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Comparative analysis of production and purification of homo- and hetero-polysaccharides produced by lactic acid bacteria

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ABSTRACT

Lactic acid bacteria (LAB) produce homopolysaccharides (HoPS) and heteropolysaccharides (HePS) with potential functional properties. In this work, we have performed a comparative analysis of production and purification trials of these biopolymers from bacterial culture supernatants. LAB strains belonging to four different genera, both natural as well as recombinant, were used as model systems for the production of HoPS and HePS. Two well characterized strains carrying the *gft* gene were used for β-glucan production, *Pediococcus parvulus* 2.6 (*P. parvulus* 2.6) isolated from cider, and the recombinant strain *Lactococcus lactis* NZ9000[pGTF] (*L. lactis* NZ9000[pGTF]). In addition, another cider isolate, *Lactobacillus suebicus* CUPV225 (*L. suebicus* CUPV225), and *Leuconostoc mesenteroides* RTF10 (*L. mesenteroides* RTF10), isolated from meat products were included in the study. Chemical analysis of the EPS revealed that *L. mesenteroides* produces a dextran, *L. suebicus* a complex heteropolysaccharide, and the β-glucan producing-strains the expected 2-substituted (1,3)-β-glucan.

1. Introduction

Many LAB synthesize extracellular polysaccharides (exopolysaccharides, EPS), which remain attached to the outer cell wall forming a capsule or are released into the environment in the form of slimy, or ropy, EPS. These bacteria produce a wide variety of EPS with different composition, structure, molecular mass, and conformation. Most LAB synthesize HePS usually composed of D-galactose, D-glucose, and L-rhamnose. These polysaccharides are produced from sugar nucleotides by the activity of intracellular glycosyltransferases (Welman & Maddox, 2003). Other LAB produce HoPS containing only D-glucose (*e.g.*: dextran, reuteran, and mutan) or D-fructose (*e.g.*: levan and inulin) (Monsan et al., 2001). HoPS are usually synthesized by extracellular glycansucrases (glucan- or fructan-sucrases) using sucrose as the glycosyl donor. EPS formation by glycansucrases has been reported for strains of *Lactobacillus* (Kralj et al., 2004; Tieking, Korakli, Ehrmann, Ganzle, & Vogel, 2003), *Leuconostoc* (Bounaix et al., 2010; Fraga Vidal et al., 2011; Seymour & Knapp, 1980), and *Weissella* (Galle, Schwab, Arendt, & Gaenzle, 2010) species. LAB strains belonging to the *Pediococcus, Lactobacillus*, and *Oenococcus* genera, isolated from cider and wine, produce a 2-substituted (1, 3)-β-D-glucan (Dols-Lafargue et al., 2008; Dueñas-Chasco et al., 1997, 1998; Ibarburu et al., 2007; Llaubères, Richard, Lonvaud, Dubourdieu, & Fournet, 1990). This β-glucan is synthesized in *Pediococcus parvulus*, *Lactobacillus suebicus*, and *Oenococcus oeni* by a single GTF glycosyltransferase, which is a membrane-bound protein that polymerizes glucosyl residues from UDP-glucose (Garai-Ibabe et al., 2010; Velasco et al., 2007; Werning et al., 2006, 2008).

The HePS from LAB play an important role in the rheology, texture and 'mouthfeel' of fermented milks (yoghurt, viili, långfil, *etc.*) (Welman & Maddox, 2003) and other fermented products, such as sorghum sourdoughs (Galle, Schwab, Arendt, & Gänzle, 2011). HoPS producers have been evaluated to a lesser extent and are used mainly for fermentation of non-dairy products. Dextrans from *Leuconostoc mesenteroides* and *Weissella* spp. as well as levan from *Lactobacillus sanfranciscensis* positively affect dough rheology and

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bread texture (Di Cagno et al., 2006; Waldherr & Vogel, 2009). The analysis of the rheological properties of 2-substituted β-D-glucan showed that it has potential utility as a biothickener (Lambo-Fodje et al., 2007; Velasco et al., 2009). Testing of this β-glucan producing LAB for the production of a fermented oat product (Martensson, Oste, & Holst, 2002), yogurt, and various beverages (Elizaquível et al., 2011; Kearney et al., 2011) indicated advantageous technofunctional properties of these strains.

Additionally, prebiotic effects of several EPS have also been demonstrated (Hongpattarakere, Cherntong, Wichienchot, Kolida, & Rastall, 2011; Korakli, Ganzle, & Vogel, 2002).

Furthermore, health benefits have been claimed for EPS from LAB because of their putative antitumoral, immunostimulatory, and blood cholesterol lowering activities (Liu et al., 2011; Welman & Maddox, 2003). In addition, β-glucan-producing *Pediococcus* (Fernández de Palencia et al., 2009) and *Lactobacillus* (Garai-Ibabe et al., 2010) are able to immunomodulate macrophages, and human consumption of oat-based food prepared with it resulted in a decrease of serum cholesterol levels, boosting the effect previously demonstrated for (1,3)-β-D-glucans in oats (Martensson et al., 2005).

