Effects of carbohydrate source on physicochemical properties of the exopolysaccharide produced by *Lactobacillus fermentum* TDS030603 in a chemically defined medium

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ABSTRACT

A thermophilic lactic acid bacterium, *Lactobacillus fermentum* TDS030603, produced about 100 mg/L of EPS in purified form when grew in de Man-Rogosa-Sharpe (MRS) broth. The 1% (w/v) solution of the purified EPS was highly viscous, exhibiting an apparent viscosity (η_{app}) of 0.88 Pa·s at a shear rate of 10/s. To investigate the impact of carbohydrate source on the production yield and chemical structure of EPS and the viscosity of EPS solution, a chemically defined medium (CDM) has been developed. Results of TLC, HPLC, and ^{1}H-NMR spectroscopy indicated that the chemical structures of EPS released in MRS and in the CDM supplemented with glucose, galactose, lactose or sucrose were very similar. All the 1% solutions of EPSs released in CDMs were highly viscous similar to the EPS released in MRS, but their viscosities appeared to differ, presumably because of the differences in their molecular mass distributions.

Keywords: Chemical structure; Chemically defined medium; Exopolysaccharide; Lactic acid bacteria; Viscosity

1. Introduction

An exopolysaccharide (EPS) is a sugar polymer that is produced mainly by bacteria and microalgae, either in a form bound to the cell-wall, a so-called capsular polysaccharide (CPS), or in a free form liberated into the culture medium, known as a slime EPS (Sutherland, 1972). It is believed that the physiological function of EPS is as the first line of biological defense against phagocytosis, phage attack, antibiotics, toxic metal ions and physical stresses such as desiccation and osmotic stress (Looijesteijn, Trapet, de Vries, Abee, & Hugenholtz, 2001; Roberts, 1996; Weiner, Langille, & Quintero, 1995; Whitfield, 1988). EPS produced by lactic acid bacteria (LAB) is very useful in the food industry, because it provides consistency to the resulting fermented milk products, such as Scandinavian ropy milks, viili and långfil (Duboc & Mollet, 2001). Furthermore, some EPSs have been claimed to show bioactivities beneficial to health, including prebiotic or anti-inflammatory effects (Salazar, Gueimonde, Hernández-Barranco, Ruas-Madiedo, & de los Reyes-Gavilán, 2008; Vinderola, Perdigón, Duarte, Farnworth, & Matar, 2007).

A large number of EPSs produced by LAB have been described (Cerning, 1990; De Vuyst & Degeest, 1999; Jolly, Vincent, Duboc, & Neeser, 2002; Laws, Gu, & Marshall, 2001; Welman & Maddox, 2003), but little is known about the effects of medium components on the chemical structure of the EPS and its rheological properties (Vaningelgem et al., 2004). Since the complexity of media composition can lead to an incorrect structural analysis of EPS (De Vuyst & Degeest, 1999), a chemically defined medium (CDM) is of great advantage when assessing the effects of medium components on the chemical structure and the physicochemical properties of EPS (Grobben et al., 1998). It has been shown, using CDM, that adenine or orotic acid stimulates both the cell growth and the yield of EPS in Lactobacilli (Petry, Furlan, Crepeau, Cerning, & Desmazeaud, 2000; Torino, Hébert, Mozzi, F., & de Valdez, 2005). Composition of carbohydrate source also exhibited significant effects on the EPS yield, but the preferences for sugar for the maximum EPS production were

strain-dependent (Cerning et al., 1994; Tallon, Bressollier, & Urdaci, 2003; Torino, Hébert, Mozzi, F., & de Valdez, 2005). On the other hand, the effects of carbohydrate source on the monosaccharide composition of EPS are still unclear: the constitutive monosaccharides were found to be the same in *L. helveticus* following alterations in the carbohydrate source (Torino, Hébert, Mozzi, F., & de Valdez, 2005), but the relative proportions of the individual monosaccharides varied in *L. delbrueckii* subsp. *bulgaricus* (Petry, Furlan, Crepeau, Cerning, & Desmazeaud, 2000). The rheological properties of the EPS produced by LAB are attributed to its molecular mass, molecular mass distribution, constituent sugar residues, linkages between the sugar monomers and the presence of side groups (Shene, Canquil, Bravo, & Rubilar, 2008). However, effects of altered medium composition on the rheological properties of EPS have been scarcely reported.

In the present paper we aimed to evaluate the effects of carbohydrate source on the yield, chemical structure and viscosity of a neutral hetero-EPS produced by *L. fermentum* TDS030603. In this context, we have developed a CDM for this strain, having modified the previously reported media (Morishita, Deguchi, Yajima, Sakurai, & Yura, 1981). The chemical structure and viscosity of EPS released into CDM were assessed using the EPS released into MRS as the reference. Possible determinants leading to rheological variations in the EPS will be discussed.

