β-Carotene-Rich Carotenoid-Protein Preparation and Exopolysaccharide Production by *Rhodotorula rubra* GED8 Grown with a Yogurt Starter Culture

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The underlying method for obtaining a β-carotene-rich carotenoid-protein preparation and exopolysaccharides is the associated cultivation of the carotenoid-synthesizing lactose-negative yeast strain *Rhodotorula rubra* GED8 with the yogurt starter culture (*Lactobacillus bulgaricus* 2-11 + *Streptococcus thermophilus* 15HA) in whey ultrafiltrate (45 g lactose/l) with a maximum carotenoid yield of 13.37 mg/l culture fluid on the 4.5th day. The chemical composition of the carotenoid-protein preparation has been identified. The respective carotenoid and protein content is 497.4 μg/g dry cells and 50.3% per dry weight, respectively. An important characteristic of the carotenoid composition is the high percentage (51.1%) of β-carotene (a carotenoid pigment with the highest provitamin A activity) as compared to 12.9% and 33.7%, respectively, for the other two individual pigments – torulene and torularhodin. Exopolysaccharides (12.8 g/l) synthesized by the yeast and lactic acid cultures, identified as acid biopolymers containing 7.2% glucuronic acid, were isolated in the cell-free supernatant. Mannose, produced exclusively by the yeast, predominated in the neutral carbohydrate biopolymer component (76%). The mixed cultivation of *R. rubra* GED8 with the yogurt starter (*L. bulgaricus* 2-11 + *S. thermophilus* 15HA) in ultrafiltrate under conditions of intracellular production of maximum amount of carotenoids and exopolysaccharides synthesis enables combined utilization of the culture fluid from the fermentation process.

Key words: Carotenoid-Protein Preparation, Exopolysaccharides, Microbial Association

Introduction

Carotenoids are naturally occurring pigments widely distributed among bacteria, yeasts, filamentous fungi, algae, and plants. Because of their biological role as vitamin A precursors in humans and animals (Olson, 1989; Ershov *et al*., 1992; Johnson and Schroeder, 1996) and owing to their antioxidant properties and suspected activity in preventing some forms of cancer as well (Edge *et al*., 1997; Hennekens, 1997; Onogi *et al*., 1998; Nishino *et al*., 1999), carotenoid pigments represent a group of most valuable molecules for industrial applications. The pharmaceutical, chemical, feed and food industries have shown increased interest in the use of carotenoids, mainly as provitamin A but also as natural food and feed colorants (De Haan *et al*., 1991; Nelis and De Leenheer, 1991; Vandamme, 1992; Johnson and Schroeder, 1996). Compared with the extraction from vegetables (Coulson, 1980) or chemical synhesis (Counsell, 1980), 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 (De Haan *et al*., 1991), and because of the economic advantages of microbial processes using natural low-cost substrates as carbohydrate sources. Accordingly, the red yeast *Phaffia rhodozyma* is currently used for the production of astaxanthin, an important carotenoid pigment that is exploited in aquaculture to give an appealing pink colour to the flesh of farmed salmonid fish (Nelis and De Leenheer, 1991; Tangeras and Slinde, 1994; Nakano *et al*., 1995; Johnson and Schroeder, 1996), and also helps to impart a desirable golden colour to the egg-yolk and flesh of poultry (Akiba *et al*., 2000). Bhosale *et al*. (2002) have proved the anticarcinogenic effect of the spray-dried carotenoid-synthesizing yeast *Rhodotorula glutinis* in mice tests.

Carotenoid biosynthesis is a specific feature of species of *Rhodotorula* (Martin *et al*., 1993; Perrier *et al*., 1995; Buzzini and Martini, 1999; Bhosale and Gadre, 2001) and *Phaffia* (Meyer and Du Preez, 1994; An *et al*., 2001; Tian *et al*., 2003; Zhu and Liang, 2003).

