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Isolation and characterization of an extracellular glucan produced by *Leuconostoc garlicum* PR

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ABSTRACT

White and highly viscous extracellular polysaccharide composed of D-glucose residues has been isolated from culture medium of *Leuconostoc garlicum* PR by acetone precipitation followed by dialysis and freeze-drying. The crude biopolymer was recovered in the yield of 50 g/L of rich culture medium and showed an apparent molecular mass over 2×10^7. Chemical and spectroscopic studies revealed almost linear character of the bacterial biopolymer composed of α-1,6-linked D-glucopyranosyl backbone carrying a low content of branched points at C-2, C-3 and C-4. Moreover, a low content (~2%) of α-1,4-linked D-glucopyranosyl residues has been determined in the extracellular biopolymer.

1. Introduction

Lactic acid bacteria (LAB) are a large group of prokaryotic microorganisms characteristic by lactic acid type fermentation of carbohydrates. Moreover, LAB are involved in many food fermentation processes and industrial applications as well due to their ability to produce extracellular biopolymers, i.e. polysaccharides or glycoproteins. They are able to synthesize homo- as well as heteropolysaccharides which can be modified for further industrial applications (De Vuyst & Degeest, 1999). The core of LAB comprises genera belonging to the order Lactobacillales, i.e. *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Pediococcus*, *Streptococcus*, *Enterococcus*, *Sporolactobacillus* and *Oenococcus*. Of them, *Streptococcus*, *Lactobacillus* and *Leuconostoc* are significant due to production of slime-forming biopolymers – dextrans, glucose-based polymers of linear or branched structure with a high molecular size. Dextrans are mostly composed of α-1,6-linked D-glucopyranosyl residues carrying a low content of branched points at C-2, C-3 or C-4 but α-1,3-linked D-glucopyranosyl backbone has been found as well. Generally, dextrans produced by bacteria differ in the type of linkages, degree and type of branching, molecular size distribution patterns and conformation of polymeric chains. Due to variable structural features, dextrans exhibit different properties such as solubility, rheology, and other physico-chemical characteristics (Monchois, Willemot, & Monsan, 1999). Some *Leuconostoc* species are commercially utilizable for the production of dextrans, slime forming α-glucans with interesting physico-chemical properties. They are used in the food industry as stabilizing, emulsifying, viscosifying, gelling, or water-binding agents and they found a large application area in the non-food industries as well (Sutherland, 1998; Welman & Maddox, 2003).

Generally, three different types of dextrans produced by *Leuconostoc* species are known in dependence on their main chain, i.e. (i) α-1,6-linked, (ii) α-1,6- and α-1,3-linked (alternan) and (iii) α-1,3-linked (mutan). A typical representative of the first group is dextran produced by *Leuconostoc mesenteroides*. It contains about 95% of α-1,6 linkages with a relatively low content (~5%) of 1,3 branches (Naessens, Cerdobbel, Soetaert, & Vandamme, 2005). Glucan produced by *Leuconostoc dextranicum* showed a highly linear structure with about 96% of α-1,6 linkages (Jeanes et al., 1954), however, the presence of low α-1,4 linkage content in this biopolymer was reported as well (Majumder, Singh, & Goyal, 2009). The backbone of dextrans of the second class (alternan) is composed of alternating α-1,3 and α-1,6-linked D-glucopyranosyl residues. This type of dextran was reported as part of soluble EPS produced by *Leuconostoc mesenteroides* (Remaud-Simeon, Willemot, Sarcabal, De Montalk, & Monsan, 2000). Dextran of class 3 (mutan) has a backbone composed of α-1,3-linked D-glucopyranosyl units containing α-1,6-linked branches. This type of dextran tends to be insoluble and it is synthesized by *Streptomyces mutans* (Cote & Leathers, 2005). The exact type of dextran produced by the particular bacterium strain depends on specificities of glucansucrase(s)

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expressed. These enzymes are excreted out of the cell and are responsible for the transfer of glucose units from saccharose to a growing polysaccharide chain (Naessens et al., 2005; Robyt, Yoon, & Mukerjea, 2008).

