Journal of Industrial Microbiology & Biotechnology (1997) 18, 272–277

Caroteno-protein and exopolysaccharide production by co-cultures of *Rhodotorula glutinis* and *Lactobacillus helveticus*

G Frengova, E Simova and D Beshkova

Laboratory of Applied Microbiology, Institute of Microbiology, Bulgarian Academy of Sciences, 4002 Plovdiv, 26 Maritza Blvd, Bulgaria

The lactose-negative yeast *Rhodotorula glutinis* 22P and the homofermentative lactic acid bacterium *Lactobacillus helveticus* 12A were cultured together in a cheese whey ultrafiltrate containing 42 g L^{-1} lactose. The chemical composition of the caroteno-protein has been determined. The carotenoid and protein contents are 248 μg g^{-1} dry cells and 48.2% dry weight. Carotenoids produced by *Rhodotorula glutinis* 22P have been identified as β-carotene 15%, torulene 10%, and torularhodin 69%. After separating the cell mass from the microbial association, the exopolysaccharides synthesized by *Rhodotorula glutinis* 22P were isolated from the supernatant medium in a yield of 9.2 g L^{-1}. The monosaccharide composition of the synthesized biopolymer was predominantly D-mannose (57.5%).

Keywords: *Rhodotorula glutinis; Lactobacillus helveticus*; cheese whey ultrafiltrate; microbial association; carotenoids; caroteno-protein; exopolysaccharides

Introduction

Yeasts in the genera *Rhodotorula* [5,21], *Rhodosporidium* [15] and *Phaffia* [2,11] synthesize carotenoid pigments. The main carotenoid pigments produced by *Rhodotorula* and *Rhodosporidium* are β-carotene, torulene, torularhodin [15,18], and astaxanthin is produced by *Phaffia* [2,11].

The pigments are in demand as a dietary supplement. When disrupted cells, without cell walls, are added to the diets of animals, astaxanthin is readily absorbed from the gut; it effectively colours the flesh of pen-reared salmonids [12], and also helps impart a desirable golden colour to the egg-yolk and flesh of poultry [13]. The yeast also contains a high level of unsaturated fat, protein and vitamins that contribute to good growth of animals [14]. These attributes enhance the potential utility of *Phaffia rhodozyma* as a source of astaxanthin in animal diets.

Yeasts can synthesize carotenoids either on synthetic monosaccharide- and disaccharide-containing substrates [5,18,21] or on natural substrates such as molasses [11]. Carotene-synthesizing yeasts which can assimilate lactose are rarely found in nature. There are few references to the use of lactose as a carbon substrate for biosynthesis of yeast carotenoids [22].

Besides being able to form carotenoid pigments intracellularly [5,21], the yeasts of the *Rhodotorula* genus also possess the ability to synthesize other bioactive substances extracellularly. Strains of *R. rubra* cultivated on synthetic substrates containing carbohydrates can synthesize exopolysaccharides [1,6]. Microbial polysaccharides have a protective role which is expressed in the enhanced specific immunobiological reactivity of the microorganism [7,10]. The bioactivity of microbial polysaccharides on the functional status of macrophages depends on the charge and polymeric properties of the molecule, the glycoside bonds and the macromolecular structure [10]. High molecular weight mannans from yeast also possess fibrinolytic properties [7]. It is these properties that make extracellular yeast-synthesized polysaccharides a target of scientific interest.

There are few reports on the production of exopolysaccharides and carotenoids by yeasts growing on lactose substrates [19,22]. No reports are available on mixed cultivation of yeast strains and lactic acid bacteria on lactose substrates for simultaneous synthesis of carotenoids and exopolysaccharides. Cultivated as monocultures, the constituent microorganisms have limited metabolic ability to synthesize carotenoid pigments and exopolysaccharides.

The present study introduces the production of caroteno-protein and exopolysaccharides from *Rhodotorula glutinis* 22P, cultivated in a natural lactose substrate (cheese whey ultrafiltrate). *R. glutinis* 22P does not assimilate lactose but readily assimilates glucose, galactose and lactic acid. The new approach in the study involves creating proper conditions for lactose assimilation by *R. glutinis* yeast by growing it together with the homofermentative lactic acid bacterium *Lactobacillus helveticus* 12A.

