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Exopolysaccharide-producing *Streptococcus thermophilus* strains as functional starter cultures in the production of fermented milks

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Abstract

Relationships between exopolysaccharide (EPS) production (amount, molecular mass and sugar composition of the EPS) by different *Streptococcus thermophilus* strains as a functional starter culture, and textural characteristics (viscosity) of fermented milk and yoghurt have been studied. Five interesting heteropolysaccharide-producing strains have been tested. Both *S. thermophilus* LY03 and *S. thermophilus* CH101 produced the highest amounts of EPS and also displayed the highest apparent viscosities in fermented milk. *S. thermophilus* ST 111 and *S. thermophilus* STD differed considerably in EPS yields, but not in apparent viscosities of fermented milk. In addition, *S. thermophilus* ST 111 displayed a high variability in EPS amounts when cultivated in milk. In milk medium, *S. thermophilus* LY03 produced two heteropolysaccharides, a high-molecular-mass (HMM) EPS and a low-molecular-mass (LMM) EPS of the same composition (Gal/Glu/GalNAc = 3.4:1.4:1.0). *S. thermophilus* ST 111 produced only a HMM-EPS (Gal/Rha = 2.5:1.0), while *S. thermophilus* CH 101 (Gal/Glu = 1.0:1.0), *S. thermophilus* ST 113 (Gal/Glu/Rha/GalNAc = 1.7:3.9:1.5:1.0) and *S. thermophilus* STD (Gal/Glu/Rha/GalNAc = 3.5:6.2:1.2:1.0) produced only LMM-EPS. Both HMM-EPS and LMM-EPS solutions (*S. thermophilus* LY03) demonstrated a pseudoplastic character; HMM-EPS solutions of 0.2% (m/v) displayed a high consistency as well. Although its production of high EPS amounts, *S. thermophilus* LY03 resulted in relatively thin yoghurts, so that texture values did not directly correlate with EPS production capacity. Once structure/function relationships are known, one can determine the molecular properties of the isolated and purified EPS (molecular size, structural characteristics) from candidate strains to predict their potential in texture formation. For a final selection of interesting EPS-producing starter strains one should test the EPS production under yoghurt manufacturing conditions.

Keywords: *Streptococcus thermophilus*; Exopolysaccharides; Functional starter cultures; Yoghurt

1. Introduction

Lactic acid bacteria (LAB) play an important role in food fermentation processes (Wood, 1997). They are very useful in the food industry, not only because of their ability to acidify and hence preserve foods from spoilage, but also for their involvement in the texture, flavour, and aroma development of the fermented food products. A recent trend is the production and application of targeted starter cultures that posses at least one inherent functional property that contributes to the organoleptical, technological, nutritional, or health properties of the fermented food. LAB producing exopolysaccharides (EPS) are a typical example of the so-called functional starter cultures (De Vuyst, 2000). The in situ use of GRAS (Generally Recognised As Safe), food-grade, EPS-producing LAB strains as functional starter cultures in fermented food products is preferred to the addition of texturisers to the food product.

Yoghurt is produced by inoculation of fortified cows' milk with *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus* as starter culture in a 1:1

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ratio. To reduce the amount of added milk solids, to improve yoghurt viscosity, to enhance texture and mouthfeel, and to avoid syneresis during fermentation or upon storage of the yoghurt, EPS-producing, functional starter cultures are interesting (De Vuyst, de Vin, Vaningelgem, & Degeest, 2001). Both *L. delbrueckii* subsp. *bulgaricus* and *S. thermophilus* strains are known to produce heteropolysaccharides, these are extracellularly secreted sugar polymers that are composed of an intracellularly synthesized repeating unit (Cerning, 1990, 1995; De Vuyst & Degeest, 1999; Marshall & Rawson, 1999; De Vuyst et al., 2001).

LAB heteropolysaccharides 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., 2001). The repeating units most often contain a combination of D-glucose, D-galactose, and L-rhamnose, albeit in different ratios. In a few cases, fucose, ribose, acetylated amino sugars (e.g. *N*-acetyl-galactosamine), glucuronic acid, or nononic acid, as well as non-carbohydrate constituents such as phosphate, acetate, and glycerol are present (De Vuyst & Degeest, 1999; De Vuyst et al., 2001; Ruas-Madiedo, Hugenholtz, & Zoon, 2002a). The molecular mass of the EPS ranges from 10 kDa to more than 1000 Da (Cerning, 1995; De Vuyst & Degeest, 1999; De Vuyst et al., 2001). Importantly, both structure and molecular mass influence the rheological properties of a polysaccharide (Faber, Zoon, Kamerling, & Vliegenthart, 1998; Tuinier et al., 2001; Ruas-Madiedo, Tuinier, Kanning, & Zoon, 2002b). In addition, environmental factors have been shown to influence the yields, monomer composition, and molecular mass of the EPS produced by LAB (Gancel & Novel, 1994a,b; Grobben et al., 1997; De Vuyst, Vanderveken, Van de Ven, & Degeest, 1998; Grobben et al., 1998; De Vuyst & Degeest, 1999; Petry, Furlan, Crepeau, Cerning, & Desmazeaud, 2000). This influence is strain dependent (Degeest, Vaningelgem, & De Vuyst, 2001a).