Up to now the greatest attention among *Leuconostocs* was paid to *L. mesenteroides*, strain NRRL B-512. Polysaccharide produced by this bacterium was intensively studied from structural point of view as well as rheological properties and it has been already commercialized (Robyt & Walseth, 1979). *Leuconostoc citreum*, strains NRRL B-742 (Monchois et al., 1999) and E4976 (Maina, Tenkanen, Maaheimo, Juvonenb, & Virkkia, 2008) have been studied in less extent. Both strains produce dextrans with an α-1,6-linked backbone carrying α-1,3 and α-1,4-linked, or α-1,2 and α-1,3-linked branches, respectively. However, only scarce information is available on extracellular polysaccharides produced by other *Leuconostoc* species.

The aim of our study was to isolate and characterize an unknown very viscous extracellular material produced by microbial strain obtained from an activation tank of waste water treatment plant of a local foodstuffs producer (Babice, Czech Republic). It has been observed that some microbial species produced enormous quantity of slime material in the tank. The preliminary identification, done after isolation and cultivation of unknown microbial agent, indicated the bacterium might belong to the Lactobacillales group. Later, the unknown strain has been identified as *Leuconostoc garlicum* (and designated as strain PR), relatively recently proposed bacterial species (Kim & Kyung, 2002). Till now, no details are available about the production of mucus material by this bacterial species and its chemical composition.

2. Materials and methods

2.1. Isolation of the L. garlicum PR strain

The strain was isolated from the sample of activated sludge showing the signs of non-filamentous bulking (slime bulking). The sample was gently homogenised in a sterile bag using a Masticator (IUL Instruments, Spain) for 15 min. Portions of 10 μL were then streaked onto tryptone yeast extract agar (TYA, HIMEDIA, India) with saccharides (tryptone 6 g/L, yeast extract 3 g/L, glucose 15 g/L and sucrose 15 g/L) and incubated for 2–7 days at 25 °C. Suspected colonies with a mucoid appearance were re-inoculated on the fresh agar plates and finally a pure *L. garlicum* PR strain was isolated. On agar medium it formed highly mucoid colonies of colourless appearance.

2.2. Identification of the L. garlicum PR strain

After Gram staining basal growth properties of the strain were determined on VL agar (Tryptone 10 g/L, yeast extract 5 g/L, beef extract 2 g/L, L-cysteine hydrochloride 0.4 g/L, NaCl 5 g/L, glucose 2 g/L, agar 18 g/L) and on TYA agars amended with glucose (15 g/L) or sucrose (15 g/L). The strain was grown at temperatures 5, 20, 25, 30, 37, 40, 45 and 58 °C aerobically and at 25 and 37 °C anaerobically (Anaerocult A, Merck). For the identification, MicroSeq Full Gene 16S rDNA PCR kit and MicroSeq Full Gene 16S rDNA Sequencing kit (Applied Biosystems) were used according to manufacturer's instruction. Sequence similarity searches were performed using BLAST at the NCBI website (http://www.ncbi.nlm.nih.gov./blast).

2.3. Cultivation of L. garlicum PR and isolation of extracellular polysaccharide (EPS)

The strain was grown in Tryptone yeast extract medium amended with sucrose (tryptone 6, yeast extract 3, sucrose 100; in g/L) at 25 °C for 10 days with vigorous shaking (100 rpm). Production of EPS was monitored by viscosity measurement of growing culture medium in Vibro Viscometer SV 10 (AND, Japan) and cell growth was followed as turbidity at 600 nm (OD_{600}). The cells were removed by centrifugation (20,000 × *g*, 25 °C, 30 min) and EPS was recovered from culture medium by precipitation with five volumes of cold acetone and left overnight at 4 °C. The crude EPS was removed by centrifugation (5000 × *g*, 25 °C, 10 min) and dried on air and in desiccator over P_2O_5. It was further purified by dialysis (MWCO 1000) and freeze-dried.