Materials and methods

Microorganisms

R. glutinis 22P was selected according to its ability to synthesize carotenoids in a medium containing: glucose, 40.0 g L^{-1}; KH_2PO_4, 8.0 g L^{-1}; $MgSO_4 \cdot 7H_2O$, 0.5 g L^{-1} and yeast extract, 3.0 g L^{-1}. The strain was identified using the Kreger van Rij determiner. The culture was maintained by monthly transfers on 2% malt extract agar slants and stored at 4°C.

Lactobacillus helveticus 12A was selected after studying the carotenoid-forming activity of the producer, *R. glutinis* 22P, grown in association with the lactic acid bacteria—*Lactobacillus bulgaricus, L. helveticus, L. casei* and *L. acidophilus* [9]. *L helveticus* 12A was identified using the determiner of Rogosa and Sharpe, and Bergey. The culture

Correspondence: Dr ED Simova, 4002 Plovdiv, 26 Maritza Blvd, Bulgaria
Received 8 July 1996; accepted 11 December 1996

was maintained in 9.0 ml of sterile skim milk and MRS broth by transferring a loopful of inoculum every week, and stored at 4°C. A mixed culture of *R. glutinis* 22P and *L. helveticus* 12A was formed for carotenoid production [9].

Medium composition and inoculum

The fermentation medium contained: whey ultrafiltrate (WU) containing lactose, 42.0 g L^{-1}; $(NH_4)_2SO_4$, 8.0 g L^{-1}; KH_2PO_4, 3.0 g L^{-1}; $MgSO_4 \cdot 7H_2O$, 0.5 g L^{-1} and yeast extract, 3.0 g L^{-1}. The pH was adjusted to 6.0 with lactic acid. The ultrafiltrate was obtained from a whey by-product from the manufacture of white brined cheese and deproteinized on a LAB 38 DDS, on GR61PP membranes. WU was utilized in native state (lactose, 42.0 g L^{-1}).

The inoculum of *R. glutinis* 22P was grown in 1000-ml Erlenmeyer flasks containing 100 ml of the culture medium with 2% malt extract, at 29–30°C, and incubated for 48 h on a rotary shaker operated at 220 rpm. The inoculum size for all fermentations was 6% (v/v) and its cell concentration was about 1.2 g dry cells L^{-1}.

The inoculum of *L. helveticus* 12A was grown in skim milk, at 37°C for 24 h. It was introduced into the fermentation medium at 1% (v/v, $4–6 \times 10^7$ cells ml^{-1}).

The mixed cultivation of *R. glutinis* 22P and *L. helveticus* 12A was performed in a bioreactor MBR AG (Zurich, Switzerland) at parameters for culture growth and yeast carotenogenesis established by us in a previous study: incubation temperature 30°C; initial pH 6.0; air flow rate 0.5 L L^{-1} min^{-1}; stirred at 220 rpm for 7 days [9]. The pH of the fermentation system was not adjusted during the growth period. The synthesis of exopolysaccharides by the carotene-producing yeast strain was studied in parallel to the carotenogenesis, under the above cultivation conditions for the microbial association of *R. glutinis* 22P and *L. helveticus* 12A.

