Available online at www.sciencedirect.com

ScienceDirect

Carbohydrate Polymers 68 (2007) 270–279

Carbohydrate Polymers

www.elsevier.com/locate/carbpol

Behavior of the rheological parameters of exopolysaccharides synthesized by three lactic acid bacteria

Nelly Canquil, Mario Villarroel, Sergio Bravo, Mónica Rubilar, Carolina Shene *

Department of Chemical Engineering, Universidad de La Frontera, Casilla 54-D, Temuco, Casilla, Chile

Received 6 September 2006; received in revised form 6 December 2006; accepted 11 December 2006
Available online 21 December 2006

Abstract

Exopolysaccharides (EPS) from *Lactobacillus delbrueckii* subsp *delbrueckii* (*L. delbrueckii*), *Bifidobacterium infantis* ATCC 15697 (*B. infantis*) and *Streptococcus thermophilus* Th4 (*S. thermophilus*) were produced and isolated form whey fermentations. A central composition rotatable design was employed to study the effects of EPS concentration, pH and ionic strength on the parameters in the Power Law. EPS and NaCl concentrations of 10.25 g/L and 0.08 M, respectively, and pH 5 were considered at the central point. Quadratic models for explaining the observed responses were determined using the surface response methodology. The three EPS presented a shear – thinning behavior. At the highest EPS concentration (20 g/L) consistencies of solutions of EPS from *B. infantis*, *L. delbrueckii* and *S. thermophilus* were 9.518, 17.706 and 13.136 mPa s, respectively. Contour plots, generated from the fitted models, were used to define the pH–ionic strength regions for higher consistencies and lower flow behavior index. Results suggested that EPS synthesized by *B. infantis* and *S. thermophilus* are polyelectrolytes while those produced by *L. delbrueckii* are neutral polysaccharides.

Keywords: Lactic acid bacteria; Viscosity; Power Law; Whey fermentation; EPS isolation

1. Introduction

Polysaccharides are often used in food products to provide a thickening effect. The corresponding enhancement of the viscosity leads to a better mouth-feel. In dairy products fermented by lactic acid bacteria (LAB) food grade polysaccharides, the exopolysaccharides (EPS), can be produced *in situ*. EPS from LAB are produced in a great variety concerning chemical composition, monomer ratio and molecular structure of the repeating unit as well as molecular mass of the polymer (De Vuyst et al., 2003). Moreover, certain LAB can produce different EPS depending on the growth conditions (temperature, pH and media composition). In spite of this diversity few reports on the rheological properties of EPS from LAB in aqueous solution are available (De Vuyst et al., 2003; Goh, Hemar, & Singh, 2005; Gorret, Renard, Famelart, Maubois, & Doublier, 2003; Navarini et al., 2001; Van den Berg et al., 1995; Vaningelgem et al., 2004). Among these, the more studied are those produced by different strains of *Lactococcus lactis* subsp *cremoris* (Higashimura, Mulder-Bosman, Reich, Iwasaki, & Robijn, 2000; Tuinier, Zoon, Cohen Stuart, Fleer, & de Kruif, 1999; Tuinier, Oomen, Zoon, Cohen Stuart, & de Kruif, 2000; Tuinier et al., 2001; Yang, Huttunen, Staaf, Widmalm, & Tenhu, 1999).

Rheological properties of EPS are important in order to predict the thickening effect in the products in which they are produced, such as fermented milks. On the other hand, the knowledge of these properties in solution allows the comparison with polysaccharides of animal, plant and microbial origin used as food additives. Shear-thinning behavior is one of the reported properties of EPS from LAB (De Vuyst et al., 2003; Goh et al., 2005; Gorret et al., 2003; Navarini et al., 2001; Tuinier et al., 1999; Van den Berg et al., 1995; Yang et al., 1999).

The aim of this work is to characterize and compare the rheological behavior of the EPS produced by different LAB

* Corresponding author. Tel.: +56 45 325491; fax: +56 45 325053.
E-mail address: cshene@ufro.cl (C. Shene).

0144-8617/$ - see front matter
doi:10.1016/j.carbpol.2006.12.012

in aqueous solutions. In order to do this the effect of pH, ionic strength and concentration of EPS on the parameters in the Power Law were determined. The effect of ionic strength on the intrinsic viscosity of EPS solutions was determined. EPS from *Lactobacillus delbrueckii* subsp *delbrueckii* (*L. delbrueckii*), *Bifidobacterium infantis* ATCC 15697 (*B. infantis*) and *Streptococcus thermophilus* Th4 (*S. thermophilus*) were the subjects of this study. These bacteria were grown in batch fermentations for the production of EPS. Deproteinized whey was used as source of lactose. Response surface methodology (RSM) was used to estimate the relationships for the consistency and flow behavior index, in terms of the independent variables. Significance of the effects was determined from the statistical analysis (ANOVA).

2. Materials and methods

2.1. Microorganisms

The *L. delbrueckii* subsp *delbrueckii* strain was isolated from a commercial yogurt obtained in the local market by selective plating on MRS agar (Difco™, Becton, Dickinson and Co., Sparks, Md, USA). The Gram morphology was determined and the carbohydrate profile was evaluated using API 50 CH test strips with CHL inoculation medium (bioMérieux, France). *Bifidobacterium infantis* ATCC 15697 (American Type Culture Collection, Rockville, MD, USA) and *S. thermophilus* Th4 (Chr Hansen, Denmark) were used. Stock cultures grown in MRS broth (Difco™, Becton, Dickinson and Co., Sparks, MD, USA) were stored at −18 °C in 50% glycerol until use.

2.2. Deproteinized Whey (DW)

Powder milk whey, purchase from local market (Colun, La Unión, Chile) was reconstituted in tap water (200 g/L). Deproteinization was carried out by heat treatment (121 °C, 15 min) of the acidified (pH 5, HCl 1 M) whey solution. Once cold it was centrifuged (10,000 rpm, 20 min, 4 °C), ultrafiltered (Pall Filtron Minisette, Pall Co., NY, USA) under 30 KDa (Minisette cassette, Pall Co., NY, USA) and autoclaved.

2.3. Culture media

Composition of culture media for *L. delbrueckii* was for 1 L and 40 g of lactose from DW: yeast extract (Bacto™, Becton, Dickinson and Co., Sparks, Md, USA), 20 g; peptone from casein (Merck, Darmstadt, Germany), 5 g; $MgSO_4 7H_2O$, 1 g; $MnSO_4 H_2O$, 0.1 g; Tween 80 (Merck, Darmstadt, Germany), 2 g. Culture media for *S. thermophilus* had the same composition but polypeptone (BBL, Becton, Dickinson and Co., Sparks, MD, USA) was used instead of the peptone from casein. Composition of the culture media for *B. infantis* was for 1 L and 20 g of lactose from DW: peptone from casein, 10 g; yeast extract, 15 g; $MgSO_4 7H_2O$, 0.1 g; $MnSO_4 H_2O$, 0.05 g; Tween 80, 1 g. These solutions were ultrafiltered under 30 KDa, autoclaved, cooled and added to the DW solutions to give the required concentration.

