Exopolysaccharides Produced by Lactic Acid Bacteria of Kefir Grains

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A *Lactobacillus delbrueckii* subsp. *bulgaricus* HP1 strain with high exopolysaccharide activity was selected from among 40 strains of lactic acid bacteria, isolated from kefir grains. By associating the *Lactobacillus delbrueckii* subsp. *bulgaricus* HP1 strain with *Streptococcus thermophilus* T15, *Lactococcus lactis* subsp. *lactis* C15, *Lactobacillus helveticus* MP12. and *Sacharomyces cerevisiae* A13, a kefir starter was formed. The associated cultivation of the lactobacteria and yeast had a positive effect on the exopolysaccharide activity of *Lactobacillus delbrueckii* subsp. *bulgaricus* HP1. The maximum exopolysaccharide concentration of the starter culture exceeded the one by the *Lactobacillus delbrueckii* subsp. *bulgaricus* HP1 monoculture by approximately 1.7 times, and the time needed to reach the maximum concentration (824.3 mg exopolysacharides/l) was shortened by 6 h. The monomer composition of the exopolysaccharides from the kefir starter culture was represented by glucose and galactose in a 1.0:0.94 ratio, which proves that the polymer synthesized is kefiran.

Introduction

Exopolysaccharides produced by lactic acid bacteria have generated increasing attention among researchers for the last few years. The lactic acid bacteria are food-grade organisms, and the exopolysaccharides that they produce contribute to the specific rheology and texture of fermented milk products and may have application in nondairy foods. When added to food products, polysaccharides function as thickeners, stabilizers, emulsifiers, gelling agents, and water binding agents (Giraffa, 1994; Crescenzi, 1995). Kefir – a unique product among the cultured milk varieties – is produced with an original native starter (kefir grains). Kefir is defined as the yogurt of the 21st century (Gorski, 1994). The kefir grains consist of slimy materials in which yeast and bacterial cells are firmly embedded. The polysaccharide matrix, forming the structure of the kefir grain, is kefiran, identified by a number of researchers (La Riviere *et al.*, 1967; Neve, 1992; Pintado *et al.*, 1996). The lactic acid bacteria, yeast and polysaccharide "kefiran" that make up the kefir grains have been described as a symbiotic community that impart unique properties to kefir (Margulis, 1996).

Investigations into the active producers of kefiran are controversial. Although La Riviere *et al.* (1967) reported that *Lactobacillus brevis*, now regarded as *Lactobacillus kefir*, was responsible for kefiran production, Kandler and Kunath (1983) concluded that *Lactobacillus kefir* was not a kefiran producer. According to other authors, the principal producer of the kefiran polymer in kefir grains is *Lactobacillus kefiranofaciens* and several other unidentified species of *Lactobacillus* (Mitsue *et al.*, 1998, 1999; Yokoi *et al.*, 1990; Toba *et al.*, 1987). Thus it remains undecided which microorganism is responsible for kefiran production in kefir grains. Exopolysaccharide production is an important feature of lactic acid bacteria characterization in forming starter cultures for fermented milk products with suitable texture and specific rheology.

The present paper reports on kefiran production by lactic acid bacteria, isolated from kefir grains, and selection of an active producer of kefiran with view of including it in a kefir starter. There is no information available about production of exopolysaccharides by single strain cultures and kefir starter cultures during kefir fermentation and storage.

Materials and Methods

Microorganisms and cultivation conditions

Lactic acid bacteria (*Lactobacillus delbrueckii* subsp. *bulgaricus* 10 strains, *Lactobacillus helveticus* 10 strains, *Lactobacillus brevis* 5 strains, *Streptococcus thermophilus* 10 strains and *Lactococcus lactis* subsp. *lactis* 5 strains) were isolated from kefir grains and identified by the methods described in an earlier publication (Simova *et al.*, 2002).