2.4. General methods

Solutions of carbohydrates were concentrated under reduced pressure at a bath temperature below 40 °C. Polysaccharides were hydrolyzed with 2 M trifluoroacetic acid for 1 h at 120 °C. Quantitative determination of the neutral sugars was carried out in the form of their trifluoroacetates by gas chromatography (Shapira, 1969). Specific optical rotation (1 mL cells, H_2O, c = 0.3%) was measured at 20 °C with a Perkin-Elmer Model 141 polarimeter. Elemental analysis was performed with EA 1108 apparatus (FISONS Instruments, UK). The uronic acid content was analyzed with the 3-hydroxybiphenyl reagent (Blumenkrantz & Asboe-Hansen, 1973).

Molecular size determination of polysaccharides were performed with Shimadzu apparatus (Vienna, Austria) using tandem HEMA-BIO 300 and 1000 columns of dimensions 8 mm × 250 mm and particle size 10 μm. As a mobile phase 0.02 M phosphate buffer pH 7.2 containing 0.1 M NaCl was used at a flow rate 0.8 mL/min. A set of pullulan standards was used for calibration of the column (Gearing Scientific, Polymer Lab. Ltd., UK). The size of EPS-derived oligosaccharides was determined on Hewlet Packard HP 1050 Series (Germany) using MetaCarbo Oligo analytical HPLC column (300 mm × 7.8 mm) using deionized water as an elution system. For isolation of pure oligosaccharides, a preparative column (300 mm × 21.5 mm) of TSKgel Amide-80 with acetonitrile–water elution system (67:33%, v/v) was used.

The quantitative determination of the neutral sugars was carried out in the form of their trifluoroacetates by gas chromatography on a Hewlett-Packard Model 5890 Series II chromatograph equipped with a PAS-1701 column (0.32 mm × 25 m), the temperature program of 110–125 (2 °C/min)–165 °C (20 °C/min) and flow rate of hydrogen 20 cm^3/min. Gas chromatography–mass spectrometry (GC–MS) of partially methylated alditol acetates was performed on FINNIGAN MAT SSQ 710 spectrometer equipped with SP 2330 column (0.25 mm × 30 m) at 80–240 °C, 70 eV, 200 A, and ion-source temperature 150 °C (Jansson, Kenne, Liedgren, Lindberg, & Lonngren, 1976). FT-IR spectra of polysaccharides were recorded with Nicolet Magna 750 spectrometer with DTGS detector and OMNIC 3.2 software. The sample was pressed into KBr pellet with a sample/KBr ratio 1/200 mg.

NMR spectra were measured in D_2O at 25 and 40 °C on Varian 400 MR spectrometer on direct 5 mm PFG AutoX probe and Varian 600 VNMRS equipped with and Triple resonance salt tolerant cold probe with ^{13}C enhanced sensitivity (both with z-gradients). Before analysis samples were freeze-dried two times from 95% D_2O and after they were dissolved in 99.98% D_2O. For both, ^1H and ^{13}C NMR spectra, chemical shifts are referenced to internal acetone (δ 2.217 and 31.07, respectively). For the assignment of signals 1D and 2D NMR techniques were used.

2.5. Gel filtration chromatography

Gel filtration chromatography of EPS was performed on columns (3 cm × 75 cm) of Sephacryl S 200, S 300, S 400 and S 1000. The sample of EPS (10 mg/1 mL) was loaded on the column and eluted with 0.1 M sodium acetate buffer (pH 4.5) at a flow rate 15 mL/h.

Fractions of 5 mL were collected and analyzed for the carbohydrate content by phenol–sulphuric acid assay (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956).