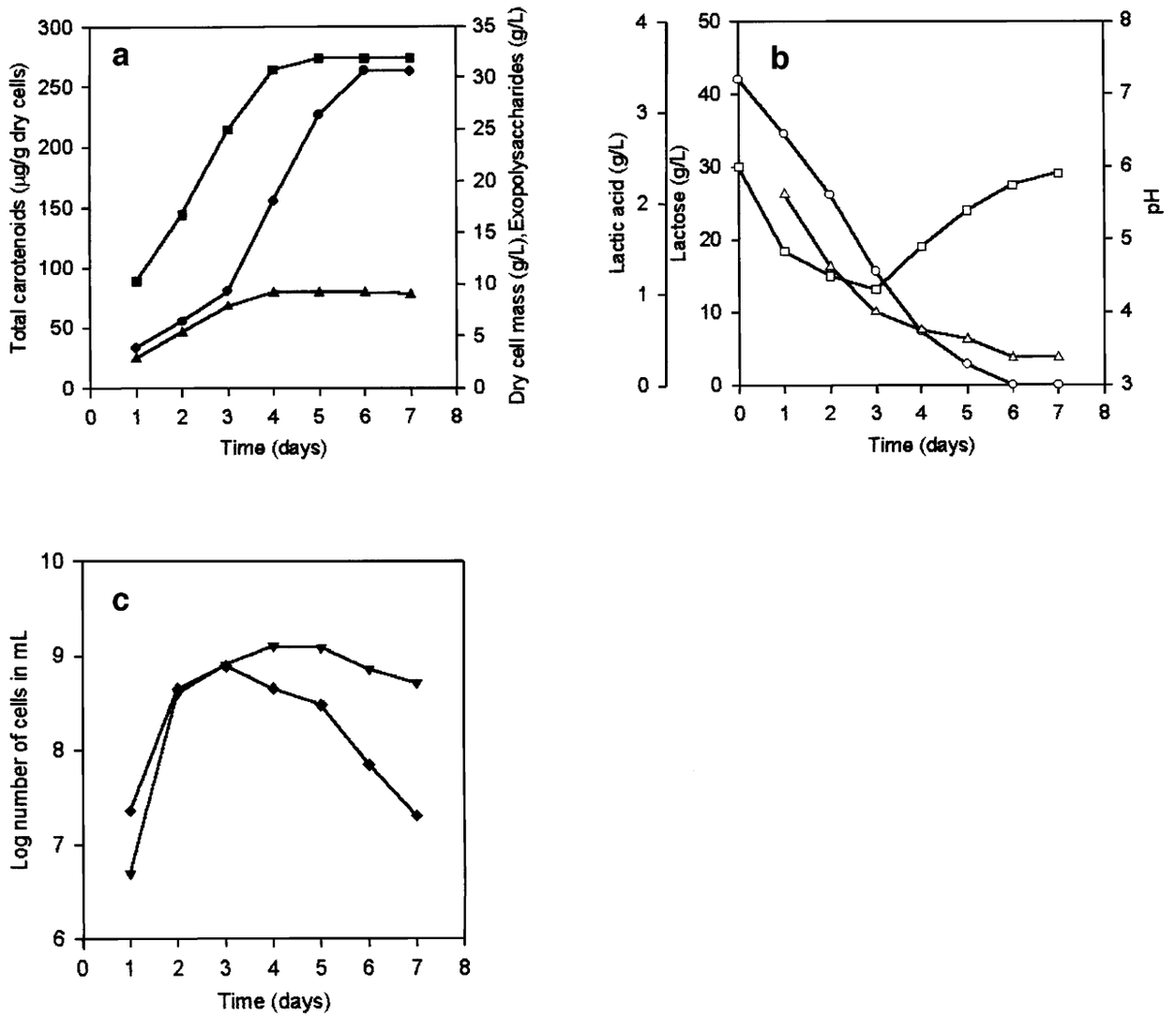

Figure 1 Time-course of growth, carotenoid formation and exopolysaccharide production in a mixed culture *R. glutinis* 22P and *L. helveticus* 12A in a cheese WU at 30°C, initial pH 6.0, air flow rate 0.5 L L^{-1} min^{-1}, agitation 220 rpm: (a) total carotenoids (■), cell dry weight (●), exopolysaccharides (▲); (b) lactose (○), lactic acid (△), pH (□); (c) *R. glutinis* 22P (▼), *L. helveticus* 12A (◆).

Table 1 Carbohydrate composition of exopolysaccharides synthesized by *Rhodotorula glutinis* 22P

Carbohydrate (% dry weight)	D-Monosaccharides (% carbohydrate)				
	Mannose	Glucose	Galactose	Xylose	Fucose
67.5	57.5	18.0	21.8	2.9	–

Analytical methods

In the mixed culture, viable counts of *R. glutinis* 22P cells were estimated on plates containing 2% malt extract and 1.2% agar after a 5-day incubation at 29°C. Viable counts of *L. helveticus* 12A were estimated on plates of a medium consisting of pancreatin-hydrolized milk (pancreatin 0.1%), agar 1.2% and china blue 0.375 g L^{-1}, after 6 days incubation at 37°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 [4].

Extraction of carotenoids from the cell, determination of total carotenoids (spectrophotometrically) and individual carotenoid pigments (by HPLC) were described earlier [9].

The exopolysaccharide content in the supernatant medium was measured after precipitating the biopolymer with acetone according to the method of Adami and Cavazoni [1].