Therefore, EPS from LAB are of great interest for Agro-Food industries since they have a vast structural diversity, which opens the way to innovations. Moreover, several LAB species have a "Generally Recognized As Safe" status by the American Food and Drug Association or a "Qualified Presumption of Safety" status by the European Food Safety Authority (Gueimonde, Frias, & Ouwehand, 2006). This fact facilitates the application of their polysaccharides either as additives or as *in situ*-produced thickeners. On the other hand, the low production of polysaccharides by the majority of LAB species has hampered both their molecular characterization and their commercial exploitation.

Thus, optimized methodologies of EPS production and recovery are required in order to facilitate their characterization, and thereby explore the structural diversity of LAB EPS and potential applications. The aim of the present study is, therefore, to identify optimized procedures for the synthesis and analysis of structurally different EPS produced by a variety of LAB species isolated from meat fermented products and beverages as well as by recombinant strains.

2. Materials and methods

2.1. Bacterial strains and growth conditions

Strains used in this study were from the authors' culture collections and had been previously characterized and identified into species. *L. mesenteroides* RTF10 was isolated from meat products (Chenoll, Macián, Elizaquível, & Aznar, 2007) at the Department of Food Biotechnology, Institute of Agrochemistry and Food Technology (C.S.I.C., Valencia, Spain). *P. parvulus* 2.6 (Dueñas-Chasco et al., 1997) and *L. suebicus* CUPV225 were isolated from ropy cider at the Department of Applied Chemistry, Faculty of Chemistry (University of the Basque Country UPV/EHU, San Sebastián, Spain). *P. parvulus* 2.6 carries the gene *gtf* that encodes the GTF glycosyltransferase, which catalyzes the synthesis of the 2-substituted-β-D-glucan. The recombinant strain *L. lactis* NZ9000[pNGTF] carries the plasmid pNGTF containing the *gtf* gene under the control of the P_{NisA} promoter, inducible by the addition of nisin to the growth medium (Werning et al., 2008). Strains were kept in Man Rogosa Sharpe (MRS) broth (Pronadisa, Madrid, Spain), except *L. lactis* NZ9000 [pNGTF], which was maintained in ESTY medium (Pronadisa, Madrid, Spain), supplemented with 20% (v/v) glycerol for long-term storage at −80 °C.

2.2. Production of EPS by natural LAB strains

To obtain inocula for EPS production, *L. mesenteroides* RTF10 was grown in MRS supplemented with 2% sucrose to an OD_{600} = 1.0 and *P. parvulus* 2.6, as well as *L. suebicus* strains, in MRS supplemented with 2% glucose, 0.05% (w/v) L-cysteine hydrochloride (Merck, Darmstad, Germany), and 0.1% (w/v) Tween 80 (Pronadisa, Barcelona, Spain) to an OD_{600} = 2.0. For EPS production, the inocula were diluted 1:100 in fresh media. Cultures were grown in the following media: defined CDM (Sánchez et al., 2008) for *L. mesenteroides*, semi-defined MST (Velasco et al., 2006) for *P. parvulus* and SMD containing glucose (20 g L^{-1}) (Dueñas-Chasco et al., 1997) for *L. suebicus*. Batch fermentations without pH control were carried out for the two lactobacillus strains. For *P. parvulus* fermentation were performed in media containing glucose (50 g L^{-1}) and ethanol 4.9% (w/v), in a 3-L fermenter (Bioflo 110, New Brunswick Scientific), at 30 °C for 96 h. The pH was controlled at 5.2 with 5 M NaOH, the agitation was set at 50–70 rpm to keep the fermentation broth homogeneous, and nitrogen gas (0.2 L h^{-1}) was sparged through the headspace continuously to maintain anaerobic conditions.

EPS production by *L. mesenteroides* RTF10 was carried out in medium supplemented with sucrose (0.8%) instead of glucose as carbohydrate source, and cultures were incubated for 12 h at 30 °C until OD_{600} = 1.0. *L. suebicus* CUPV225 was grown in the SMD medium (pH 5.5) for 72 h at 28 °C in an atmosphere containing 5% CO_2 until OD_{600} = 3.4.

2.3. Production of EPS by L. lactis NZ9000[pNGTF]

A frozen culture of the recombinant strain was used after thawing to inoculate 1 L of CDM medium supplemented with glucose (0.5%) and chloramphenicol (5 μg mL^{-1}). The culture was grown at 30 °C to OD_{600} = 0.6. Then, it was centrifuged at 6816 × *g* for 20 min at 4 °C and re-suspended in fresh CDM medium without antibiotic. For EPS production, expression of *gft* gene was induced by the addition of nisin (0.25 ng mL^{-1}) and further incubation during 24 h at 30 °C. Batch fermentation without pH control was performed.

