plantarum* – at 30 °C for 19 h; *L. helveticus* – at 37 °C for 11 h and *L. casei* – at 30 °C for 20 h. The inoculum amount for the fermentation medium was 1% (v/v) ( $5.0\text{--}7.5 \times 10^8$  cells/ml). The inocula of each yeast and lactic acid cultures were introduced simultaneously into the fermentation medium.

The microbial associations (yeasts + lactic acid bacteria) were grown on a rotary shaker with 220 rpm at 30 °C in 1000 ml Erlenmeyer flasks containing 100 ml fermentation medium. The composition of the fermentation medium was as follows; whey ultrafiltrate (WU), containing 35.0 g

lactose/l;  $(\text{NH}_4)_2\text{SO}_4$  – 6.0 g/l;  $\text{KH}_2\text{PO}_4$  – 5.5 g/l;  $\text{Na}_2\text{HPO}_4$  – 3.0 g/l;  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  – 0.5 g/l; yeast extract – 5.0 g/l, pH = 6.0. 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 (Nakskow, Denmark), with GR61PP (Nakskow, Denmark) membranes. WU was utilized in its native state (35.0 g lactose/l) or brought to lactose concentration (45.0–75.0 g/l) using a DDS RO-System LAB 20 (Nakskov, Denmark) with a CA995PP 540–0.16 membrane (Nakskov, Denmark). The association *Rh. rubra* GED2 + *L. casei* Ha1 was grown in WU with lactose concentrations (35.0, 45.0, 55.0, 65.0, 75.0 g/l) and pH of the fermentation medium 6.0 at a temperature of 30 °C; in fermentation medium with pH 4.0, 4.5, 5.0, 5.5, 6.0, 6.5 and lactose concentration 55.0 g/l at 30 °C; at cultivation temperatures 20, 25, 30, 35 °C in fermentation medium with pH = 5.5 and lactose concentration 55.0 g/l.

The two strains were cultivated in association batchwise in a 15 l MBR AG fermentor (Zurich, Switzerland) (7.5 l working volume) under the following conditions: lactose concentration in the WU 55.0 g/l; pH = 5.5; temperature 30 °C; air flow rate 1.0 l/l min, agitation 220 rpm.

The pH of the cultivation system was not adjusted during the growth period.

### Analytical methods

In the mixed culture, viable counts (in colony forming units: cfu/ml) of *Rh. rubra* GED2 were estimated on plates with 2% malt extract and 1.2% agar after a 5-day incubation at 29 °C. Viable counts (in colony forming units: cfu/ml) of *L. casei* Ha1 were estimated on plates of a medium consisting of pancreatin-hydrolyzed milk (pancreatin 0.1%), agar 1.2% and China blue 0.375 g/l, after 6 days incubation at 30 °C. Cell dry weight was determined after heating them at 105 °C to a constant weight. Lactose, glucose, galactose and lactic acid were determined by enzymatic methods as described by Boehringer Mannheim (1983). Extraction of carotenoids from cell, determination of total carotenoids (spectrophotometrically) and individual carotenoid pigments (by HPLC) were described earlier (Frengova *et al.*, 1994). Total protein content was calculated from the total nitrogen

Table I. Carotenoid and cell mass production by *Rh. rubra* strains co-cultured with lactic acid bacteria in WU with 35.0 g lactose/l, at 30 °C, initial pH = 6.0 for 6 days.

Microbial association (yeasts + lactic acid bacteria)	Final pH	Residual lactose [g/l]	Dry cell mass, [g/l]	Total carotenoids, mg/l culture medium
<i>Rh. rubra</i> GED2+ <i>L. casei</i> B1	5.46 ± 0.09	1.60 ± 0.02	12.1 ± 0.65	2.50 ± 0.09
<i>Rh. rubra</i> GED2+ <i>L. casei</i> Ha1	5.52 ± 0.04	1.40 ± 0.10	13.3 ± 0.65	2.78 ± 0.03
<i>Rh. rubra</i> GED4+ <i>L. casei</i> B1	5.39 ± 0.02	1.84 ± 0.05	11.1 ± 0.46	2.12 ± 0.007
<i>Rh. rubra</i> GED4+ <i>L. casei</i> Ha1	5.41 ± 0.04	1.80 ± 0.09	11.3 ± 0.36	2.04 ± 0.02
<i>Rh. rubra</i> GED5+ <i>L. casei</i> B5	5.38 ± 0.02	1.90 ± 0.05	10.1 ± 0.26	2.13 ± 0.03
<i>Rh. rubra</i> GED5+ <i>L. casei</i> Ha4	5.30 ± 0.03	1.92 ± 0.03	10.3 ± 0.26	2.06 ± 0.03

Note: The data about the remaining microbial associations of *Rh. rubra* strains with *L. casei* and *L. lactis* strains can be obtained directly from the authors

content (Nx6.25) and determined by the conventional method of Kjeldahl System 1028 (Osborne and Voogh, 1978).