2. Materials and methods

2.1. Bacterial strain and chemicals

L. fermentum TDS030603 was obtained from the bacterial collection of our own laboratory (Leo et al., 2007). MRS was from Oxoid (Cambridge, UK). DEAE-Sephadex A-50 and Toyopearl HW-55F was from GE Healthcare (Uppsala, Sweden) and Tosoh (Tokyo, Japan), respectively. D_2O (99.99% atom % D) was from Sigma-Aldrich (St. Louis, USA). All the chemicals used were analytical grade.

2.2. Development of CDM and culture condition

Following the previous report (Morishita, Deguchi, Yajima, Sakurai, & Yura, 1981), we firstly tested a prototype CDM consisting of 48 constituents, of which 6 were non-essential amino acids (see below) and the other 42 are listed in Table 1. Essential or important chemical compounds for cell growth were determined by checking the cell density of culture medium from which one of the above constituents had been omitted. After static culture in MRS for 24 h at 30°C under aerobic conditions, the cells were harvested, washed thoroughly with sterilized phosphate buffered saline, and inoculated into 1 L of either MRS or CDM to yield an optical density (OD) of 0.2 at 600 nm. Cell growth ($OD_{600\ nm}$) and pH of the static culture were monitored. Simultaneously, cultivable cell numbers were counted on MRS-agar plates; after a given time period, a 1-ml aliquot of culture medium was collected, diluted with MRS and spread on a MRS-agar plate, which was incubated at 30°C for 24 h under anaerobic condition. Colonies appearing on the plate were counted as cultivable cells. To measure the amount of EPS produced, a 100-ml aliquot of the culture medium was collected, and the EPS was purified following the procedure described below.

2.3. Production and isolation of EPS

Glucose, galactose, lactose, or sucrose was used in the CDM as carbohydrate source at the final concentration of 1% (w/v). After cultivation as described above, the cells were removed by centrifugation (17,000 *g*, 1 h, 4°C). Crude EPS released in CDM was precipitated by addition of an equal volume of ice-cold ethanol to the supernatant. The ethanol precipitate was collected by centrifugation (17,000 *g*, 30 min, 4°C), dissolved in 30 ml of water, dialyzed overnight with water at 4°C, and lyophilized. The lyophilized crude EPS (from 100 ml of the culture medium) was dissolved in 10 ml of 50 mM Tris-HCl (pH 8.7), and purified by a batch method using a 20-ml slurry of DEAE-Sephadex A-50 equilibrated with the same buffer. The non-adsorbed fraction was collected, thoroughly dialyzed against water, and lyophilized. Crude EPS released in MRS was subjected to the same procedure described above, however, it required further purification using a Toyopearl HW-55F column (2.6 × 100 cm, 15 ml/h) equilibrated with water. The polymer dry mass of purified EPS was determined by measuring the weight. The lyophilized EPS was stored in a desiccator until used.

2.4. Estimation of EPS molecular mass

The molecular mass distribution of EPS was estimated using high performance liquid chromatography (HPLC). The purified EPS was dissolved in water (1 mg/ml), and 100 μl of this solution was loaded onto a TSKgel G6000PWXL column (7.8 × 300 mm, Tosoh). Elution was done with water at 40°C at a flow rate of 1 ml/min. The EPS was detected by measuring the refractive index of the eluate using a refractive index monitor RI-8020 (Tosoh). Shodex Standard P-82 (Showa Denko, Tokyo, Japan), a series of pullulans with known molecular masses ranging from 0.59×10^4 to 7.88×10^5 Da, was used as the standard.

2.5. Monosaccharide composition of EPS

The purified EPS (2 mg) was hydrolyzed in 250 μl of 2 M trifluoroacetic acid (TFA) at 100°C for 5 h. Excess TFA was removed by rotary evaporation, and the hydrolysate was washed thoroughly with water and lyophilized. The lyophilized powder was dissolved in 100 μl of water, and a 5-μl aliquot was used for thin-layer chromatography (TLC). Development was done twice on a silica gel TLC plate (20 × 20 cm) using a developing solvent of *n*-butanol:ethanol:water (2:1:1, v/v). Carbohydrates were visualized by heating the TLC plate after spraying with 5% (v/v) sulfuric acid in ethanol. Glucose, galactose, and mannose were used as standard monosaccharides.

The molar ratio of the monosaccharides of the hydrolysate was analyzed using an HPLC. Prior to the analysis, the hydrolysate was labeled with 2-aminobenzoic acid using a modified method of Anumula & Dhume (1998). For the labeling reaction, labeling reagent A (4% sodium acetate trihydrate, 2% boric acid, in methanol, w/v) and labeling reagent B (0.32 M 2-aminobenzoic acid, 1 M sodium cyanoborohydride, in the labeling reagent A) were used. The hydrolysate (1.4 mg of EPS) in 10 μl of water was mixed with 50 μl of the labeling reagent B, and the mixture was heated at 80°C for 50 min. After cooling to room temperature, 1 ml of chloroform and 0.5 ml of water were added, mixed well, and then an aqueous phase was collected by centrifugation (900 *g*, 10°C, 10 min). The solvent was removed by rotary evaporation, and the dried sample was dissolved in 500 μl of water. The sample was diluted 50 times with 150 mM trisodium citrate (pH 4.5)/7.5 % (v/v) acetonitrile, and passed through an ODS-100Z column (4.6 × 250 mm, Tosoh) at a flow rate of 0.75 ml/min, using the same buffer as eluant. Glucose and galactose, labeled with 2-aminobenzoic acid, were used as standards. The molar ratio of glucose and galactose was calculated from the peak areas. Each analysis was performed in triplicate.