The aim of this paper was a systematic study of the growth and EPS production characteristics of five different *S. thermophilus* strains, and of the impact of the molecular characteristics and amount of their EPS on the rheological properties of EPS solutions and fermented milk. The species *S. thermophilus* has been chosen given its low production of EPS and its minor role in proteolysis during milk fermentation as compared with *L. delbrueckii* subsp. *bulgaricus*, hence requesting a rational choice of the strain to be implemented in industrial fermentations. Finally, a pilot plant trial has been performed with a selected *S. thermophilus* strain (LY03) to evaluate its performance in yoghurt manufacturing.

2. Materials and methods

2.1. Microorganisms and media

A preliminary screening of 26 *S. thermophilus* strains revealed five strains showing interesting EPS production characteristics. These strains were used throughout this study: *S. thermophilus* LY03 (isolated from an English industrial starter culture; De Vuyst et al., 1998), *S. thermophilus* CH101 (isolated from a Polish industrial starter culture), *S. thermophilus* ST 111 (isolated from an artisan Romanian yoghurt), *S. thermophilus* ST 113 (isolated from an artisan Romanian yoghurt), and *S. thermophilus* STD (isolated from Romanian raw milk). *S. thermophilus* SY60 (commercial yoghurt producer) and *L. delbrueckii* subsp. *bulgaricus* LY58 (low EPS-yielding strain) were used for the pilot plant yoghurt production. All strains were stored at −80°C in MRS medium (Oxoid, Basingstoke, UK) containing 25% (v/v) glycerol as cryoprotectant. To obtain fresh cultures for the experiments the bacteria were propagated at 42°C for 12 h, first in MRS medium (Oxoid), followed by two subcultivations in the medium used later on (see below). The inoculum for the subcultivations and fermentation experiments was always 1.0% (v/v).

The media used in this study were milk medium (10.0% skimmilk powder, m/v; Dairy Industry Inco, Kallo, Belgium), and enriched milk medium (10.0% skimmilk powder, Dairy Industry Inco; 1.0% peptone, Oxoid; and 0.5% yeast extract, VWR International, Darmstadt, Germany; m/v). The initial pH was always adjusted to pH 6.3. LAPT agar, composed of 1.0% (m/v) yeast extract (VWR International), 1.5% (m/v) peptone (Oxoid), 1.0% (m/v) tryptone (Oxoid), 1.0% (m/v) glucose, 0.1% (v/v) Tween 80, and 1.5% (m/v) agar (Oxoid), was used for cell counting.

2.2. Fermentation experiments and analyses

Bacterial growth and EPS production of different strains was assessed after 12 h of fermentation in 1-L flasks that contained milk medium or enriched milk medium. Incubations were performed under static conditions without pH control. Fermentations took place at 42°C for 12 h. Measurements of pH (acidifying properties) and cell counts (after plating on LAPT agar) were performed. The number of viable cells was expressed as colony forming units (CFU) per mL. The isolation of EPS from fermented (enriched) milk medium was performed using the two-step precipitation protocol outlined by De Vuyst et al. (1998). Distinction was made between floating and non-floating EPS material for molecular purposes (Degeest & De Vuyst, 1999). Total EPS amounts were determined gravimetrically by measuring the polymer dry mass (PDM) after 48 h of drying at 42°C.

To correlate EPS production and viscosity, fermentations were performed in a 7.5-L laboratory fermentor (Biostat CT; B. Braun Biotech International, Melsungen, Germany), filled with 5 L of milk medium. Fermentations were carried out with free pH at 42°C and continuous stirring at 100 rpm to keep the fermentation medium homogeneous. Samples were taken after 12 h of fermentation to determine apparent viscosity (see below) and amount of EPS produced (as PDM; see above).

Small-scale fermentations were carried out to get additional information concerning EPS yield and texture characteristics (firmness, consistency, cohesiveness, resistance to flow, viscosity, pH) for the yoghurt manufacture (see below). Twice steam sterilised skim milk (16% and 15% of total solids for the single and mixed cultures, respectively; m/v) was used, 16 h fermentations were employed, 24 h storage at 4°C, and all experiments were performed in triplicate. A single culture consisted of *S. thermophilus* LY03 or *S. thermophilus* SY60 only; a mixed culture was a combination of *S. thermophilus* LY03 and *L. delbrueckii* subsp. *bulgaricus* LY58 in a 3:1 ratio of cocci:rods with respect to inoculation percentage.

2.3. Determination of EPS molecular mass

EPS isolations were performed on 1-L aliquots of fermented milk medium (see above). For further purification, isolated EPS were dissolved in ultra pure water (MilliQ; Millipore Corp., Bedford, Massachusetts, USA), adjusted to pH 7.0. They were dialysed against distilled water at 4°C during 7 days with water replacement twice a day, using Spectra/Por membranes (VWR International) with a molecular-mass-cut-off (MMCO) of 3500 kDa. The purified EPS were lyophilised, dissolved in potassium phosphate buffer (50 mM, pH 6.8) at a concentration of maximally 50 mg lyophilised EPS per mL, and the molecular mass was determined by gel permeation chromatography (GPC), making use of a 1.6 × 100 cm Sephacryl S-400 column (Amersham Biosciences AB, Uppsala, Sweden). A dextran standard series (molecular masses of 80, 150, 270, 670, and 1800 kDa; Sigma, St. Louis, Missouri, USA) was used as molecular size marker. A 50 mM potassium phosphate/NaOH buffer (pH 6.8) with 0.15 M NaCl was used for elution, at a flow rate of 1.0 mL min^{-1}. The polysaccharide content was determined on line with a Waters 2410 Refractive Index Detector (Waters Corp., Milford, Massachusetts, USA) at an internal temperature of 40°C.