2.4. Fermentations

One milliliter of the respective stock culture was suspended in 5 mL of MRS broth and incubated at 37 °C for 24 h. Incubation of *B. infantis* was carried out under anaerobic conditions (95% H_2 and 5% CO_2, GasPak Plus, BBL, Sparks, MD, USA). Grown culture of *B. infantis* was transferred to 50 mL of sterile culture media, incubated for 18 h at 37 °C under anaerobic conditions. This second grown culture was used to inoculate 1.7 L of sterile culture media. Fermentations were carried out in a laboratory fermentor (Biostat M, B. Braun, Melsungen, Germany) at 36 °C. Stirring was set to 100 rpm and pH was kept 6.5 through the controlled addition of NaOH 10 M. Fermentations of *L. delbrueckii* and *S. thermophilus* were carried out at 36 and 42 °C, respectively, in a laboratory fermentor (BioFlo, 2000; New Brunswick, USA). Pre-inoculum (5 mL) was transferred to 100 mL of sterile culture media. This grown culture was used to inoculate 5 L of sterile culture media. In the fermentations of *L. delbrueckii* and *S. thermophilus*, pH was kept at 6 and 6.2, respectively, through the controlled addition of NaOH 10 M. Fermentations were carried out until the biomass concentration reached its maximum.

2.5. Isolation of EPS

The grown culture was centrifuged (4000 rpm, 20 min) in order to eliminate the biomass. The cell-free culture was filtered under 0.20 μm and 30 KDa. Retentate was supplemented with trichloroacetic acid (Merck, Darmstadt, Germany) to give a concentration of 20% w/v. After 24 h at 4 °C the precipitates were eliminated by centrifugation (10,000 rpm, 4 °C for 30 min). One volume of chilled ethanol (99.7%) was added to the supernatant. The solution was kept 24 h at −18 °C. Precipitates were recovered by centrifugation (10,000 rpm, 4 °C for 30 min) and dissolved in hot distilled water. EPS solution was neutralized with NaOH (1 M), centrifuged (10,000 rpm, 4 °C for 30 min) and exhaustively dialyzed (MWCO 6000 – 8000, Spectra/Por, Spectrum Laboratories, Inc., CA, USA) against distilled water for 4 days at 4 °C. The dialyzed solution was centrifuged (10,000 rpm, 4 °C for 30 min,) and freeze-dried. The total sugar concentration in the dry sample was measured according to the method of Dubois, Giles, Hamilton, Reberts, and Smith (1956) with glucose as standard. Protein concentration was determined according to the method of Bradford (1976) using BSA as the standard. Freeze-dried EPS powders from *L. delbrueckii*, *S. thermophilus* and *B. infantis* contain 72%, 94% and 55% of glucose equivalents; protein contents were 1.1%, 0.6% and 0.7%, respectively.

2.6. Intrinsic viscosity

Measurements were carried out using a glass capillary viscometer (Cannon-Fenske 75) immersed in a constant temperature bath at 25 °C. Stock solutions (5 g/L *B. infantis*, 2 g/L *L. delbrueckii* and 0.5 g/L *S. thermophilus*) were prepared by dissolving the EPS in 0.01, 0.1 and 0.2 M NaCl. Twenty milliliters of the EPS solutions filtered under 0.20 μm were loaded into the viscometer. Relative viscosities (η_{rel}) were calculated by dividing the flow times of the EPS solutions by that of the different salt solutions. Elution time of each solutions was taken as the average of five concordant readings. Dilutions to yield at least four lower concentrations were made by adding the appropriate aliquots of the salt solutions. All the experiments were performed in the range $1.05 < \eta_{rel} < 1.5$.

2.7. Apparent viscosity

Viscosity measurements were carried out on 1 mL samples using a cone-plate Brookfield Digital Rheometer Model DV III (Brookfield Engineering Laboratories Inc., Stoughton, Mass, USA). The rheometer was equipped with a flat spindle, type CP 42 (Brookfield Engineering Laboratories Inc.) that rotated in the sample-containing chamber connected to a temperature-controlled water bath. Radius of the cone spindle was 2.4 cm with a cone angle of 1.565°. The rheometer was controlled with the Brookfield Rheocalc software (Brookfield Engineering Laboratories Inc.). Apparent viscosity measurements were performed at 25 °C. Spindle speeds of 2–100 rpm were applied. All viscosity measurements, expressed in mPa s, were performed in triplicate and averaged. Parameters in the Power Law ($\eta = K \cdot \gamma^{n-1}$; K, consistency, n, flow behavior index) were estimated through the minimization of the error between the experimental and calculated flow curves.

2.8. Design of experiments

Response surface methodology (RSM) was used to estimate the relationship between the independent variables, EPS concentration, pH and ionic strength (NaCl concentration), and the observed responses Y (consistency, K, and flow behavior index, n). A three-factor central composition rotatable design (CCRD) was employed to study the responses. At the central point three replicates were performed to allow estimation of error. All experiments were carried out in randomized order to minimize the effect of unexplained variability in the observed responses due to extraneous factors. Coded variables used in the CCRD are shown in Table 1. A solution having EPS and NaCl concentrations of 10.25 g/L and 0.08 M, respectively, at pH 5, was used as the central point.

The behavior of the system was explained by the following second-degree polynomial equation:

$$Y_P = b + \sum_{i=1}^{3} a_i X_i + \sum_{i=1}^{3} \sum_{j=1, j\neq i}^{3} a_{ij} X_i X_j + \sum_{j=1}^{3} a_{jj} X_j^2 \tag{1}$$

Where Y_P is the predicted response, X_i the coded value of the i-independent variable; b, a_i, a_{ii} and a_{ij} are the intercept, linear, quadratic and interaction regression coefficients, respectively. When a central composite design is used, the full quadratic model can be estimated, but often some of the terms are not significant. In order to identify an appropriate reduced quadratic model, the significance of each source of variation was obtained from statistical analysis (ANOVA). Sources that were not statistically significant ($p > .05$) were sequentially dropped and its effect on the coefficient of determination (R^2) was evaluated. Statgraphics Plus Statistical graphic system version 7.0 was used for multiple regression analysis and analysis of variance (ANOVA).

3. Results

In order to isolate the EPS produced by the different LAB, culture media was ultrafiltered under 30 KDa before the autoclaving. In this way polysaccharides in the deproteinized whey and those in the nitrogen sources, yeast extract and peptone [glucomammans, molecular weight of 75 KDa (Vaningelgem et al., 2004)] were eliminated. EPS were isolated from the cell-free fermented culture media that was retained over 30 KDa. Through the exhaustive dialysis against distilled water, lactose and galactose were eliminated.