2.6. Partial acid hydrolysis and HPLC and gel filtration chromatography

The sample of EPS (500 mg) was partially hydrolyzed with 1 M TFA (50 mL) for 60 min at 100 °C. TFA was evaporated and a hydrolyzed mixture was analyzed on MetaCarbo Oligo analytical HPLC column (300 mm × 7.8 mm) using deionized water as an elution system. For preparative purposes the hydrolyzed mixture was separated on Bio-Gel P2 column (200 cm × 2.5 cm) into ten fractions, i.e. monosaccharides, oligosaccharides of dp 2–8 and partly hydrolyzed polymeric residues. Fractions of 5 mL were collected and analyzed for the carbohydrate content (Dubois et al., 1956). The degree of polymerization (dp) of oligomers was identified by comparison with the elution profile of maltooligosaccharides (Serva, Germany) which were used as reference standards. Oligosaccharides of dp 2–7 were further purified on a preparative HPLC column (21.5 mm × 30.0 cm) of TSKgel Amide-80 with acetonitrile–water elution system (67:33%, v/v). Fractions of 5 mL were collected and pure oligomeric fractions were concentrated and freeze-dried to give two dimers (2_1 and 2_2), trimer (3), tetramer (4), pentamer (5), hexamer (6) and heptamer (7).

2.7. Sugar linkage analysis

The dry samples of polysaccharide and oligosaccharides (1–5 mg) were dissolved in dry dimethyl sulfoxide (1 mL) and methylated by the Hakomori procedure (Hakomori, 1964). The methylated carbohydrates were purified using the Sep-Pak C18 cartridge (Waters Assoc.), hydrolyzed with 90% formic acid at 100 °C for 1 h and with 2 M trifluoroacetic acid at 120 °C for 1 h, reduced with sodium borodeuteride, acetylated and analyzed in GC–MS (Jansson et al., 1976).

3. Results and discussion

3.1. Isolation and physico-chemical characteristics of EPS

The *L. garlicum* PR strain was grown in Tryptone yeast extract medium rich in sucrose at 25 °C for 10 days. Extracellular polysaccharide (EPS) was harvested from culture medium at a stationary phase of growth. Microbial biomass was separated by centrifugation and EPS was recovered from viscose culture medium by acetone precipitation and air drying. A crude EPS was further dissolved in distilled water and purified by dialysis, and freeze-dried to give a white fluffy material. The yield of EPS recovered from the culture medium was 50 g/L (Fig. 1). Compositional analysis of EPS revealed the presence of glucose residues only, indicating thus that a homopolymer of glucan type is produced by *L. garlicum*. Gel filtration chromatography of EPS on Sephacryl S 200, S 300 and S 400 columns (not shown) was found to be not efficient as it afforded one dominant peak eluted in the void volume and one small peak (supposed as a contaminant) in the total volume of Sephacryl columns. Successive chromatography on Sephacryl S 1000 column (Fig. 2) suggested relatively high molecular mass of EPS which was much higher than that of dextran used as a reference standard (M_w 2 × 10^6). Followed HPLC analysis of EPS revealed molecular mass over 2 × 10^7. CHN analysis of EPS did not confirm the presence of nitrogen content and it indicated the carbohydrate-based EPS only. Its specific optical rotation, determined to be +131° (c = 0.3, H_2O), indicated α-configuration of glycosidic linkage in EPS.

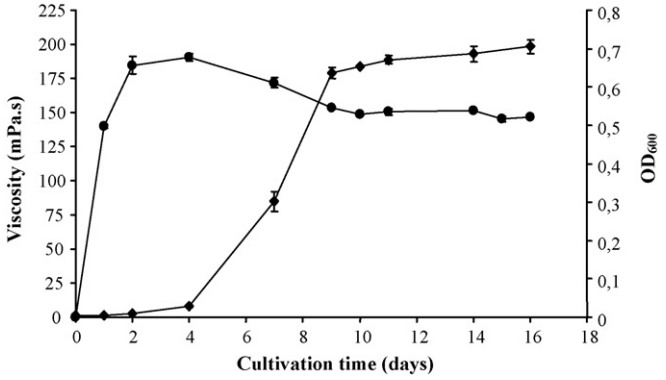

Fig. 1. Growth of *L. garlicum* PR and polymer production in a liquid medium; (♦) the growth of the strain followed as OD_{600}; (●) viscosity of a culture medium.