2.4. EPS isolation and purification from P. parvulus 2.6 culture supernatant

Bacterial cells were removed from fermented media by centrifugation at 16,000 × *g* for 30 min at 4 °C. The clear supernatant was collected and the EPS precipitated by adding three volumes of cold absolute ethanol, and maintained overnight at 4 °C. The precipitate was recovered by centrifugation at 14,000 × *g* for 10 min at 4 °C. The resulting EPS pellet was dissolved in ultrapure water, and the EPS was recovered by precipitation with ethanol (three times). The final precipitate was dissolved in and dialysed for 2 days against ultrapure water (changed twice each day), using a dialysis membrane (Medicell International, Ltd., London, U.K.) having a cut-off of 12–14 kDa. After dialysis, the solution was frozen at −80 °C and lyophilized (Telstar Cryodos equipment, Spain) at −50 °C for up to 3 days to completely remove the solvent. The lyophilized solid was then stored in a desiccator at room temperature. To eliminate impurities the EPS preparation was fractionated by size-exclusion chromatography (SEC). Dry EPS was dissolved in 0.3 M NaOH (to eliminate extra contaminants and to improve the EPS dissolution) and centrifuged to eliminate insoluble material. The supernatant was loaded into a column (60 cm × 2.6 cm) of Sepharose CL-6B equilibrated with 0.3 M NaOH, which was also used as eluent (0.3 mL min^{-1}). Fractions were collected, and monitored for carbohydrate content by the phenol–sulphuric acid method (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956). A calibration curve was obtained by using standards (Blue Dextran; Dextrans: T500, T70, and T10 (Pharmacia), and vitamin B12). From this curve, the

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apparent molecular mass (Mr) of the EPS was estimated. The fractions corresponding to the same chromatographic peak were pooled together, concentrated to a small volume, dialysed against ultrapure water using a dialysis membrane (12–14 kDa cut-off) until neutrality, and, after feezing, lyophilized for up to 3 days.

2.5. EPS isolation and purification from L. suebicus CUPV225 culture supernatant

Bacterial cells were removed from fermented medium by centrifugation at 16,000 × *g* for 30 min at 4 °C. The clear supernatant was collected and the EPS precipitated by adding three volumes of cold absolute ethanol. A floating fraction was first removed by winding around a glass rod and resuspended in ultrapure water (fraction H). The remaining cloudy ethanol mixture was maintained overnight at 4 °C, then centrifugation at 11,000 × *g* for 30 min at 4 °C to collect the suspended polysaccharide material (fraction L). This fraction was washed 3 times with 70% (v/v) ethanol and centrifuged. The floating and precipitated polysaccharides were re-suspended in and dialysed against ultrapure water, using a membrane (12–14 kDa cutoff), for 2 days (changed twice). After dialysis, both fractions were frozen at −80 °C and lyophilized for up to 3 days. The floating fraction (H) was very insoluble and was not further analyzed. Fraction L was subjected to SEC and the Mr of the EPS was estimated as described in Section 2.4.

2.6. EPS isolation and purification from L. mesenteroides RTF10 culture supernatant

Cells were removed from culture supernatant by centrifugation at 16,000 × *g* for 30 min at 4 °C. Then, cold absolute ethanol (v/v) was added to the supernatant, allowing the EPS to precipitate at 4 °C for 24 h. After centrifugation at 10,651 × *g* for 60 min at 4 °C, the supernatant was removed, and the EPS was re-suspended in and dialysed for 2 days against ultrapure water (changed twice), using a membrane (12–14 kDa cutoff). The EPS was frozen at −80 °C and lyophilized for up to 3 days. The dry biopolymer was dissolved in ultrapure water and centrifuged to eliminate insoluble material. The supernatant was loaded into a column (60 cm × 2.6 cm) of Sepharose CL-6B equilibrated with ultrapure water, which was also used as eluent (0.3 mL min^{-1}). The Mr of the EPS was estimated as described in Section 2.4.

2.7. EPS isolation and purification from L. lactis NZ9000[pGTF] supernatants

To improve the release of EPS from bacterial surface, the cultures were heated at 60 °C for 20 min in a water bath. Then, for removal of the bacteria, the culture was centrifuged at 10,651 × *g* for 60 min at 4 °C. The EPS present in the supernatant was recovered by overnight precipitation at −20 °C with three volumes of absolute ethanol and re-suspension in ultrapure water (three times). After each precipitation, the EPS was recovered by centrifugation at 10,651 × *g* for 60 min at 4 °C. Then, the precipitate was re-suspended in and dialysed for 2 days against ultrapure water (changed twice) in a membrane (12–14 kDa cutoff). The EPS was frozen at −80 °C and lyophilized for up to 3 days. The dry biopolymer, after being dissolved in 0.3 M NaOH, was subjected to SEC and its Mr was estimated as described in Section 2.4.

2.8. Determination of EPS concentration and presence of contaminants

The concentration of EPS in the different steps of purification was estimated as neutral carbohydrate content determined by the phenol–sulphuric acid method (Dubois et al., 1956). The amount of EPS was determined from culture supernatants: (i) after concentration, precipitation with two volumes of absolute ethanol (to remove the carbon source added to the medium), and washing of the EPS with 70% (v/v) ethanol, vacuum drying and suspension in water; (ii) after precipitation with ethanol, dialysis, freeze-drying and EPS suspension in water (solutions 1 or 2 mg mL^{-1}, depending on their solubility); and (iii) after chromatographic purification, dialysis, freeze-drying and resuspension in 1 or 2 mg mL^{-1} water solutions of the EPS. Contamination by DNA, RNA, and proteins was measured with the Qubit® 2.0 fluorometer in the same solutions for the purified samples or directly from the culture supernatant. This technique allows the detection of more than 0.5 μg mL^{-1} of DNA, 20 ng mL^{-1} of RNA, and 1 μg mL^{-1} of proteins.