2.6. ^{1}H-NMR spectroscopy

Exchangeable protons in the purified EPS (2 mg) were replaced by deuterium in 99.99% D_2O. Using a 500 MHz FT-NMR spectrometer, Jeol ECP-500 (Jeol, Tokyo, Japan), ^{1}H-NMR spectra were recorded at a probe temperature of 343 K that allowed us to observe the chemical shifts in the range of 4.75-4.35 ppm, which were overlapped by a large signal of HDO at ambient temperature. The spectrum was measured by reference to internal acetone (δ=2.225), but chemical shifts (ppm) were represented by reference to internal sodium 2,2-dimethyl-2-silapentane-5-sulphonate.

2.7. Viscosity measurement

Using a Dynamic Analyzer RDA II (Rheometric Scientific, Piscataway, USA) equipped with a cone-and-plate attachment (diameter, 25 mm; angle, 0.1 radian; gap, 65 μm), the shear stress of a 1% (w/v) solution of the purified EPS was monitored for 300 s at 22°C up to 300/s, in a steady shear testing mode. The apparent viscosity, η_{app}, was calculated from the shear stress at a certain point of shear rate.

3. Results and discussion

3.1. Development of CDM and the growth of L. fermentum *TDS030603 in CDM*

By subtracting each component from the prototype CDM, out of 48 chemicals, we have determined 25 essential and 17 important but not essential compounds for the cell growth of *L. fermentum* TDS030603 (Table 1). When the strain was cultivated in the prototype CDM which did not contain one of the essential components, no cell growth was observed (results not shown). Optimum cell growth was not obtained even in a medium that contained the 25 essential compounds, so we added other components individually and thereby determined 17 compounds that were important for optimum cell growth. Six amino acids, L-alanine, L-cysteine, glycine, L-lysine, L-proline and L-threonine, were not required for the growth of *L. fermentum* TDS030603, indicating that the biosynthetic pathways of these 6 amino acids were functionally active (Morishita, Deguchi, Yajima, Sakurai, & Yura, 1981). In the developed CDM consisting of 42 chemicals, the bacterial population only reached an OD_{600nm} of 1.5 units whereas 4.5 units were detected after the bacterial growth in MRS (Fig. 1A). At the end of the exponential phase, the great difference of bacterial population between CDM and MRS cultures was confirmed by cultivable cell counts and pH values reflecting acidification of the culture medium (Fig. 1B and 1C).

3.2. EPS production in MRS and CDM supplemented with various carbohydrate sources

EPS production was investigated using MRS and the CDM supplemented with either glucose (CDM_{Glc}), galactose (CDM_{Gal}), lactose (CDM_{Lac}) or sucrose (CDM_{Suc}), each at a concentration of 1% (w/v). Neither cell growth nor EPS production was observed when maltose and/or fructose were used as carbohydrate sources (results not shown). After 72 h cultivation, the best EPS production (97.1 mg/L) in purified form was found in MRS. The

206 EPS production of *L. fermentum* TDS030603 grown in MRS and CDM supplemented with
207 various carbohydrate sources reaches the maximal concentration at the beginning of the
208 stationary phase, in accordance with other EPS producing LAB (Petry, Furlan, Crepeau,
209 Cerning, & Desmazeaud, 2000; Torino, Hébert, Mozzi, F., & de Valdez, 2005). Of the
210 carbohydrates tested, glucose gave the second highest EPS production (69.0 mg/L), while
211 lactose, galactose or sucrose yielded 73%, 51% or 19%, respectively, of the EPS released in
212 CDM_{Glc}. There is no consistency in the reported preference for carbohydrates regarding EPS
213 production (Cerning et al., 1994; Tallon, Bressollier, & Urdaci, 2003; Torino, Hébert, Mozzi,
214 F., & de Valdez, 2005), and thus this seems to be strain-dependent. When the strain was
215 cultivated in MRS, CDM_{Glc}, or CDM_{Gal}, the EPS production reached to the highest at 24-48 h,
216 but a decline of the EPS yield was observed at 72 h (Fig. 1D). The decline of EPS production
217 during prolonged fermentation has been observed in *L. rhamnosus* R being attributed to the
218 enzymatic degradation of EPS (Pham, Dupont, Roy, Lapointe, & Cerning, 2000). Therefore,
219 EPS degrading enzymes might be expressed in *L. fermentum* TDS030603 in 72 h cultivation.
220
221 *3.3. Chemical structure of EPS*
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223 All the EPSs that were released in MRS and the CDM supplemented with various
224 carbohydrates consisted of glucose and galactose; no other monosaccharide was detected on
225 TLC (Fig. 2). The monosaccharide composition of EPS was also investigated by an HPLC
226 experiment. As the result, the molar ratio of glucose to galactose ranged from 2.6 to 2.8
227 (Table 2). This result was similar to the value of 2.5 which had been previously determined
228 for the EPS produced by *L. fermentum* TDS030603 in MRS (Leo et al., 2007). The ^{1}H-NMR
229 spectra of the EPSs released in MRS and CDMs were very similar (Fig. 3). In all spectra,
230 typical chemical shifts that represent (i) an H-2 signal of glucose, which was substituted at
231 OH-2 and OH-3 (δ=5.661), (ii) α-anomeric configuration of the glucose (δ=4.978 and 5.314),
232 and (iii) β-anomeric configuration of the glucose (δ=4.510 and 4.725) were identified. All

