# Effect of Aeration on the Production of Carotenoid Pigments by *Rhodotorula rubra-lactobacillus casei* Subsp. *casei* Co-Cultures in Whey Ultrafiltrate

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Under intensive aeration (1.3 l/l min) the associated growth of *Rhodotorula rubra* GED2 and *Lactobacillus casei* subsp. *casei* in cheese whey ultrafiltrate (55 g lactose/l) proceeded effectively for both cultures with production of maximum carotenoids (12.4 mg/l culture fluid). For maximum amount of carotenoids synthesized in the cell, the yeast required more intensive aeration than the aeration needed for synthesis of maximum concentration of dry cells. Maximum concentration of carotenoids in the cell (0.49 mg/g dry cells) was registered with air flow rate at 1.3 l/l min, and of dry cells (27.0 g/l) at 1.0 l/l min. An important characteristic of carotenogenesis by *Rhodotorula rubra* GED2 + *Lactobacillus casei* subsp. *casei* was established – the intensive aeration (above 1.0 l/l min) stimulated β-carotene synthesis (60% of total carotenoids).

*Key words:* Carotenoid Pigments, Yeast, Lactic Acid Bacteria

## Introduction

The microbial production of carotenoids, when compared with extraction from vegetables (Coulson, 1980) or chemical synthesis (Counsell, 1980), seems to be 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 (De Haan *et al.*, 1991), and because of the economic advantages of microbial processes using natural low-cost substrates as carbohydrate source.

Carotenoid biosynthesis is a specific feature of the *Rhodotorula* species (Martin *et al.*, 1993a; Perrier *et al.*, 1995; Buzzini and Martini, 1999; Bhosale and Cadre, 2001; Buzzini, 2001; Vijayalakshmi *et al.*, 2001), *Rhodosporidium* (Kvasnikov *et al.*, 1978) and *Phaffia* genera (Longo *et al.*, 1992; Martin *et al.*, 1993b; Meyer and Du Preez, 1994; An *et al.*, 2001). Carotenoid-synthesizing yeasts are aerobes and the air flow rate in the culture is an essential factor to assimilate the substrate as well as for growth rate, cell mass and carotenoids synthesis. The effect of aeration is dependent on the species of the microorganism, which frequently manifests itself in quantitative variation of the synthesized carotenoids (Longo *et al.*, 1992; Martin *et al.*, 1993a; Kvasnikov *et al.*, 1978). Investigations in this direction are particularly important in cases of carotenoid synthesis by a mixed culture, consisting of microorganisms of two taxonomic groups with different needs of oxygen for their growth and metabolism. Carotenoid synthesis by lactose-negative yeast of the *Rhodotorula* genus in lactose substrates can be accomplished only by creating conditions in which the lactic-acid bacteria transform lactose into carbon sources (glucose, galactose, lactic acid) that are easily assimilated by the yeast in a process of co-cultivation of yeast and bacterial cultures (Frengova *et al.*, 1994). Carotenoid-synthesizing yeasts having the ability to assimilate lactose are rarely found in nature (Zalashko, 1990). Lactic-acid bacteria are microaerophils and do not require oxygen to grow and metabolise in natural nutrient media like milk and whey. Our previous studies showed that some strains of yogurt bacteria could grow and metabolise at relatively high concentrations of oxygen dissolved in milk (up to 30%) (Beshkova *et al.*, 2002). Carotenogenesis of the yeast *Rhodotorula glutinis* 22P was studied using the associated culture *Lactobacillus helveticus* 12A capable of growing in cheese whey ultrafiltrate under intensive aeration (0.5 l/l min) (Frengova *et al.*, 1994). The growth of

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the lactic-acid bacteria in conditions of intensive aeration is probably related to the stimulating effect of the products from yeast metabolism.

The present work reports on the production of carotenoid pigments by *Rhodotorula rubra* GED2 co-cultivated with *Lactobacillus casei* subsp. *casei* Ha1 in cheese whey ultrafiltrate (WU) under various intensities of aeration.

## Materials and Methods

### *Microorganisms and cultivation conditions*

The strain *Lactobacillus casei* subsp. *casei* Ha1, capable of producing maximum amount of lactic acid in WU (27 g/l for 26 h) under intensive aeration (1.5 l/l min), was selected out of 58 strains of the species *Lactobacillus delbrueckii* subsp. *bulgaricus*, *Lactobacillus helveticus*, *Lactobacillus casei* subsp. *casei* in various aeration conditions. The strains *Rhodotorula rubra* GED2 and *Lactobacillus casei* subsp. *casei* were associated after preliminary screening of the carotenoid-forming activity of yeast strain-producers grown in association with different lactic-acid cultures in WU.

