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Rheological characterization of the EPS produced by *P. acidi-propionici* on milk microfiltrate

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Abstract

The EPS produced by *Propionibacterium acidi-propionici* DSM 4900 has been investigated on a biochemical and a rheological basis. The EPS was mainly composed of galactose, mannose, and glucosamine, with traces of glucose, galactosamine and phosphate. It could be fractionated by anion-exchange chromatography into a major (78%) acidic fraction and a minor neutral component. Viscosity determinations in the absence and presence of salt showed a polyelectrolyte behavior and an intrinsic viscosity of 22 dl/g in 0.01 M NaCl. EPS solutions displayed a shear-thinning behavior with limiting zero-shear viscosity (η_0). Variations of the specific limiting viscosity (η_{sp0}) as a function of concentration indicated three concentration regimes with $c^* = 0.2$ and $c^{**} = 2$ g/l. At relatively low concentration (4–18 g/l), G' and G'' showed variations with limiting slopes of 2 and 1, respectively, at low frequency and a crossover of these two moduli at high frequency. Master curves of the flow curves as well as of the viscoelastic functions could be built. At the lowest concentrations, the Cox–Merz rule was obeyed while at the highest ones the superposition of $|\eta^*(\omega)|$ and $\eta(\dot{\gamma})$ curves was obtained only at low frequencies. All these rheological determinations show that aqueous solutions of EPS behave as entangled polymer solutions. The polysaccharide does not display gelling properties and could be used as a highly viscous thickener.

Keywords: Exopolysaccharides; Rheology; Chemical structure

1. Introduction

Polysaccharides are widely used as food thickening and gelling agents. However, these are regarded as additives despite their natural origin. In view of the growing interest in 'safety' food, production of new polysaccharides by food-grade bacteria may be considered. If the polysaccharide is produced in situ during processing of the product, it is not considered as an additional ingredient. At present, the food-grade bacteria known for their ability to produce EPS are dairy bacteria; mainly lactic acid bacteria (Cerning, 1995; Cerning, Bouillanne, Landon, & Desmazeaud, 1990), but also propionibacteria (Crow, 1988; Racine, Dumont, Champagne, & Morin, 1991; Skogen, Reinbold, & Vedamuthu, 1974) and bifidobacteria (Andaloussi, Talbaoui, Marczak, & Bonaly, 1995; Roberts et al., 1995). These investigations were mostly directed towards the biosynthesis, growth conditions and chemical characterization. A wide range of chemical structures has been described, some polysaccharides being neutral while others are polyelectrolytes (Bouzar, 1997; de Vuyst & Degeest, 1999). On the other hand, the rheological characterization of these polysaccharides has received poor attention despite intrinsic viscosity determinations suggest that high molecular weight polysaccharides can be produced (Bouzar, Cerning, & Desmazeaud, 1997; Cerning, 1995; Cerning et al., 1990; Docco, Wieruszeski, & Fournet, 1990; de Vuyst & Degeest, 1999). Actually, most of the rheological characterizations which have been performed in the past 10 years have been devoted to the description of the properties of EPS-containing yoghurt (Bouzar et al., 1997; Hess, Robert, & Ziegler, 1997; Marshall & Rawson, 1999; Rawson & Marshall, 1997; Schellhaass & Morris, 1985; Teggatz & Morris, 1990). However, the role of EPS in the texture of yoghurt is far from being understood.

Recently, a polysaccharide produced by *Lactococcus lactis* ssp. *cremoris* B40 has been investigated in detail. This

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polysaccharide is composed of galactose, glucose, rhamnose and phosphate with molar ratios of 2:2:1:1 (Nakajima et al., 1990; Oba et al., 1999; Tuinier, 1999). Differences in the viscosity in the absence and presence of salt confirmed its polyelectrolyte character. The polysaccharide was shown to be a high molecular weight polymer with an intrinsic viscosity of 32 dl/g in 10^{-1} M NaCl and a weight average molar mass of the order of 1.5×10^6 g/mol (Tuinier, Zoon, Cohen Stuart, Fleer, & de Kruif, 1999). The rheology of solutions of this polysaccharide was investigated by viscometry and dynamic measurements (Tuinier, Oomen, Zoon, Cohen Stuart, & de Kruif, 2000; Tuinier et al., 1999). In a quite similar work, Oba et al. (1999) described the rheological properties of the same polysaccharide, which they called 'viilian', but neither the intrinsic viscosity nor the molecular weight were determined. From these two investigations, it is clear that this purified EPS in aqueous solution exhibits the properties of a macromolecular solution and is not gelling.

Dairy propionibacteria are also food-grade microorganisms and EPS-producing bacteria. However, these EPS have been much less investigated than those from lactic acid bacteria. Only four papers have been published on this topic (Crow, 1988; Racine et al., 1991; Reddy, Washam, Reinbold, & Vedamuthu, 1973; Skogen et al., 1974). No rheological characterization of the polysaccharide has been performed. Nevertheless, Skogen et al. (1974) used viscosity measurements to monitor EPS production in the medium. Crow (1988) also isolated a polysaccharide produced by a strain of propionibacterium, *P. freudenreichii* ssp. *shermanii* ATCC 9614, the composition of which was galactose (39%), glucose (4%), methylpentose (39%). Racine et al. (1991) obtained viscosity values of 20 mPa s at 60 rev/min at 25 °C on the medium containing EPS produced by *Propionibacterium acidi-propionici* ATCC 25562, which is the same strain as *P. acidi-propionici* DSM 4900. The EPS comprised two components: a water-soluble fraction composed of rhamnose (22%) (w/w), mannose (10%) (w/w) and both galactose and glucose (34%) (w/w), and a water insoluble fraction composed of fucose (7%) (w/w), mannose (22%) (w/w), galactose (40%) (w/w) and glucose (31%) (w/w). These authors also noticed the presence of phosphate groups in the soluble fraction but they did not conclude on the presence of phosphate groups on the EPS backbone. The water-soluble fraction accounted for 15% (w/w) while the water-insoluble fraction was estimated at 27% (w/w), the remaining being composed of ash (65%) (w/w). Molecular weight of the polysaccharide of the soluble fraction was estimated at 5800 g/mol by gel filtration.