Intrinsic viscosities ([η]) of the EPS produced by the lactic acid bacteria were determined by the Huggins–Kramer extrapolation of the regression lines. Table 2 shows the values of the intrinsic viscosity for the different ionic strengths (I). This data was used to compute the stiffness parameter B ($B = S/([\eta]_{0.1})^{1.3}$) (Smithrød & Haug, 1971). To do this, the slope of linear relationship between [η] and $I^{0.5}$, S, was computed; values of S and B, for the different EPS are presented in Table 2.

Table 1
Variables used in the CCRD

Independent variable	Coded variable	Levels				
		$-\alpha$	-1	0	1	α
EPS concentration (g/L)	X_1	0.5	4.45	10.25	16.05	20
pH	X_2	2.5	3.5	5	6.5	7.5
Ionic strength NaCl (M)	X_3	0	0.032	0.08	0.128	0.16

Table 2
Intrinsic viscosity [η], slope of the linear relationship between [η] and $I^{-0.5}$, S, and stiffness parameter, B of EPS from lactic acid bacteria at different ionic strength, I

EPS	Ionic strength (M)			S	B
	0.01 [η]/dL/g	0.1 [η]/dL/g	0.2 [η]/dL/g		
B. infantis	0.20	0.19	0.18	0.003	0.022
L. delbrueckii	0.82	0.75	0.74	0.010	0.015
S. thermophilus	14.21	4.23	2.90	1.458	0.224

Consistency (K) and flow behavior index (n), were estimated from the flow curves of the aqueous solutions of EPS whose concentration, ionic strength and pH are shown in Table 3. The different levels in the CCDR were chosen as follow: pH values in the neutral–acidic range were considered since pH of most food products is found in here. At the central point in the CCDR the ionic strength was that of milk, 0.08 M. Highest EPS concentration was 20 g/L, chosen due to the availability of EPS. Rheological parameters in the Power Law for each of the aqueous solution in the CCRD are shown in Table 3.

At the intermediate shear rates tested aqueous solutions of EPS from *B. infantis*, *L. delbrueckii* and *S. thermophilus* exhibited shear-thinning ($0 < n < 1$) behavior. Graphs in Fig. 1 show the effect of EPS concentration (0.5, 10.25 and 20 g/L) on the flow curves obtained at ionic strength and pH equal to 0.08 M and 5, respectively. For the highest EPS concentration (20 g/L) tested (pH of 5 and ionic strength of 0.08 M) consistencies of the solutions of EPS from *B. infantis*, *L. delbrueckii* and *S. thermophilus* were 9.518, 17.706 and 13.136 mPa s, respectively. Due to the limitation of the rheometer the limiting zero shear-rate viscosity and the upper limit shear rates were not measured. Flow behavior index of EPS solutions was limited to different ranges depending on the source of the EPS. Flow behavior index of the solutions of EPS from *B. infantis* was found between 0.563 and 0.722. The corresponding values for the solutions of EPS from *L. delbrueckii* were between 0.882 and 0.966 while those of solutions of EPS from *S. thermophilus* were more widely distributed (between 0.606 and 0.928).

Significance of the different effects (concentration, pH and ionic strength) and their interactions on the quadratic models for describing the rheological parameters was determined from the statistical analysis.

Analysis of variance for the terms in the full quadratic model for the consistency of solutions of EPS from *B. infantis* is shown in Table 4. These results suggested that the model had an insignificant lack-of-fit ("Prob > F" = 0.120) and an adequate level of R^2 (0.978). Highly significant effect was that of the EPS concentration (X_1). Quadratic EPS concentration (X_1^2), quadratic pH (X_2^2) and ionic strength (X_3) were significant ($p < .05$). The only significant interaction term was that between pH and ionic strength (X_2X_3). Sequential drop of the following terms: interaction between EPS concentration and ionic strength (X_1X_3), interaction between EPS concentration and pH (X_1X_2), and quadratic ionic strength (X_3^2) resulted in values of R^2 equal to 0.978, 0.978 and 0.974, respectively. The reduced quadratic model for the consistency of solutions of EPS from *B. infantis*, shown in Table 7, had an insignificant lack-of fit ("Prob > F" = 0.158).

The full quadratic model for the flow behavior index of aqueous solutions of EPS from *B. infantis* had a satisfactory level of R^2 (0.673) although it showed a significant lack-of-fit ("Prob > F" = 0.010) (Table 4) suggesting that the second order model was not adequate. In spite of the results obtained from the statistical analysis, it was considered that the model was a first approximation for

Table 3
Observed values of the parameters in the Power Law model (consistency, K and flow behavior index, n) for the aqueous solutions of EPS from *L. delbrueckii*, *B infantis* ATCC 15679 and *S. thermophilus* Th4 for the different points in the CCRD

Variables			*B. infantis*		*L. delbrueckii*		*S. thermophilus*	
X_1	X_2	X_3	K	n	K	n	K	n
−1	−1	−1	4.148	0.699	3.414	0.892	5.017	0.709
−1	−1	1	5.065	0.619	2.205	0.964	5.540	0.658
−1	1	−1	4.817	0.650	2.171	0.965	7.047	0.606
−1	1	1	4.700	0.666	2.107	0.966	8.280	0.641
1	−1	−1	7.131	0.597	11.808	0.905	10.099	0.861
1	−1	1	7.977	0.563	11.652	0.910	9.325	0.804
1	1	−1	7.611	0.574	10.947	0.923	14.256	0.818
1	1	1	7.413	0.600	12.644	0.882	13.535	0.753
−α	0	0	3.927	0.681	1.581	0.903	2.272	0.775
α	0	0	9.518	0.619	17.706	0.895	13.136	0.862
0	−α	0	7.122	0.722	5.345	0.929	9.068	0.824
0	α	0	5.910	0.621	4.859	0.945	16.782	0.605
0	0	−α	5.122	0.653	5.810	0.915	7.066	0.928
0	0	α	5.701	0.646	6.012	0.918	11.789	0.648
0	0	0	5.754	0.649	5.776	0.922	4.530	0.923
0	0	0	5.473	0.641	5.531	0.920	6.468	0.826
0	0	0	5.618	0.642	6.130	0.902	6.104	0.837