The carotenoid-synthesizing strain (*Rh. rubra* GED2) contaminating a commercially-fermented yogurt was isolated and used in the present investigation. It was identified as *Rhodotorula rubra* according to Kreger van Rij's determiner (1984). The culture was maintained by monthly transfers onto 2% malt extract agar slants and stored at 4 °C.

The lactic acid bacteria were supplied by the Milk Technology Department 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 RdH, Buchs, Switzerland) by transferring a loopful of inoculum every week, and stored at 4 °C.

The composition of the fermentation medium was as follows: WU, containing 55.0 g lactose/l; $(NH_4)_2SO_4$ – 6.0 g/l; $KH_2PO_4$ – 5.5 g/l; $Na_2HPO_4$ – 3.0 g/l; $MgSO_4.7H_2O$ – 0.5 g/l; yeast extract – 5.0 g/l, pH = 5.5. 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 (Nakskov, Denmark), on GR61PP (Nakskov, Denmark) membranes. WU was brought to a lactose concentration of 55.0 g/l using a DDS RO-SYSTEM LAB 20 (Nakskov, Denmark ) with a CA995PP 540–0.16 membranes (Nakskov, Denmark).

The inoculum of *Rh. rubra* GED2 was 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 for all fermentations was 5% (v/v) and its cell concentration was about 1.4 g dry cells/l.

The inoculum of *L. casei* Ha1 was grown statically in skim cow's milk, at 30 °C, in the course of 20 h. It was introduced into the fermentation medium in a quantity of 1% (v/v) ($5.0–5.7 \times 10^8$ cells/ml).

The association *Rh. rubra* GED2 + *L. casei* Ha1. was cultivated in a 15 l MBR AG fermentor (Zurich, Switzerland) at 30 °C using a 7.5 l working volume, an air flow rate of (0.4, 0.7, 1.0, 1.3, 1.6 l/l min) and agitation at 220 rpm for 8 days. The yeast and *Lactobacillus* cultures were inoculated simultaneously. The pH of the fermentation system was not adjusted during the growth period.

### *Analytical methods*

Dry cell weight was determined at 105 °C to a constant weight.

Lactose was determined by enzymatic methods as described by Boehringer Mannheim (1983).

The extraction of carotenoids from the cell, determination of total carotenoids (spectrophotometrically) and individual carotenoid pigments (by HPLC technique) have been described previously (Frengova *et al.*, 1994).

## Results and Discussion

The dynamics of synthesis of carotenoids and dry cells during cultivation of the microbial association *Rh. rubra* GED2 + *L. casei* Ha1 with air flow rate from 0.4 to 1.6 l/l min and agitation 220 rpm showed that the maximums for dry cell accumulation and carotenoid formation do not coincide (Fig. 1a–e). It was established that the carotenoid content in the cells reached a maximum after growth of the cultures had terminated, *i.e.* in the stationary phase of the growth cycle of the yeast. With an air flow rate of 1.3 l/l min a maximum yield of carotenoids 12.35 mg/l culture fluid was obtained. For a maximum amount of carotenoids