Twince a week, the lactobacilli and lactococci were propagated in sterilized skim milk (115 °C, 15 min) in test tubes as follows: *L. bulgaricus* at 43 °C; *L. helveticus* at 37 °C; *L. brevis* at 30 °C; *L. lactis* at 28 °C, and *S. thermophilus* at 43 °C up to pH = 4.7. They were stored at 4 °C.

The lactobacillus and coccus strains were used in the first stage of screening for exopolysaccharide synthesis by cultivation at 22 °C for 22 h in 500 ml flasks with screw caps, containing 400 ml autoclaved homogenized milk (3% milk fat) and 2.0% v/v inoculum. The *L. bulgaricus* HP1 culture, which manifested the highest exopolysaccharide activity, was investigated for kefiran production in the second stage of screening as a component of kefir starter. The *L. bulgaricus* HP1 strain was cultivated in association with strains of the species *S. thermophilus*, *L. lactis*, *L. helveticus* and the yeast culture *S. cerevisiae.* A13 under the following conditions of kefir manufacture: Milk (3% milk fat), homogenized at 12.5–17.5 MPa (55 °C), pasteurized at 92 °C for 15 min and supplemented with 0.45% sucrose, was inoculated with the bacterial starters: yogurt culture (*S. thermophilus* + *L. bulgaricus* HP1) – 2%; *L. helveticus* – 2%; *L. lactis* – 1% and with the yeast starter *S. cerevisiae* A13 – 0.5%. After thorough stirring, the inoculated milk was distributed in 500-ml glass bottles (400 ml per bottle) closed with crown caps and incubated at 22 °C until a pH of 4.7 (about 16 h). There were two brief intermittent stirrings during incubation. The coagulum was cooled slowly for 12 h to 10 °C and kefir was stored at 4 °C for 24 h.

The primary bacterial cultures required for the preparation of the inoculum (starter) were propagated in autoclaved homogenized milk (3% milk fat) as follows: *L. bulgaricus* HP1 and *S. thermophilus* were grown as a mixed yogurt culture – inolulum 3% (*L. bulgaricus* HP1: *S. thermophilus* = 1:1), and incubated at 43 °C up to pH = 4.7 (for approx 150 min); *L. helveticus* – inoculum 3%, incubation at 37 °C up to pH = 4.7 (for approx 16 h); *L. lactis* – inoculum 1%, incubation at 28 °C up to pH = 4.7 (for approx 18 h). The cultures were transferred every three days and stored at 4 °C.

The yeast culture required for the preparation of the inoculum was propagated in clarified and autoclaved apple juice + grape juice (1:1, 10% dry matter), with addition of 1% of a 20% $(NH_4)_2HPO_4$ solution. Inoculation was made with loops of growth from the agar slant and incubation was performed at 20 °C on a reciprocating water shaker bath for 48 h.

The yeast strain was maintained on malt extract agar slants at 28 °C, stored at 4 °C and transferred every 4 weeks.

The required quantities of bacterial inocula were prepared in milk (3% milk fat), homogenized at 12.5–17.5 MPa (55 °C) and pasteurized at 92 °C for 15 min, using the same amounts of inoculum and conditions of incubation as for the primary bacterial cultures.

The yeast inoculum was prepared by inoculating with 6% of the aerated 48-h yeast culture the required quantity of apple juice + grape juice (1:1) supplemented with 1% of a 20% $(NH_4)_2HPO_4$ solution followed by stationary incubation at 20 °C for 48 h.

The monoculture *L. bulgaricus* HP1 and the starter culture (*L. bulgaricus* HP1 + *S. thermophilus* T15 + *L. helveticus* MP12 + *L. lactis* C15 + *S. cerevisiae* A13) were cultivated in a 15 l fermenter MBR AG (Zurich, Switzerland) using a 10 l working volume under the conditions of kefir manufacture described above.

Analytical methods

Viable cells (in colony forming units: CFU/ml) were determined from the colony counts on specific agar: Streptococcus selective agar (Merck) for cocci; LB-agar (Fluka) for lactobacilli and YM agar (Fluka) for yeasts after incubation at 30 °C and 37 °C (for lactic acid bacteria), and at 28 °C (for yeasts).