these data indicated that composition in monosaccharides of *L. fermentum* TDS030603 EPS were not dependent on the nature of carbohydrates supplemented in the culture medium as was the case for other *Lactobacillus* strains (Petry, Furlan, Crepeau, Cerning, & Desmazeaud, 2000; Torino, Hébert, Mozzi, F., & de Valdez, 2005; van den Berg et al., 1995).

3.4. Molecular mass of EPS

The molecular masses of the major EPSs (peak I, Fig. 4) released either in MRS or CDM supplemented with various carbohydrates showed similar values. Only the major EPS produced in CDM_{Gal} yielded a higher molecular mass than the others. Referring to the standard pullulan and considering the size exclusion limit of the column (approx. 5×10^7 Da), the molecular mass of peak I was estimated to be more than 10^6 Da. The EPS produced in MRS contained a lower molecular mass fraction (peak III, Fig 4), whose molecular mass was 2.8×10^4 Da. An apparent shoulder was observed in the vicinity of peak I in the EPSs released in CDMs supplemented with different carbohydrate sources, although it was not apparent in the EPS released in MRS (peak II, Fig. 4). The molecular mass distributions of peak II were clearly divided into two groups: one includes the EPSs released in CDM_{Glc} and CDM_{Lac} and the other the EPSs released in CDM_{Gal} and CDM_{Suc} (Fig. 4). The peak II fraction was higher in the former group than in the latter.

3.5. Viscosity of EPS

Rheological analysis revealed that the EPS solutions exhibited pseudoplastic behavior that was typical of aqueous solutions of high molecular mass biopolymers. Among the EPS solutions, however, the viscosities were obviously different, especially in the low shear rate range (Fig. 5). At a shear rate of 10/s, the solution of EPS released in MRS yielded an apparent viscosity, η_{app} of 0.88 Pa·s. Compared with this value, only the EPS in CDM_{Glc} had a

higher viscosity, η_{app} of 1.27 Pa·s. Other carbohydrates yielded EPSs whose apparent viscosities at the same shear rate were much lower, namely 43 to 65% of that of the EPS produced in MRS.

3.6. Possible determinant of the viscosity of EPS solution

In contrast to the effect on chemical structure of EPS, carbohydrates had a significant influence on its viscosity (Fig. 5). Generally, the viscosity of EPS is affected by (i) electrostatic interactions between charged residues, (ii) entanglement of long sugar chains, and (iii) the effect of branching. The electrostatic interaction is not the case for *L. fermentum* TDS030603 producing EPS, since it was revealed to be a neutral polysaccharide (Leo et al., 2007). ^{1}H-NMR and monosaccharide composition analysis demonstrated that the chemical structures of the EPSs were very similar (Fig. 2 and Fig. 3). Furthermore, no correlation was observed between the viscosity of EPS solution and the monosaccharide composition ratios of EPS; therefore variations in monosaccharide composition are not a cause of the variations in viscosity. Although no significant variations could be found in the chemical structures of the EPSs, some differences were demonstrated in the molecular mass distribution (Fig. 4). Even though the major EPS fraction (peak I) in CDM_{Gal} had the highest molecular mass, EPS in CDM_{Gal} showed the lowest viscosity. A low molecular mass fraction (peak III) could be found only in the EPS from MRS indicating that differences in EPS chain length are unlikely to cause variations in viscosity. In *Lactobacillus rhamnosus* R lowering molecular mass of EPS caused a decline of the viscosity of EPS solution (Pham, Dupont, Roy, Lapointe, & Cerning, 2000). Therefore, the viscosity of EPS solution might be affected by the ratio of low molecular mass EPS. A certain relationship between viscosity and molecular mass distribution of EPS has been observed in peak II (Fig. 4). Degradation of peak II fraction may cause the lower viscosity of EPS released in CDM_{Gal} and CDM_{Suc}. It was difficult to find a clear relationship between molecular mass distribution and viscosity; nevertheless,

heterogeneity of molecular mass distribution was the most probable cause for the variation of EPS viscosity.