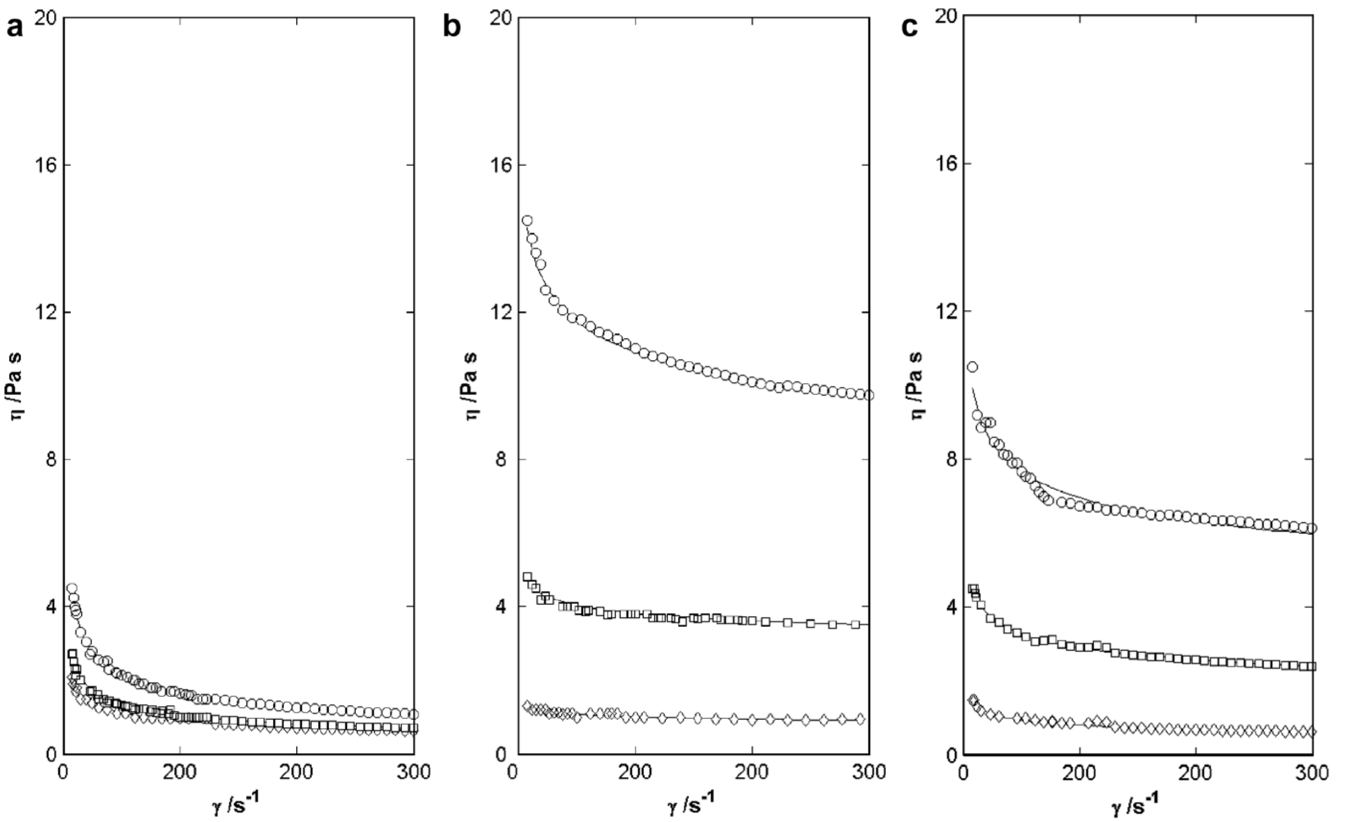

Fig. 1. Effect of the concentration on the apparent viscosity (η) of solutions of EPS from (a) *B. infantis* ATCC 15697 (b) *L. delbrueckii* and (c) *S. thermophilus* Th4 as a function of shear rate. 0.5 g/L, ◆; 10.25 g/L, □; 20 g/L, ○. Ionic strength, 0.05 M; pH, 5.

Table 4
Analysis of variance of the second order model for parameters K and n in the Power Law for solutions of EPS from *B. infantis* ATCC 15679

Source of variation	K					n				
	Sum of squares	*df*	Mean squared	*F*-value	*P*	Sum of squares	*df*	Mean squared	*F*-value	*P*
X_1	31.6972	1	31.6972	1605.24	.001	0.0119	1	0.0119	664.38	.002
X_2	0.2419	1	0.2419	12.25	.073	0.0019	1	0.0019	103.79	.010
X_3	0.4295	1	0.4295	21.75	.043	0.0005	1	0.0005	29.52	.032
X_1X_2	0.0190	1	0.0190	0.96	.440	0.0000	1	0.0000	1.59	.334
X_1X_3	0.0029	1	0.0029	0.15	.742	0.0004	1	0.0004	22.74	.041
X_2X_3	0.5393	1	0.5393	27.31	.035	0.0031	1	0.0031	170.41	.006
X_1^2	1.4547	1	1.4547	73.67	.013	0.0004	1	0.0004	23.08	.041
X_2^2	0.9230	1	0.9230	46.74	.021	0.0000	1	0.0000	1.44	.353
X_3^2	0.1234	1	0.1234	6.25	.130	0.0004	1	0.0004	24.31	.039
Lack-of-fit	0.7564	5	0.1513	7.66	.120	0.0090	5	0.0018	100.86	.010
Pure error	0.0395	2	0.0197			0.0000	2	0.0000		
Total (corr.)	36.4048	16				0.0278	16			
R^2	0.978					0.673				

describing the behavior of this rheological parameter. Table 7 shows the quadratic model for the flow behavior index of the aqueous solutions of EPS from *B. infantis*.

Statistical analysis of the factors in the quadratic models for the rheological parameters of aqueous solutions of EPS from *L. delbrueckii* is shown in Table 5. The full quadratic model for the consistency of solutions of EPS from *L. delbrueckii* was considered adequate because it had an insignificant lack-of-fit ("Prob > F" = 0.764) and a high level of R^2 (0.999). Highly significant effects were those of the linear (X_1) and quadratic (X_1^2) EPS concentration ($p < .05$). All of the other sources of variation were not significant. The reduced quadratic model for the consistency of solutions of EPS from *L. delbrueckii*, presented in Table 7, had a satisfactory level of R^2 (0.988) and an insignificant lack-of-fit ("Prob > F" = 0.862).

The full quadratic model for the flow behavior index of solutions of EPS from *L. delbrueckii*, was considered adequate because of the satisfactory level of R^2 (0.836) and its insignificant lack-of-fit ("Prob > F" = 0.299) (Table 5).

Table 5
Analysis of variance of the second order model for parameters K and n in the Power Law for solutions of EPS from *L. delbrueckii*

Source of variation	K					n				
	Sum of squares	*df*	Mean squared	*F*-value	*P*	Sum of squares	*df*	Mean squared	*F*-value	*P*
X_1	302.4885	1	302.4885	3340.08	.000	0.0024	1	0.0024	20.76	.045
X_2	0.3011	1	0.3011	3.32	.210	0.0006	1	0.0006	5.49	.144
X_3	0.0270	1	0.0270	0.30	.645	0.0001	1	0.0001	1.10	.404
X_1X_2	0.2704	1	0.2704	2.99	.226	0.0009	1	0.0009	7.92	.107
X_1X_3	0.9895	1	0.9895	10.93	.081	0.0015	1	0.0015	12.77	.070
X_2X_3	1.1236	1	1.1236	12.41	.072	0.0017	1	0.0017	14.99	.061
X_1^2	21.8592	1	21.8592	241.37	.004	0.0002	1	0.0002	1.37	.362
X_2^2	0.5186	1	0.5186	5.73	.139	0.0011	1	0.0011	9.21	.094
X_3^2	0.0581	1	0.0581	0.64	.515	0.0001	1	0.0001	0.54	.545
Lack-of-fit	0.2315	5	0.0463	0.51	.764	0.0015	5	0.0003	2.61	.299
Pure error	0.1811	2	0.0906			0.0002	2	0.0001		
Total (corr.)	332.7841	16				0.0106	16			
R^2	0.999					0.836				

The only significant effect in this model was that of the EPS concentration (X_1) ($p < .05$). Drop of the following terms: quadratic ionic strength (X_3^2) and quadratic EPS concentration decreased the level of R^2 to 0.830 and 0.807, respectively. Drop of any other term reduced the predictive capability of the model. Table 7 shows the reduced quadratic model for the flow behavior index of solutions of EPS from *L. delbrueckii* which had an insignificant lack-of-fit ("Prob > F" = 0.342).