In previous works, we described the conditions of EPS production by fermentation of *P. acidi-propionici* DSM 4900 on milk microfiltrate (Gorret, Maubois, Engasser, & Ghoul, 2001a; Gorret, Maubois, Ghoul, & Engasser, 2001b). The production varied from 0.0 to 0.6 g/l depending upon the fermentation conditions. We also observed that even for a low amount of EPS produced, i.e. 0.3 g/l, a shear-thinning behavior of the fermented medium was exhibited suggesting production of a high molecular weight EPS.

In the present work, we characterized the rheological behavior of the purified polysaccharide in relation to its biochemical composition. For this purpose, together with chemical methods to determine the composition of the polysaccharide, we described flow and viscoelastic properties of the aqueous solutions by means of steady and oscillatory shear measurements over a wide range of polysaccharide concentrations.

2. Materials and methods

2.1. EPS production

Bacterial strain and culture condition. *P. acidi-propionici* DSM 4900 (ATCC 25562) were chosen. The growth medium consisted of skim milk microfiltrate. It was obtained by tangential filtration on 0.1 μm membrane as described by Fauquant, Maubois, and Pierre (1988). Continuous fermentation was realized in a 2 l SGI (Toulouse, France) fermentation reactor at 23 °C, pH 6 and 3 g/l yeast extract. EPS concentration in the reactor varied from 0.1 to 0.3 g equiv. glucose/l.

Extraction and purification of the EPS. The culture medium was concentrated by tangential ultra-filtration. The module of filtration was the Millipore equipment (Molsheim, France) containing a spiral membrane (Millipore, France) (30 KD; area, 0.23 m^2). The transmembrane pressure was 2 bars and temperature was 25 °C. After concentration, the medium was centrifuged (20 min at $16{,}000 \times g$) to remove the cells. The supernatant was treated with a protease from *Streptomyces griseus* (Sigma, France) at 37 °C pH 7 during one night in presence of Thimerosal (1/1000) to prevent growth of microorganism. The enzyme was inactivated by heating at 100 °C for 10 min. EPS was precipitated by adding five volumes of chilled ethanol 95% followed by an overnight storage at 4 °C. The precipitate, collected after centrifugation (10 min at $5000 \times g$), was redissolved in distilled water, dialyzed (cellulose dialysis tube, Polylabo, cut-off 2 KDa) against water at 4 °C during 5 days to eliminate residual sugars from the culture medium (water was changed three times a day) and then freeze-dried.

2.2. EPS characterization

Analytic ion-exchange chromatography. Crude EPS was subjected to analytic anion-exchange chromatography on a column (75×7.5 mm^2) of TSK DEAE 5 PW (Toyosoda, Japan). Elution was performed using a gradient of ammonium acetate buffer at pH 6 from 0.05 to 0.5 M. The elution flow rate was 0.6 ml/min. The eluate was continuously monitored using the automated orcinol method (Tollier & Robin, 1979).

Preparative ion-exchange chromatography. Crude EPS was subjected to preparative anion-exchange chromatography on a column (2.5 × 40 cm) of DEAE Sepharose CL6B (Pharmacia). The column was equilibrated in 0.05 M ammonium acetate buffer, pH 6. Elution was performed using a gradient of ammonium acetate buffer at pH 6 from 0.05 to 0.5 M. The flow rate was 2 ml/min. Fractions were collected; the eluate was continuously monitored as above. Peak-forming fractions were pooled, dialyzed and freeze-dried.

High performance size exclusion chromatography (HPSEC). Crude EPS was subjected to size exclusion chromatography on Progel TSK G5000PWXL, G4000PWXL, G3000PWXL, G2500PWXL (300 × 7.8 mm) (Toyosoda, Japan) mounted in series and fitted with a precolumn TSK XL (75 × 7.5 mm) (Toyosoda, Japan). Elution was performed using 0.4 M sodium acetate buffer at pH 3.6. The elution flow rate was 0.8 ml/min at 35 °C. The eluate was continuously monitored as above.

Sugar composition. Each fraction was hydrolyzed with TFA 0.2N in sealed vials at 120 °C for 12 h. The liberated sugars and amino-sugars were converted to alditol acetate (Kiho et al, 1986). Derivatized samples were analyzed on a DP-225 (30 m × 0.15 mm) capillary column (J & W Scientific, Folsom, USA). The oven temperature was set at 215 °C and the flame ionization detector at 250 °C. Hydrogen at 0.8 bar was used as carrier gas. Inositol was used as internal standard.

Cation-exchange capacity (CEC). The charge of the crude polysaccharide was estimated by measuring its cation-exchange capacity. Freeze-dried EPS (100 mg) was dissolved in 10 ml 18 MΩ/Cm ultra-pure water (Millipore, Molsheim, France) and stirred overnight at 4 °C. The solution was then transferred to a 2 KDa cellulose dialysis tube (SpectraPore 6, PolyLabo) and dialyzed against a solution of 0.1N HCl at 4 °C during 16 h (solution was changed once). In a second stage, the EPS was dialyzed against 18 MΩ/Cm ultra-pure water at 4 °C during 3 days (water was changed three times a day). This acidic EPS solution was freeze-dried. A sample of 50 mg of freeze-dried acidic polysaccharide was dissolved in 25 ml 0.1 M NaCl and titrated with a freshly prepared and titrated solution of approximately 0.02N KOH.