4. Conclusions

To conclude, a CDM for *L. fermentum* TDS030603 has been developed. The ability of the strain to produce a highly viscous EPS was observed in the CDM as well as in MRS. The production of the EPS reaches the maximal concentration at the beginning of the stationary phase, but degradation of the EPS was observed during 72 h cultivation. Carbohydrates did not affect the chemical structure of the EPS, but did affect the production yields. Moreover, the viscosity of the EPS was also affected by carbohydrates, possibly owing to the heterogeneity in the molecular mass distribution of the EPS.

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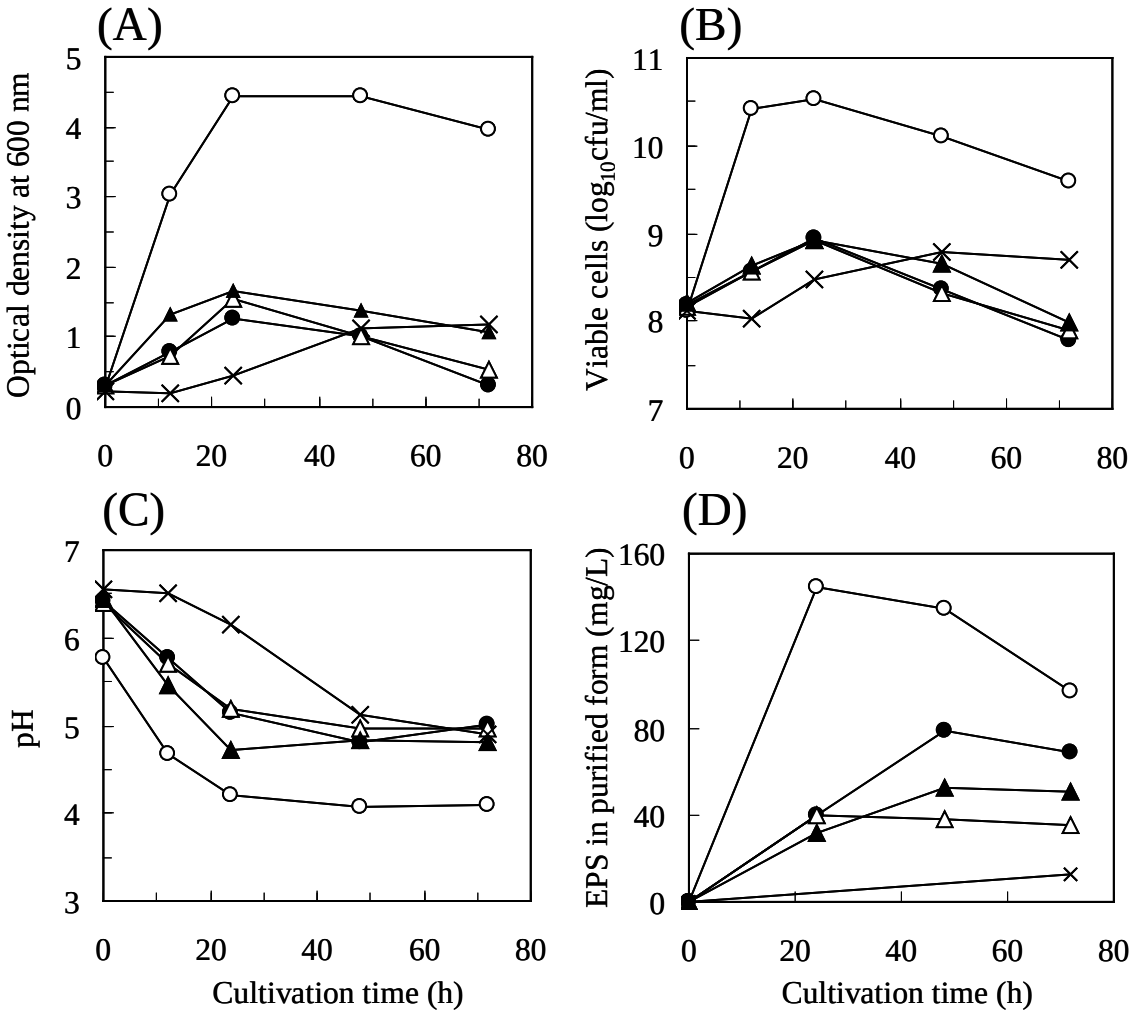

Fig. 1. Cell growth and EPS production of *L. fermentum* TDS030603 and pH profile of the culture broth. The cell growth was monitored by OD_{600nm} (A) and cultivable cell count (B). (C), pH profile of the culture broth. EPS was purified and its dry mass was weighed (D). Symbols; open circle, EPS released in MRS; closed circle, EPS released in CDM supplemented with glucose; open triangle, EPS released in CDM supplemented with galactose; closed triangle, EPS released in CDM supplemented with lactose; cross, EPS released in CDM supplemented with sucrose.