Statistical analysis of the factors in the quadratic models for the rheological parameters of solutions of EPS from *S. thermophilus* is shown in Table 6. These results suggested that the model for the consistency was adequate because it had an insignificant lack-of-fit ("Prob > F" = 0.315) and a satisfactory level of R^2 (0.940). EPS concentration (X_1) was highly significant. Linear (X_2) and quadratic pH (X_2^2) were significant ($p < .05$). No statistically significant interaction existed between any two of the three factors. The sequential drop of the following effects: interaction between pH and ionic strength (X_2X_3), interaction between EPS concentration and ionic strength (X_1X_3), interaction between EPS concentration and pH (X_1X_2), quadratic EPS concentration resulted in values of R^2 of 0.939, 0.934, 0.928 and 0.918, respectively. The reduced quadratic model for the consistency of solutions of EPS from *S. thermophilus* (Table 7) presented an insignificant lack-of-fit ("Prob > F" = 0.389).

The full quadratic model for the flow behavior index of solutions of EPS from *S. thermophilus* was considered adequate because it had an insignificant lack-of-fit ("Prob > F" = 0.323) and a satisfactory level of R^2 (0.783) (Table 6). However, all the terms in the model were not significant ($p > .05$). Drop of the three interaction terms and the quadratic EPS concentration (X_1^2) decreased the level of R^2 to 0.736. The reduced quadratic model (Table 7) for the flow behavior index of solutions of EPS from *S. thermophilus* presented an insignificant lack-of-fit ("Prob > F" = 0.434).

The statistical analysis showed that EPS concentration was significant for the consistency of the different EPS solutions. Moreover, consistency was positively related to the EPS concentration (Table 3). However, the effects of pH and ionic strength on the consistency of the EPS solutions were difficult to visualize from results in Table 3. In order

Table 6
Analysis of variance of the second order model for parameters K and n in the Power Law for solutions of EPS from *S. thermophilus* Th4

Source of variation	K					n				
	Sum of squares	*df*	Mean squared	*F*-value	*P*	Sum of squares	*df*	Mean squared	*F*-value	*P*
X_1	114.8228	1	114.8228	108.24	.009	0.0433	1	0.0433	15.20	.060
X_2	49.9125	1	49.9125	47.05	.021	0.0248	1	0.0248	8.72	.098
X_3	4.9222	1	4.9222	4.64	.164	0.0272	1	0.0272	9.54	.091
X_1X_2	1.6178	1	1.6178	1.52	.342	0.0001	1	0.0001	0.03	.880
X_1X_3	1.3215	1	1.3215	1.25	.381	0.0014	1	0.0014	0.49	.563
X_2X_3	0.0730	1	0.0730	0.07	.820	0.0008	1	0.0008	0.27	.662
X_1^2	2.3035	1	2.3035	2.17	.279	0.0062	1	0.0062	2.18	.278
X_2^2	59.6032	1	59.6032	56.18	.017	0.0411	1	0.0411	14.43	.063
X_3^2	12.7091	1	12.7091	11.98	.074	0.0133	1	0.0133	4.68	.163
Lack-of-fit	12.9954	5	2.5991	2.45	.315	0.0337	5	0.0067	2.37	.323
Pure error	2.1217	2	1.0609			0.0057	2	0.0028		
Total (corr.)	250.4886	16				0.1815	16			
R^2	0.940					0.738				

Table 7
Reduced quadratic models for the rheological parameters of aqueous solutions of EPS from *L. delbrueckii*, *B. infantis* ATCC 15697 and *S. thermophilus* Th4

	Model	R^2
B. infantis	$K = 5.49088 + 1.524148 \cdot X_1 - 0.133154 \cdot X_2 + 0.177425 \cdot X_3 - 0.259637 \cdot X_2 \cdot X_3 + 0.390559 \cdot X_1^2 + 0.317377 \cdot X_2^2$	0.974
	$n = 0.646348 - 0.029539 \cdot X_1 - 0.011675 \cdot X_2 - 0.006227 \cdot X_3 + 0.001888 \cdot X_1 \cdot X_2 + 0.007138 \cdot X_1 \cdot X_3 + 0.019538 \cdot X_2 \cdot X_3 - 0.006066 \cdot X_1^2 + 0.001517 \cdot X_2^2 - 0.006225 \cdot X_3^2$	0.673
L. delbrueckii	$K = 5.660388 + 4.708373 \cdot X_1 + 1.426903 \cdot X_1^2$	0.988
	$n = 0.912824 - 0.013202 \cdot X_1 + 0.006791 \cdot X_2 + 0.003045 \cdot X_3 - 0.01065 \cdot X_1 \cdot X_2 - 0.013525 \cdot X_1 \cdot X_3 - 0.01465 \cdot X_2 \cdot X_3 + 0.010003 \cdot X_2^2$	0.807
S. thermophilus	$K = 6.354384 + 2.900884 \cdot X_1 + 1.912586 \cdot X_2 + 0.600612 \cdot X_3 + 2.169294 \cdot X_2^2 + 0.929994 \cdot X_3^2$	0.918
	$n = 0.834412 + 0.056326 \cdot X_1 - 0.042668 \cdot X_2 - 0.044625 \cdot X_3 - 0.053537 \cdot X_2^2 - 0.027513 \cdot X_3^2$	0.736

to represent graphically these effects on the consistency, the reduced quadratic models were used to generate the contour plots for a solution having an EPS concentration of 10.25 g/L. Fig. 2 shows the contours representing specific consistencies above the plane defined for the combination of the levels of pH and ionic strength. Results in Fig. 2a show two regions of high consistency for the solutions of EPS from *B. infantis*. One was defined by high pH and low ionic strength; the other was defined by low pH and high ionic strength. On the other hand, consistency of solutions of EPS from *S. thermophilus* presented a minimum in the region defined by pH ranging between 3 and 5.5 and ionic strength ranging between 0.01 and 0.12 M (Fig. 2b).