Analytical procedures. Total polysaccharide was estimated by the phenol sulfuric acid method (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956) using glucose as a standard. The EPS concentrations are thus expressed in g equiv. glucose/l. Protein has been determined by bicinconinic acid assay kit (Kit Sigma No. BCA-1) with a BSA standard. Phosphorus has been determined after mineralization by the colorimetric method using sodium molybdate and hydrazine sulfate (FIL, 1982). Determination of esterified organic acids was performed after EPS saponification with 0.4 M NaOH followed by an injection on Dionex DX-500 high performance anion-exchange chromatographic system (Jouy-en-Josas, France) as described by Gaucheron, Le Graet, Piot, and Boyaval (1996). Uronic acids were estimated by the method described by Blumenkrantz and Asboe-Hansen (1973).

2.3. Rheological characterization of the EPS

The lyophilized crude EPS was solubilized in ultra-pure water while stirring. Agitation was maintained overnight at 4 °C. Determination of the EPS concentration of this stock solution was realized by the phenol sulfuric acid method (Dubois et al., 1956). This stock solution was diluted to the desired concentration (ranging from 18 g/l down to 0.01 g/l) and again homogenized. To remove air bubbles, all polysaccharide solutions were centrifuged at 5000*g* for 5 min prior to the measurements.

Rheological measurements were carried out at 25 °C; for the concentration lower than 1 g/l, low shear viscometers were used (LS 30 and LS 40 from Contraves) with coaxial cylinders geometry ($r_1 = 5.5$ mm; $r_2 = 6$ mm, $h = 8.0$ mm in case of LS 30 and $r_1 = 6$ mm; $r_2 = 6.5$ mm, $h = 18.0$ mm in case of LS 40). In both cases, the shear rate range was the same (0.017–128 s^{-1}). A rheometrics RSF II fluids spectrometer equipped with a cone-plate attachment (diameter, 50 mm; angle, 0.04 rad; truncation, 50 μm) (Rheometrics Inc., Piscataway, USA) was employed for the higher concentrations at 25 °C. Dynamic oscillatory shear (viscoelasticity) and steady shear (flow behavior) were performed successively. A thin layer of Paraffin oil covered the sample to prevent evaporation. For dynamic measurements, the imposed strain was 10% and was chosen to be in the linear domain. The range of frequency was 0.01–100 rad/s with five points per decade. As to steady shear measurements, the shear sensitivity was first assessed by performing a 'thixotropic' loop from a linear programmation of the shear rate from 0 to 100 s^{-1} and then back to zero, both for 2 min. Then, the flow behavior was described by performing steady shear measurements stepwise from 100 s^{-1} down to 0.025 s^{-1}.

3. Results and discussion

3.1. Chemical characterization of EPS

The freeze-dried partially purified EPS from *P. acidipropionici* DSM 4900 was analyzed by size exclusion chromatography (Fig. 1). The chromatographic profile clearly showed one main population with an elution time of 25 min and one minor population, composed of polysaccharides of lower molecular weight and eluted at an elution time of about 28 min. Both polysaccharides appeared to elute as wide peaks, which therefore suggests a wide distribution of molecular weights. Analytical ion-exchange chromatography on a TSK-DEAE column (Fig. 2) confirmed that the EPS was a mixture of at least two main polysaccharide fractions, one neutral, and one highly

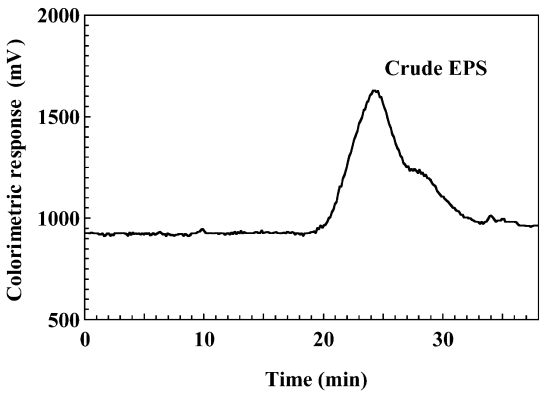

Fig. 1. Size exclusion chromatography of crude EPS.

charged, eluting at an ionic strength of almost 0.5 M. The fractions were shown to be devoid of uronic acids, i.e. the charge of the main fraction was probably due to a substituent of the sugar (such as phosphate or sulfate).

Examples of phosphate group substitution have already been given for EPS produced by lactic acid bacteria, mainly for the strain *L. lactis* ssp. *cremoris* (Marshall, Cowie, & Moreton, 1995; Nakajima et al., 1990; Oba et al., 1999; Tuinier et al., 1999; Van Casteren, Dijkema, Schols, Beldman, & Voragen, 1998; Yang, Huttunen, Staaf, Widmalm, & Tenhu, 1999) but also for *Lactobacillus sake* (Robijn et al., 1996a) and *L. paracasei* (Robijn et al., 1996b). To our knowledge, sulfate substitution has not been described for EPS produced by lactic acid bacteria. An estimation of the charge of the crude EPS was realized by the cation-exchange capacity. The number of charged groups (CEC), determined at the inflection point, was 660 μmol/g of powder, confirming the polyelectrolyte nature of the polymer and its high charge density.

A preparative DEAE chromatography has been performed to purify the two fractions. Sugar recovery yields with this preparative chromatography were low, only 40% of the initial sugars. As before, two main fractions have been obtained. A minor neutral fraction (22% of the total sugars recovery) and a major charged fraction (78% of the total

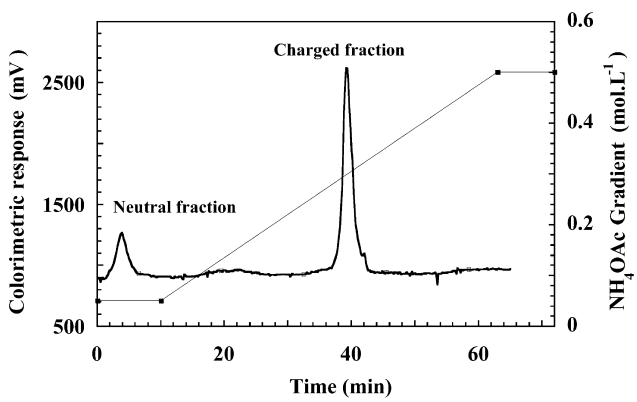

Fig. 2. Analytical ion-exchange chromatography of crude EPS powder with increasing NH_4Oac gradient on TSK DEAE 5 PW.

sugars recovery) have been purified. The major charged fraction could be further fractionated in a minor fraction (25%) and a major one (75%). The minor charged fraction could correspond to the slight colorimetric response noticeable on the previous analytical DEAE chromatography at elution times (18–24 min). The protein content has been determined for the crude polysaccharide and for the various fractions. Protein contamination was low for all the samples, crude EPS (6% (w/w)), neutral fraction (5% (w/w)), minor charged fraction (7% (w/w)) and the major charged one (6% (w/w)).