The carbohydrate composition was determined by gas-chromatography using Fractovap 2407 (Carlo Erba, Milan, Italy). Chromatography conditions: flame ionization detector temperature 350°C, 4 × 2000-mm steel column, 2% SE-54 W80/100 mesh silanised chromosorb, carrier gas N_2 at 35 cc min^{-1}, programmed temperature 160°C increased to 300°C by 4°C steps, injector temperature 350°C, Autolab 6300-02 injector and chart speed 10 mm min^{-1}.

The cell mass formed by the co-cultivation of *R. glutinis* 22P and *L. helveticus* 12A was separated by microfiltration on a GRM 20PP membrane, disintegrated on a Gaulin homogenizer (10% microbial suspension, P = 50 MPa, v = 23.3 L h^{-1}, T = 8–10°C), spray-dried (inlet T 185°C, outlet T 90°C) and characterized for proteins, lipids, carotenoids, vitamins, minerals and amino acids.

Total protein content was calculated from the total nitrogen content (N × 6.25) and determined by the conventional method of Kjeldahl System 1028 [17]. Total lipids in the cell mass were determined by Soxhlet's method after ethyl-ether extraction of the lipids from the test material [17]. Vitamin B_1 and B_6 contents in the cell mass were determined microbiologically using an auxoheterotrophic yeast *Debaryomyces disporous* [16]. Vitamin B_2 was measured by the lumiflavine method by extracting the vitamin from the test material with 2 N hydrochloric acid followed by chloroform extraction [20].

The minerals P, K, Na, Ca and Mg in the cell mass were estimated by the conventional methods (P colorimetrically [17]; K and Na flame photometrically [17]; Ca and Mg by EDTA titration methods [3]). The amino acid concentration was measured on a H-1200 E aminoanalyser (Prague, Czech Republic) after hydrolyzing the protein with 6 N hydrochloric acid [17].

Table 2 Chemical composition of a caroteno-protein product

Component	Content
Total carotenoids (μg g^{-1} dry cell mass)	248.00
Carotenoid pigments (μg g^{-1} dry cell mass):	
β-carotene	37.20
Torulene	25.50
Torularhodin	171.10
Protein (% dry weight)	48.20
Lipids (% dry weight)	10.30
Minerals (% dry weight):	
P	2.60
K	1.70
Mg	0.30
Ca	0.50
Na	0.20
Vitamins of the B-complex (μg g^{-1} dry weight):	
Vitamin B_1	18.00
Vitamin B_2	26.30
Vitamin B_6	29.00
Moisture (%)	5.00

Results

By growing *R. glutinis* 22P and *L. helveticus* 12A under the conditions described, it was established that the synthesis maxima for cell mass, exopolysaccharides and carotenoid pigments do not coincide (Figure 1a). In this fermentation system, synthesis of exopolysaccharides correlated with synthesis of cell mass, and their maximum yields on day 4 were 8.2 g L^{-1} and 30.9 g L^{-1}, respectively. The maximum

Table 3 Amino acid composition of a caroteno-protein product

Amino acid	Content (% dry weight)
Lysine	3.67
Histidine	1.00
Arginine	3.35
Asparagine	4.66
Threonine	2.15
Serine	2.35
Glutamic acid	9.25
Proline	2.02
Glycine	2.30
Alanine	3.21
Valine	2.27
Methionine	0.47
Isoleucine	1.25
Leucine	3.21
Tyrosine	1.09
Phenylalanine	1.70