Contours representing specific flow behavior index above the plane defined for the combination of the levels of pH and ionic strength were drawn. Results for an EPS solution having a concentration of 10.25 g/L are presented in Fig. 3. Aqueous solutions of EPS from *B. infantis* had low flow behavior index (more shear-thinning behavior) in two regions (Fig. 3a). One defined by high pH and low ionic strength and the other defined by low pH and high ionic strength. Lower values of the flow behavior index of aqueous solutions of EPS from *L. delbrueckii* were in the region defined by low pH and low ionic strength (Fig. 3b). Flow behavior index of the aqueous solutions of EPS from *S. thermophilus* presented a maximum (Fig. 3c) in region defined by pH ranging between 3 and 6, and ionic strength lower than 0.1 M.

4. Discussion of the results

Different studies have shown that EPS from LAB affect positively the rheological properties of fermented milk in which they are synthesized *in situ*. However, due to large variability of composition, charge, spatial arrangement, rigidity and the ability to interact with proteins and ions no clear correlation between EPS concentration and apparent viscosity of the product has been established (Petry et al., 2003). At present EPS yields in LAB fermentations are still too low to consider them alternative to polysaccharides from animal and plant sources. Nevertheless, the search for over producing strains and the better understanding of the mechanisms that regulate their synthesis could improve the yields to economically feasible levels. In this scenario and due to the attractive rheological and health properties of EPS from LAB, their use in other food products could be suggested.

Intrinsic viscosity of aqueous solutions of EPS from *B. infantis*, *L delbrueckii* and *S. thermophilus* presented significant differences. Since in theory the intrinsic viscosity represents the effective hydrodynamic specific volume of a polymer, size of the different EPS, could be sorted as follow: *B. infantis* < *L. delbrueckii* < *S. thermophilus*. Ionic strength had a significant effect on the intrinsic viscosity of solutions of EPS from *S. thermophilus* for which highest value of $[\eta]$ was obtained at lowest ionic strength. According to the values of parameter B (Table 2), EPS

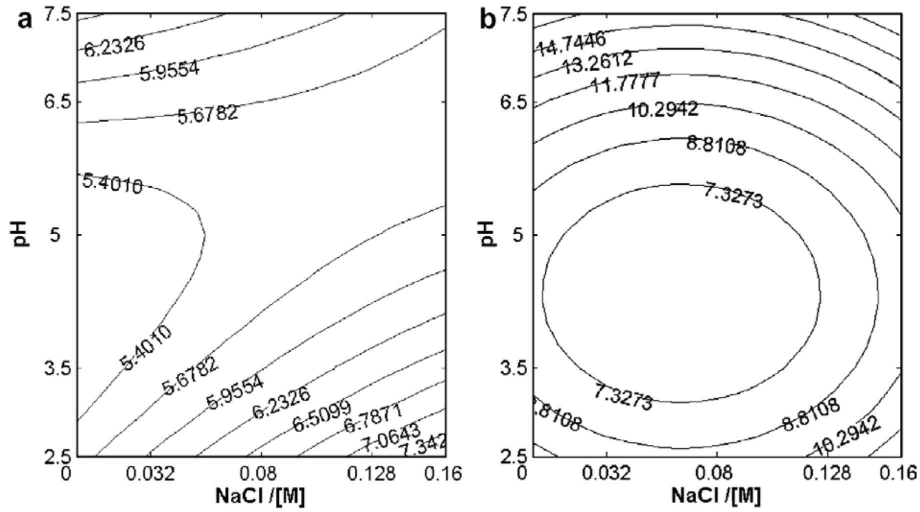

Fig. 2. Contour plots at the combination of levels of pH and ionic strength for the consistencies of solutions of EPS from (a) *B. infantis* ATCC 15697 and (b) *S. thermophilus* Th4.

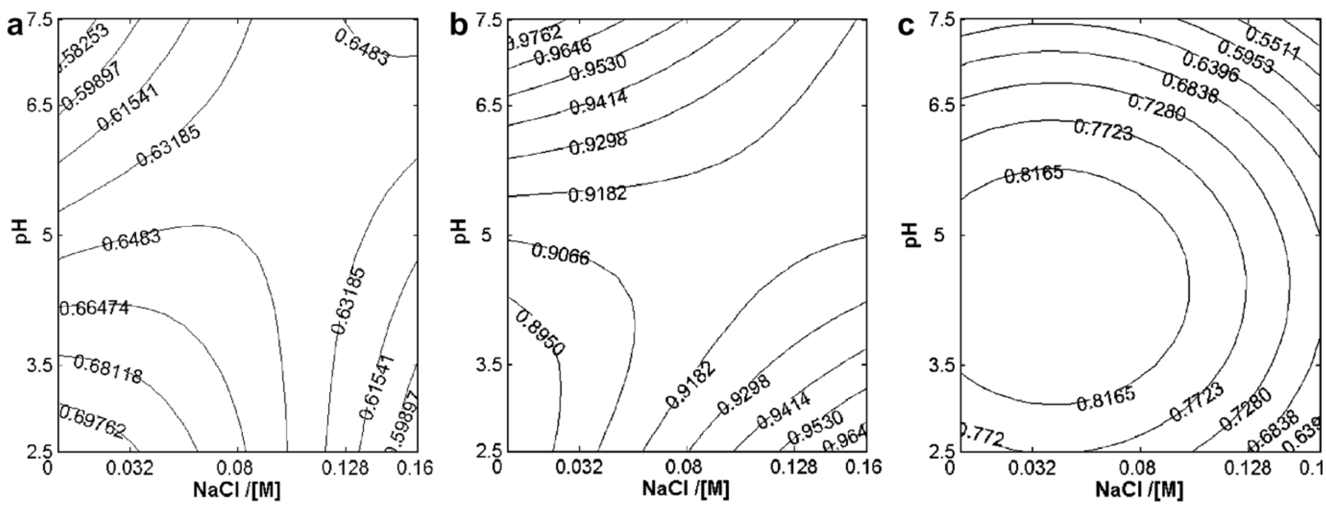

Fig. 3. Contour plots at the combination of levels of pH and ionic strength for the flow behavior index of solutions of EPS from (a) *B. infantis* ATCC 15697, (b) *L. delbrueckii* and (c) *S. thermophilus* Th4.

from *S. thermophilus* was less stiff than those produced by *B. infantis* and *L. delbrueckii*.

The significance of the effects of EPS concentration, pH and ionic strength and their interactions on the parameters in the Power Law for viscosity was established from statistical analysis and quadratic models for explaining the observed responses were determined using the surface response methodology.

According to the level of R^2 the flow behavior index of the different EPS solutions can be satisfactory described by the quadratic models. Reduced quadratic models for describing the flow behavior index of the solutions of EPS from *B. infantis*, *L. delbrueckii* and *S. thermophilus* presented R^2 of 0.671, 0.807 and 0.736, respectively. However, quadratic model for flow behavior index of aqueous solutions of EPS from *B. infantis* has a significant lack-of-fit. Aqueous solutions of the different EPS display a shear-thinning behavior ($0 < n < 1$). This rheological parameter presents behaviors that depend on pH, ionic strength, concentration and source of the EPS. Depending on the source of the EPS, values for this rheological parameter are constrained to different ranges.