The results represent the average of three independent experiments.

Results and Discussion

40 strains of lactic-acid bacteria from the *L. lactis*, *L. plantarum*, *L. helveticus* and *L. casei* species were subjected to preliminary large-scale screening in order to select strains of lactic acid bacteria for associated growth with the yeast strain-producers *Rh. rubra* GED2, *Rh. rubra* GED4, *Rh. rubra* GED5 and carotenoid synthesis. Out of the 120 associations formed, 5 strains of lactococci (*L. lactis* C21, *L. lactis* C24, *L. lactis* C25, *L. lactis* C27, *L. lactis* C28) and 5 strains of lactobacilli (*L. casei* B1, *L. casei* B5, *L. casei* B6, *L. casei* Ha1, *L. casei* Ha4) were selected on the basis of their carotenoid-synthesizing activity. The strain *R. rubra* GED2 cultivated in association with *L. casei* Ha1 manifested the highest carotenoid-synthesizing activity – 2.78 mg total carotenoids/l culture medium (Table I). The mixed culture *Rh. rubra* GED2 + *L. casei* Ha1 also synthesized the highest

yield of dry cells – 13.3 g/l. The associations with *L. lactis* strains manifested relatively lower carotenoid-synthesizing activity. The culture media with these associations had lower pH values and higher concentrations of residual lactose (2.25–2.73 g/l) against 1.40–1.95 g/l with the associations including *L. casei*. The microscope preparations of the culture media with *Rh. rubra* and *L. casei* showed predomination of the yeast. In the culture media with *Rh. rubra* and *L. lactis* the cocci prevailed.

The association *Rh. rubra* GED2 + *L. casei* Ha1 developed actively with maximum production of carotenoids at a lactose content in the WU 55.0 g/l (4.47 mg/l culture medium or 0.24 mg/g dry cells) (Table II). The increased lactose concentrations (65.0 and 75.0 g/l) inhibited carotenoid formation and the carotenoids recorded per g dry cells were less than those at lower lactose concentrations (35.0 and 45.0 g/l). The increased lactose concentration resulted in increased dry cell yield of 21.8 g/l at 75.0 g lactose/l. At initial lactose concentration 75.0 g/l, 67% were transformed against 96–98% at initial lactose concentration 35.0–55.0 g/l. No correlation between biomass accumulation, carotenoid formation, and lactose contents in the natural substrate was established.

Table II. Effect of lactose concentration on the growth and carotenoids formation by *Rh. rubra* strains co-cultured with *L. casei* Ha1, at 30 °C, initial pH = 6.0 for 6 days.

Initial lactose conc. [g/l]	Final pH	Residual lactose [g/l]	Dry cell mass [g/l]	Total carotenoids, mg/g dry cells	Total carotenoids, mg/l culture medium
35.00	5.52 ± 0.04	1.40 ± 0.10	13.3 ± 0.65	0.21 ± 0.009	2.78 ± 0.03
45.00	5.60 ± 0.04	3.23 ± 0.09	16.5 ± 1.18	0.23 ± 0.01	3.76 ± 0.06
55.00	5.72 ± 0.02	6.60 ± 0.10	18.4 ± 0.53	0.24 ± 0.007	4.47 ± 0.14
65.00	5.43 ± 0.05	13.71 ± 0.61	20.6 ± 0.60	0.20 ± 0.003	4.18 ± 0.12
75.00	5.31 ± 0.04	24.80 ± 1.09	21.8 ± 0.98	0.15 ± 0.008	3.33 ± 0.09

The pH values of the fermentation medium had greater influence on the synthesis of dry mass and less influence on the production of carotenoids by *Rh. rubra* GED2 + *L. casei* Ha1. The low initial pH values of the fermentation medium (pH = 4.0 and pH = 4.5) inhibited considerably the biomass synthesis and carotenoid formation. A maximum concentration of dry cells (19.3 g/l) was recorded at initial pH = 5.5 and maximum concentration of carotenoids (0.24 mg/g dry cells) at pH = 5.0 and pH = 6.0. At pH = 6.5 the cell growth inhibition was greater than the carotenoid formation.