Composition of the crude EPS and of the three main fractions was determined by GLC (Table 1). Three neutral sugars (glucose, galactose and mannose) and two aminosugars (glucosamine and galactosamine) were found. The neutral fraction was composed mainly of glucose and mannose whereas the charged fraction contained mainly galactose, with mannose and glucosamine, and traces of glucose and galactosamine. The minor charged fraction was composed mainly of mannose, glucose, glucosamine and galactose with traces of galactosamine. Such composition has not been described in the literature for EPS produced by dairy bacteria.

Phosphate groups determination has also been performed on the crude EPS in the acidic form. Phosphate groups have been detected at a low level 21 μmol/g of powder, i.e. much lower than would be expected from the CEC (660 μmol/g). Due to that discrepancy, the presence of other potential anionic groups was examined. Organic acids groups were not observed in significant amount after saponification of the EPS. Absence of uronic acids has also been established by the absence of characteristic colored reaction. Determination of phosphate content in the purified fractions could not be carried out due to low amount. Racine et al. (1991) also noticed the presence of phosphate groups in the soluble fraction but they did not demonstrate that was integral to the polysaccharide.

It can be concluded from the present results that the main polysaccharide fraction is a polyelectrolyte with a high molar weight. Differences between our result and those of Racine et al. (1991), who worked on the same strain may be due to the different methodologies used. Bacterial exopolysaccharides often exhibit a repetitive structure with repetitive units comprising typically 5–10 monomers. Their structures are highly variable and cannot easily be classified, as would be the case for plant polysaccharides, which fall in a restricted number of classes. Controlled hydrolysis experiments (results not shown) suggested that this was the case here also as discrete fractions appeared after mild acidic treatments. A complete structural investigation would have required not so much linkage analysis as isolation and identification of these repetitive units and was beyond the scope of this work.

Table 1
Composition of the crude EPS and the three main fractions

Sugars	Glucose	Galactose	Mannose	Glucosamine	Galactosamine
Crude EPS	1.0	2.4	2.0	1.6	0.1
Neutral fraction	3.0		0.9	0.3	
Minor charged fraction	1	0.6	4.1	0.7	0.3
Major charged fraction	0.5	4.7	2.1	2.3	0.4

3.2. Rheological characterization of EPS

3.2.1. In dilute solution

In the dilute regime, typically at concentrations less than 0.05 g/dl, the behavior was Newtonian. Fig. 3 shows the variations of the reduced viscosity as a function of concentration in aqueous solution (no salt added). The upward curvature as concentration decreased is typical of a polyelectrolyte behavior, as expected from the chemical structure of the major fraction. Measurements in the presence of salt (0.01 M NaCl) resulted in the curves shown in Fig. 4 and the double extrapolation to zero concentration of the Huggins and Kramer plots gave an intrinsic viscosity of 22 dl/g. The ionic strength used here, though admittedly still low, was sufficient as evidenced from the linear plots/absence of upward curvature of the reduced viscosity at low concentrations and the existence of an intercept for the Huggins and Kramer plots. Although the ionic content was far from infinite ionic strength conditions, this intrinsic viscosity value may be compared to literature data of other polysaccharides, let they be neutral polymers or polyelectrolytes. This is relatively a high value which compares to sodium hyaluronate (10–30 dl/g) (Yanaki & Yamaguchi, 1990), guar (10–20 dl/g) and pectins (< 10 dl/g) (Lapasin & Pricl, 1995). It is quite close to that reported for an EPS from *Cyanospira capsulata* (~22 dl/g in 0.1 M NaCl) (Navarini, Cesaro, & Ross-Murphy, 1992); an EPS from *L. lactis* subsp. *cremoris* B40 whose weight averaged molar mass was estimated at 1.47×10^6 g/mol, also gave a comparable high value (~30 dl/g in NaCl 10^{-1} M) (Tuinier et al., 1999). It is worth noting that an intrinsic viscosity as high as 20 dl/g does correspond to weight averaged molar weights of the order of 10^6 g/mol. The present intrinsic viscosity value, like the HPSEC data, indicate a much higher molar weight than the very low value (5800 g/mol) that has been reported by Racine et al. (1991) for EPS from *P. acidi-propionici*.

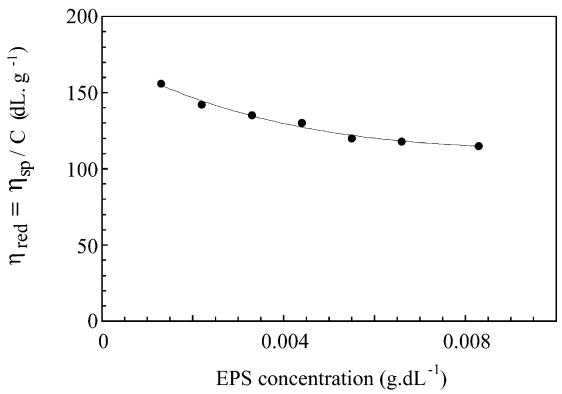

Fig. 3. Reduced viscosity as a function of concentration of the EPS produced by *P. acidi-propionici* DSM 4900 in salt-free solution.

