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Use of Whey Ultrafiltrate as a Substrate for Production of Carotenoids by the Yeast *Rhodotorula Rubra*

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Abstract

Carotenogenesis of the lactose-negative yeast *Rhodotorula rubra* GED5 was studied by cocultivation with *Kluyveromyces lactis* MP11 in whey ultrafiltrate (WU) (35, 50, and 70 g of lactose/L). Maximum yields of cell mass (24.3 g/L) and carotenoids (10.2 mg/L of culture fluid or 0.421 μ g/g of dry cells) were obtained by growing the microbial association in WU (50 g of lactose/L) in a fermentor with an airflow rate of 0.8 L/(L·min), agitation of 220 rpm, and temperature of 30°C. The identified carotenoid pigments—β-carotene, torulene, and torularhodin—reached maximum concentrations (133, 26.9, and 222.3 μg/g of dry cells, respectively) on d 5 for torulene and d 6 for β-carotene and torularhodin.

Index Entries: Carotenogenesis; microbial association; *Rhodotorula rubra*; *Kluyveromyces lactis*; cocultivation; whey.

Introduction

Perhaps the best-known function of carotenoids such as α-carotene, β-carotene, β-cryptoxanthin, torulene, and torularhodin is that of provitamin A *(1,2)*. Humans and animals convert these carotenoid pigments into retinal and further into retinol (vitamin A).

Carotenoid-synthesizing yeasts represent an alternative within the potential of biotechnologies for obtaining natural pigments that could be used as flavoring and coloring food additives. When included in animal diets, carotenoid-containing yeasts with destructured cell walls improve animal growth *(3)*, effectively color fish meat *(4)*, and enhance the desired golden color of egg yolk and poultry meat *(5)*. Carotenoid biosynthesis is a specific feature of the species of the *Rhodotorula (6–9)*, *Rhodosporidium (10)*,

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and *Phaffia (8,11,12)* genera. The major carotenoid pigments produced by the yeasts *Rhodotorula* and *Rhodosporidium* are β-carotene, torulene, and torularhodin in various proportions *(6,7,9,10)* and astaxanthin by *Phaffia rhodozyma (11–13)*. The yeasts can synthesize carotenoids during cultivation in synthetic media containing various monosaccharides or disaccharides *(7,14,15)*. Studies on carotenogenesis reveal a growing interest in the use of natural substrates such as grape juice, glucose syrup, grape must, soybean flour extract, maize flour extract, peat extract, peat hydrolysate, and molasses *(6,8,11,12,16)*. Generally, these are agroindustrial byproducts and surpluses that create environmental problems and could be successfully utilized as low-cost sources of carbohydrate substrate for the microbial fermentations.

A widespread natural substrate, a residuum from the cheese manufacture, is milk whey containing lactose as a carbon source. A few bibliographic sources indicate that lactose may be a source of carbon for carotenoid biosynthesis by yeast *(17)*.

The carotenoid producer strain of *Rhodotorula rubra* GED5 used in the present study does not assimilate lactose but actively assimilates glucose, galactose, and saccharose. Studies on carotenogenesis by lactose-negative yeasts in milk whey are certainly interesting not only for their economic and ecologic aspects, but also for the possibility of using lactose as a carbon substrate for synthesis of carotenoids. The search for ways to biotransform lactose led to the idea of creating an association of microorganisms that, otherwise as monocultures, have a limited capacity to produce this metabolite. Carotenoid synthesis by lactose-negative yeasts can only be accomplished by enzymatic hydrolysis of lactose to assimilable carbon sources, thus providing the method of cocultivation with lactose-positive yeasts, producers of β-galactosidase. Our earlier studies *(18)* reported results obtained from carotenoid synthesis by *Rhodotorula glutinis* 22P grown in association with homofermentative lactic acid bacteria.

There are no reports on carotenoid synthesis by lactose-negative yeasts grown in association with species of lactose-positive yeasts. In this article, we report the results from a study on carotenogenesis of *R. rubra* GED5 cultivated in association with *Kluyveromyces lactis* MP11 in whey ultrafiltrate (WU).

Materials and Methods

Microorganisms

A carotenoid-synthesizing strain (*R. rubra* GED5) contaminating a commercially prepared yogurt was isolated and used. It was identified as *R. rubra* according to Kreger van Rij *(19)*. The culture was maintained by monthly transfers on 2% malt extract agar slants and stored at 4°C.