2.9. Detection of EPS by electron microscopy

The presence of the EPS attached to the bacteria either capsular, tightly associated with the cell surface, and/or as slime or rope secreted into the extracellular environment, was determined by transmission electron microscopy (TEM) using either the ruthenium red staining method (Akin & Rigsby, 1990) or by negative staining with uranyl acetate (Maeyama, Mizunoe, Anderson, Masao Tanaka, & Matsuda, 2004). (i) The ruthenium red staining method was used for *L. mesenteroides* RTF10 basically as described by Akin and Rigsby (1990). The bacterium was grown to early stationary phase in MRS medium (Pronadisa, Madrid, Spain) at 30 °C. Cells were sedimented by centrifugation, washed in phosphate buffer saline (PBS, pH 7.4), and concentrated fivefold in 0.1 M sodium cacodylate buffer (pH 7.4). Cells were fixed in freshly prepared 2.5% (v/v) glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4), embedded in 2% agarose, and sections were cut with a scalpel. Cells were post fixed in 1.5% (w/v) OsO_4 in 0.1 M cacodylate buffer (pH 7.4) containing 0.075% (w/v) ruthenium red. Then, they were washed three times in 0.1 M sodium cacodylate buffer (pH 7.4), dehydrated using a graded ethanol series (30, 50, 70, 95, and 100% ethanol, 5 min each), and embedded in epoxy resin. Thin sections (60 nm) were made with a Diatome glass knife, using an Ultracut Leica UC6 ultramicrotome and examined with a JEOL JEM1010 transmission electron microscope after uranyl acetate staining. Images were captured and digitized using a MegaView III camera with Software "AnalySIS". (ii) The negative staining with uranyl acetate was used for *P. parvulus* 2.6, *L. suebicus* CUPV225, *L. lactis* NZ9000[pNGTF], and for purified EPSs. *P. parvulus* 2.6 and *L. suebicus* CUPV225 were grown to early stationary phase in MRS medium at 30 °C and *L. lactis* NZ9000[pNGTF] cultures were induced with nisin for 24 h as indicated in section 2.3. The EPS previously purified from *L. lactis* NZ9000[pNGTF] was re-suspended in PBS pH 7 at 1 mg mL^{-1} prior to negative staining. Samples were prepared as follows. Glow-discharged carbon-coated Formvar grids were placed facedown over a droplet of each culture concentrated fivefold in 0.1 M NH_4Ac, pH 7 or PBS pH 7. After 1 min, each grid was removed, blotted briefly with filter paper, and without being dried, negatively stained with 2% uranyl acetate for 40 s and then blotted quickly and air-dried. Samples were examined using a JEOL 1230 transmission electron microscope operated at 100 kV. Images were digitalized using an Epson Perfection 4870 Photo scanner at 1200 dpi final resolution.

2.10. Infrared (IR) spectroscopy

IR spectra were obtained by the KBr technique. In brief, approximately 2 mg of dry sample were thoroughly mixed in a mortar with 300 mg of KBr and maintained in a desiccator. The pellet was prepared by using a hydraulic press, applying a pressure of 2 tons for 2 min and then 10 tons for 6 min. The spectra were recorded in a FTIR 4200 type A instrument (Jasco Corporation, Tokyo, Japan).

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Light source of transmittance was in the middle range infrared 400–4000 cm^{-1}. The detector used was triglycine-sulfate (TGS) with resolution 4 cm^{-1}.

2.11. Determination of monosaccharide composition and phosphate content

For analysis of neutral sugars, the polysaccharides (approximately 1 mg) were first hydrolyzed with 3 M TFA (121 °C, 1 h). The monosaccharides were converted into their corresponding alditol acetates by reduction with $NaBH_4$ and subsequent acetylation (Laine, Esselman, & Sweeley, 1972). Identification and quantification were performed by gas-liquid chromatography (GLC) on a 6890A instrument (Agilent) equipped with a flame-ionization detector, using a HP5 fused silica column (30 m × 0.25 mm I.D. × 0.2 μm film thickness) with He as the carrier gas. Injector and detector were set at 250 °C. Samples (1 μL) were injected with a split ratio of 1:50, with a temperature program: 160 °C for 5 min, then 3.5 °C min^{-1} to 205 °C and finally 210 °C for 0.5 min. Identification was performed on the basis of the coincidence of the retention time of sample components with those previously measured for standards analyzed in identical conditions, using inositol as internal standard. Phosphate content was deduced from inorganic phosphate determination on a 5500 Inductively Coupled Plasma instrument (Perkin Elmer).