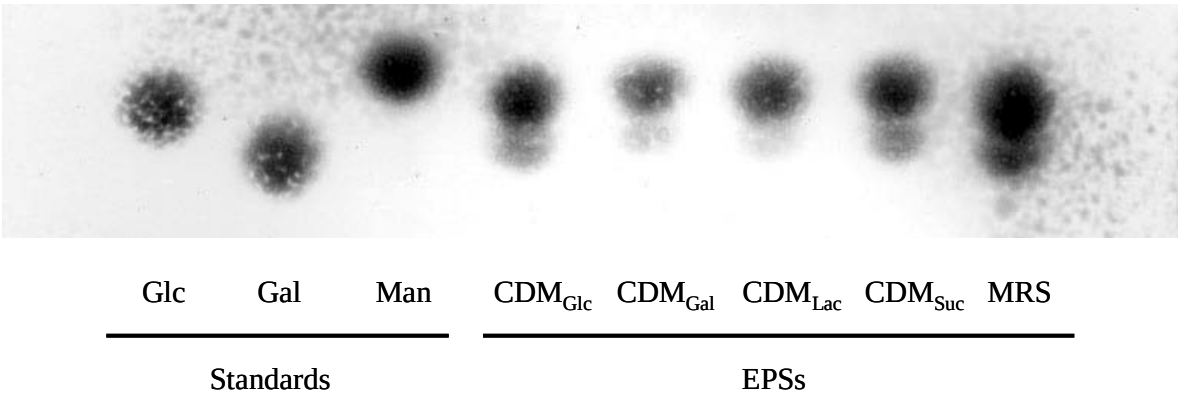

Fig. 2. Monosaccharide composition of EPSs produced by *L. fermentum* TDS030603 in MRS and CDM supplemented with various carbohydrate sources. Glucose (Glc), galactose (Gal), and mannose (Man) were used as standards. MRS, EPS released in MRS broth; CDM_{Glc}, EPS released in CDM supplemented with glucose; CDM_{Gal}, EPS released in CDM supplemented with galactose; CDM_{Lac}, EPS released in CDM supplemented with lactose; CDM_{Suc}, EPS released in CDM supplemented with sucrose.

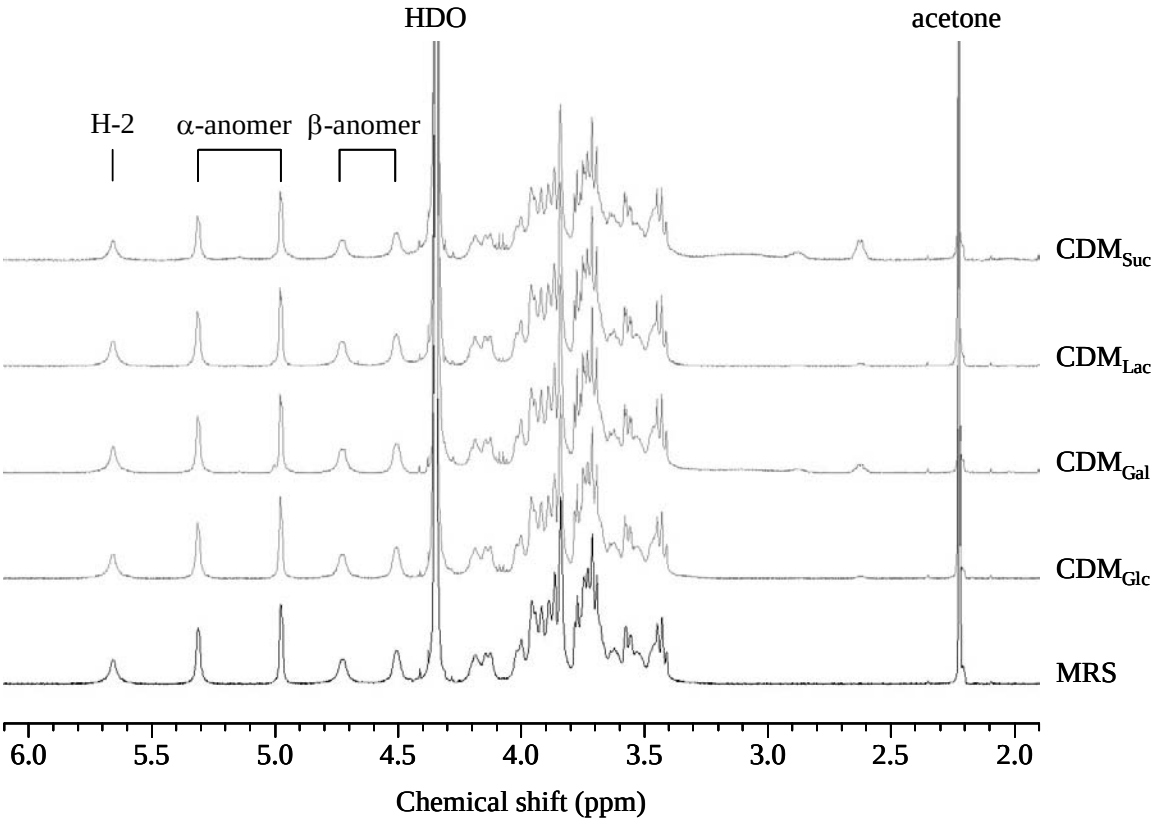

Fig. 3. NMR spectra of EPSs produced by *L. fermentum* TDS030603 in MRS and CDM supplemented with various carbohydrate sources. Chemical shifts derived from a H-2 signal of glucose, which was substituted at OH-2 and OH-3 (δ = 5.661), α-anomer (δ = 4.978 and 5.314), and β-anomer (δ = 4.510 and 4.725) were observed. Chemical shifts of heavy water (HDO) and acetone were δ = 4.348 and 2.225, respectively.