K. lactis MP11 strain was selected as an active producer of intracellular β-galactosidase from 15 cultures of yeasts. Our earlier investigation proved that *K. lactis* MP11 synthesized β-galactosidase intercellularly, but after h 12

from the moment of cultivation about 30% of β-galactosidase left the cell and hydrolyzed the carbon substrate (lactose) in the culture medium to glucose and galactose, which are entirely able to ensure yeast growth and carotenogenesis *(20)*. The yeast cultures were supplied by the collection of the Laboratory of Applied Microbiology at the Institute of Microbiology, Bulgarian Academy of Sciences. The strain was identified according to Kreger van Rij *(19)*. It was maintained by monthly transfers in a medium containing 40 g/L of lactose, 6.0 g/L of $(NH_4)_2HPO_4$, 0.5 g/L of $MnSO_4 \cdot 7H_2O$, 1.0 g/L of yeast extract, 12.0 g/L of agar, and stored at 4°C.

A microbial association of *R. rubra* GED5 + *K. lactis* MP11 was formed for the purpose of carotenoid synthesis.-

Composition of Medium and Inoculum

WU supplemented with 8.0 g/L of $(NH_4)_2SO_4$, 3.0 g/L of KH_2PO_4, 0.5 g/L of $MgSO_4 \cdot 7H_2O$, 0.5 g/L of $MnSO_4 \cdot 7H_2O$, and 3.0 g/L of yeast extract was used as fermentation medium. The pH was adjusted to 6.0 with lactic acid. The ultrafiltrate was prepared from a whey obtained from the manufacture of white brined cheese and deproteinized on a Lab 38 DDS on GR61PP membranes. WU was utilized in its native state (35 g/L of lactose) or brought to lactose concentration (50 and 70 g/L) using a DDS RO-SYSTEM LAB 20 with a CA995PP 540-0.16 membrane.

The inoculum of *R. rubra* GED5 was grown in 1000-mL Erlenmeyer flasks containing 100 mL of culture medium containing 20 g/L of malt extract at 29–30°C for 48 h on a rotary shaker at 220 rpm. The size of the inoculum for all fermentations was 5% (v/v), and its cell concentration was about 1.3 g of dry cells/L.

The *K. lactis* MP11 inoculum was grown by cultivation on a rotary shaker at 220 rpm in 1000-mL Erlenmeyer flasks containing 100 mL of culture medium with the following composition: 40 g/L of WU lactose, 6.0 g/L of $(NH_4)_2HPO_4$, 0.5 g/L of $MnSO_4 \cdot 7H_2O$, 1.0 g/L of yeast extract, pH 5.0, at 29°C for 24 h. The size of the inoculum for all fermentations was 3% (v/v), and its cell concentration was about 1.5 g of dry cells/L.

Flask mixed cultures were grown for 10 d on a rotary shaker at 220 rpm and 30°C in 1000-mL Erlenmeyer flasks containing 100 mL of culture medium. Fermentor mixed cultures were grown in a 15-L MBR AG (Zurich, Switzerland) fermentor at 30°C using a 7.5-L working volume, an airflow rate of 0.8 L/(L·min), and an agitation of 220 rpm for 8 d. The yeasts *R. rubra* GED5 and *K. lactis* MP11 were inoculated simultaneously. The pH of the fermentation system was not adjusted during the growth period.

Analytical Methods

Viable cell counts (expressed in colony-forming units/milliliter) of *R. rubra* GED5 and *K. lactis* MP11 in the mixed culture were taken on a dish medium containing 20 g/L of malt extract and 12 g/L of agar following a 5-d incubation at 29°C. Dry cell weight was determined at 105°C to a con-

stant weight. Lactose, glucose, and galactose were determined by enzymatic methods as described by Boehringer Mannheim *(21)*. β-Galactosidase was determined by a method described by Roger et al. *(22)*. Total protein content was calculated from the total nitrogen content (Nx6.25) and determined by the conventional method of Kjeldahl System 1028 *(23)*. Extraction of carotenoids from the cell and determination of total carotenoids (spectrophotometrically) and individual carotenoid pigments (by high-performance liquid chromatography) were done as described previously *(18)*.