The lactic acid was determined by enzymatic methods as described by Boehringer Mannheim (1983).

The exopolysaccharides were isolated according to the method of Garcia-Garibay and Marshall

(1991). The lactic acid culture was treated with 17% (v/v) of 80% trichloracetic acid solution and centrifuged at 16,000 × *g* at 4 °C for 30 min. The clarified supernatant was concentrated 5 times by evaporation using a rotavap evaporator. The exopolysaccharides were precipitated by adding 3 volumes of cold absolute ethanol, and stored overnight at 4 °C. Finally, the recovered precipitates were redissolved with distilled water and dialysed against the same solution for 24 h at 4 °C to remove residual lactose from the medium. The polysaccharides were freeze-dried and stored at 4 °C. The exopolysaccharide production was exptessed as mg/l after measuring the weigh of the isolated and dried polymers.

The total amount of carbohydrates in the polysaccharides was determined by the phenol-sulfuric acid method described by Dubois *et al.* (1956). The carbohydrate composition was determined by gaschromatography using Fractovap 2407 (Carlo Erba) after hydrolysis with 4M H_2SO_4 for 8 h at 105 °C. The gas chromatograph was equipped with a flame ionization detector and a steel column (0.4 × 200 cm) packed with 2% SE-54 on silanized Chromosorb W80/100 mesh. The temperature of injector and detector was 350 °C. The analysis was performed using temperature programming from 160 °C to 300 °C at a heating rate of 4 °C/min. The carrier gas was N_2 (35 ml/min). The Autotab 6300–02 injector was programmed from 350 °C, chart speet 0.1 cm/min.

Viscosity (cst) was measured on an Oswald (Sibata, Kyoto, Japan) cinematic viscometer.

The data given are average of three independent triplicate experiments.

Table I. Production of exopolysaccharides by lactic acid bacteria of kefir grains.

Lactic acid bacteria	Exopolysaccharides, mg/l
L. bulgaricus HP1	460.27±7.02
L. bulgaricus HP2	147.23±6.85
L. bulgaricus HP3	46.93±4.94
L. bulgaricus HP4	195.37±3.97
L. bulgaricus HP5	51.60±4.84
L. bulgaricus HP6	298.83±8.79
L. bulgaricus HP7	370.30±9.14
L. bulgaricus HP8	66.63±4.89
L. bulgaricus HP9	112.23±6.45
L. bulgaricus HP10	38.17±2.97
L. helveticus MP10	15.37±3.85
L. helveticus MP11	19.23±3.34
L. helveticus MP12	–
L. helveticus MP13	10.40±1.55
L. helveticus MP14	22.63±2.67
L. helveticus MP15	16.90±2.39
L. helveticus MP16	–
L. helveticus MP17	20.67±4.07
L. helveticus MP18	–
L. helveticus MP19	–
L. brevis B1	–
L. brevis B2	–
L. brevis B3	–
L. brevis B4	–
L. brevis B5	–
S. thermophilus T10	31.40±4.71
S. thermophilus T11	15.77±2.93
S. thermophilus T12	–
S. thermophilus T13	27.80±3.38
S. thermophilus T14	–
S. thermophilus T15	–
S. thermophilus T16	19.13±2.12
S. thermophilus T17	–
S. thermophilus T18	–
S. thermophilus T19	23.83±3.26
L. lactis C11	–
L. lactis C12	–
L. lactis C13	–
L. lactis C14	–
L. lactis C15	–

Results and Discussion

The investigations in the first stage of screening for exopolysacccharide synthesis by lactic acid bacteria isolated from kefir grains showed that more than 50% of the studied *L. bulgaricus* strains are active producers of exopolysaccharides (Table I). The *S. thermophilus* and *L. helveticus* strains manifested poor production activity, while some of them did not possess exopolysaccharide activity. The strains of the *L. lactis* and *L. brevis* species also did not reveal any exopolisaccharide activity. According to some authors, however, the formation of kefiran matrix in the kefir grain is due to *L. brevis* (La Riviere *et al.*, 1967). The highest concentration of exopolysaccharides was measured in the culture *L. bulgaricus* HP1, which exceeded that in the cultures with comparatively high exopolysaccharide activity – *S. thermophilus* T10 and *L. helveticus* MP14 – by approx 15–20 times.