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Besides being able to form carotenoid pigments intracellularly, the yeasts of the *Rhodotorula* genus also possess the ability to synthesize other bioactive substances extracellularly. Strains of *R. rubra* and *R. glutinis* cultivated on synthetic substrates containing carbohydrates can synthesize exopolysaccharides (Adami and Cavazzoni, 1990; Cho *et al.*, 2001). The microbial exopolysaccharides have generated increasing attention among researchers for the last few years. When added to food products, polysaccharides function as thickeners, stabilizers, emulsifiers, gelling agents and water-binding agents (Crescenzi, 1995). Exopolysaccharides are synthesized by microorganisms of different taxonomy (bacteria, fungi, yeast). Glucose is a primary carbon substrate in synthetic nutrient media for exopolysaccharide synthesis.

There is a growing interest in using natural substrates of agro-industrial origin that are viewed not only as a low-cost alternative carbohydrate source for the production of microbial metabolites, but also in terms of minimizing environmental and energy 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. There are few reports on the production of exopolysaccharides and carotenoids by yeasts growing on lactose substrates (Staufer and Leeder, 1978; Igoshi *et al.*, 1990; Zalashko, 1990). Carotenoid-synthesizing yeasts which can assimilate lactose are rarely found in nature (Zalashko, 1990). The yeast *Rhodotorula rubra* GED8 used in this study did not assimilate lactose. Substrates containing lactose used for high-efficiency synthesis of carotenoids and exopolysaccharides by lactose-negative yeast are of certain interest. The synthesis can occur in associated cultivation of the yeast strain producers and lactose-transforming microorganisms (Frengova *et al.*, 1994; Simova *et al.*, 2000). There are no other reports available on the associated growth of yeasts with microorganisms of other species in lactose substrates for synthesizing carotenoids and exopolysaccharides simultaneously.

The present work reports on the production of a β-carotene-rich carotenoid-protein preparation and exopolysaccharides by *Rhodotorula rubra* GED8, co-cultivated with a yogurt starter culture (*Lactobacillus bulgaricus* 2-11 + *Streptococcus thermophilus* 15HA) in natural lactose substrate – cheese whey ultrafiltrate (WU).

Materials and Methods

Microorganisms and cultivation conditions

Two cultures, a yeast (*R. rubra* GED8) and a yogurt starter (*L. bulgaricus* 2-11 + *S. thermophilus* 15HA), were selected for associated growth in WU and active synthesis of carotenoids (Simova *et al.*, 2004a). The carotenoid-synthesizing strain *R. rubra* GED8 was maintained by monthly transfers on 2% malt extract agar slants and stored at 4 °C. 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 inoculum for yeast culture was 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 the fermentation medium was 5% (v/v) and the concentration was 1.2–1.4 g dry cells/l. 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 starter yogurt culture for the fermentation medium was 1% (v/v). Inocula of yeast and yogurt starter culture were introduced simultaneously into the fermentation medium.

WU supplemented with (g/l): $(NH_4)_2SO_4$ (6.0); KH_2PO_4 (5.5); Na_2HPO_4 (3.0); $MgSO_4 \cdot 7H_2O$ (0.5), and yeast extract (5.0) was used as fermentation medium. The pH value was adjusted to 6.0 with lactic acid. WU was utilized in its native state (45.0 g lactose/l). The ultrafiltrate was obtained from a whey byproduct (Milk Industry, Plovdiv, Bulgaria) from the manufacture of white brined cheese and deproteinized on a Lab38DDS, on GR61PP membranes (Nakskov, Denmark).

The mixed cultivation of *R. rubra* GED8 and yogurt starter culture (*L. bulgaricus* 2-11 + *S. thermophilus* 15HA) was performed in a 15-l MBR AG fermentor (Zurich, Switzerland) with a working volume of 7.5 l at parameters for culture

growth and yeast carotenogenesis established by us in a previous study: incubation temperature 30 °C; initial pH 5.5; air-flow rate 1.3 l/min; agitation 220 rpm for 5.5 d (Simova *et al*., 2004a). The pH value of the fermentation system was not adjusted during the growth period. The synthesis of exopolysaccharides by the carotenoid-producing yeast strain was studied in parallel to the carotenogenesis under the above cultivation conditions for the microbial association of *R. rubra* GED8 and (*L. bulgaricus* 2-11 + *S. thermophilus* 15HA).