Results and Discussion

For *R. rubra* GED5 grown in association with *K. lactis* MP11 in ultrafiltrate containing 35, 50, and 70 g/L of lactose and cultured in shake flasks, it was established that the maximal cell mass and carotenoid did not coincide for the three concentrations studied. Carotenoid contents in the yeasts reached the highest levels in the stationary phase of the yeast culture. No correlation was found among the cell mass formation, carotenoids, and lactose contents in the WU medium. Carotenogenesis was at its most active when cultivating in ultrafiltrate with 50 g/L of lactose. The maximum concentration of carotenoids, 4.4 mg/L of culture fluid or 249 µg/g of dry cells, was established on d 8. For the given lactose concentration, a maximum yield of cell mass of 17.7 g/L was obtained on d 6. Lower amounts of carotenoids and cell mass were synthesized when the lactose concentration in the ultrafiltrate was diminished to 35 g/L. The highest cell mass yield (23.8 g/L) was obtained for the highest studied lactose content in WU (70 g/L). At this lactose concentration in WU, carotenogenesis was prolonged by 24 h and a lower carotenoid-forming activity was recorded. The synthesized carotenoid concentration (143.0 µg/g of dry cells) was approx 1.5–2 times lower than at lactose concentrations of 35 and 50 g/L. Since there was a considerable increase in the yield of synthesized cell mass at a lactose concentration of 70 g/L, a higher carotenoid yield (3.4 mg/L of culture fluid) than the yield (3.1 mg/L of culture fluid) from WU containing 35 g of lactose/L was recorded, despite the observed lower carotenoid-forming activity. The curves of lactose assimilation reveal a steady decrease in substrate concentration for the three fermentation systems and incomplete assimilation of substrate at the highest concentration of the carbon carrier.

The lactose-negative strain *R. rubra* GED5 increased its carotenoid-synthesizing activity when grown in association with *K. lactis* MP11 in ultrafiltrate. The activity was 1.4 times higher than that of an *R. glutinis* 22P + *Lactobacillus helveticus* 12A mixed culture grown in ultrafiltrate, and twice as high as the activity of *R. rubra* GED5 monoculture grown in a synthetic medium with glucose, or *Rhodotorula lactosa* monoculture grown in whey as reported in the literature *(17,18)*.

The growth and synthesis of carotenoids in a fermentor batch mixed culture of *R. rubra* GED5 + *K. lactis* MP11 on ultrafiltrate containing 50 g of

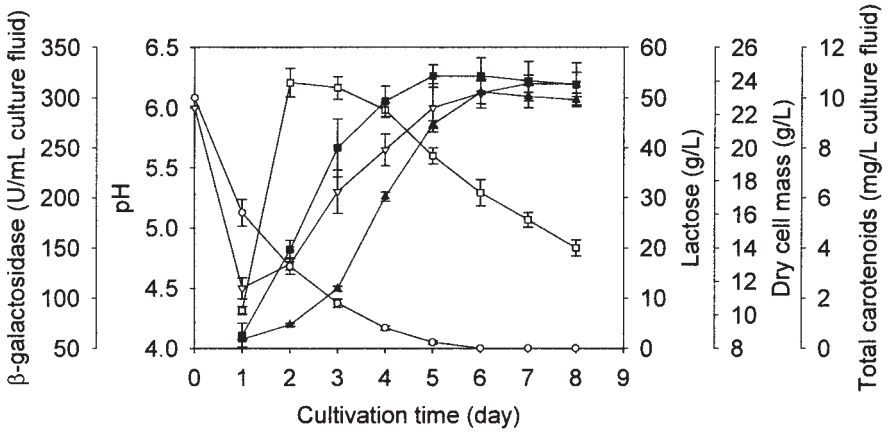

Fig. 1. Growth, production of carotenoids, and β-galactosidase activity of microbial association of *R. rubra* GED5 + *K.* lactis MP11 in WU with 50 g of lactose/L in fermentor batch culture: Dry cell mass (■); total carotenoids (▲); β-galactosidase (□); pH (▽); and lactose (○). Bars represent SD.