2.4. Determination of EPS composition and structure

Monomer analysis of purified EPS was carried out by acid hydrolysis followed by High Performance Anion Exchange Chromatography with Pulsed Amperometric Detection (HPAEC-PAD) as described previously (Degeest, Vaningelgem, Laws, & De Vuyst, 2001b). Structure elucidation was performed by one-dimensional NMR spectroscopy as described previously (Degeest et al., 2001b).

2.5. Apparent viscosity measurements

Apparent viscosity measurements were carried out on 0.5-mL samples, using a cone-plate Brookfield Digital Rheometer Model DV III (Brookfield Engineering Laboratories Inc., Stoughton, Massachusetts, USA). The rheometer was equipped with a flat spindle, type CP 40 (Brookfield Engineering Laboratories Inc.), that rotated in a sample-containing chamber connected to a temperature-controlled cryostat water bath (Thermomix®, B. Braun Biotech International). The rheometer was controlled with the Brookfield Rheocalc software (Brookfield Engineering Laboratories Inc.).

Apparent viscosity measurements of the fermented milk samples were performed at 42°C. Spindle speeds of 0, 10, 15, 20, 30, 50, 70, 90, 100, and 120 rpm were applied during 90 s, provided that the torque to rotate the spindle in the fluid was between 15.0% and 85.0% of the maximum torque (0.0673 mN m). Values out of this range were not valid and resulted in misinterpretation of the data. Therefore, to compare the samples between the different strains a spindle speed of 50 rpm was chosen. It corresponded with a shear rate of 375 s^{-1}. All viscosity measurements were expressed in mPa s and performed in triplicate, averaged, and standard deviations were determined.

To compare the rheology of purified EPS solutions, crude EPS solutions were adjusted to pH 7.0, extensively dialysed against MilliQ for 7 days, using membranes with a MMCO of 1000 Da. After dialysis, the EPS solutions were freeze-dried, and redissolved in MilliQ. To have an idea of the purity of the EPS material, the percentage of proteins was determined applying a Bio-Rad DC protein assay kit (Bio-Rad Laboratories, Hercules, California, USA). The protein concentration of all EPS samples was less than 3.0%. All measurements were performed at 25°C. First, a series of short measurements (90 s) was performed on solutions with varying EPS concentrations to determine the range of possible programmable measurements, that is a range where the torque was comprised between 15.0% and 85.0% of the maximum torque. EPS concentrations were expressed as glucose equivalents that were measured colorimetrically according to the Anthron method (Scott & Melvin, 1953). It turned out that an EPS concentration of 0.3% (m/v) was the only concentration for which the viscosity of all EPS fractions could be measured using the CP40 spindle (data not shown). Second, at this concentration of 0.3%

(m/v), programmable measurements were carried out by gradually increasing the spindle velocity with a constant step after a constant time interval (90 s) to find a shear rate where both high-molecular-mass EPS (HMM-EPS) and low-molecular-mass EPS (LMM-EPS) solutions could be compared, and to build flow curves. If possible, measurements were programmed to build flow curves composed of 10–20 experimental points. The shear rate was increased with a constant step of 2.5 and 25 s^{-1} for the HMM-EPS and LMM-EPS solutions, respectively. To return to low shear rates, one single measuring point was taken into account at minimum shear rate. The latter was 2.5 and 25 s^{-1}, respectively. Third, to compare solutions of different EPS from different strains, excluding the influence of shear rate, the consistency of EPS solutions at a concentration that was strain dependent (to fall within the range of 15.0–85.0% of the maximum torque) was calculated based on the power-law model (Walter, 1998). Guar gum from the leguminous plant *Cyamopsis tetragonolobus* (molecular mass of approximately 220 kDa; Sigma) was used as reference polysaccharide during these rheological experiments. Guar gum is a texturiser that is often used in yoghurt manufacture.

2.6. Yoghurt manufacture

Yoghurts were prepared using a 'miniplant' available at a commercial dairy (Uniq plc., Wootton Basset, Wiltshire, UK). The milk used was the standard factory recipe without stabilisers (15% skimmilk powder, hydrated for 1 h, preheated to 65°C, homogenised at 138 bar, pasteurised at 95°C for 5 min, and cooled to 43°C). Three 5-L vessels were used and inoculated (1%, v/v) with a mixed culture of *S. thermophilus* LY03 or *S. thermophilus* SY60, combined with *L. delbrueckii* subsp. *bulgaricus* LY58 in a 3:1 ratio of cocci:rods (v/v of hydrated lyophilised culture). The temperature of the fermentation vessels was maintained at 43°C and the pH was monitored until the pH value fell to 4.45–4.50, at which point the yoghurt was pitched and cooled to 16–18°C within the miniplant. The yoghurts were then packed into plastic containers (5 kg), stored below 4°C, and transported in boxes containing dry ice for texture and sensory evaluation. Two further replicate experiments were carried out over the following two weeks.