2.12. Methylation analysis

The polysaccharides (1–5 mg) were methylated according to the method of Ciucanu and Kerek (1984). The permethylated polysaccharides were hydrolyzed with 3 M trifluoroacetic acid (TFA) at 121 °C for 1 h. The resulting partially methylated monosaccharides were converted into their corresponding alditol acetates by reduction with $NaBD_4$ and subsequent acetylation with 250 μL of pyridine:acetic anhydride (1:1) for 1 h at 100 °C, as described by Laine et al. (1972). The partially methylated alditol acetates obtained were analyzed by gas chromatography–mass spectrometry (GC–MS) on a 6890A/5975C instrument from Agilent, with He as the carrier gas. The injector was programmed at 250 °C. Samples (1 μL) were injected with a split ratio of 1:50 and their components separated in a HP5MS (Agilent) fused silica column (30 m × 0.25 mm I.D. × 0.2 μm film thickness), with a temperature program starting at 160 °C (1 min) and then rising 2 °C min^{-1} up to 200 °C. An m/z range between 40 and 450 amu was scanned. Identification was done on the basis of the retention time and mass spectra of the compounds. Quantification was performed according to peak area.

3. Results and discussion

3.1. Production of EPS by LAB

The amount and composition of the EPS produced by LAB is strongly influenced by culture and fermentation conditions such as pH, temperature, and medium composition (Dueñas, Munduate, Perea, & Irastorza, 2003). In general, higher yields of HoPS and HePS are obtained when complex media are used for bacterial growth. However, the use of these media results in EPS preparations with higher levels of contaminants (Ruas-Madiedo & de los Reyes-Gavilán, 2005). Thus, in this work, specific conditions for the production of each EPS produced by different LAB have been set up by using defined or semi-defined media, to diminish, during purification, the co-precipitation of interfering compounds (*e.g.* polysaccharides from the culture medium) together with the bacterial EPS.

The four strains analyzed secreted variable amounts of EPS into the culture media, as quantified by the phenol sulfuric acid method: *L. suebicus* CUPV225 144 ± 1 mg L^{-1}, *P. parvulus* 2.6 378 ± 3 mg L^{-1}, *L. lactis* NZ9000[pGTF] 561 ± 18 mg L^{-1}, and *L. mesenteroides* RTF10 1870 ± 180 mg L^{-1}. The proportion of the EPS in the supernatants relative to protein, RNA, and DNA contaminants ranged from 9 to 97% from *L. suebicus* CUPV225 to *L. mesenteroides* RTF10 cultures, the highest contamination being due to proteins (Table 1).

The production of the exocellular polymers was also detected by TEM analysis of bacterial cultures (Fig. 1). *P. parvulus* 2.6 (Fig. 1A), *L. lactis* NZ9000[pGTF] (Fig. 1B), and *L. suebicus* CUPV225 (Fig. 1D) preparations revealed their EPS as amorphous masses, either attached to the bacterial cells or free in the preparation. Similarly, the EPS was visualized in aqueous solutions of the purified EPS. As an example, the EPS produced by the recombinant *L. lactis* strain is depicted in Fig. 1C. In the case of *L. mesenteroides* RTF10, the EPS was predominantly concentrated around the exterior of the cells in a putative capsule, with fibrous structures interconnecting adjacent cells. There were also cell-free isolated clumps of EPS in the medium (Fig. 1E). The detection of a low proportion of molecules that were not attached to the cells and the high yield of EPS production by the bacterium indicated that most of the biomolecules produced by *L. mesenteroides* were lost by sedimentation of the bacterial culture prior to TEM analysis.

3.2. Recovery and initial purification of EPS

Bacterial EPS are generally recovered from culture broth by precipitation, by adding a water-miscible organic solvent (Kumar, Mody, & Jha, 2007). Here, ethanol precipitation was used for the recovery and initial purification of EPS from bacterial culture supernatants. The concentration of neutral carbohydrates, protein, DNA, and RNA was analyzed in aqueous solutions of the EPS

Table 1
Detection of biomolecules in successive steps of the purification process.

Strain	Culture supernatant[b]				After precipitation and dialysis[c]				After SEC[c]			
	EPS (%)	Protein (%)	DNA (%)	RNA (%)	EPS (%)	Protein (%)	DNA (%)	RNA (%)	EPS (%)	Protein (%)	DNA (%)	RNA (%)
P. parvulus 2.6	65.3	34.7	0	0	98.5	<0.1	0	0	100	0	0	0
L. lactis NZ9000 [pGTF]	95.5	4.5	<0.1	<0.1	98.5	1.4	<0.1	0	100	0	0	0
L. suebicus CUPV225[a]	9	83.3	0	7.7	29.6	4	0	7.9	37.3	3	0	0
L. mesenteroides RTF10	97	2.9	<0.1	<0.1	99.1	0.9	<0.1	<0.1	100	0	0	0

[a] In addition to neutral sugars, this sample contained phosphate and aminosugars. Consequently, low EPS levels were detected using the phenol-sulfuric acid method, which is appropriate for neutral sugars but not for aminosugars.
[b] Protein, DNA and RNA concentrations were measured directly from supernatants. EPS concentration was determined from neutral sugars estimation after ethanol precipitation from culture supernatants. 100% corresponds to the total concentration of detected biomolecules (EPS, protein, RNA, and DNA) in each sample.
[c] Solutions were prepared in water at 2 mg mL^{-1} for *L. mesenteroides* EPS and 1 mg mL^{-1} for the other EPS.