Analytical methods

In the mixed culture, viable counts [colony forming units (cfu)/ml] 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) were determined from the colony counts on *Streptococcus* selective agar for *S. thermophilus* 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 (1983). Extraction of carotenoids from cells and the determination of total carotenoids (spectrophotometrically) and individual carotenoid pigments (by HPLC) were described earlier (Frengova *et al*., 1994). The exopolysaccharide content in the supernatant medium was measured after precipitating the biopolymer with acetone according to the method of Adami and Cavazzoni (1990). The carbohydrate composition in the polysaccharides was determined after hydrolysis with 2 N H_2SO_4 for 8.0 h at 105 °C. Determination of neutral sugars in the hydrolysate (by gas chromatography) was described earlier (Simova *et al*., 2000). Determination of glucuronic acid in the hydrolysate (by HPLC) was described earlier (Simova *et al*., 2004b).

The cell mass formed by the co-cultivation of *R. rubra* GED8 and (*L. bulgaricus* 2-11 + *S. thermophilus* 15HA) was separated by microfiltration on a GRM2.0PP membrane (DDS, Nakskov), desintegrated on a Gaulin homogenizer (Hilversum, Holland) (10% microbial suspension, $P = 50$ MPa, $v = 23.3$ l/h, $t = 8–10$ °C), spray-dried (intel $t =$ 185 °C, outlet $t = 90$ °C) and characterized for proteins, lipids, carotenoids, vitamins, minerals and amino acids. The total content of proteins, lipids, vitamins (B_1, B_2, B_6), minerals (P, K, Na, Ca, Mg) and the amino acid concentration in the carotenoid-protein preparation were determined by methods described earlier (Frengova *et al*., 1997).

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

Results and Discussion

The data about the growth dynamics of the microbial association *R. rubra* GED8 + (*L. bulgaricus* 2-11 + *S. thermophilus* 15HA) cultivated in WU (45 g lactose/l) at 30 °C, air flow rate 1.3 l/min, agitation 220 rpm, show that the maximums for synthesis of carotenoids, exopolysaccharides and cell mass do not coincide in time (Fig. 1A). The carotenoid content in the cells reached a maximum of 510.6 μg/g dry cells on the 4.5th day after the cultures had stopped growing, *i. e.* in the stationary phase of the growth cycle of the yeast. A correlation between exopolysaccharide production and cell mass synthesis was established in the fermentation system, with maximum yields registered on the 4th day – 12.8 g/l and 26.2 g/l, respectively. Both biosynthetic processes – carotenogenesis and exopolysaccharide production – followed the natural pH change in the fermentation medium (Fig. 1A). The yogurt bacteria actively transformed lactose to monosaccharides and lactic acid during their associated cultivation with the yeast strain producer. Lactose was fully assimilated by the 4.5th day. The two monosaccharides and lactic acid were easily assimilated by *R. rubra* GED8 thereby allowing the yeast culture to grow exponentially in a medium in which the carbon substrate lactose was not assimilated (Fig. 1B).

The main carotenoid pigments constituting the total carotenoids synthesized by *R. rubra* GED8, co-cultivated with the yogurt starter, were β-carotene, torulene and torularhodin in the following contents: 216.4, 67.9 and 171.1 μg/g dry cells. These results were taken on the 4.5th day after the time of cultivation of the microbial association, *i.e.* at the time of a maximum amount of synthesized carotenoids – 510.6 μg/g dry cells – with a maximum carotenoid yield of 13.37 mg/l culture fluid. The carotenoid yield is significantly higher than the yields obtained by the lactose-positive yeast *R. lactosa* cultivated in whey and by the lactose-negative yeast *R. glutinis* 22P cultivated in association with the homofermentative lactobacillus *L. helveticus* 12A in WU with respective levels of 2.32 mg total