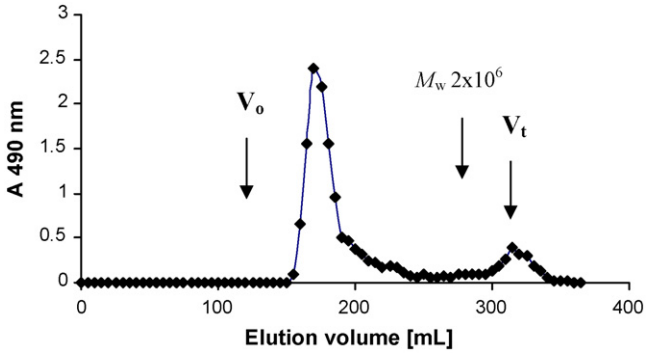

Fig. 2. Sephacryl S-1000 chromatography of EPS produced by *L. garlicum* PR.

3.2. Depolymerization of EPS and isolation of oligomers

The partial degradation of EPS with diluted acid yielded a hydrolyzate mixture containing a polymeric residue, series of oligosaccharides and monosaccharide fractions. The analytical elution profile of depolymerized EPS is shown in Fig. 3. The size of oligomers was identified by comparison with the elution profile of maltooligomers used as reference standards. As it can be seen from the Fig. 3 the major oligomeric components of the degraded EPS were dimers, trimers, tetramers and pentamers. The content of higher oligomers was decreasing with increasing length of their chains. For preparative purposes depolymerized EPS was separated on Bio-Gel P-2 column into ten fractions, i.e. partly hydrolyzed polymeric residue, oligosaccharides of dp 2–8 and monosaccharides. Oligosaccharides of dp 2–7 were further

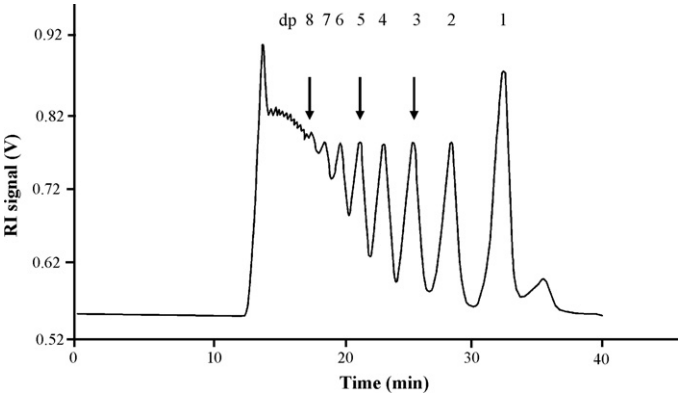

Fig. 3. HPLC profile of *L. garlicum* PR EPS-derived oligosaccharides.

Table 1
Sugar linkage analysis of extracellular polysaccharide (EPS) from *L. garlicum* PR.

Sugar derivative	Mole (%)	Mode of linkage	Relative proportion of bonds (%)	
2,3,4,6-Me_4-Glc[a]	7.2	Glcp-(1→		
2,3,4-Me_3-Glc	84.0	→6)-Glcp-(1→	1→6	91.2
2,3,6-Me_3-Glc	1.9	→4)-Glcp-(1→	1→4	2.5
2,4-Me_2-Glc	5.7	→3,6)-Glcp-(1→	1→3	5.7
3,4 + 2,3-Me_2-Glc	1.2	→2,6)-Glcp-(1→	1→2	0.6
		→4,6)-Glcp-(1→		

[a] 2,3,4,6-Me_4-Glc = 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-glucitol, etc.

purified using a preparative HPLC column into two dimers (2_1 and 2_2), trimer (3), tetramer (4), pentamer (5), hexamer (6) and heptamer (7). Glucooligomers of dp 2–7 were chosen for chemical and spectroscopic characterization. Their sugar compositional analyses revealed glucose residue only as the sugar component.