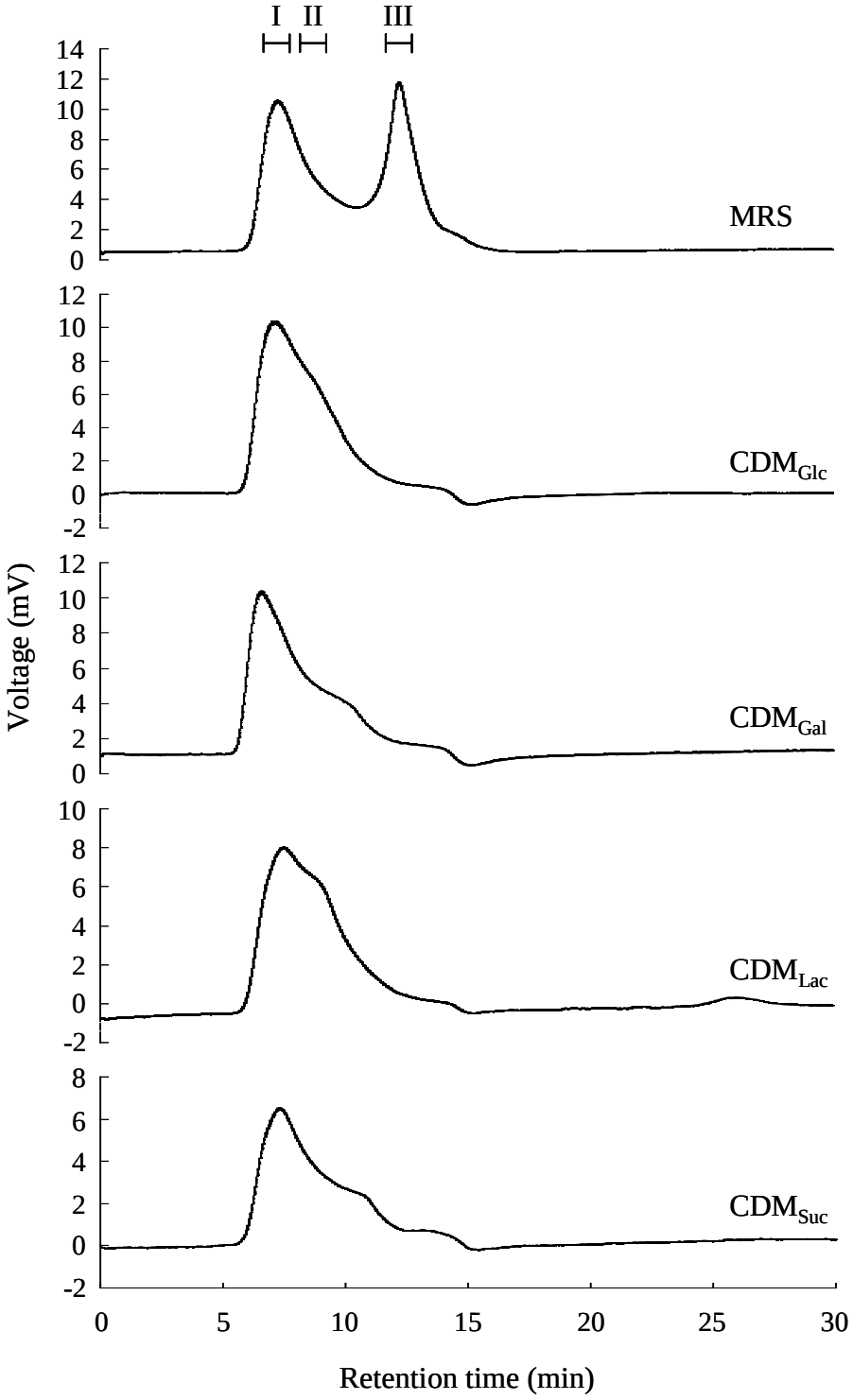

Fig. 4. Molecular mass distribution of EPSs produced by *L. fermentum* TDS030603 in MRS and CDM supplemented with various carbohydrate sources. 100 μg of the each purified EPS were used. I, II, and III indicate corresponding peaks in the chromatograms. The elution of the EPS was monitored by refractive index of the eluent.