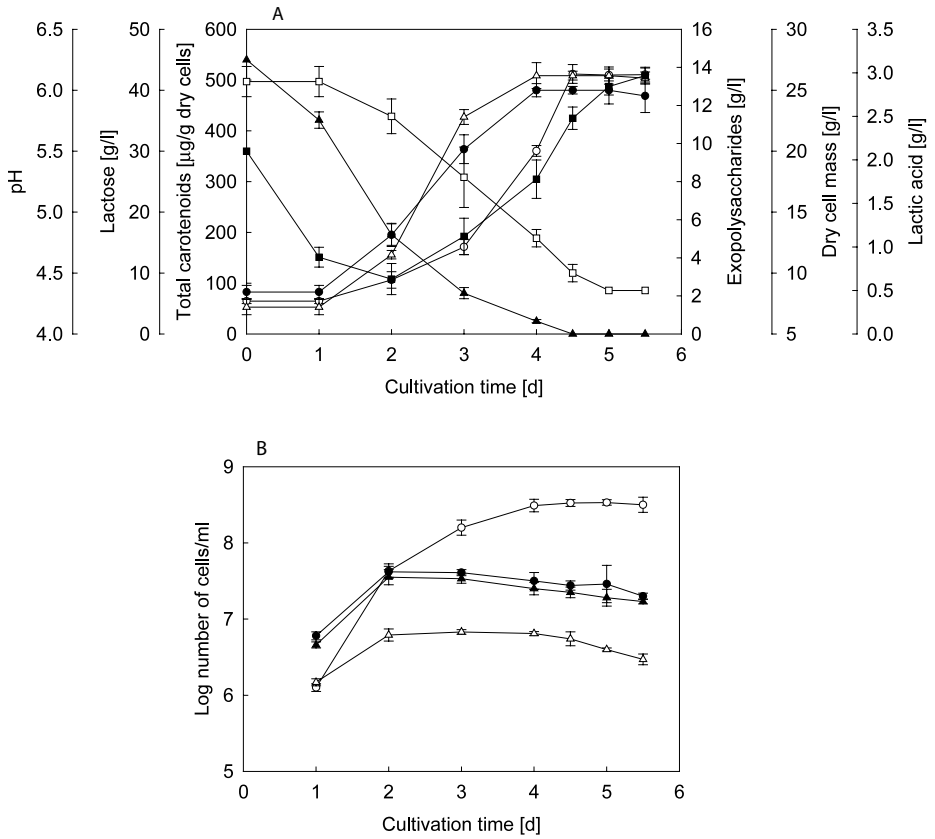

Fig. 1. Profile of carotenoid formation, exopolysaccharide production and growth of the microbial association *R. rubra* GED8 + (*L. bulgaricus* 2-11 + *S. thermophilus* 15HA) in whey ultrafiltrate: (A) total carotenoids (○), exopolysaccharides (●), dry cell mass (△), pH (■), lactose (▲), lactic acid (□); (B) *R. rubra* GED8 (○), *L. bulgaricus* 2-11 (△), *S. thermophilus* 15HA (▲), *L. bulgaricus* 2-11 + *S. thermophilus* 15HA (●). Bars represent standard deviation.

carotenoids/l culture fluid and 8.38 mg total carotenoids/l culture fluid (Zalashko, 1990; Frengova *et al.*, 1997). The identified individual pigments are typical of the species of the *Rhodotorula* genus, which is supported by other authors (Perrier *et al.*, 1995; Buzzini and Martini, 1999; Bhosale and Gadre, 2001; Buzzini, 2001).