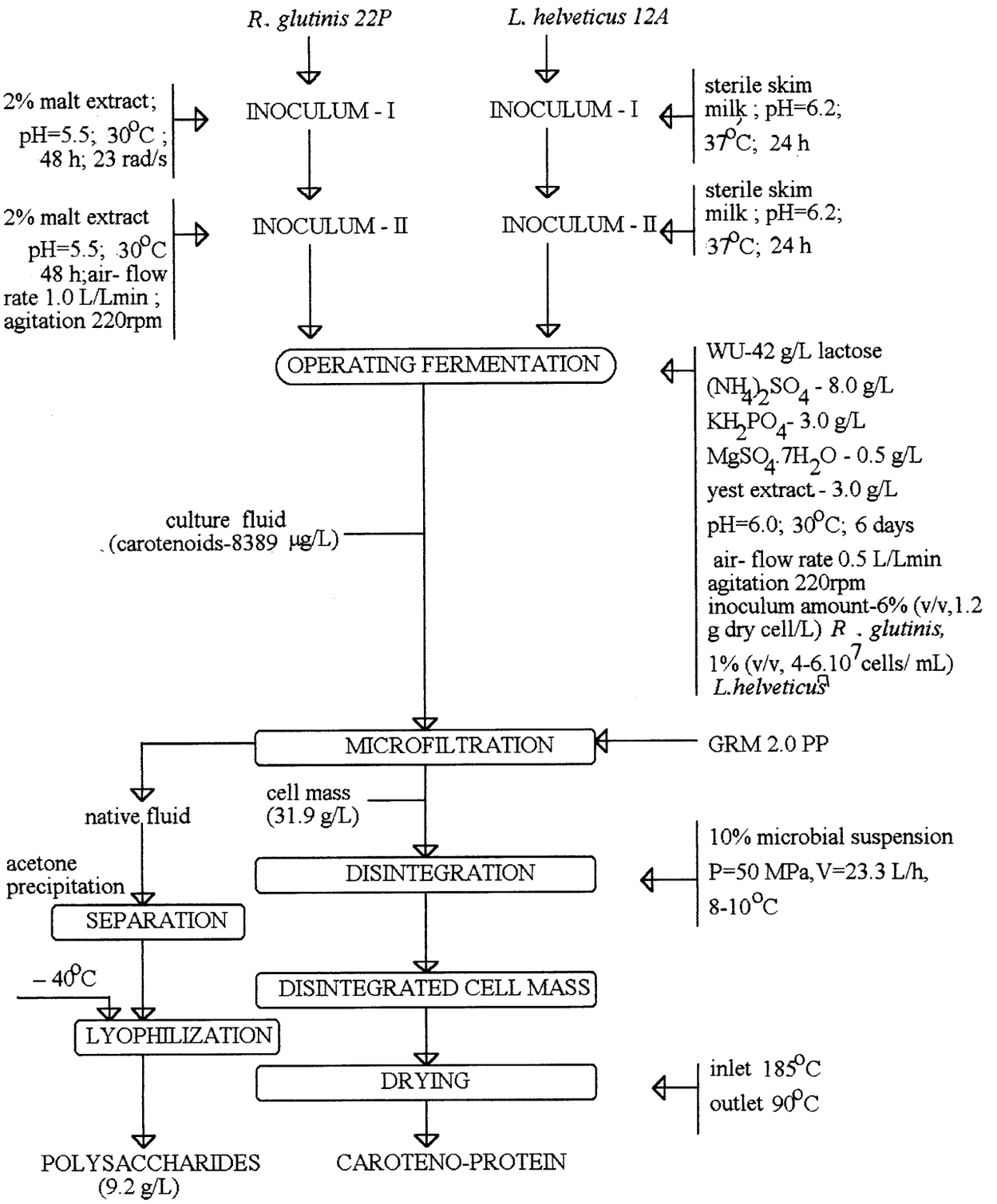

Figure 2 Proposed scheme for producing caroteno-protein and exopolysaccharides by *R. glutinis* 22P grown in association with *L. helveticus* 12A in cheese WU.

carotenoid concentration in the cells (263 μg g^{-1} dry cells) was reached on day 6 when growth had been completed, ie in the stationary phase of the growth cycle of the yeast.

The biosynthesis of carotenoids and exopolysaccharides followed the natural pH change of the fermentation medium (Figure 1b). The dynamics of pH change show a decrease by day 3, the time when the yeast actively synthesized exopolysaccharides. From day 3 to day 7, pH of the

medium increased, coinciding with carotenogenesis. Lactose from the medium (42 g L^{-1}) had been assimilated by the mixed culture by day 6 (Figure 1b).

The homofermentative lactic acid bacteria actively transformed lactose into glucose, galactose and lactic acid. The analysis of glucose and galactose in the process revealed traces of galactose and absence of glucose. Lactic acid concentrations were of the order of 2.1–0.3 g L^{-1} (Figure 1b). Both the monosaccharides, which are easily assimilated, and the lactic acid acted as substrates for the yeast's development, so that the latter quickly entered an exponential growth phase in a medium in which the carbon substrate lactose was not assimilated (Figure 1c). By day 3, viable counts of lactic acid bacteria were found in the culture medium. The yeast stayed in a phase of active growth by day 5, during which period the main amount of carotenoids accumulated in the cells, and maximum amount of exopolysaccharides was produced (Figure 1a,c).