The total carbohydrate content in the exopolysaccharides from strains with high exopolysaccaride activity was about 71–75%. These results agree with the results about exopolysaccarides from lactic acid bacteria provided by other authors (Cerning *et al.*, 1994). Glucose and galactose were the basic structural units of the biopolymers

Table II. Carbohydrate composition of exopolysaccharides, produced by lactic acid bacteria of kefir grains.

Lactic acid Bacteria	Monosaccharides,%		
	Glucose	Galactose	Mannose
L. bulgaricus HP1	50.48±0.59	48.80±0.49	–
L. bulgaricus HP2	50.76±0.33	49.85±0.44	–
L. bulgaricus HP4	51.79±0.26	47.34±0.34	–
L. bulgaricus HP6	51.36±0.36	47.95±0.24	–
L. bulgaricus HP7	50.87±0.39	49.35±0.32	–
L. helveticus MP14	65.23±0.27	34.44±0.41	–
L. helveticus MP17	68.66±0.29	30.72±0.24	–
S. thermophilus T10	41.52±0.46	57.12±0.60	1.24±0.19
S. thermophilus T16	43.63±0.34	55.41±0.43	0.75±0.16

(Table II). The polysaccharides synthesized by *S. thermophilus* contained mannose as well. The monomer composition of the exopolysaccarides synthesized by *S. thermophilus* differed from the reports by other authors on this species (Cerning *et al.*, 1990; Ariga *et al.*, 1992; Stingele *et al.*, 1996). The lactobacilli synthesized biopolymers whose compositions contained glucose and galactose. In the exopolysaccharides synthesized by *L. helveticus* the glucose:galactose ratio was 2:1, which supports the results found by other authors (Robbin *et al.*, 1995; Yang *et al.*, 2000). In the exopolysaccharides synthesized by *L. bulgaricus* the glucose:galactose ratio was 1.0:0.91–1.0:0.98 (w/w) in accord with the data provided by other researchers (Toba *et al.*, 1987; Mukai *et al.*, 1988, 1990; Yokoi *et al.*, 1990). Of all studied lactic acid bacteria, isolated from kefir grains, only the *L. bulgaricus* strains synthesized the polymer kefiran, which contains equivalent amounts of glucose and galactose (Toba *et al.*, 1987; Mukai *et al.*, 1990; Yokoi *et al.*, 1990). The studies so far do not report on the production of kefiran polymer by *L. bulgaricus.* Authors report on production of kefiran by the species *L. brevis*, *L. kefiranofaciens* and *Lactobacillus* sp. (La Riviere *et al.*, 1967; Toba *et al.*, 1987; Yokoi *et al.*, 1990; Mukai *et al.*, 1990; Mitsue *et al.*, 1998; Micheli, 1999).

The study of exopolysaccharide production by the *L. bulgaricus* HP1 monoculture during lactic acid fermentation and subsequent refrigeration and storage (under conditions of kefir manufacture) found high exopolysaccharide activity in the phase of increased acidification and intensive cell growth (Fig. 1). Reduced polysaccharide content was recorded during refrigeration and storage of the culture at 4 °C, which was possibly related to the interaction of polysaccharides with milk casein as suggested by other authors (Cerning *et al.*, 1990; Teggatz and Morris, 1990).