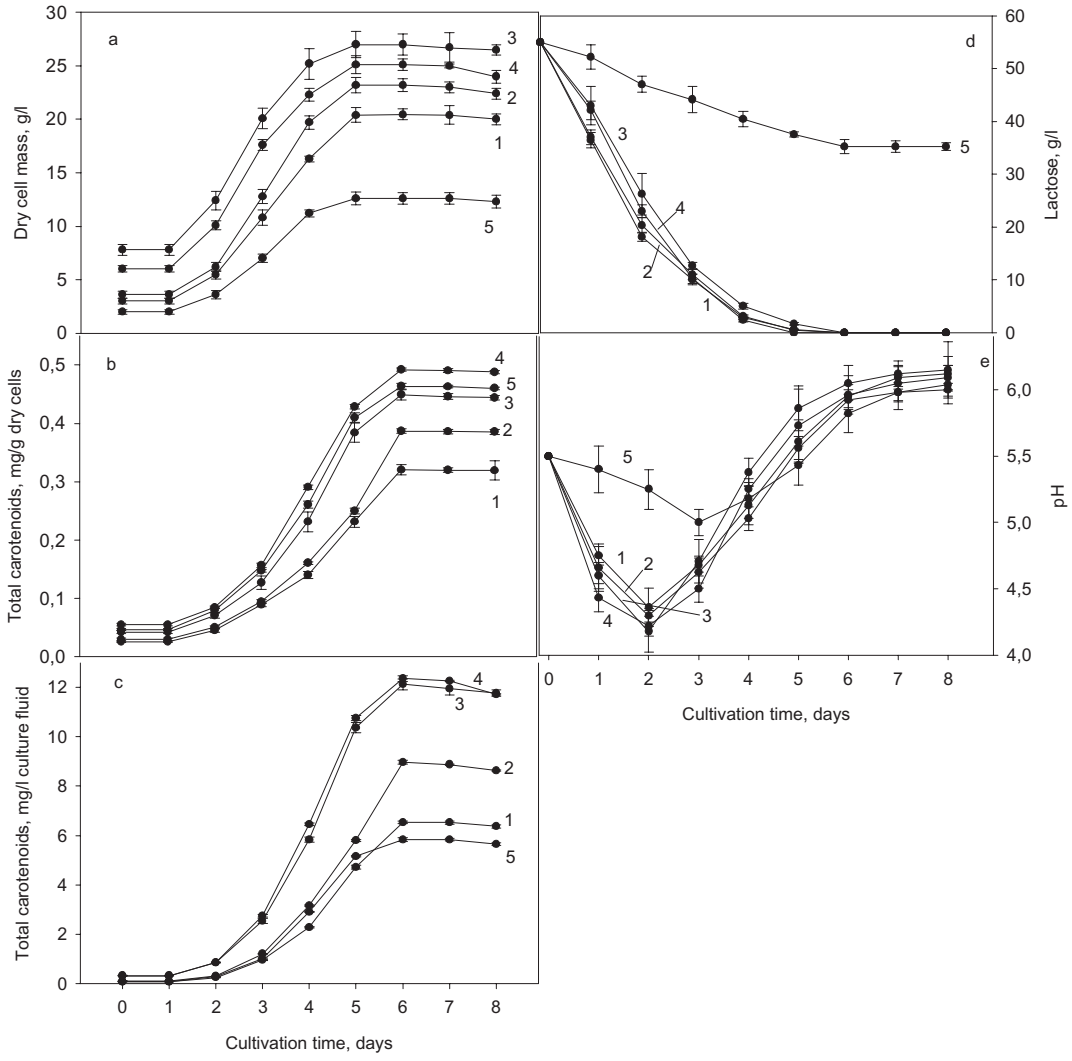


Fig. 1. Profile of carotenoid formation and growth of a microbial association *Rh. rubra* GED2 + *L. casei* Ha1 in WU, at 30 °C and different air flow rates: 0.4 l/l min (1), 0.7 l/l min (2), 1.0 l/l min (3), 1.3 l/l min (4), 1.6 l/l min (5); dry cell mass (a), total carotenoids, mg/g dry cells (b); total carotenoids, mg/l culture fluid (c), lactose (d), pH (e). WU – cheese whey ultrafiltrate.

synthesized in the cell, the yeast needed more intensive aeration in comparison with that required for maximum concentration of dry cells. A maximum concentration of carotenoids in the cell (0.49 mg/g dry cells) was recorded at an air flow rate of 1.3 l/l min, and of dry cells (27.0 g/l) at 1.0 l/l min (Fig. 1a, b,). Dry cell synthesis and carotenoid formation proceeded less intensively with air flow rate above 1.3 l/l min. Cell growth in a culture aerated with 1.6 l/l min was affected to a greater extent, where the recorded yield of dry cells (12.6 g/l) was 1.9 times less than that with 1.3 l/l min. At an air flow rate 1.6 l/l min the growth of *Lactobacillus* was inhibited. During the fermentation process certain changes in the morphology of *L. casei* Ha1 was observed. The cells had become thinner, elongated, forming long chains and their number was comparatively smaller. *L. casei* Ha1 showed considerably lower activity of acidification – 36% of lactose was transformed. With an air flow rate 0.7–1.0 l/l min there was complete assimilation of the carbon car-

rier by the 6th day (Fig. 1d). Although after a 5-day cultivation the lactose in the culture aerated with an air flow rate of 1.3 l/l min was used up, carotenoid production continued and reached a maximum concentration on the 6th day. The highest air flow rate (1.6 l/l min) did not cause significant decrease in pH to values of 4.2–4.4 (on the 2nd day), which is characteristic of the growth of the microbial association at air flow rate 0.4–1.3 l/l min (Fig. 1e).

The aeration of the mixed culture influenced not only the amount of carotenoids produced, but also the composition of individual pigments making up the total carotenoids (Fig. 2a–e). An important characteristic of the carotenogenesis by *Rh. rubra* GED2 + *L. casei* Ha1 was established, namely that intensive aeration of the cultures stimulated β-carotene synthesis. Increasing the air flow rate to 1.6 l/l min the relative proportion of β-carotene increased from 42.0% to 60.0%, the proportion of torularhodin decreased from 44.0% to 29.0%, while the proportion of torulene changed only slightly (9.5–11.0%). Further increase of the air flow rate to 2.0 l/l min resulted in an increase of β-carotene content in the total carotenoids to 64.0% and at the same time to a considerable decrease in the carotenoid yield (up to 0.38 mg/g dry cells). Some authors have registered changes only in the amount of total carotenoids depending on the aeration (Kvasnikov *et al.*, 1978; Longo *et al.*, 1992; Martin *et al.*, 1993a), other authors have recorded decrease in the torularhodin concentration (Zalashko, 1990). The identified individual pigments that form total carotenoids