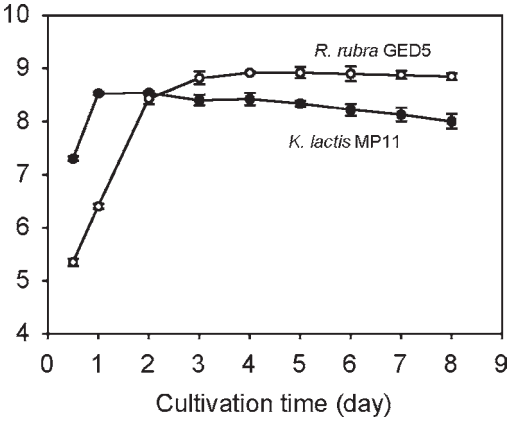

Fig. 2. Growth of mixed culture of *R. rubra* GED5 and *K. lactis* MP11 in WU with 50 g of lactose/L in fermentor. Bars represent SD.

lactose/L are presented in Figs. 1 and 2. Preliminary experiments on the influence of aeration intensity of the medium on culture growth and yeast carotenogenesis determined that the most suitable airflow rate for active carotenoid synthesis in the system was 0.8 L/(L·min) with an agitation speed of 220 rpm. The growth curves show that the lactose-negative yeast *R. rubra* GED5 rapidly entered a phase of exponential growth in a culture medium in which the carbon substrate was directly unassimilable. After d 2, actively budding *R. rubra* GED5 cells dominated up to d 3 that grew up to physiologic maturity by d 4. In aeration-stimulated growth, there was an active synthesis of cell mass with a maximum yield of 24.3 g/L. Carotenogenesis was shortened by 48 h, and carotenoid concentration was 2.3 times

higher (10.2 mg/L of culture fluid) in comparison with the shake-flask culture. The production of carotenoids followed the natural course of change in pH of the fermentation medium. The pH interval for active synthesis of β-galactosidase established agrees with the pH interval for intensive synthesis of carotenoids (pH 4.77–6.12).

In our previous studies, monoculture produced a maximum amount of β-galactosidase (1300 U/mL of culture fluid) up to h 22–24 when grown in conditions optimal for active enzyme production (poor aeration of the medium: 0.15 L/[L·min]) *(24)*. Monoculture of *K. lactis* MP11 grown in WU under intensive aeration (airflow rate of 0.8 L/[L·min], optimal for development of the microbial association) synthesized β-galactosidase to a lesser extent with a maximum concentration of 450 U/mL of culture fluid for 30 h. Under conditions of mixed cultivation of the two yeast species, β-galactosidase synthesis was prolonged up to d 4, with a maximum amount on d 2 to 3 (approx 320 U/mL of culture fluid). Through β-galactosidase hydrolysis of lactose, the lactose-negative carotenoid producer *R. rubra* GED5 is provided with easily assimilated glucose and galactose, which are indispensable for its growth and carotenoid formation. On the d 1, 50% of lactose was assimilated by the yeast cultures, and by the end of the process it was entirely used up. During the fermentation process, there was no glucose in the culture medium and no galactose after d 2. The absence of glucose in the process of mixed cultivation can be explained by its fast assimilation by both yeast species. The results from preliminary studies on the growth of *K. lactis* MP11 monoculture in conditions identical to those used for growing the microbial association showed that *K. lactis* MP11 actively assimilated lactose and by h 30 about 97% of lactose was utilized; the residual glucose and galactose concentrations in the culture medium were 0.4 g/L and 0.9 g/L at h 35. The mixed culture assimilated about 65 of the lactose in h 48. The slower assimilation of lactose by the mixed culture was probably owing to poorer hydrolytic activity of β-galactosidase, on the one hand, and the possible inhibition of the hydrolysis action of β-galactosidase by some compounds from the reciprocal metabolism of the two cultures, on the other. Notwithstanding the certain inhibition of the hydrolytic process, the β-galactosidase activity produced was quite sufficient for inducing active carotenogenesis by the mixed culture of *R. rubra* GED5 + *K. lactis* MP11.

The microbial association activity can be determined by the physiologic state of the microorganisms and the number of viable cells in the course of the process. The morphologic differences of the two yeast species made their differentiation easier in the course of the process. The shape of *K. lactis* MP11 cells is elliptical to oblong and their size is (2.0–6.6) × (3.0–8.0) μm, and *R. rubra* GED5 cells are oval and measure (4.5–10.0) μm. Until h 24, we observed intensive growth of *K. lactis* MP11 (young budding cells) and slow growth of *R. rubra* GED5 (single, nonbudding but well-shaped cells). After d 2, *R. rubra* GED5 dominated.