The *L. bulgaricus* HP1 strain, which had shown the highest exopolysaccharide activity, was used in the second stage of screening – formation of kefir starter. In associated cultivation of the cultures (*L. bulgaricus* HP1 + *S. thermophilus* T15 + *L. lactis* C15 + *L. helveticus* MP12 + *S. cerevisiae* A13) making up the kefir starter the exopolysaccharide activity was increasing up to the 22nd h

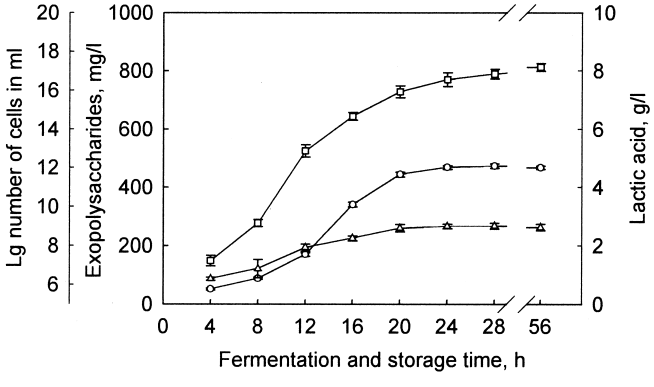

Fig. 1. Profile of exopolysaccharides and lactic acid production and growth of monoculture *L. bulgaricus* HP1 during fermentation and storage. Bars represent standard deviation; ○ -exopolysaccharides; □ -lactic acid; △ -lg N.

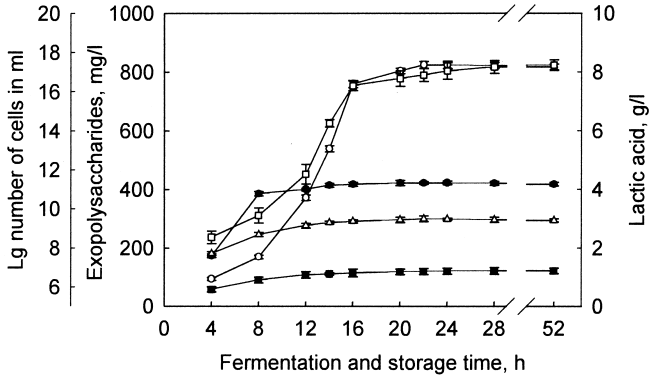

Fig. 2. Profile of exopolysaccharides and lactic acid production and growth of a mixed culture during fermentation and storage. Bars represent standard deviation; ○ -exopolysaccharides; □ -lactic acid; △ -lg N of lactobacilli; ■ -lg N of yeast; ● -lg N of cocci.

with a maximum concentration of 824.3 mg EPS/l against 472.6 mg EPS/l at the 28th h by the monoculture (Fig. 2, Fig. 1). Other authors also recorded a positive effect from the associated cultivation of lactobacilli and yeast, isolated from kefir grains, in intended synthesis of kefiran (Mitsue *et al.*, 1999).

The time change in exopolysaccharide production in the kefir starter culture is analogous to that in the *L. bulgaricus* HP1 monoculture (Fig. 2, Fig. 1).

The maximum exopolysaccharide concentration obtained from the monoculture and the mixed culture is higher than the one given by other authors for *Lactobacillus.* sp. (Yokoi *et al.*, 1990) and lower than the one produced by *L. kefiranofaciens* (Mitsue *et al.*, 1999). It should be noted that the exopolysaccharide activity by the *L. bulgaricus* HP1 strain is starting activity, while Mitsue *et al.* (1998) subjected the strain to UV radiation to boost the exopolysaccharide activity for intended synthesis.

The exopolysaccharides produced by the kefir starter culture contained glucose and galactose in a 1.0:0.94 ratio, which is determinative for the kefiran polymer. The viscosity of kefir with starter culture (1.089 cst) is similar to that of traditional kefir with kefir grains (1.082 cst) (Simova *et al.*, 2002).

A *L. bulgaricus* HP1 strain, producing the polymer kefiran, was selected. The exopolysaccharide activity of the strain increased (1.7 times approx) in its associated growth with lactobacteria strains + a yeast strain, isolated from kefir grains. The reported results expand the understanding of "kefiran polymer" production by lactic acid bacteria isolated from kefir grains. They can form the basis for further studies on how to increase the exopolysaccharide activity of *L. bulgaricus* HP1, create conditions for intended synthesis of kefiran, as well as define the structure and physicochemical characteristics of the biopolymer.

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