The temperature regimen for active growth of the microbial association *Rh. rubra* GED2 + *L. casei* Ha1 and active carotenogenesis is a principal factor for the course of both processes – lactic acid fermentation and carotenoid formation. At cultivation temperature of 30 °C there was active microbial growth with maximum yield of dry cells 19.3 g/l and active carotenoid formation in the yeast cell with maximum yield of 0.24 mg/g dry cells and 4.69 mg/l culture medium. When the temperature was lowered to 20 °C, the growth of both cultures was considerably inhibited, which resulted

in approximately twofold decrease of the dry cells yield. The residual lactose concentration was high – 52% of the initial lactose had been transformed against 88% at 30 °C. At 35 °C there was active growth of *L. casei* Ha1 and relatively lower carotenoid formation than that at 30 °C. The cultivation temperature and pH of the fermentation medium are dependant on the species affiliation and the strain peculiarity of the carotenoid-synthesizing yeast. The pH optimum for a great part of the yeast, producing carotenoids in substrates with different carbon sources, is in the interval of pH 4.5–7.5 while the optimum temperature of carotenoid formation is 20–30 °C (Martin *et al.*, 1993b; Buzzini and Martini, 1999; Bhosale and Gadre, 2001; Vijayalakshmi *et al.*, 2001; Buzzini, 2001).

Under the given conditions (temperature 30 °C, initial pH = 5.5, lactose concentration in the WU 55.0 g/l, and air flow rate 1.0 l/l min at agitation 220 rpm) the mixed culture grew intensively and there was active carotenoid formation with maximum yields of carotenoids (12.12 mg/l culture medium on the 6<sup>th</sup> day) and dry cells (27.0 g/l on the 5<sup>th</sup> day) (Fig. 1a,b,c). It was observed that the nat-

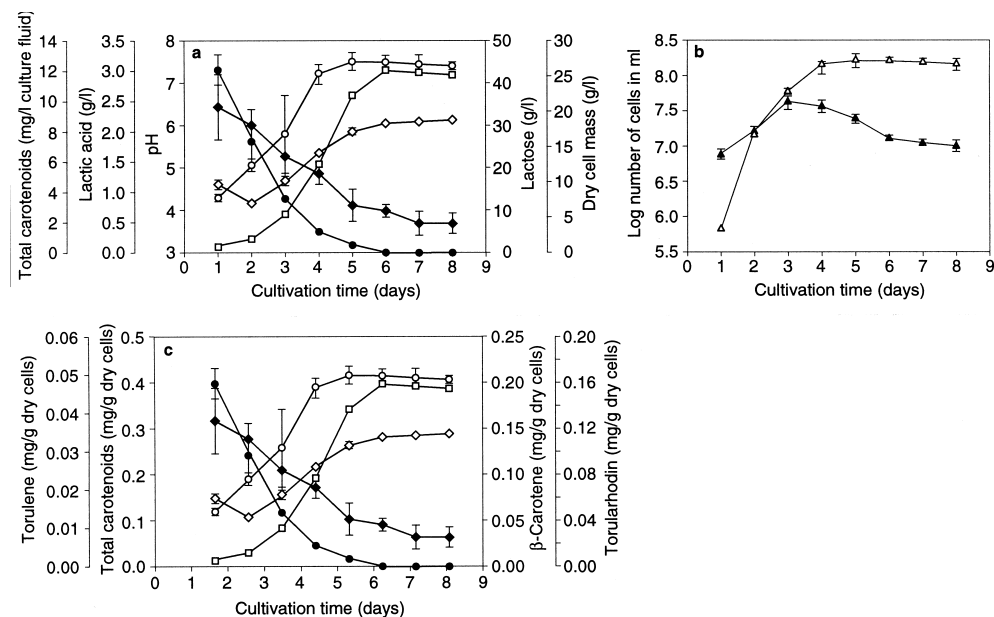


Fig. 1. Profile of carotenoids formation and growth of a microbial association *Rh. rubra* GED2 + *L. casei* Ha1 in WU with 55.0 g lactose/l, at 30 °C, initial pH = 5.5, air flow rate 1.0 l/l min and agitation 220 rpm: (a) total carotenoids (□), dry cell mass (○), pH (◇), lactose (●), lactic acid (◆); (b) *Rh. rubra* GED2 (△), *L. casei* Ha1 (▲); (c) total carotenoids (■), β-carotene (▽), torulene (◇), torularhodin (▼).