Texture analysis and apparent viscosity measurements were carried out using the TA-XT2 texture analyser (Stable Microsystems, Surrey, UK) and the Brookfield viscometer Model DV II+ connected with Wingather software version 1.1 (Brookfield Viscometers Ltd., Harlow, UK), respectively. Texture analysis was performed on 100 g of yoghurt sample in a cylinder (diameter of 48 mm) with a 45-mm disc. The tests were performed with the measuring force in compression mode and a measuring cycle of 30 s was set. The apparent viscosities were measured at 1.5 rpm after 90 s of testing. Four days following the date of manufacture, a trained panel carried out the sensory evaluation. Therefore, 11 assessors (five male and six female between 18 and 60 yr old) were trained over a period of 7 weeks, including a pre-test assessment to evaluate each of the yoghurt samples. Selection criteria were based on those described by Meilgaarde, Civille, and Carr (1991). Testing was carried out in sensory booths, and approximately 50 g of randomly coded samples were presented in clear plastic containers, straight from the fridge (4°C). Assessors were asked to make a mark on an unstructured, anchored line (0–100 mm long). The anchors were determined using known reference samples, which were also included in each of the evaluation sessions. The samples were first visually evaluated for smoothness, viscosity, ropiness and stickyness, and results were compared and statistically analysed. For statistical analysis 79 data points were included. Panellists were then asked to assess texture characteristics (see above) by determining the texture of the product when introduced into the mouth. Means were determined from three replicates from each of the three different experiments. Paired *t*-tests were carried out on the mean values to determine differences of significance.

3. Results

3.1. Growth and EPS production in milk and enriched milk media

Growth of all *S. thermophilus* strains was enhanced in enriched milk medium; a lower pH and higher cell counts were obtained (Table 1). Also, a higher concentration of EPS for the five *S. thermophilus* strains tested was observed in enriched milk medium compared with milk medium, except for *S. thermophilus* LY03. However, for the latter strain not all EPS was recovered from the medium. Although it has been shown before that the higher EPS content in enriched milk medium is also due to the presence of glucomannans derived from the complex medium ingredients yeast extract and peptone (Degeest et al., 2001b), both yeast extract and peptone stimulated growth and hence growth-associated EPS production, confirming earlier observations (Degeest & De Vuyst, 1999). Differences in EPS yields were not reflected in the concomitant apparent viscosities (Table 2). However, both *S. thermophilus* LY03 and *S. thermophilus* CH101 produced the highest amounts of EPS and also displayed the highest apparent viscosities in fermented milk. On the other hand, *S. thermophilus* ST 111 and *S. thermophilus* STD differed considerably in EPS yields, but not in apparent viscosities of fermented milk. In addition, *S. thermophilus* ST 111

Table 1
Growth and exopolysaccharide production characteristics of different *Streptococcus thermophilus* strains in milk medium and in enriched milk medium

Streptococcus thermophilus strain	Milk medium			Enriched milk medium			Molecular mass[a] (kDa)
	pH after 12 h	Cell count (CFU mL^{-1})	Total EPS (mg PDM L^{-1})	pH after 12 h	Cell count (CFU mL^{-1})	Total EPS (mg PDM L^{-1})	
LY03	4.5	1.0×10^7	29	4.1	3.3×10^8	32[b]	HMM (1800) LMM (470)
CH101	4.4	5.6×10^8	60	4.5	6.3×10^8	185	LMM (850)
ST 111	4.6	2.5×10^8	50	4.4	4.7×10^9	173	HMM (> 2000)
ST 113	4.6	3.0×10^7	23	4.7	4.1×10^8	284	LMM (210)
STD	4.8	1.5×10^7	<5	4.5	1.5×10^8	144	LMM (4)

CFU=colony forming units; EPS=exopolysaccharides; PDM=polymer dry mass; HMM=high molecular mass; LMM=low molecular mass.
[a] The molecular mass of the EPS was revealed by gel permeation chromatography (GPC) (see Materials and Methods).
[b] Both floating and non-floating EPS material appeared after the first and second precipitation step, but only the floating EPS material was measured.

Table 2
Exopolysaccharide production and apparent viscosity of fermented milk medium for different *Streptococcus thermophilus* strains

S. thermophilus strain	Total EPS (mg PDM L^{-1})	Apparent viscosity (mPa s) (SD)
LY03	30	3.3 (0.3)
CH101	72	3.0 (0.2)
ST 111	18	2.2 (0.1)
ST 113	17	1.7 (0.1)
STD	<5	2.1 (0.5)

displayed a high variability in EPS amounts when cultivated in milk (Tables 1 and 2).

3.2. Molecular mass determination of the EPS produced

In milk medium, *S. thermophilus* LY03 produced two heteropolysaccharides of a different size, corresponding with the recovered floating and non-floating EPS material, respectively. Both heteropolysaccharides possessed the same monomer composition (see below). GPC of the floating and non-floating EPS material resulted in a molecular mass of 1800 kDa for the HMM-EPS and 470 kDa for the LMM-EPS, respectively (Table 1).

S. thermophilus ST 111 produced a HMM-EPS corresponding with the floating EPS material recovered from fermented milk medium and a molecular mass of >2000 kDa as revealed by GPC (Table 1). When this strain was grown in enriched milk medium, two peaks were detected in the chromatogram, one corresponding with a HMM-EPS (>2000 kDa) and one that corresponded with a MM of 75 kDa. The latter peak was absent when the strain was grown in milk medium. It contained the polysaccharide material derived from the medium components of enriched milk medium, in particular glucomannans with a molecular size around 75 kDa (Degeest et al., 2001a).