Gas-chromatographic studies of the monosaccharide composition of the exopolysaccharides synthesized by the carotenoid-forming yeasts showed that D-mannose, D-glucose, D-galactose and D-xylose are structural elements of the polysaccharide macromolecule (Table 1). Of the monosaccharides detected, only the amount of D-xylose was insignificant (2.9%). D-Mannose dominated in the biopolymer (57.5%), accounting for the biological activity of the exopolysaccharides produced by *R. glutinis* 22P. According to some authors, a mannose content of more than 50% in the polysaccharide macromolecule is a premise for bioactivity [7].

The disintegrated and dried cell mass synthesized by the co-culture was named 'caroteno-protein', which contained 248 μg of total carotenoids per g dry cell mass. Torularhodin was the major carotenoid, with lesser amounts of β-carotene and torulene (Table 2). *In vitro* tests with β-carotene-15-15′ dioxygenase established that torulene and torularhodin synthesized by *Phaffia rhodozyma* possess provitamin-A activity like β-carotene, which is a vitamin A precursor [8]. Thus, the microbial biomass received from the mixed cultivation of *R. glutinis* 22P and *L. helveticus* 12A can be used as a vitamin, protein or coloring additive. Alongside the carotenoids localized in the yeast cell, cytoplasmic components of high biological value (vitamins, minerals, lipids, proteins) are also being released during disintegration (Table 2). Sixteen amino acids were identified and their concentrations were determined in the amino acid composition of the caroteno-protein (Table 3). As illustrated in Tables 2 and 3, the dried disintegrated cell mass yielded caroteno-protein of rich chemical composition, which presupposes high nutritional value and easy assimilation of the product.

Discussion

During the mixed cultivation of *R. glutinis* 22P and *L. helveticus* 12A, the homofermentative lactic acid bacteria actively transform lactose into glucose, galactose and lactic acid. Consequently, there is a vigorous yeast growth and active formation of carotenoids by *R. glutinis* 22P in which lactose cannot undergo direct assimilation. On the other hand, it is assumed that vitamins and amino acids produced by the yeast stimulate the lactic acid bacteria throughout the period of carotenoid formation.

Study of the biosynthesis of exopolysaccharides concomitantly with carotenogenesis by the yeast strain grown in association with *L. helveticus* 12A revealed extra metabolic activities in the lactose-negative yeast. Milk whey has also been used as a substrate for synthesizing exopolysaccharides [19]. Whey lactose is hydrolyzed to glucose and galactose with the commercially produced enzyme preparation β-galactosidase before cultivation of monocultures from *Hansenulla holstii* and *Cryptococcus laurentii*. The maximum concentrations of biopolymers were reached after a 5-day incubation and were 9.4 g L^{-1} and 5.7 g L^{-1}, respectively.

Mixed cultivation of the lactose-negative yeast *R. glutinis* 22P with the homofermentative lactic acid bacterium *L. helveticus* 12A produced caroteno-protein with significantly greater content of carotenoids, proteins, vitamins, minerals and amino acids than that of the yeast biomass synthesized by *Rhodotorula lactosa* [22]. The rich chemical composition of the caroteno-protein determines the trends in its future application. The final complex product contains carotenoids, proteins, lipids, vitamins, minerals and amino acids, and, therefore, can be used as a nutritious ingredient. Since carotenoids are vitamin A precursors, they can have a positive effect on the growth and productivity of animals, poultry and fish when included in their diet; supplying them to poultry diet could result in intensive pigmenting of the egg yolk. In the food industry, the caroteno-protein can be added to bread dough to enhance the protein and vitamin value of various bakery products.

Based on the results from the mixed cultivation of the lactose-negative yeast *R. glutinis* 22P with the homofermentative lactic acid bacteria *L. helveticus* 12A in a cheese whey ultrafiltrate, we propose a scheme for production of caroteno-protein and exopolysaccharides (Figure 2).