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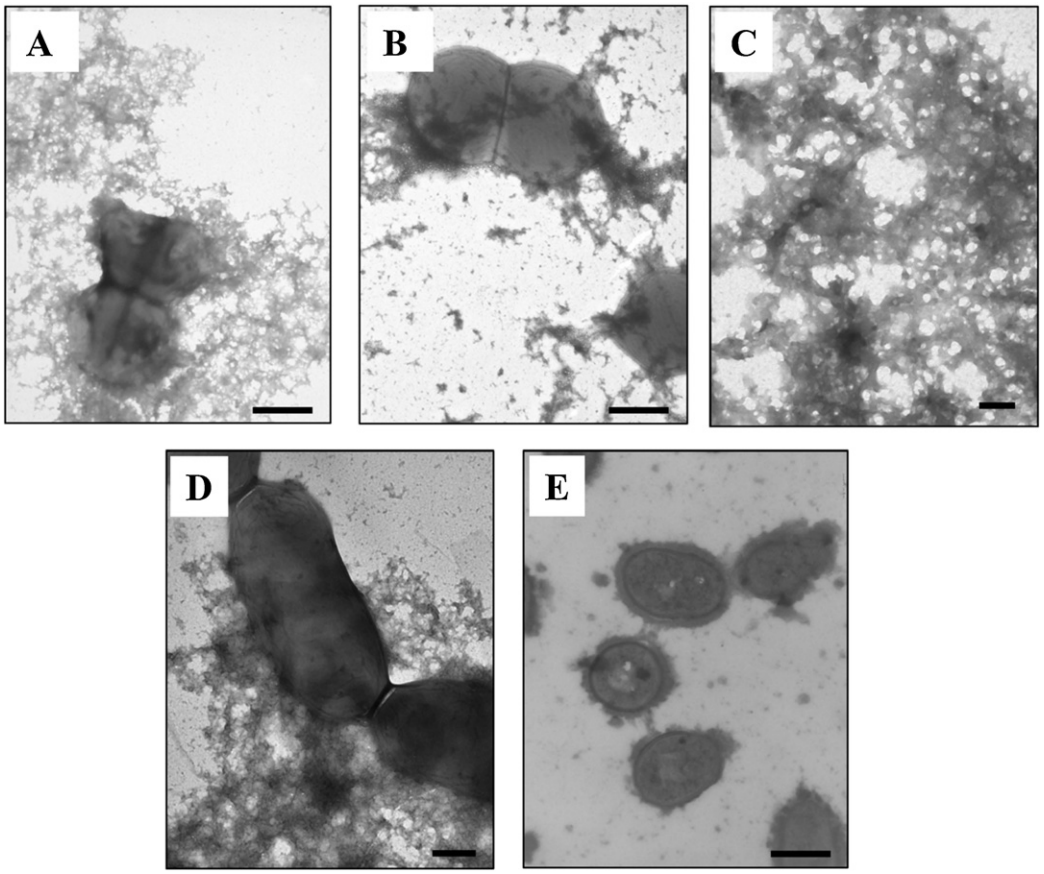

Fig. 1. Transmission electron micrographs of EPS and bacterial cells. Negative staining with uranyl acetate of whole cells of *P. parvulus* 2.6 (A), *L. lactis* NZ9000[pNGTF] (B), and *L. suebicus* CUPV225 (D) as well as of EPS purified from *L. lactis* NZ9000[pNGTF] (C). Ultrathin section of *L. mesenteroides* RTF10 cell stained with ruthenium red (E). All bars correspond to 500 nm except (C), 100 nm.

preparations to check their purity after ethanol precipitation, dialysis, and lyophylisation (Table 1).

The amounts of absolute ethanol needed to achieve the recovery of the EPS from the culture supernatants depended on the type of polymer released. For example, the EPS from *P. parvulus* and the recombinant *L. lactis*, which are β-glucans, needed three volumes of ethanol, while the EPS from *L. mesenteroides* precipitated easily with only one volume of this solvent. In the case of *L. suebicus* two EPS fractions were separated from the culture supernatant, one that precipitated quickly after ethanol addition (fraction H) and other that flocculated after a longer time under cold conditions (fraction L). Fraction H turned out to be very insoluble after drying, and was not further analyzed. In addition, the EPS from *P. parvulus* and *L. lactis*, either directly from supernatants or after initial purification or chromatographic fractionation (see below), generated gel-like solutions at concentrations higher than 3 mg mL^{-1} and had to be diluted to 1 mg mL^{-1} to get a homogeneous suspension. The EPS from *L. mesenteroides*, which was produced in the highest yield and precipitated with the lowest ethanol proportion (1:1, v/v), contained a negligible amount of impurities. A similar yield was observed for the EPS produced by the recombinant *L. lactis* strain, for which optimized polymer production was better controlled. By contrast, the EPS preparations from *P. parvulus* and *L. suebicus* strains had variable amounts of contaminating proteins and nucleic acids.

The results show that purification resulted in all cases in a decrease of protein contamination and it had variable effects to remove the residual DNA and RNA contamination presumably due to cell lysis.