S. thermophilus CH101 and *S. thermophilus* ST113 produced LMM-EPS corresponding with the non-floating EPS material recovered from fermented enriched milk medium and a molecular mass of 850 and 210 kDa, respectively, as revealed by GPC (Table 1). The peak of the 210-kDa EPS and the contaminating medium polysaccharides (75 kDa) could not be separated. However, after comparing the monomer composition of different eluted fractions in the molecular mass range 14–1700 kDa with the monomer composition of the native EPS material isolated from fermented milk medium (and hence not contaminated with glucomannans), the 210-kDa peak could be allocated to the LMM-EPS produced by *S. thermophilus* ST113.

S. thermophilus STD produced no HMM-EPS neither in milk medium nor in enriched milk medium. Only a LMM-EPS (4 kDa), derived from fermented milk medium, could be shown by GPC (Table 1).

3.3. Monomer composition of the EPS produced

The composition of the five heteropolysaccharides studied, as revealed by acid hydrolysis followed by HPAEC-PAD, differed. EPS from *S. thermophilus* CH101 consisted of only galactose and glucose in a 1.0:1.0 ratio, while EPS from *S. thermophilus* ST 111 consisted of galactose and rhamnose in a 2.5:1.0 ratio. The EPS from *S. thermophilus* LY03 was composed of galactose, glucose, and *N*-acetyl-galactosamine in a 3.4:1.4:1.0 ratio; no difference was observed between HMM-EPS and LMM-EPS. EPS from *S. thermophilus* ST113 and *S. thermophilus* STD consisted of galactose, glucose, rhamnose, and *N*-acetyl-galactosamine in a ratio of 1.7:3.9:1.5:1.0 and 3.5:6.2:1.2:1.0, respectively.

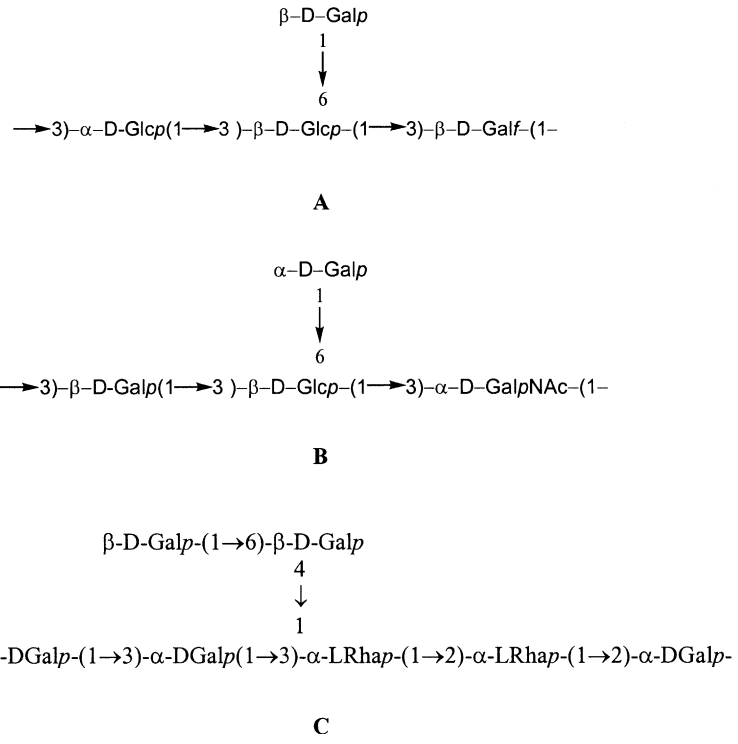

Fig. 1. Structure of the repeating unit of the heteropolysaccharides produced by *S. thermophilus* STD and *S. thermophilus* CH101 (A), *S. thermophilus* LY03 (B), and *S. thermophilus* ST 111 (C).

3.4. Structure elucidation of the EPS produced

The ^{1}H-NMR spectra recorded for the heteropolysaccharides of *S. thermophilus* CH101 and *S. thermophilus* STD (data not shown) were the same as those from *S. thermophilus* Sfi39 (Lemoine et al., 1997). The spectra for the EPS of *S. thermophilus* LY03 and *S. thermophilus* ST 111 (data not shown) were the same as the ones reported for the EPS from *S. thermophilus* CNCMI 733 (Doco et al., 1990) and *S. thermophilus* OR901 (Bubb, Urashima, Fujiwara, Shinnai, & Ariga, 1997), respectively. The structures of the EPS subunits are represented in Fig. 1. The structure of the heteropolysaccharide of *S. thermophilus* ST 113 has not been elucidated.

3.5. Rheological properties of purified EPS solutions

At one defined shear rate of 7.5 s^{-1}, the apparent viscosity of a 0.3% (m/v) solution of the HMM-EPS from *S. thermophilus* LY03 was higher (113.1 mPa s) than that of its LMM-EPS (50 mPa s). The latter was lower than that of guar gum (77.5 mPa s) while the molecular mass of the LMM-EPS of *S. thermophilus* LY03 was higher (470 kDa) than the molecular mass of the guar gum (220 kDa). This confirms the general statement that there is no straightforward relationship between the molecular mass and the solution viscosity of any polysaccharide, including guar.

When the apparent viscosity was measured as a function of shear rate, both HMM-EPS and LMM-EPS solutions (*S. thermophilus* LY03) as well as the guar gum solution clearly demonstrated a pseudoplastic or 'shear-thinning' character, that is a less pronounced (non-linear) decrease of the apparent viscosity for increasing shear rates (Fig. 2). All EPS solutions tested also displayed a thixotropic character, that is a decrease in apparent viscosity after a certain time at a constant shear rate. When returning to the initial state at low shear the apparent viscosities did not reach their original values (Fig. 2). Similar flow curves were obtained for all EPS from all strains tested as well as for guar gum.