ural change in pH became lower by the 3<sup>rd</sup> day (pH = 4.18) since start of cultivation, accompanied by intensive lactic-acid fermentation; an increase followed during the intensive carotenogenesis – the maximum production of carotenoids proceeded within the pH interval 5.38–6.05, after which pH remained about 6.15 – an indication to the preclusion of the fermentation process (Fig. 1a). The lactose in the WU was completely assimilated by the mixed culture on the 6<sup>th</sup> day. The homofermentative lactobacilli actively transformed lactose into glucose, galactose and lactic acid. The analyses of glucose and galactose presence in the course of the process showed traces of galactose and absence of glucose, while the lactic-acid concentrations were 0.5–2.4 g/l (Fig. 1a). Both the monosaccharides, which are easily assimilated, and the lactic acid acted as substrates for development of the yeast, so that the latter quickly entered an exponential growth phase in a medium in which the carbon substrate lactose was not assimilated (Fig. 1b). Active growth of the lactobacillus was observed, which predominated over the yeast cells up to the 3<sup>rd</sup> day (Fig. 1b). Microscope preparations showed short rods sized  $[0.9 \times (3.0–8.0)] \mu\text{m}$  occurring singly, in pairs and in short chains. The observation of yeast growth during the first two days established slight growth of non-budding but well-formed cells of oval shape sized (5.0–10.0  $\mu\text{m}$ ). Intensive growth of the yeast with prevailing young budding cells was registered from the 3<sup>rd</sup> to the 6<sup>th</sup> day. After the 3<sup>rd</sup> day the yeast population exceeded the population of the lactobacilli. After the 5<sup>th</sup> day the bacterial microflora considerably decreased, the cells grew older, elongated and formed long chains. The active synthesis of carotenoids coincided with the active growth of yeast. During the stationary phase of yeast growth (after the 4<sup>th</sup> day), however, the production of carotenoids continued (Fig. 1a, b). The high carotenoid-forming ability of the lactoso-negative strain *Rh. rubra* GED2 during cultivation in WU with *L. casei* Ha1 on the one hand, and the active growth and acidification on the other, is evidence of the reciprocally positive interaction between the two strains, *i.e.* of symbiotic effect (Fig. 1a, b).

The lactoso-negative strain *Rh. rubra* GED2 showed high carotenoid-synthesizing activity in associated cultivation with *L. casei* Ha1 in WU un-

der intensive aeration (Fig. 1a). The carotenoid-producing activity was (1.7 times) higher than that of a mixed culture *Rh. glutinis* 22P + *L. helveticus* 12A grown in WU; (2.5 times) higher than that of *Rh. rubra* GED2 monoculture grown in synthetic medium with glucose; (about 5 times) higher than the lactoso-positive strain *Rh. lactosa* BKM-1264 cultivated in whey reported in literature (Frengova *et al.* 1994; Zalashko, 1990).

The major carotenoid pigments making up the total carotenoid synthesized by *Rh. rubra* GED2 in association growth with *L. casei* Ha1 were  $\beta$ -carotene, torulene and torularhodin (Fig. 1c). During carotenoid formation the time to reach the maximum concentration of total carotenoids (0.45 mg/g dry cells) coincided with the time for maximum accumulation of  $\beta$ -carotene (0.21 mg/g dry cells). Torulene was formed earlier in the growth cycle of the yeast and a maximum concentration (0.05 mg/g dry cells) was recorded on the 5<sup>th</sup> day. Torularhodin synthesis followed the path of  $\beta$ -carotene production. The relative share of individual pigments in the total carotenoids was  $\beta$ -carotene 46.6%, torulene 10.7%, and torularhodin 36.9%. The identified individual pigments that form total carotenoids were typical of the species of the *Rhodotorula* genus reported by other authors (Buzzini and Martini, 1999; Bhosale and Gadre, 2001; Perrier *et al.*, 1995; Buzzini, 2001; Buzzini *et al.*, 2001). Our previous work and the data on the carotenogenesis of the strain *Rh. rubra* GED2 showed that the amount and correlation between the separate pigments depended on the species peculiarity of the strain-producer (Frengova *et al.*, 1994). *Rh. rubra* GED2 cultivated in association with *L. casei* Ha1 synthesized carotenoids with high  $\beta$ -carotene content (46.6% of the total carotenoids). The lactoso-negative strain *Rh. rubra* GED2 cultivated with *L. casei* Ha1 produced 2.8 times more  $\beta$ -carotene than the lactoso-negative strain *Rh. glutinis* 22P cultivated in WU with *L. helveticus* 12A (Frengova *et al.*, 1994) and approximately 2.5 times more than the reported lactoso-positive strain *Rh. lactosa* BKM-1264 (Zalashko, 1990). Our preliminary studies on carotenoid formation by the monoculture *Rh. rubra* GED2 cultivated in glucose medium showed approximately 3 times lower yield of total carotenoids and nearly 3.5 times lower concentrations of  $\beta$ -carotene. The obtained higher yields of caro-



tenoids and  $\beta$ -carotene, synthesized by *Rh. rubra* GED2 grown in a substrate (lactose) that it did not assimilate, showed the possibilities of mixed cultivation with reciprocal stimulation of cultures for efficient synthesis of carotenoids.

The protein content in the dry cells synthesized by *Rh. rubra* GED2 + *L. casei* Ha1 was significantly higher than the one reported by other authors for the monoculture *Rh. lactosa* BKM 1264 cultivated in whey (45.6% against 33.8%) (Zalashko, 1990).

On the basis of the data obtained, it can be concluded that carotenoid formation by *Rh. rubra* GED2 + *L. casei* Ha1. can yield carotene-protein concentrates with application as vitamin, colouring and protein supplements to foods and feeds.

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