3.3. SEC of the EPS preparations and analysis of sugar composition of the purified EPS

To further purify the polysaccharides and to estimate their average molecular mass, the lyophilized samples were fractionated by SEC after being dissolved in the appropriate solvent, according to their solubility. Thus, for *P. parvulus*, *L. lactis*, and *L. suebicus*, the use of 0.3 M NaOH was required, in order to get a better dissolution of the samples. On the other hand, the more soluble EPS from *L. mesenteroides* was analyzed using a water solution. Analysis of neutral sugar content of EPS preparations, after SEC fractionation, revealed for all isolates a single peak. EPS from *P. parvulus* 2.6, *L. lactis* NZ9000[pGTF], and *L. mesenteroides* RTF10 had a Mr higher than 10^6 Da and were composed exclusively of glucose (results not shown). However, the EPS collected in fraction L from *L. suebicus* CUPV225 had a Mr around 52,000 Da and contained galactose, glucose, and glucosamine in the proportions 1.1:1.8:1, respectively, and 4.5% phosphate. Analysis of EPS, after SEC, did not reveal contamination by RNA and DNA (Table 1). Moreover, protein contamination at the level of 3% was only detected in the *L. suebicus* EPS preparation. As previously mentioned, this HePS was composed of neutral sugars and glucosamine. Since the phenol–sulfuric acid method does not give positive reaction with aminosugars, carbohydrate content in this sample (37.3%) was underestimated by using this technique.

SEC is a chromatographic procedure currently used to purify in one step HePS from fungal cell walls (Leal, Prieto, Bernabé, & Hawksworth, 2010). Moreover, this method has been successfully used to purify extracellular neutral HePS from Bifidobacteria

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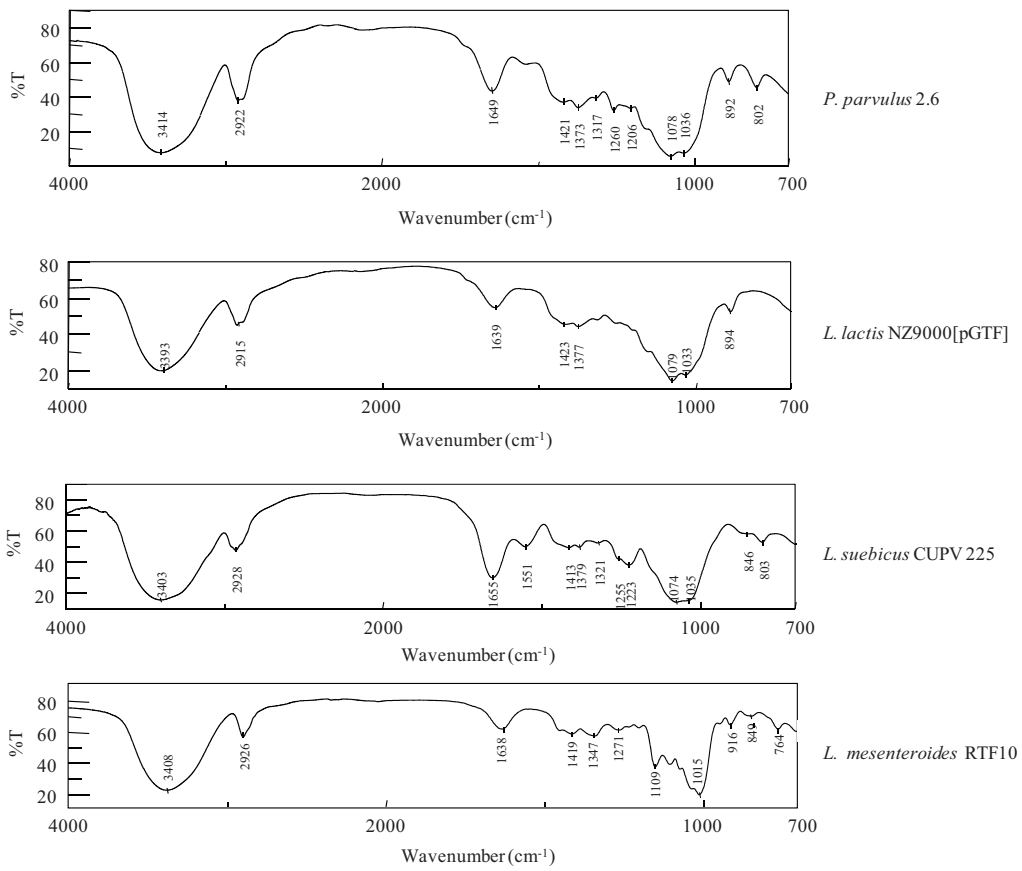

Fig. 2. Infrared spectra of homo- and hetero-polysaccharides synthesized by LAB.

(Salazar, Ruas-Madiedo, Prieto, Calle, & de los Reyes-Gavilaĭn, 2012). Our results validate the use of this method to purify HoPS produced by LAB. However, they show that this method is not sufficient to remove all impurities from the HePS from *L. suebicus*, probably due to interactions of its aminosugars with the Sepharose bed and/or with other components of the preparation. In any case, this fact did not prevent the characterization of the preparation by IR spectroscopy and chemical analysis (see below). Nevertheless, if a high purity HePS is required for further uses, other chemical or enzymatical deproteinization treatments and/or other chromatographic fractionation methods, such as ion exchange chromatography, could be used (Ruas-Madiedo & de los Reyes-Gavilán, 2005; Kumar et al., 2007; Freitas, Alves, & Reis, 2011).