Finally, the consistencies of different EPS solutions were extrapolated from the flow curves, using a power-law model (Table 3). The highest consistencies were found for guar gum and EPS from *S. thermophilus* LY03 (0.2% HMM-EPS solutions). A ten-fold increase in concentration of LMM-EPS resulted in a similar range of the consistency for EPS solutions from *S. thermophilus* ST 113. At a concentration of 0.2%, *S. thermophilus* ST 111 HMM-EPS displayed a lower consistency. A high concentration of EPS (LMM-EPS, 3.0%) from *S. thermophilus* CH101 and *S. thermophilus* STD displayed a low-range consistency.

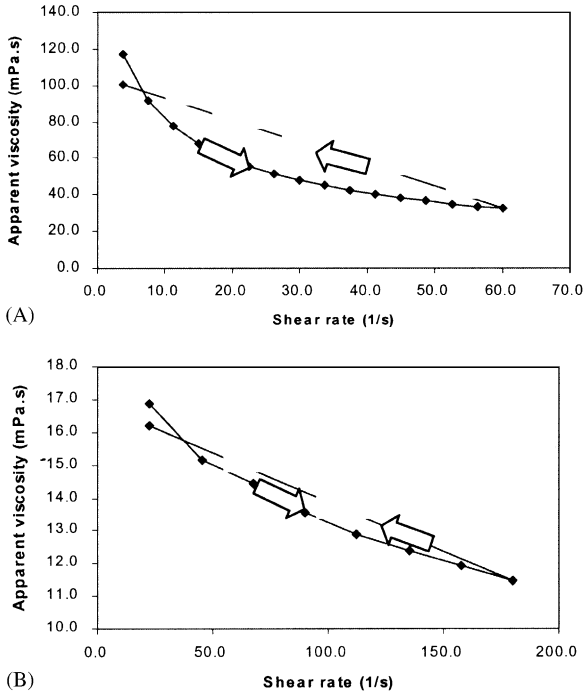

Fig. 2. Flow curve of a 0.3% (m/v) solution of the HMM (A) and the LMM (B) heteropolysaccharide from *S. thermophilus* LY03, indicating a pseudoplastic, thixotropic behaviour. The arrows indicate the direction of the flow curve measurements. The broken line represents the return of the shear rate (one step) to the sole single measuring point corresponding with its initial value (minimum shear rate). The minimum shear rate applied on the HMM and LMM EPS solutions was 2.5 s^{-1} and 25 s^{-1}, respectively. The shear rate was increased with a constant step of 2.5 s^{-1} and 25 s^{-1}, respectively.

Table 3
Consistencies of different EPS solutions. The consistency was calculated from the intercept of the linearised values of the shear stress as a function of increasing shear rates using the power-law model

Origin of EPS	EPS concentration as % glucose equivalents	Consistency in mPa s (SD)
LY03	0.2	829 (27)
CH101	3.0	13 (3)
ST 111	0.2	36 (16)
ST 113	2.0	240 (9)
STD	3.0	3 (0)
Guar gum	0.2	1308 (93)

3.6. Yoghurt manufacture

Yoghurt manufacture was performed with *S. thermophilus* LY03 because of its high production of a HMM-EPS and its high EPS amounts. Statistical analysis (ANOVA) showed that there was no difference between the batches (data not shown). The pH was well controlled and consistent and made no contribution to any differences observed. There was no difference between the texture attributes of the pilot plant samples as measured by the TA-XT2 texture analyser, but viscosity using the Brookfield viscometer was significantly higher for the pilot scale samples using *S. thermophilus* SY60 (Table 4).

Concerning the visual/physical evaluation, the samples were immediately perceived as different concerning viscosity and ropiness (based on stirring of the sample and lifting it with a spoon) when compared with the reference samples. They were less viscous and seemed to be non-ropy; there were no lumps or graininess. However, there was no significant difference in the smoothness and stickyness of the pilot plant samples (Table 4). Only a small difference could be detected for both viscosity and ropiness. That produced from *S. thermophilus* SY60 was more viscous and ropy than that produced from *S. thermophilus* LY03.

Concerning the sensory evaluation (Table 4), the panellists found the pilot plant samples similar for all attributes. Panellists could not detect differences between the products in terms of smoothness, creaminess, and mouthfeel. Results were not significantly different for any values. The samples however were unexpectedly thin. There were few lumps and the samples were smooth and showed no wheying off. A visual evaluation only was able to discern differences between the pilot plant samples fermented with *S. thermophilus* LY03 and with *S. thermophilus* SY60 (see above). Some differences were picked up from mouthfeel evaluation also, but these were less clear. It was anticipated that EPS would be present in the samples although it was not measured at the time. However, EPS content of products of small-scale fermentations using mixed cultures which included *L. delbrueckii* subsp. *bulgaricus* LY58 showed that production of EPS from strains of *S. thermophilus* LY03 was maintained or even enhanced (Table 4). It was also seen that texture values did not directly correlate with the quantity of the EPS produced (data not shown).