The study of the biosynthesis of exopolysaccharides concomitantly with the carotenogenesis by the yeast strain *R. rubra* GED8 grown in association with the yogurt starter (*L. bulgaricus* 2-11 + *S. thermophilus* 15HA) revealed extra metabolic activities in the lactose-negative yeast. The exopolysaccharide yield (12.8 g/l) by the mixed culture *R. rubra* GED8 + (*L. bulgaricus* 2-11 + *S. thermophilus* 15HA) was higher than the amounts of exopolysaccharides synthesized by the monocultures *Cryptococcus laurentii* var. *flavescens* and *Hansenula holstii* (5.7 and 9.4 g/l, respectively) grown in whey containing lactose priorly hydrolyzed with β-galactosidase (Staufer and Leeder, 1978). The microbial association yeast + yogurt starter produced exopolysaccharides in a concentration that was higher than the concentration of the exopolysaccharides synthesized by *Bullera alba* (5.8 g/l) and was close to the concentration of the exopolysaccharides synthesized by *Candida humicola* (13.0 g/l) cultivated in native whey as monocultures (Ananyeva *et al.*, 1995; Igoshi *et al.*, 1990). The mixed culture *R. rubra* GED8 + (*L. bulgaricus* 2-11 + *S. thermophilus* 15HA) manifested a higher exopolysaccharide production level than that of the mixed culture *R. glutinis* 22P + *L. helveticus* 12A in previous studies – *ca.* 1.4 times higher (Frengova *et al.*, 1997) and 1.2 times higher activity than the monoculture *R. rubra* grown in a glucose synthetic

Table I. Carbohydrate composition of exopolysaccharides produced by the mixed culture *R. rubra* GED8 + (*L. bulgaricus* 2-11 + *S. thermophilus* 15HA) and by the yogurt starter (*L. bulgaricus* 2-11 + *S. thermophilus* 15HA).

Mixed culture	Composition of exopolysaccharides (%)					
	Mannose	Glucose	Galactose	Xylose	Arabinose	Glucuronic acid
R. rubra GED8 + (*L. b.*[a] 2-11 + *S. t.*[b] 15HA)	76.00 ± 1.00	8.18 ± 0.49	6.47 ± 0.67	1.69 ± 0.25	0.32 ± 0.06	7.20 ± 0.28
L. b.[a] 2-11 + *S. t.*[b] 15HA	3.98 ± 0.20	48.52 ± 1.85	43.89 ± 2.57	1.36 ± 0.18	2.27 ± 0.33	–

[a] *L. bulgaricus* 2-11. [b] *S. thermophilus* 15HA.

medium (Adami and Cavazzoni, 1990). Along with its main function – biotransformation of lactose to hydrolytic carbon-containing products (glucose, galactose and lactic acid) that are easily assimilated by lactose-negative yeast – the yogurt starter (*L. bulgaricus* 2-11 + *S. thermophilus* 15HA) performs the exopolysaccharide synthesis in parallel with the yeast strain *R. rubra* GED8 during mixed cultivation in WU. The comparative studies of the monosaccharide composition of the biopolymers showed that the yogurt starter synthesized neutral exopolysaccharides, and the association yeast + yogurt starter produced acidic exopolysaccharides with glucuronic acid content (7.2%) (Table I). Mannose, produced exclusively (95%) by the yeast, predominated in the neutral carbohydrate biopolymer component (76%). Notwithstanding the results of the authors differ significantly in terms of carbohydrate composition and monosaccharides ratio in the biopolymers (depending on the growth conditions and the specific properties of the strain producer), a number of authors showed the prevailing presence of mannose in exopolysaccharides produced by *Rhodotorula* (Adami and Cavazzoni, 1990; Simova *et al.*, 2000; Cho *et al.*, 2001).