3.3. Methylation analysis of EPS and its oligomers

Sugar linkage analysis of *L. garlicum* PR polysaccharide revealed the presence of characteristic linkages found commonly in dextran type of polymers produced by *Leuconostoc* species (Table 1). Obtained data show that 1,6-linked glucopyranose was found as a dominant component of methylation analysis, indicating thus a linear character of glucopyranose backbone in EPS. In addition, small amounts of 2,6-linked, 3,6-linked and 4,6-linked Glc residues due to branching points have been determined. Thus the total amount of 1,6 linked Glc represents over 91% of all linkages present in EPS. As it can be seen from the Table 1, totally 5.7% of 1,3 linkages, 2.5% of 1,4 linkages and 0.6% of 1,2 linkages only have been determined in EPS. The presence of above mentioned linkages can be observed normally in dextrans class 1 (Jeanes et al., 1954; Lewis, Cyr, & Smith, 1968). Linkage analyses of EPS-derived oligosaccharides of dp 2_2–7 showed the presence of two components only, i.e. terminal and 6-linked glucopyranose residues confirming thus their linear 6-linked backbone. However, methylation analysis of 2_1 dimers revealed in addition to terminal and 6-linked glucose (as a contaminant) as well 4-linked, 3-linked and 2-linked glucose residues indicating thus the presence of four glucose disaccharides, i.e. isomaltose, maltose, nigerose and kojibiose, respectively.

3.4. NMR and FT-IR spectroscopies of EPS and EPS-derived oligomers

The ^{1}H NMR spectrum of EPS (Fig. 4) produced by *L. garlicum* PR showed one dominant H1 signal at δ 4.99, typical for anomeric protons of α1,6-linked dextrans, and three low intensity signals at δ 5.11, 5.18 and 5.33 indicating the branching of some glucose units in the polysaccharide backbone. Relative intensities of these anomeric protons were found to be 94.8%, 1.3%, 0.8% and 3.1%, respectively, and their $^3J_{1,2}$ coupling constants gave an evidence about α-type glycosidic linkages. The ^{1}H NMR spectral pattern of EPS produced by *L. garlicum* PR resembled that of *L. citreum* E497. In the ^{1}H NMR spectrum of *L. citreum* E497 chemical shift of internal 1,6-linked αGlc units was observed at δ 4.98, that ones due to 1,2,6- and 1,3,6-linked αGlc at δ 5.18 and 5.32, respectively, while the signal due to the terminal αGlc units linked to O_2 of neighbouring αGlc was found at δ 5.11 (Robyt et al., 2008). Comparison of intensities of anomeric signals in the ^{1}H NMR spectra of EPSs from both strains showed differences indicating different degree of branching. EPS produced by *L. citreum* E497 showed ~75% of non-substituted α-1,6-linked D-glucopyranosyl units, ~11% of 2,6-linked, 3-4% of 3,6-linked and ~11% of terminal glucose units. However, the total amount of 1,6 linked Glc represents ~90% of all linkages present in EPS. In *L. garlicum* PR dextran, about 95% of α-1,6-linked Glc residues were found, while only 5% of those involved in others linkages. However, their low quantity did not allow detection in 2D ^{1}H-^{1}H homonuclear correlated COSY and ^{1}H-^{13}C heteronuclear HSQC experiments (Figs. 5 and 6). Thus only NMR data due to 1,6-linked αGlc units in the backbone were assigned (4.99/98.79 (H1/C1), 3.59/72.40 (H2/C2), 3.73/74.33 (H3/C3), 3.54/70.61 (H4/C4), 3.91/71.12 (H5/C5) and 3.76, 3.99/66.53 (H6, H6′/C6)). They were in full accordance with data published for *L. citreum* E497 (Robyt et al., 2008).