Quadratic models for describing the consistency of the different EPS solutions present satisfactory levels of R^2 (higher than 0.91) and insignificant lack-of-fit. Eventhough consistency of the EPS solutions is positively related to the EPS concentration, the magnitude of the increase depends on the source of the EPS. At 20 g/L consistencies of aqueous solutions of EPS from *B. infantis*, *L. delbrueckii* and *S. thermophilus* were 142%, 1020% and 478% higher than those at 0.5 g/L, respectively; the one showing the higher increase is that having the lowest consistency at the lowest concentration.

Viscosity of EPS solutions depends on a number of characteristics related to the EPS structure (composition of the polysaccharide, chain stiffness, branches and side groups in the polysaccharide chain) (Ruas-Madiedo, Hugenholtz, & Zoon, 2002; Tuinier et al., 2001; Yang, Staaf, Huttunen, & Widmalm, 2000). However, a common trend is that higher intrinsic viscosities are those of EPS having high molecular weight (Vaningelgem et al., 2004).

Of the three EPS under study the lower consistencies are those of solutions of EPS from *B. infantis*. EPS isolated from *B. infantis* cultures are also those having the lowest glucose equivalent content (51%) although the purification method was the same employed for obtaining the others EPS. According to the intrinsic viscosity the EPS having the highest hydrodynamic specific volume are those produced by *S. thermophilus*. In solution these EPS present a consistency of 13.1362 mPa s at the highest concentration, value that is lower than that of the solution of EPS from *L. delbrueckii* at the same concentration.

Effect of ionic strength and quadratic pH are significant for the consistency of the solutions of EPS form *B. infantis*. The effect of the ionic strength on the intrinsic viscosity of EPS produced by *Lactobacillus sake* O-1 (Van den Berg et al., 1995) and *Lactococcus lactis* subsp *cremoris* B40 (Tuinier et al., 1999) was explained due to a charged group in the polysaccharide. In solution the intramolecular repulsion forces in a charged polymer chain would increase the hydrodynamic volume and thus the intrinsic viscosity is relatively high (Van den Berg et al., 1995). The addition of small amount of salt decreases the repulsion effects, which leads to a smaller hydrodynamic volume and thus a lower intrinsic viscosity. According to the reduced quadratic model for the consistency of solutions of EPS from *B. infantis* the effect of the ionic strength depends on the pH. At pH lower than 5 the higher consistencies of these EPS solutions are obtained at higher ionic strengths.

Structure of EPS from *B. infantis* ATCC 15697 has not been reported. However, a polysaccharide from the cell wall of this bacterium has been purified (Habu, Nagaoka, Yokokura, & Azuma, 1987). This polysaccharide seems to have an octasaccharide repeating unit with β-galactofuranoside and three branches.

EPS synthesized by different *S. thermophilus* strains differ in their molecular weight, ranging from 10 to more than 2000 KDa (Vaningelgem et al., 2004). Differences between consistencies of solutions of EPS from different strains were assigned to differences in their masses and structures (Vaningelgem et al., 2004). Primary structure of EPS secreted by different *S. thermophilus* strains have been elucidated (Bubb, Urashima, Fujiwara, Shinnai, & Ariga, 1997; Doco et al., 1990; Faber, Zoon, Kamerling, & Vliegenthart, 1998; Faber, van den Haak, Kamerling, & Vliegenthart, 2001; Faber, van Haaster, Kamerling, & Vliegenthart, 2002; Lemoine et al., 1997; Marshall et al., 2001; Nordmark, Yang, Huttunen, & Widmalm, 2005; Vincent, Faber, Neeser, Stingele, & Kamerling, 2001). None of the reported structure contains charged groups. However, the EPS produced by *S. thermophilus* S8 has the ability to form a lactone in the repeating unit. It was suggested that this capability might alter the physical properties in response to pH (Faber et al., 2002). Our results showed that consistency of solutions of EPS from *S. thermophilus* Th4 depends on pH and the ionic strength.

On the opposite side, effects of pH and ionic strength are not significant for the consistency of solutions of EPS from *L. delbrueckii*. Viscosity of solutions of EPS from *Lactobacillus delbrueckii* subsp *bulgaricus* NCFB 2483, a related LAB strain, was not affected by the presence of salt (Goh et al., 2005) and it was suggested that this is a neutral polysaccharide. A general feature of the EPS produced by different strains of *L. bulgaricus* subsp *delbrueckii* is that they consist in two fractions of different molecular weights. While the production of the high molecular weight fraction depends, in addition to the strain, on the culture conditions, the low molecular weight fraction would be produced more continuously (Grobben et al., 1997; Petry et al., 2003).

4.1. Conclusions

The surface response methodology allows to identify the effects of environmental conditions (pH and ionic strength), that of the EPS concentration and their interactions on the rheological parameters of aqueous solutions of the EPS synthesized by three LAB. Consistency of aqueous solutions of the EPS from *L. delbrueckii* can be explained in terms of the concentration of the polysaccharide. Nevertheless, the flow behavior index of these EPS solutions depends on pH and ionic strength. On the other hand, rheological parameters of solutions of EPS from *B. infantis* and *S. thermophilus* are explained, in addition to the concentration of the polysaccharide, in terms of pH and ionic strength. These results suggest that the EPS synthesized by these bacteria are polyelectrolytes. Results confirm the large variability in the viscosifing properties of EPS produced by LAB. Contour plots generated from the fitted models allow to define the pH–ionic strength regions at which the higher consistency and lower flow behavior index of the EPS solutions are obtained. The contour plots show that consistency and flow behavior index of aqueous solutions of EPS from *B. infantis* and *S. thermophilus* are inversely related. pH–ionic strength regions that define higher consistencies are also the regions at which lowest flow behavior index are obtained.

Since regions for highest consistencies are different for the different EPS, it could be expected that mixtures of these EPS show rheological properties that differ from that of the components. The study of the rheological behavior of mixtures of EPS from different sources is now under investigation.

Acknowledgement

Authors acknowledge the financial support given by Conicyt through Fondecyt project 1050602.

References

Bradford, M. M. (1976). A rapid and sensitive method for quantification of microgram quantities of protein utilizing the principles of protein dye binding. *Analytical Biochemistry, 72*, 248–254.

Bubb, W. A., Urashima, T., Fujiwara, R., Shinnai, T., & Ariga, H. (1997). Structural characterisation of the exocellular polysaccharide produced by *Streptococcus thermophilus* OR 901. *Carbohydrate Research, 301*, 41–50.

De Vuyst, L., Zamfir, M., Mozzi, F., Adriany, T., Marshall, V., Degeest, B., et al. (2003). Exopolysaccharide-producing *Streptococcus thermophilus* strains as functional starter cultures in the production of fermented milks. *International Dairy Journal, 13*, 707–717.