3.2.2. Flow behavior of concentrated solutions

Thixotropic loop. The thixotropic behavior of polysaccharide solutions is illustrated in Fig. 5 for three EPS concentrations (6, 12, and 18 g/l), with a clear shear-thinning behavior. No hysteresis that is, shear-, or time-dependent effect was visible for the lowest concentration (6 g/l), while for the two highest concentrations (12 and 18 g/l) an overshoot together with an hysteresis loop were seen. The hysteresis was, however, limited to the low shear range (typically up to 5 s^{-1} at 12 g/l and 20 s^{-1} at 18 g/l) and the overshoot was more pronounced at 18 g/l than at 12 g/l. This is the evident result of the non linear viscoelastic behavior. However, it is clear that a quantitative analysis of these time-dependent properties in the non linear regime requires the performance of transient experiments, that is measurements of shear stress as a function of time at a given shear rate as has been performed for xanthan (Richardson & Ross-Murphy, 1987a), guar galactomannan (Richardson & Ross-Murphy, 1987b) and also an EPS of *C. capsulata* (Navarini, Bertocchi, Cesaro, & Lapasin, 1990). Whatsoever, the present results provide clear indications that pronounced viscoelastic properties are exhibited by the EPS at a high enough concentration, namely higher than 6 g/l.

Flow curves. Flow curves of EPS solutions over a wide

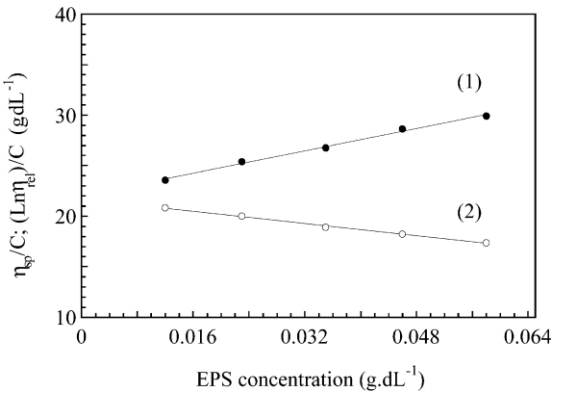

Fig. 4. Determination of the intrinsic viscosity of the EPS produced by *P. acidi-propionici* DSM 4900 and NaCl 10^{-2} M. Curve 1: Huggins' equation; curve 2: Kraemer's equation.

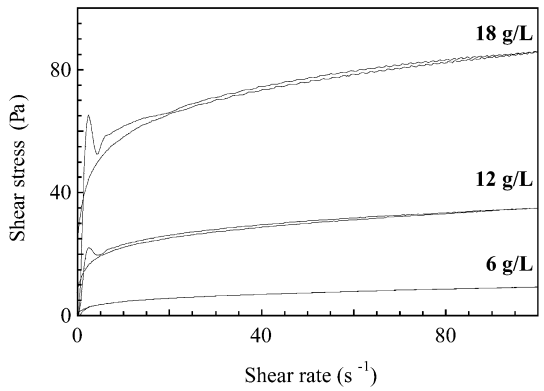

Fig. 5. Thixotropy loops for various EPS concentrations, 6, 12, and 18 g/l.

concentration range (2–18 g/l) are shown in Fig. 6. Shear-thinning behavior was clearly exhibited whatever the concentration may be. Each flow curve was characterized by a Newtonian plateau (η_0) at low shear rate followed by a decrease as the shear rate increased. The onset of the non Newtonian zone shifted to higher shear rates as the concentration decreased. Such a behavior is typical of macromolecular solutions and has been reported for most of non gelling polysaccharides (Doublier & Launay, 1981; Lapasin & Pricl, 1995; Morris, Cutler, Ross-Murphy, & Rees, 1981). All the flow curves could be fitted using the so-called Cross equation:

$$\eta = \frac{\eta_0}{1 + (\tau\dot{\gamma})^m} \quad (1)$$

where $\tau = 1.32$ and $m = 0.83$. Parameters of the Cross model are reported in Table 2. Quality of the fit as estimated from the mean relative deviation (M.R.D.) was fair whatever the concentration. At high concentrations, the value of the exponent (m) was between 0.8 and 0.9. This actually corresponded to a limiting slope of the flow curve at intermediate shear rate. Measurements at higher shear rates

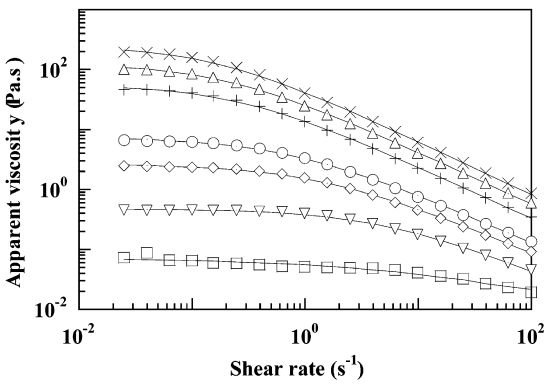

Fig. 6. Flow curves of EPS solutions. 18 g/l, ×; 16 g/l, Δ; 12 g/l, +; 8 g/l, ○; 6 g/l, ◇; 4 g/l, ▽; 2 g/l, □. Symbols: experimental values; continuous lines: fit using the Cross model.

Table 2
Parameters of the Cross model

EPS (g/l)	Cross model				Master curve	
	M.R.D. (%)[a]	η_0 (Pa s)	τ (s)	m	X shift	Y shift
1.0	0.0	0.03	0.01	0.30	0.008	0.0041
2.0	1.8	0.07	0.08	0.45	0.07	0.0096
3.0	0.9	0.18	0.11	0.73	0.09	0.0246
3.5	1.3	0.27	0.11	0.81	0.10	0.037
4.0	0.8	0.47	0.19	0.80	0.14	0.0643
5.0	0.7	1.22	0.39	0.79	0.27	0.167
6.0	0.7	2.63	0.69	0.80	0.50	0.36
7.0	0.7	4.53	0.95	0.82	0.75	0.62
8.0	0.8	7.31	1.32	0.83	1.00	1
11.0	0.7	33.75	3.25	0.86	2.90	4.62
12.0	0.8	56.08	3.78	0.87	3.30	7.67
14.0	0.8	72.72	3.57	0.90	3.30	9.95
16.0	0.8	125.40	4.93	0.87	3.90	17.2
18.0	0.8	252.71	6.34	0.85	5.40	34.6
Master curve	0.0	1.06	1.32	0.83		

η_0, zero-shear rate Newtonian viscosity (Pa s); τ, structural relaxation time (s) and values of the shift of the master curve (reference concentration, 8 g/l).