3.4. IR spectra of exopolysaccharides

The overall appearance of the spectra (Fig. 2) is typical of those from carbohydrates. Observation of the signals showed similarities and differences among the EPSs analyzed in the present work and allowed certain chemical characteristics of these compounds to be deduced. The spectra from the EPS produced by *P. parvulus* 2.6 and *L. lactis* NZ9000[pGTF] were very similar, displaying an absorption band around 890 cm^{-1} attributable, as expected, to β-anomers. On the other hand, the spectrum from the EPS from *L. mesenteroides* RTF10 did not show this absorption band, but instead had a shoulder at 849 cm^{-1} and a more intense absorption band at 916 cm^{-1}, both characteristic of α-anomers.

The spectrum of the EPS from *L. suebicus* CUPV225 showed two absorption bands, at 1551 and 1655 cm^{-1}, characteristic of the amide linkage, confirming that the glucosamine residues detected by GC were N-acetylated. However, nothing could be clearly deduced regarding the anomeric configuration of sugars, probably due to the presence of more than one anomeric type in the EPS preparation. Only a slight band at 846 cm^{-1} was observed in the IR spectrum.

3.5. Methylation analysis of exopolysaccharides produced by LAB

Methylation analysis of the polysaccharides (Table 2) proved the similarity of the biopolymers produced by *L. lactis* NZ9000[pGTF] and *P. parvulus* 2.6, giving the expected products for a (1 → 3)-glucan partially branched at positions *O*-2 in agreement with previous results (Dueñas-Chasco et al., 1997; Werning et al., 2008).

The EPS from *L. mesenteroides* RTF10 gave the partially methylated and partially acetylated derivatives of a (1 → 6)-glucan with approximately 6% of substitutions at positions *O*-3 by side chains composed of a single residue of glucose. Since the band for α anomers was observed in the IR spectrum of this polymer, it can be described as a dextran-type polysaccharide. The production of dextrans from *Leuconostoc* strains grown in medium supplemented with sucrose is well known and widely documented (Korakli & Vogel, 2006; Monsan et al., 2001; Sarwat, Qader, Aman, & Ahmed, 2008; Van Hijum, Kralj, Ozimek, Dijkhuizen, & Van Geel-Schutten, 2006), although this is the first report on the production of dextrans from a *Leuconostoc* strain isolated from meat.

Finally, the EPS from *L. suebicus* CUPV225 gave a complicated pattern of derivatives from methylation analysis, suggesting that the strain is producing a complex HePS. The possibility of dealing with a mixture of polymers, which eluted together in SEC, cannot be ruled out. Glucose residues were found to be mainly as terminal residues, or (1 → 6)-, (1 → 3)- and (1 → 4) linked, although a small proportion of (1 → 3,6)-glucose was also detected. Galactose was mostly found as galactofuranose (1 → 2,6)-linked. Aminosugars

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Table 2
Linkage types and their percentages, deduced from methylation analysis of homo and hetero-polysaccharides synthesized by LAB and purified by SEC.

Linkage type	*P. parvulus* 2.6	*L. lactis* NZ9000 [pGTF]	*L. mesenteroides* RTF10	*L. suebicus* CUPV 225
Galp-(1→	0.0	0.0	0.0	6.4
Galf-(1→	0.0	0.0	0.0	2.9
→2)-Galp-(1→	0.0	0.0	0.0	3.4
→6)-Galf-(1→	0.0	0.0	0.0	3.4
→2,6)-Galf-(1→	0.0	0.0	0.0	14.4
Glcp-(1→	30.1	31.1	6.3	24.7
→3)-Glcp-(1→	37.9	36.7	0.0	17.5
→4)-Glcp-(1→	0.0	0.0	0.0	7.0
→6)-Glcp-(1→	0.0	0.0	86.5	15.4
→3,6)-Glcp-(1→	32.0	32.2	7.2	4.7

were detected as non-methylated compounds, probably due to an incomplete dissolution of the sample in DMSO.

4. Conclusions

In this work we have used a combination of conditions to produce and purify high yields of homo- and hetero-polysaccharides from LAB. The strategy was based on identifying defined or semi-defined media as well as growth conditions to minimize contamination by other carbohydrates and bacterial molecules. Moreover, experimenting with different conditions of ethanol precipitation of high molecular mass EPS, which seems to depend on EPS composition and conformation, led to simple isolation methods suitable for determination of their primary structure. In addition, the method was combined with dialysis to remove low molecule weight contaminants such as residual media components and metabolites. The final purification of the EPS preparations was achieved by chromatographic SEC eluting with 0.3 M NaOH in all cases except for the α-glucan, which had good solubility in water. This method yielded pure HoPS and a partially purified HePS. In addition, chemical analyses have allowed us to describe a dextran-producing *L. mesenteroides* strain from a fermented meat product as well as a heteropolysaccharide-producing *L. suebicus* strain isolated from cider, whose HePS merits further investigation.

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