4. Discussion

The functional behaviour of heteropolysaccharide-producing strains of *S. thermophilus* grown in milk medium differs considerably. First of all, EPS production yields in milk medium are low and strain dependent. In milk, the production of EPS by the *S. thermophilus* strains tested never reached 100 mg of PDM L^{-1}, unless in a mixed culture with *L. delbrueckii* subsp. *bulgaricus*. The higher EPS production in a mixed milk culture is well known (Cerning, 1990). However, EPS production by *S. thermophilus* can be enhanced using more complex media (Degeest & De Vuyst, 1999). The better growth and higher EPS production in for instance enriched milk medium reflects both the limited proteolysis of *S. thermophilus* strains and the dependence of both growth and EPS production on nitrogen compounds and particular growth factors such as

Table 4
Texture and sensory characteristics of small-scale and pilot plant yoghurt samples

Yoghurt characteristics	Single culture		Mixed culture	
	LY03	SY60	LY03	SY60
Small-scale fermentations				
EPS yield (mg PDM L^{-1})	13	ND	170	ND
Texture characteristics[a]				
Firmness (g)	16.6	23.0	161.4 (13.3)	ND
Consistency (g s)	158.0	232.6	2548.1 (530.5)	ND
Cohesiveness (g)	3.3	1.7	123.1 (12.5)	ND
Resistance to flow (g s)	43.9	79.6	1744.5 (151.2)	ND
Viscosity (Pa s)	ND	ND	43660.0 (607.6)	ND
pH	4.20	4.40	4.35	ND
Pilot plant fermentations				
Texture characteristics[a]				
Firmness (g)	ND	ND	69.0 (5.5)	70.0 (7.8)
Consistency (g s)	ND	ND	1162.3 (123.5)	1091.1 (231.7)
Cohesiveness (g)	ND	ND	38.9 (4.0)	40.9 (6.7)
Resistance to flow (g s)	ND	ND	615.5 (64.4)	607.5 (152.8)
Viscosity (Pa s)	ND	ND	12829.0 (4636.8)	18317.5 (6157.4)
pH	ND	ND	4.30	4.30
Visual/physical characteristics[b,c]				
Smoothness	ND	ND	29.2 (36.0)	31.2 (13.7)
Viscosity	ND	ND	22.4 (19.4)	29.7 (29.7)
Ropy	ND	ND	16.4 (14.0)	23.8 (25.5)
Sticky	ND	ND	15.9 (20.9)	18.4 (19.2)
Sensory characteristics[b,d]				
Viscosity	ND	ND	31.5 (23.4)	30.0 (21.8)
Adhesiveness	ND	ND	27.0 (20.4)	26.3 (21.7)
Smoothness	ND	ND	59.7 (40.3)	63.0 (40.0)
Thickness	ND	ND	30.2 (24.5)	31.8 (23.0)
Creaminess	ND	ND	52.6 (26.6)	50.9 (25.4)
Mouthfeel	ND	ND	33.0 (25.4)	34.5 (24.7)

Visual/physical and sensory characteristics of pilot plant samples were determined with a trained panel. Standard deviations are indicated between brackets. ND=not determined.

[a] Texture characteristics were determined with a TA-XT2 texture analyser. Firmness is the maximum positive force needed to penetrate the fermented milk. Consistency is the force needed to attain a given deformation during the compression cycle of the plunger (area of positive peak). Cohesiveness is the maximum negative force required to overcome the attractive forces between the surface of the fermented milk and the probe. The resistance to flow is the force necessary to pull the plunger up through the sample (area of the negative peak).

[b] Results were recorded on unstructured anchored line scales (100 mm long). Reference samples had been presented in training, which represented the maximum and minimum anchor points. Figures given in the table are the mean values of perception by the panellists.

[c] Standard deviations were large and may reflect the need for further training of the panellists in assessment. Panellists were inexperienced in material from pilot plant production.

[d] Standard deviations were large, because panellists appeared less confident using 'mouthfeel' to assess differences in texture.

vitamins (Grobben et al., 1998; Degeest & De Vuyst, 1999; Deutsch et al., 2000).

A large biodiversity exists among the known heteropolysaccharides produced by LAB with respect to their monomer composition and molecular mass (De Vuyst et al., 2001). This was confirmed by the analysis of the monomer composition of the EPS from the five *S. thermophilus* strains studied in this paper. The differences in monomer composition, revealed by acid hydrolysis analysis and NMR structure elucidation as reported in this paper, are ascribed to differences in EPS solubility (NMR only detects soluble EPS), EPS recovery (EPS isolation from low producing strains is often hindered by extraction of peptidoglycan-associated cell wall polymers that contain glucose, galactose, rhamnose, and *N*-acetyl-glucosamine), and the underestimation of the amount of acetylated aminosugars due to the harsh acid hydrolysis conditions applied. The biodiversity may be ascribed to the sugar nucleotide biosynthesis routes and genetic potential of these strains (Grobben, Smith, Sikkema, & de Bont, 1996; Stingele, Neeser, & Mollet, 1996; Escalante, Wacher-Rodarte, García–Garibay, & Farrés, 1998; Stingele, Newell, & Neeser, 1999; van Kranenburg, Vos, van Swam, Kleerebezem, & De Vos, 1999; Degeest & De Vuyst, 2000; Degeest et al., 2001b; Mozzi, Rollán, Savoy de Giori, & Font de Valdez, 2001). The potential of genetic or metabolic engineering for directed changes in the EPS

structure and EPS yield is very promising (van Kranenburg et al., 1999; Stingele et al., 1999; Germond, Delley, d'Amico, & Vincent, 2001; Jolly & Stingele, 2001; Jolly, Newell, Porcelli, Vincent, & Stingele, 2002; Levander, Svensson, & Rådström, 2002).