[a] The agreement between calculated and experimental values was estimated by the mean relative deviation (MRD). $\mathrm{MRD} = \left(\sqrt{[(\eta_{calc} - \eta_{exp})/\eta_{calc}]^2/N}\right) \times 100$, where η_{exp} is the experimental value, η_{calc}, the calculated value and N, the total number points.

should result in a flattening of the flow curve and ultimately in a limiting viscosity η_∞. The limiting slope corresponds therefore to an inflection point in the flow curve, and has been reported for several polymers in the melt or in concentrated solution. For example, limiting m values equal to 0.79–0.80 have been quoted for guar galactomannan (Doublier & Launay, 1981; Robinson, Ross-Murphy, & Morris, 1982). This value also compares with that reported by Milas, Rinaudo, Knipper, and Schuppiser (1990) for xanthan gum (0.8). Same results have been found for EPS of *C. capsulata* (Navarini et al., 1992) and for EPS of *L. cremoris* SBT 095 (Oba et al., 1999). In other respects, τ can be taken as a structural relaxation time. Its increase with concentration can be ascribed to the fact that the degree of coil overlap increases with concentration. As a result, the freedom of movement of individual chains is progressively restricted, with consequent increase in the time required to form new entanglements to replace those disrupted by externally imposed shear. Therefore, the shear rate at which the behavior becomes non Newtonian shifts to a lower value as concentration increases.

By shifting all the flow curves along the X and Y axis in log scale, it was possible to generate a master flow curve. The resulting composite curve shown in Fig. 7 illustrates this procedure which allows one to describe the flow curve at a given concentration (here 8 g/l concentrated solution is taken as the reference concentration) over a wide range of shear rates (from 10^{-4} to 10^3 s^{-1}). This procedure has been

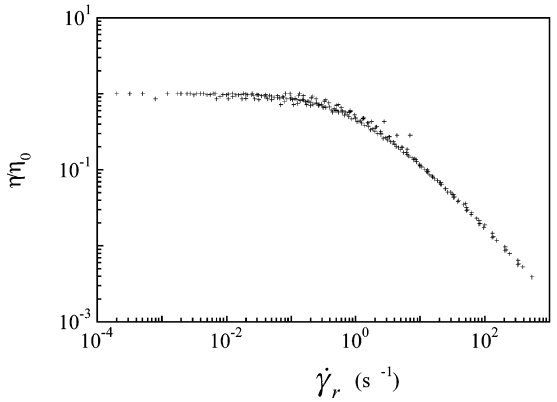

Fig. 7. Master curve by superposition of flow curves of EPS solution at various concentrations with *X* and *Y* shifts. Reference concentration, 8 g/l.

applied successfully to other polysaccharide solutions (Morris, 1984). This composite curve could be described by a generalized Cross equation:

$$\eta/\eta_0 = \frac{1}{1+(\tau\dot{\gamma})^m} \quad (2)$$

where $t = 1.32$ and $m = 0.83$.

Concentration dependence of the zero-shear viscosity. The logarithmic representation of η_{sp0} as a function of EPS concentration (Fig. 8) shows the typical behavior exhibited by polysaccharide solutions. The curve can be arbitrarily divided into three parts each being assimilated to a straight line. Three different zones could be distinguished by this way separated by two critical concentrations $c^* = 0.2$ and $c^{**} = 2$ g/l. The slopes were 0.7 in the dilute regime, 1.5 in the intermediate one and 3.9 in the higher concentration range, respectively. The slope lower than unity in the dilute regime ($c < 0.2$ g/l) was related to the polyelectrolyte character of the polysaccharide since measurements have been performed in the absence of salt. c^* corresponds to the

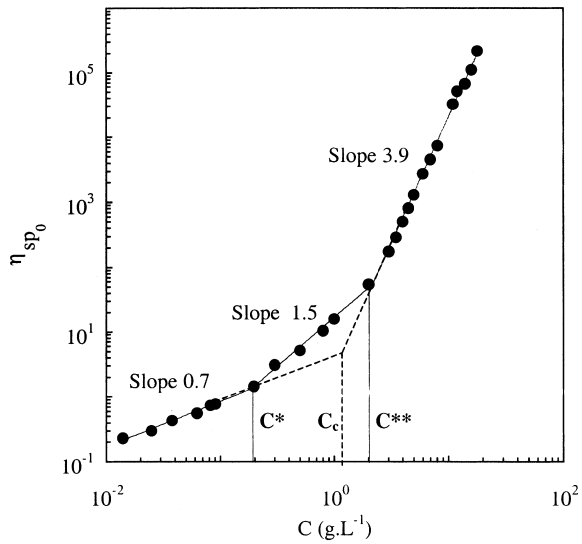

Fig. 8. Specific zero-shear viscosity as a function of EPS concentration in salt-free conditions.

upper limit of the dilute regime and is related to the onset of significant coil overlap. c^{**} determines the beginning of the so-called semi-dilute regime, where the thickening behavior of the EPS is fully experienced. The value of this parameter is quite low, and could be related to the high intrinsic viscosity of the polymer. Actually, as emphasized by Lapasin and Pricl (1995), a close look at the results evidences a continuity from the dilute to the concentrated regime and the overall curve could be described as well by an analytical expression like the Martin equation. The discontinuous representation has no true physical meaning and should be only regarded as a convenient way to estimate these characteristic parameters. Another critical concentration ($c_c = 1.22$ g/l) can be defined from the intercept of the two linear branches, respectively, in the dilute and the semi-dilute regime. Its value, 1.22 g/l, is comparable to the value reported by Navarini et al. (1990) for the EPS produced by *C. capsulata* and again is quite low by comparison with most of the polysaccharides (Lapasin & Pricl, 1995) hence suggesting good thickening properties.