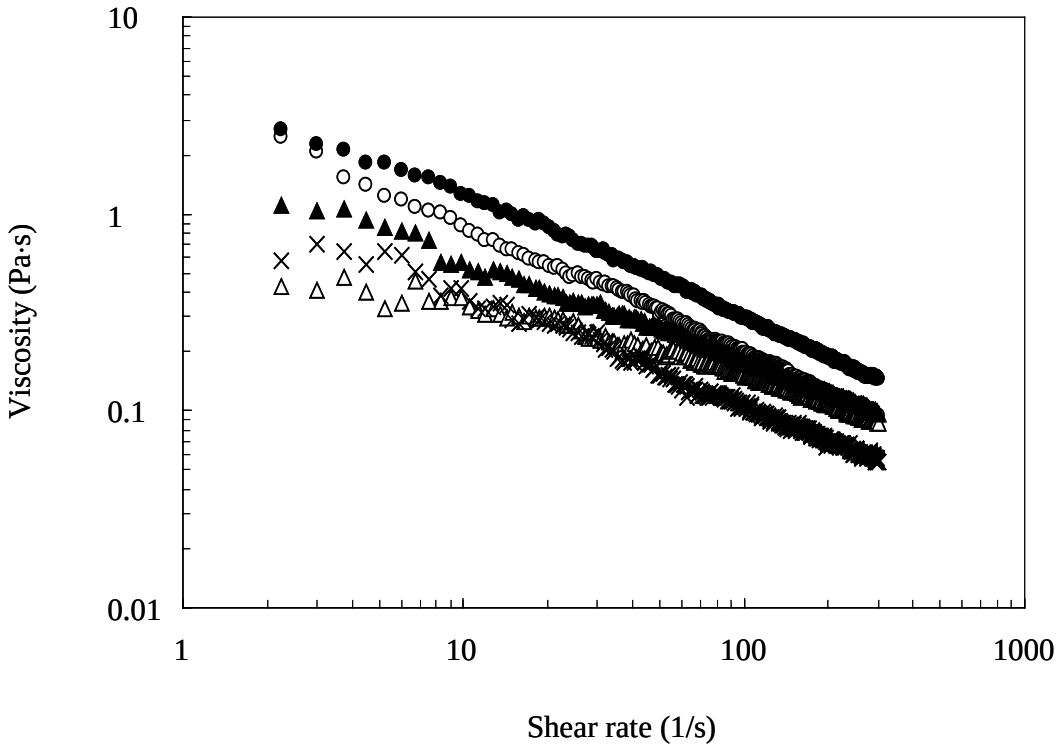

Fig. 5. Viscosity of EPSs produced by *L. fermentum* TDS030603 in MRS and CDM supplemented with various carbohydrate sources. Symbols; open circle, EPS released in MRS; closed circle, EPS released in CDM_{Glc}; open triangle, EPS released in CDM_{Gal}; closed triangle, EPS released in CDM_{Lac}; cross, EPS released in CDM_{Suc}.

Table 1

Chemical composition of the CDM for *L. fermentum* TDS030603

Components	Concentration (g/L)
D-Glucose	10.0[a]
DL-Alanine	0.2[b]
L-Arginine	0.1[a]
L-Aspartic acid	0.1[b]
L-Glutamic acid	0.2[a]
L-Histidine	0.1[a]
L-Isoleucine	0.1[a]
L-Leucine	0.1[a]
L-Methionine	0.1[a]
L-Phenylalanine	0.1[a]
L-Serine	0.1[b]
L-Tryptophan	0.1[a]
L-Tyrosine	0.1[a]
L-Valine	0.1[a]
p-Aminobenzoic acid	0.002[b]
Biotin	0.00001[a]
Folic acid	0.0001[b]
Nicotinamide	0.001[b]
Nicotinic acid	0.001[b]
Pantotheic acid	0.002[a]
Pyridoxal	0.002[a]
Pyridoxol	0.001[b]
Riboflavin	0.0002[b]
Adenine	0.01[a]
Guanine	0.01[b]
Thymine	0.005[a]
Uracil	0.01[a]
Xanthine	0.01[b]
Adenylic acid	0.02[a]
Cytidylic acid	0.05[a]
2'-Deoxyguanosine	0.01[a]
Ammonium citrate	1.0[a]
Sodium acetate	6.0[b]
Sodium citrate	0.5[b]
Sodium thioglycolate	0.5[b]
$FeSO_4 \cdot 7H_2O$	0.02[b]
K_2HPO_4	3.0[a]
KH_2PO_4	3.0[a]
$MgSO_4 \cdot 7H_2O$	0.5[b]
$MnSO_4 \cdot 5H_2O$	0.2[a]
Spermidine phosphate	0.005[b]
Tween80	1.0[a]

[a] Essential.

[b] Important but not essential.

Table 2

Monosaccharide composition of *L. fermentum* TDS030603 releasing EPS in MRS and CDM supplemented with various carbohydrate sources

Media	Molar ratio[a] (Glucose/Galactose)
MRS	2.6 ± 0.05
CDM_{Glc}	2.7 ± 0
CDM_{Gal}	2.6 ± 0.03
CDM_{Lac}	2.6 ± 0.02
CDM_{Suc}	2.8 ± 0

[a]Mean±S.D. (*n*=3).