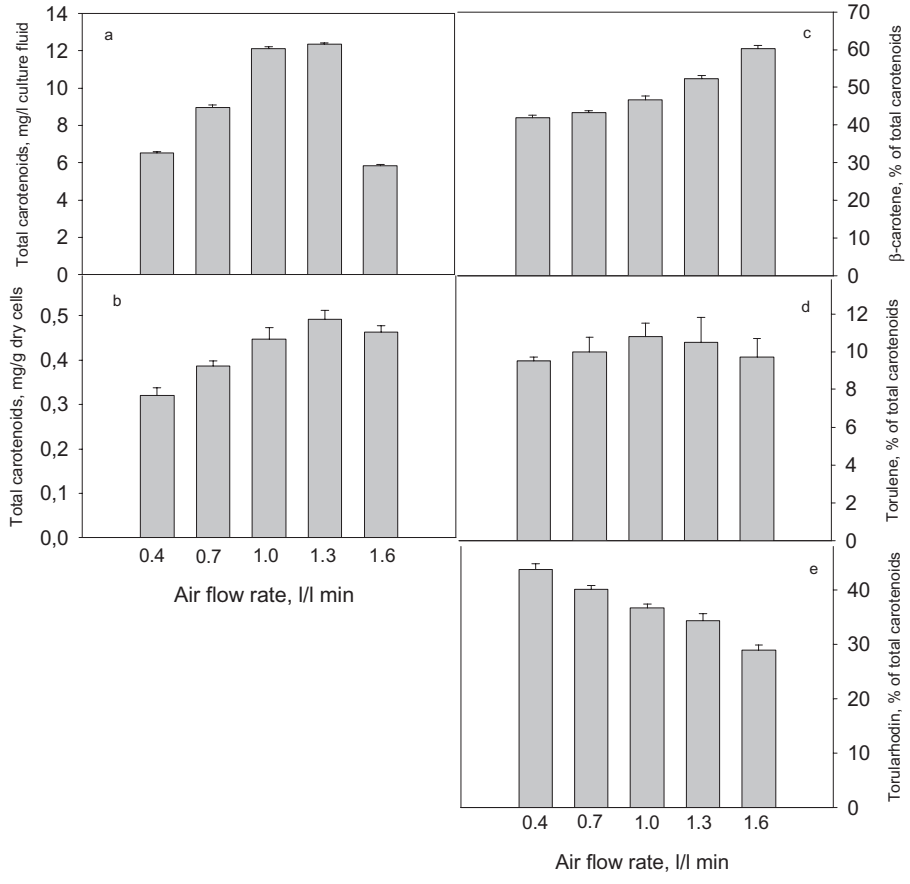


Fig. 2. Concentrations of individual pigments in total carotenoids synthesized by *Rh. rubra* GED2 co-cultivated with *L. casei* Ha1 in WU, at 30 °C and different air flow rates: total carotenoids, mg/l culture fluid (a); total carotenoids, mg/g dry cells (b); β-carotene (c); torulene (d); torularhodin (e). WU – cheese whey ultrafiltrate.

produced by *Rh. rubra* GED2 + *L. casei* Ha1 are typical for the species of the *Rhodotorula* genus reported by other authors (Perrier *et al.*, 1995; Buzzini and Martini, 1999; Buzzini, 2001; Bhosale and Gadre, 2001). These results revealed that the amount and ratio of separate pigments depend on the species peculiarity of the strain-producer (Frengova *et al.*, 1994).

Carotenoid production activity was 1.8 times higher than the activity of the mixed culture *Rh. glutinis* 22P + *L. helveticus* 12A grown in WU; 2.7 times higher than that of monoculture *Rh. rubra* GED2 grown in glucose-rich synthetic medium; 5.7 times higher than the lactose-positive strain *Rh. lactosa* BKM-1264 cultivated in whey, according to literature (Frengova *et al.*, 1994; Zalashko, 1990).When cultivating the aerobic culture *Rh. rubra* GED2 together with the facultative anaerobe *L. casei* Ha1, the aeration level should create the conditions for intensive metabolism of the lactic acid bacteria which transform lactose into glucose, galactose and lactic acid – a precondition for *Rh. rubra* GED2 growth and active carotenogenesis. Despite the fact that the independent cultivation of the homofermentative lactic-acid bacteria, *L. casei* Ha1, does not require oxygen it was found out that under intensive aeration the associated cultivation of the yeast and lactic-acid bacteria proceeded effectively for both cultures (Fig. 1a–e).

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