The slope of 3.9 is within the range found for polysaccharides; typically, 3.3 in the work by Morris et al. (1981) on several random coil polysaccharides, 3.7–4.0 for some others, (galactomannans, xanthan, hyaluronate) (Launay, Cuvelier, & Martinez-Reyes, 1997; Milas et al., 1990; Morris et al., 1981). Similar values or even slightly higher have been obtained for charged EPS from bacteria: 4.0 for a polysaccharide produced by *C. capsulata* (Navarini et al., 1990) and 4.5 for an EPS produced by *L. lactis* ssp. *cremoris* SBT 0495 (Oba et al., 1999). Tuinier (1999) also found a slope of 3.5 for another strain of *L. lactis cremoris*. It has been suggested that large values of this parameter are to be ascribed to the presence of more specific polymer–polymer interactions (Morris et al., 1981; Robinson et al., 1982). However, it should be realized that small differences in this value may have no real meaning since the determination of this slope depends strongly on the highest concentration that is accessed.

3.2.3. Viscoelastic properties

The viscoelastic behavior of the EPS solution from dynamic oscillatory shear measurements is illustrated in Fig. 9 for three concentrations: 4, 8, and 18 g/l. Both $G'(\omega)$ and $G''(\omega)$ showed a continuous increase with ω. At low frequency, the loss modulus $G''(\omega)$ was higher than the storage modulus $G'(\omega)$. In this frequency range, both parameters varied sharply with ω with slope values tending to 1 for $G''(\omega)$ and 2 for $G'(\omega)$, respectively. With increasing ω, $G'(\omega)$ crossed $G''(\omega)$ at a given frequency ω_c. Such a behavior is typical of macromolecular solutions with topological entanglements. The frequency dependence at low frequency corresponds to the terminal zone of the viscoelastic spectrum while at high frequency when $G'(\omega)$ is higher than $G''(\omega)$ the plateau zone is reached (Ferry, 1980). By increasing the concentration from 4 to 18 g/l, the cross-over frequency, ω_c, shifted from 10 to 0.3 rad/s (Table

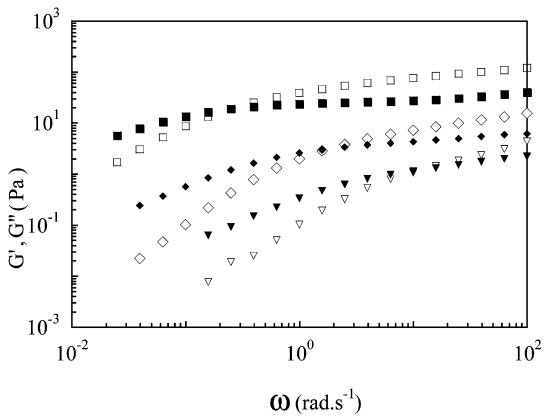

Fig. 9. Viscoelastic spectra of EPS solutions at three concentrations (salt-free conditions): 18 g/l, □; 8 g/l, ◇; 4 g/l. Filled symbols: storage modulus (G'), empty symbols: loss modulus (G'').

3). The G' and G'' variations with frequency were slighter particularly towards the high frequency range where $G' > G''$, that is when accessing to the plateau zone. Again, comparable results have been reported in the case of the EPS from *C. capsulata* (Navarini et al., 1992). This change in the mechanical spectrum is a mere result of the shift of the accessed frequency window as the concentration increases. ω_c can be regarded as a measure of an effective junction time ($\tau_c = 1/\omega_c$), which thus varied from 0.1 to 3.3 s for an increase of concentration from 4 to 18 g/l (Table 3).

Furthermore, by using the Graessley model it was possible to determine the zero-shear viscosity, η_0, the steady shear compliance, J_e^0 and the terminal relaxation time τ_0 (Graessley, 1993).

$$\eta_0 = \lim_{\omega \to 0} \frac{G''(\omega)}{\omega} \qquad J_e^0 = \frac{1}{\eta_0^2} \lim_{\omega \to 0} \frac{G'(\omega)}{\omega^2} \qquad \tau_0 = \eta_0 J_e^0 \tag{3}$$

Table 3
Parameters of the Graessley model

EPS (g equiv. glucose/l)	ω (rad/s)	τ (s)	Graessley model			
			η_0 (Pa s)	τ_0 (s)	J_e^0 (Pa)	M_e ($\times 10^3$ mol/g)[a]
4	10.0	0.1	0.4	0.7	1.86	575
5	6.0	0.2	1.0	1.2	1.14	375
6	3.0	0.3	2.3	1.7	0.72	269
7	2.5	0.4	3.7	1.9	0.51	210
8	2.0	0.5	6.2	2.4	0.39	161
11	0.8	1.3	34.4	5.6	0.16	63
12	0.6	1.7	51.1	6.6	0.13	53
14	0.5	2.0	77.3	8.5	0.11	39
16	0.4	2.5	121.4	9.5	0.08	30
18	0.3	3.3	233.5	12.9	0.06	21

η_0 is the zero-shear rate Newtonian viscosity (Pa s); τ_0, the terminal relaxation time (s); J_e^0, the steady-state shear compliance and M_e is the estimation of the molar weight between entanglements.

[a] M_e from Eq. (4).

τ_0 is known to reflect the intrinsic flexibility of the macromolecular chains. The η_0 value is to be compared to the value obtained from the Cross model (Tables 2 and 3).