References

1 Adami A and V Cavazzoni. 1990. Exopolysaccharides produced by some yeast strains. Ann Microbiol 40: 245–253.
2 An G, D Schuman and E Johnson. 1989. Isolation of *Phaffia rhodozyma* with increased astaxanthin content. Appl Environ Microbiol 55: 116–124.
3 Anonymous. 1975. Elementary analysis. In: Official Methods of Analysis (Horwitz W, ed), pp 5–6, Association of Official Analytical Chemists, Washington.
4 Anonymous. 1983. Lactose/galactose, lactic acid. In: Methods of Enzymatic Food Analysis Using Test Combinations, pp 33–35, Boehringer Mannheim GmbH Biochemica, Mannheim, Germany.
5 Costa I, H Martelli, I Da Silva and D Pomeroy. 1987. Production of β-carotene by a *Rhodotorula* strain. Biotechnol Lett 9: 373–375.
6 Elinov N, G Vitovskaya, V Marikin and A Kozlov. 1979. Mannan produced by *Rhodotorula rubra* strain 14. Carbohydr Res 75: 185–190.
7 Elinov N, E Ananyeva, G Vitovskaya, M Chlenov, O Trushina, S Gurina and A Karavaeva. 1988. Investigation of fractions and fibrinolytic activity of yeast mannan. Antibiot Hymioter 33: 359–362.
8 Ershov Y, A Dmitrovsky, O Polulyakh, O Podoprigova and V Bykhovky. 1992. Enzymatic conversion of torulene and torularhodin to retinal. Prikl Biochim Microbiol 28: 680–684.
9 Frengova G, E Simova, K Pavlova, D Beshkova and D Grigorova. 1994. Formation of carotenoids by *Rhodotorula glutinis* in whey ultrafiltrate. Biotechnol Bioeng 44: 888–894.
10 Gurina S, N Elinov, M Kashkina and E Ananyeva. 1988. Effect of yeast polysaccharides on functional activity of albino mice macrophages. Antibiot Hymioter 33: 842–845.

11 Haard N. 1988. Astaxanthin formation by the yeast *Phaffia rhodozyma* on molasses. Biotechnol Lett 10: 609–614.
12 Johnson E, D Conklin and M Lewis. 1977. The yeast *Phaffia rhodozyma* as a dietary pigment source for salmonids and crustaceans. J Fish Res Board Canada 34: 2417–2421.
13 Johnson E, M Lewis and C Grau. 1980. Pigmentation of egg-yolks with astaxanthin from the yeast *Phaffia rhodozyma*. Poultry Sci 59: 1777–1782.
14 Johnson E, T Villa and M Lewis. 1980. *Phaffia rhodozyma* as an astaxanthin source in salmonid diets. Aquaculture 20: 123–134.
15 Kvasnicov E, T Grinberg, V Kaskivnjuk, S Nagorvaja, V Sudenko and I Stelokova. 1978. Yeasts synthesizing carotenoids. Izv ANSSR Seria Biologicheskaja 4: 565–575.
16 Nachev L. 1963. Using yeasts for determination of some group B vitamins. Izv Microbiol Ins BAN 15: 43–48.
17 Osborne D and P Voogh. 1987. Methods for the analysis of nutrients in food. In: The Analysis of Nutrients in Foods (Stewart G *et al*, eds), pp 113–116, 155–156, 171–173, Academic Press, London, New York, San Francisco.
18 Simpson K, T Nakayama and G Chichester. 1964. Biosynthesis of yeast carotenoids. J Bacteriol 88: 1688–1694.
19 Stauffer K and Y Leeder. 1978. Extracellular microbial polysaccharide production by fermentation on whey or hydrolyzed whey. J Food Sci 43: 756–758.
20 Strohecker R and H Henning. 1965. Lumiflavine method. In: Vitamin Assay Tested Methods, pp 113–115, Verlag chemie, GMBH, Weinheim/Bergstr.
21 Tada M and M Shiroishi. 1982. Mechanism of photoregulated carotenogenesis in *Rhodotorula minuta* I. Photocontrol of carotenoid production. Plant Cell Physiol 23: 541–547.
22 Zalashko M. 1990. Processing of milk whey based on lactose oxidation. In: Biotechnology of Milk Whey Processing (Sokolova E, ed), pp 161–163, Science Press, Moscow.