The disintegrated and dried cell mass synthesized by the microbial association *R. rubra* GED8 + (*L. bulgaricus* 2-11 + *S. thermophilus* 15HA) was named "carotenoid-protein preparation", which contained 497.4 μg total carotenoids/g dry cells and 50.3% protein (Table II). An important characteristic of the carotenoid composition is the high percentage (51.1%) of β-carotene (a carotenoid pigment with the highest provitamin A activity) as compared to 12.9% and 33.7%, respectively, for the other two individual pigments – torulene and torularhodin. *In vitro* tests with β-carotene-15,15′-dioxygenase established that torulene and torularhodin synthesized by *Ph. rhodozyma* also possess provitamin-A activity (Ershov *et al.*, 1992). Besides the greater relative amount of β-carotene (nearly 3 times) in the total carotenoids synthesized by *R. rubra* GED8 co-cultivated with the yogurt starter, there were significantly higher β-carotene yields (5.4 and 8.9 times, respectively) compared to the amounts obtained for *R. glutinis* 22P in associated growth with *L. helveticus* 12A in WU, and for the lactose-positive yeast strain *R. lactosa*, grown as a monoculture in whey (Frengova *et al.*, 1997; Zalashko, 1990). In addition to the active carotenoid synthesis, an important advantage of the studied microbial association is that the carotenogenesis of the lactose-negative yeast *R. rubra* GED8 is shorter by 36 h as compared to the carotenogenesis of the lactose-negative yeast *R. glutinis* 22P from the mixed culture with *L. helveticus* 12A (Frengova *et al.*, 1997). Alongside with

Table II. Chemical composition of the carotenoid-protein preparation.

Component	Content
Total carotenoids [μg/g dry cells]	497.40 ± 8.43
Carotenoid pigments [μg/g dry cells]	
β-Carotene	254.20 ± 9.46
Torulene	63.90 ± 2.68
Torularhodin	167.6 ± 9.91
Protein (% dry weight)	50.3 ± 1.04
Lipids (% dry weight)	11.9 ± 1.15
Minerals (% dry weight)	
P	2.25 ± 0.47
K	2.10 ± 0.38
Mg	0.23 ± 0.04
Ca	0.40 ± 0.08
Na	0.36 ± 0.06
Vitamins of the B-complex [μg/g dry cells]	
Vitamin B_1	23.2 ± 2.75
Vitamin B_2	19.5 ± 2.90
Vitamin B_6	34.7 ± 0.26
Water (%)	5.4 ± 0.26

Table III. Amino acid composition of the carotenoid-protein preparation.

Amino acid	Content (% dry weight)
Lysine	3.65 ± 0.31
Histidine	0.74 ± 0.05
Arginine	3.87 ± 0.23
Asparagine	6.42 ± 0.51
Threonine	2.63 ± 0.17
Serine	2.91 ± 0.12
Glutamic acid	11.22 ± 0.58
Proline	2.85 ± 0.25
Glycine	1.73 ± 0.03
Alanine	3.02 ± 0.30
Valine	1.76 ± 0.11
Methionine	0.40 ± 0.05
Isoleucine	0.82 ± 0.13
Leucine	3.84 ± 0.17
Tyrosine	1.31 ± 0.05
Phenylalanine	1.45 ± 0.18

the carotenoids localized in the yeast cell, cytoplasmic components of high biological value (vitamins, amino acids, minerals, proteins, lipids) are released during the disintegration (Tables II, III), which predetermines the high nutritive value and improved assimilation of the carotenoid-protein preparation. The obtained composite has enhanced properties not only in terms of total carotenoid and β-carotene content but also in terms of protein and B-group vitamins content compared to the chemical composition of the carotene-protein obtained by the association *R. glutinis* 22P + *L. helveticus* 12A cultivated in WU and of the yeast biomass obtained by the lactose-positive culture *R. lactose* cultivated in whey (Frengova *et al.*, 1997; Zalashko, 1990). The rich chemical composition of the carotenoid-protein preparation determines its utilization as a vitamin, protein and pigmenting supplement in foods and feeds.

The associated cultivation of the lactose-negative yeast *R. rubra* GED8 with the yogurt starter (*L. bulgaricus* 2-11 + *S. thermophilus* 15HA) in WU under conditions for intracellular production of maximum amounts of carotenoids and exopolysaccharides synthesis enables the complex utilization of the culture fluid from the fermentation process by obtaining two bioproducts – a carotenoid-protein preparation and exopolysaccharides.

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