Doco, T., Wieruszeski, J. M., Fournet, B., Carcano, D., Ramos, P., & Loones, A. (1990). Structure of an exocellular polysaccharide produced by *Streptococcus thermophilus*. *Carbohydrate Research, 198*, 313–321.

Dubois, M., Giles, K. A., Hamilton, J. K., Reberts, P. A., & Smith, F. (1956). Colorimetric method for determination of sugar and related substances. *Analytical Chemistry, 28*, 349–356.

Faber, E. J., van den Haak, M. J., Kamerling, J. P., & Vliegenthart, J. F. G. (2001). Structure of the exopolysaccharide produced by *Streptococcus thermophilus* S3. *Carbohydrate Research, 331*, 173–182.

Faber, E. J., van Haaster, D. J., Kamerling, J. P., & Vliegenthart, J. F. G. (2002). Characterization of the exopolysaccharide produced by *Streptococcus thermophilus* 8S containing an open chain nononic acid. *European Journal of Biochemistry, 269*, 5590–5598.

Faber, E. J., Zoon, P., Kamerling, J. P., & Vliegenthart, J. F. G. (1998). The exopolysaccharides produced by *Streptococcus thermophilus* Rs and Sts have the same repeating unit but differ in viscosity of their milk cultures. *Carbohydrate Research, 310*, 269–276.

Goh, K. K. T., Hemar, Y., & Singh, H. (2005). Viscometric and static light scattering studies on an exopolysaccharide produced by Lactobacillus delbruekii subspecies bulgaricus NCFB 2483. *Biopolymers, 77*, 98–106.

Gorret, N., Renard, C. M. G. C., Famelart, M. H., Maubois, J. L., & Doublier, J. L. (2003). Rheological characterization of the EPS produced by *P. acidi-propionici* on milk microfiltrate. *Carbohydrate Polymers, 51*, 149–158.

Grobben, G. J., van Casteren, W. H. M., Schols, H. A., Oosterveld, A., Sala, G., Smith, M. R., et al. (1997). Analysis of the exopolysaccharide produced by *Lactobacillus delbrueckii* subsp *bulgaricus* NCFB 2772. *Applied Microbiology Biotechnology, 48*, 516–521.

Habu, Y., Nagaoka, M., Yokokura, T., & Azuma, I. (1987). Structural studies of cell wall polysaccharides from *Bifidobacterium breve* YIT 4010 and related *Bifidobacterium* species. *Journal Biochemistry, 102*, 1423–1432.

Higashimura, M., Mulder-Bosman, B. W., Reich, R., Iwasaki, T., & Robijn, G. W. (2000). Solution properties of villan, the exopolysaccharides from *Lactococcus lactis* subsp *cremoris* SBT 0495. *Biolpolymers, 54*, 143–158.

Lemoine, J., Chirat, F., Wieruszeski, J. M., Strecker, G., Favre, N., & Neeser, J. R. (1997). Structural characterization of the exocellular polysaccharides produced by *Streptococcus thermophilus* SFi39 and SFi12. *Applied Environmental Microbiology, 63*, 3512–3518.

Marshall, V. M., Dunn, H., Elvin, M., McLay, N., Gu, Y., & Laws, A. P. (2001). Structural characterisation of the exopolysaccharide produced by *Streptococcus thermophilus* EU20. *Carbohydrate Research, 331*, 413–422.

Navarini, L., Abatangelo, A., Bertocchi, C., Conti, E., Bosco, M., & Picotti, F. (2001). Isolation and characterization of the exopolysaccharides produced by *Streptococcus thermophilus* SFi20. *International Journal Biological Macromolecules, 28*, 219–226.

Nordmark, E. L., Yang, Z. N., Huttunen, E., & Widmalm, G. (2005). Structural studies of an exopolysaccharide produced by *Streptococcus thermophilus* THS. *Biomacromolecules, 6*, 105–108.

Petry, S., Furlan, S., Waghorne, E., Saulnier, L., Cerning, J., & Maguin, E. (2003). Comparison of the thickening properties of four *Lactobacillus delbrueckii* subsp *bulgaricus* strains and physicochemical characterization of their exopolysaccharides. *FEMS Microbiology Letters, 221*, 285–291.

Ruas-Madiedo, P., Hugenholtz, J., & Zoon, P. (2002). An overview of the functionality of exopolysaccharide produced by lactic acid bacteria. *International Dairy Journal, 12*, 163–171.

Smithrød, D., & Haug, A. (1971). Estimation of the relative stiffness of the molecular chain in polyelectrolytes from measurements of viscosity at different ionic strengths. *Biopolymers, 12*, 1213–1219.

Tuinier, R., Oomen, C. J., Zoon, P., Cohen Stuart, M. A., & de Kruif, C. G. (2000). Viscoelastic properties of an exocellular polysaccharide produced by a Lactococcus lactis. *Biomacromolecules, 7*, 219–223.

Tuinier, R., van Casteren, W. H. M., Looijesteijn, P. J., Schols, H. A., Voragen, A. G. J., & Zoon, P. (2001). Effects of structural modification on some physical characteristics of exo-polysaccharides from Lactococcus lactis. *Biopolymers, 59*, 160–166.

Tuinier, R., Zoon, P., Cohen Stuart, M. A., Fleer, G. J., & de Kruif, C. G. (1999). Concentration and shear rate dependence of the viscosity of an exocellular polysaccharide. *Biopolymers, 50*, 641–646.

Van den Berg, D. J. C., Robijn, G. W., Janssen, A. C., Giuseppin, M. L. F., Vreeker, R. V., Kamerling, J. P., et al. (1995). Production of a novel extracellular polysaccharide by *Lactobacillus sake* 0-1 and characterization of the polysaccharide. *Applied Environmental Microbiology, 61*, 2840–2844.

Vaningelgem, F., Zamfir, M., Mozzi, F., Adriany, T., Vancanneyt, M., Swings, J., et al. (2004). Biodiversity of exopolysaccharides produced by *Streptococcus thermophilus* strains is reflected in their production and their molecular and functional characteristics. *Applied Environmental Microbiology, 70*, 900–912.

Vincent, S. J. F., Faber, E. J., Neeser, J. R., Stingele, F., & Kamerling, J. P. (2001). Structure and properties of the exopolysaccharide produced by *Streptococcus macedonicus* Sc136. *Glycobiology, 11*, 131–139.

Yang, Z. N., Huttunen, E., Staaf, M., Widmalm, G., & Tenhu, H. (1999). Separation, purification and characterization of extracellular polysaccharide produced by slime-forming *Lactococcus lactis* ssp *cremoris* strains. *International Dairy Journal, 9*, 631–638.

Yang, A., Staaf, M., Huttunen, F., & Widmalm, G. (2000). Structure of a viscous exopolysaccharide produced by *Lactobacillus helveticus* K16. *Carbohydrate Research, 329*, 465–469.