On the other hand, by using the expression (Ferry, 1980; Yanaki & Yamaguchi, 1990),

$$G_e^0 = \frac{g_N \rho RT}{M_e} \tag{4}$$

it is possible to have an estimate of the molecular weight between entanglement points, M_e. g_N is a numerical factor not far from unity, ρ is the density, R is the gas constant and T is the temperature. Despite G_e^0 could not be estimated with accuracy since this would require measurements at frequency higher than accessed (>100 rad/s), we took the G' value at 100 rad/s as a rough estimate. The values are reported in Table 3. These appear quite consistent with regards to the molar weight of the EPS assuming this is of the order of 10^6 mol/g. The molecular weight between entanglement points decreases as the EPS concentration increases. This was expected since increasing the number of macromolecules should result in a reduction of the distance between entanglement points. At the lowest concentration considered (4 g/l), only a few entanglements would be considered, which is consistent with viscosity measurements ($c^{**} = 2$ g/l).

Moreover, by shifting all the G' (ω) and G'' (ω) curves along the X and Y axis in log scales, it was possible to generate a master curve. The resulting composite curve shown in Fig. 10 illustrates this procedure which allows one to describe the variation of G' (ω) and G'' (ω) at a given concentration (here 4 g/l concentrated solution is taken as the reference concentration) over a wide angular frequency range (from 10^{-1} to 10^4 rad/s). The general aspect of the two curves and the presence of a cross-over point above which G'/C becomes higher than G''/C are very similar to the behavior observed in concentrated entangled solutions of polystyrene (Ferry, 1980) and also in xanthan solution (Milas et al., 1990).

3.2.4. Cox–Merz rule

From dynamic measurements, the variations of the complex dynamic viscosity $|\eta^*|$ (given by $|\eta^*| = \sqrt{(G'2 + G''2)}/\omega$) as a function of angular frequency (in rad/s), can be compared to the flow curves (Fig. 11). For the 4 g/l concentration, the curves were totally superimposed showing that the Cox–Merz rule was obeyed (Cox & Merz, 1958). This superposition is generally exhibited for random coil polymers. In contrast, for the 18 g/l concentration, the superposition of the curves was only seen at the lowest values of the frequency (or shear rate) and then, from 0.5 to 0.6 rad/s, the slopes of $|\eta^*(\omega)|$ became slightly lower than that of $\eta(\dot{\gamma})$. The frequency at which the deviation took place actually corresponds to the beginning of the plateau zone. Same deviations have been reported for xanthan (Milas et al., 1990) and for EPS produced by *C. capsulata* (Navarini et al., 1992). This has been interpreted in terms of

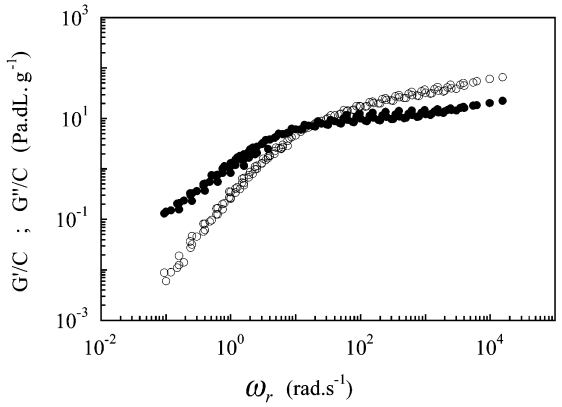

Fig. 10. Superposition of viscoelastic spectra of EPS solution a different concentrations with Y and Y shifts. Storage modulus G', ○; loss modulus G'', •. Reference concentration: 4 g/l.

specific interactions between chain segments, occurring in addition to normal topological entanglements (Richardson & Ross-Murphy, 1987a).

4. Conclusions

P. acidi-propionici DSM 4900 seems to produce at least two polysaccharides which differ by their composition, their charge and their molar weight. The major one is a high molar weight polyelectrolyte rich in galactose, which accounts for more than 78% of the total polysaccharide. Given this complex composition, it appears difficult to describe the overall rheological properties on a detailed macromolecular basis. Therefore, our main objective was to evaluate the performance of this polysaccharide as a thickener with respect to the classical industrial polysaccharides. The rheological properties indicated that aqueous solutions of EPS behave as an entangled polymer solution. Overall, they are mostly governed by the high molar weight polyelectrolyte fraction. Nevertheless, concentrations of polysaccharides have to be taken with precaution because they were estimated on the basis of the total concentrations and not specific concentrations of the implicated polysaccharide. EPS solutions displayed a shear-thinning behavior with limiting zero-shear viscosity (η_0) and viscoelastic properties typical of a macromolecular solution. Because of its high intrinsic viscosity, the polysaccharide could be used as highly viscous thickener; moreover, this does not display gel properties. In spite of the presence of a mixture of polysaccharide in the crude powder, we were in presence of a homogeneous macromolecular solution. Even if all the polysaccharides were not involved in the rheological behavior, none of them has an antagonist effect. In terms of performance, as a thickening agent, this EPS appears at least as efficient as the usual industrial polysaccharides.

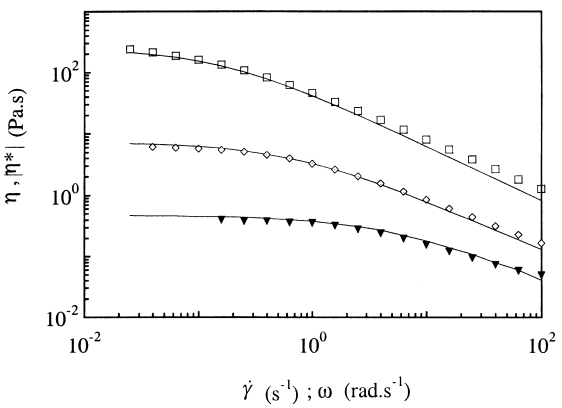

Fig. 11. Apparent viscosity as a function of shear rate as compared to dynamic viscosity as a function of angular frequency. Apparent viscosity η; symbols, dynamic viscosity $|\eta^*|$, continuous lines; concentrations, 18 g/l, □; 8 g/l, ◇; 4 g/l, ▼.

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