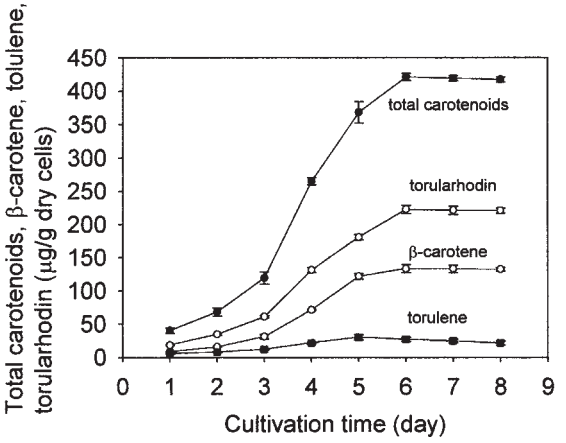

Fig. 3. Changes in individual concentration of the three major carotenoid pigments and total pigments as function of time in mixed culture of *R. rubra* GED5 and *K. lactis* MP11 in WU with 50 g of lactose/L in fermentor. Bars represent SD.

The major carotenoid pigments making up the total carotenoids synthesized by *R. rubra* GED5 in associated growth with *K. lactis* MP11 are β-carotene, torulene, and torularhodin. Figure 3 shows the formation kinetics of the three pigments. The time for reaching maximum concentration by total carotenoids (421 μg/g of dry cells on d 6) coincided with that for individual pigments: β-carotene (133 μg/g of dry cells) and torularhodin (222.3 μg/g of dry cells). The maximum concentration of torulene (26.9 μg/g of dry cells) was recorded 24 h earlier (on d 5). The identified individual pigments that form total carotenoids are typical of the species of the *Rhodotorula* genus reported by other investigators *(6,9,15,18)*. The present results revealed that the amount and ratio between separate pigments depend on the generic specificity of the producer strain *(18)*.

Table 1 compares the protein and carotenoid contents obtained for *R. rubra* GED5 in the present study with previous reports on *R. glutinis* 22P and *R. lactosa* cultivated in lactose substrates. The values obtained for *R. rubra* GED5 cocultivated with *K. lactis* MP11 are significantly higher than those for *R. lactosa* monoculture, which tends to confirm previous observations *(18)* that mixed cultivation in a natural lactose substrate (WU) has the effect of promoting microbial protein and carotenoid contents of lactose-negative yeasts. Of both microbial associations (*R. glutinis* 22P + *L. helveticus* 12A and *R. rubra* GED5 + *K. lactis* MP11) cultivated in ultrafiltrate, *R. rubra* GED5 synthesized a higher yield of carotenoids and a larger relative share of β-carotene, whose provitamin A activity has been well known and proven for a long time.

The results on the cell mass synthesized by growing the microbial association of *R. rubra* GED5 + *K. lactis* MP11 can be used in further studies as a basis for making carotenoprotein concentrates. These concentrates can find application not only as protein or coloring, but also as vitamin nutri-

Table 1
Comparison of Carotenoid and Protein Contents
of *R. rubra* GED5 With Those of Other Species of Yeasts Cultivated in Lactose Substrates

	Total carotenoids		Major carotenoid pigments (% of total carotenoids)			Protein	
Yeast	(mg/L culture fluid)	(μg/g dry cells)	β-Carotene	Torulene	Torularhodin	(mg/g dry wt)	Reference
R. lactosa[a]	2.3	86.7	19.1	11.3	69.9	338	*17*
R. glutinis 22P[b]	8.1	268.0	16.3	8.5	67.9	468	*18*
R. rubra GED5[c]	10.2	421.0	31.6	6.4	52.8	497	This work

[a]Monoculture of *R. lactosa* grown in whey.
[b]*R. glutinis* 22P cocultivated with *L. helveticus* 12A in WU.
[c]*R. rubra* GED5 cocultivated with *K. lactis* MP11 in WU.

tive supplements owing to the familiar function of carotenoid pigments—that of provitamin A.

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