The interpretation of the contribution of heteropolysaccharides to structure/function relationships is very complex. It has been postulated that for high intrinsic viscosities stiffer chains are required, which are more likely in the case of $\beta(1-4)$ linkages (Tuinier et al., 2001). This will in turn lead to a higher consistency of the EPS solutions. Also, the degree of branching may contribute to the stiffness of the polymer (Tuinier et al., 2001). In addition, the complexity of the primary structure (size, monomer composition and side groups, α- and β-linkages, branching) will influence the viscosifying effects of EPS solutions (Yang, Staaf, Huttunen, & Widmalm, 2000; Tuinier et al., 2001). This has been shown in this study for EPS with a similar molecular mass, for instance HMM-EPS from *S. thermophilus* ST 111 and *S. thermophilus* LY03, but a different chemistry. Further, the molecular mass of an EPS is of primary importance for the intrinsic viscosity of the polymer (Faber et al., 1998). Moreover, a positive correlation exists between the molecular mass of the EPS and the consistency of the EPS solution. This has been shown in this study for EPS with an identical structure but a different molecular mass, for instance the LMM-EPS from *S. thermophilus* STD and *S. thermophilus* CH101. Hence, both molecular structure and molecular mass will dictate the functional properties of the EPS as well as the functional behaviour of the EPS-producing strains during milk fermentation. In addition, some strains like *S. thermophilus* ST 111 produce only HMM-EPS, others like *S. thermophilus* CH101, *S. thermophilus* ST 113 and *S. thermophilus* STD produce only LMM-EPS, while still other strains, for instance *S. thermophilus* LY03, produce both types of EPS. They differ in corresponding milk viscosities, and no clear-cut relationship between amount of EPS produced and medium viscosity occurs when comparing different strains. Earlier work on fermented milks also reported the lack of a clear relationship between EPS production and viscosity (Wacher-Rodarte et al., 1993; van Marle & Zoon, 1995; Bouzar, Cerning, & Desmazeaud, 1997; Sebastiani & Zelger, 1998; Ruas-Madiedo et al., 2002b). Finally, the influence of pH on the rheology of the end product may not be overlooked.

Several authors already reported on the existence of LAB strains producing more than one type of EPS with respect to size, depending on the culture conditions used (Grobben et al., 1996; Degeest & De Vuyst, 1999). Grobben et al. (1997) also observed a higher viscosity for media containing the HMM-EPS (MM of 1700 kDa) compared to the LMM-EPS (MM of 440 kDa) as was the case for solutions of the HMM-EPS and LMM-EPS from *S. thermophilus* LY03. Both HMM-EPS and LMM-EPS solutions displayed a pseudoplastic and thixotropic character. However, the LMM-EPS showed a less-pronounced thixotropic character as compared to the HMM-EPS. The shear-thinning behaviour is an important property in view of the role of *S. thermophilus* heteropolysaccharides as biothickener in milk products. However, the occurrence of only HMM-EPS, LMM-EPS, or both types of EPS in milk products fermented with EPS-producing strains may drastically influence the viscosity of the end product. For instance, it has been reported that two different *S. thermophilus* strains producing equal amounts of EPS with an identical composition and structure but with a different molecular mass can differ in their effects on the viscosity of stirred yoghurts due to differences in intrinsic viscosity as mentioned above (Faber et al., 1998). Hence, various EPS-producing strains may enhance the viscosity of yoghurt differently (Sebastiani & Zelger, 1998). As shown in this paper, although its production of an EPS with a high molecular mass with both α- and β-linkages resulting in solutions with high consistency, as well as the high EPS amounts produced, *S. thermophilus* LY03 fermentations resulted in relatively thin yoghurts. Consequently, texture values did not directly correlate with EPS production characteristics of this strain. It is further suggested that complex interactions between the bacterial cells (all or not surrounded by EPS, encapsulated or not), the protein network (spatial structure, interactions, pH, ionic strength, gel strength), and the EPS (localisation, aggregation) play an important role in texture build up (van Marle & Zoon, 1995; Duboc & Mollet, 2001; Hassan, Frank, & Qvist, 2002). For instance, the most important textural characteristics of yoghurt are firmness and the ability to retain water (Zoon, Roefs, de Cindio, & van Vliet, 1990; Rohm & Kovac, 1994; Hassan, Frank, Schmidt, & Shalabi, 1996; Rawson & Marshall, 1997; Marshall & Rawson, 1999). These properties are related with the gel structure and might be influenced by the type of culture and EPS.

Given the complex interactions and no clear-cut relationships of both EPS yields and functional properties, rational selection of EPS-producing starters for fermented milk production is necessary if one wants to influence yoghurt viscosity and texture through in situ EPS production. Concerning heteropolysaccharide-producing strains of *S. thermophilus* this will mainly depend on structure/function relationships of their EPS produced. Molecular size (chain length and molecular mass) and structure (monosaccharide composition, type and strength of the glycosidic linkages, and degree, type, and length of the branches) will certainly play a key role in this process. Once structure/function relationships are known, one can determine the molecular properties of the isolated and purified EPS (molecular size, structural

characteristics) from candidate strains to predict their potential in texture formation. For a final selection of interesting EPS-producing starter strains one should test the EPS production under yoghurt manufacturing conditions.

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