Signals present in the anomeric region of ^{1}H NMR spectra of oligosaccharide fractions (Fig. 7) with dp 2_2-7, indicated the presence of 1,6-linked α glucose oligomers. Signals due to reducing end αGlc and βGlc appeared at δ 5.241 and 4.675, respectively, while those due to internal Glc units were found at δ 4.980. They were partly overlapped by the signal due to the terminal Glc at δ 4.965. In all spectra signals of very low intensities, indicating the presence of other type of linkages could be observed, but their total quantity was less than 2-3% in each fraction. The dimer fraction 2_2

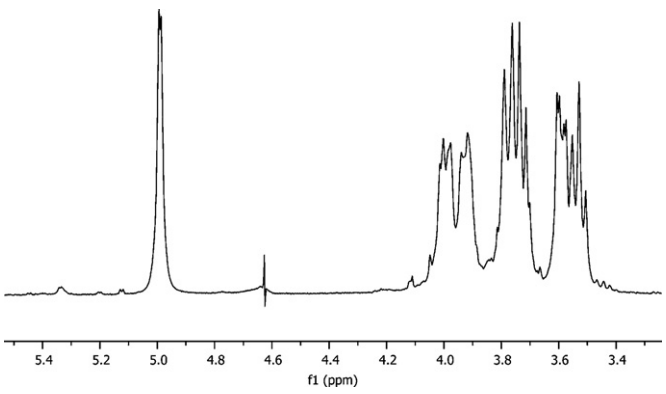

Fig. 4. The ^{1}H NMR spectrum of EPS produced by *L. garlicum* PR.

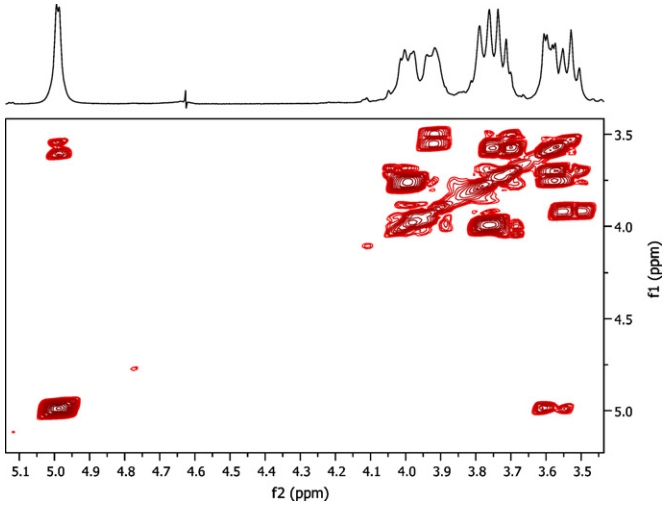

Fig. 5. The COSY spectrum of *L. garlicum* PR EPS.

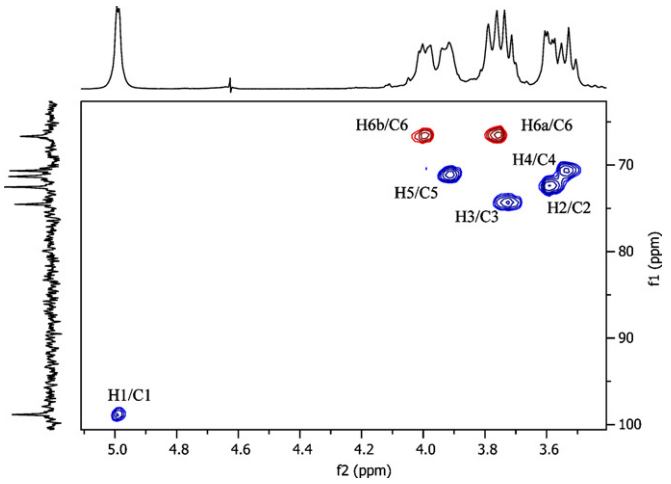

Fig. 6. The HSQC spectrum of *L. garlicum* PR EPS.

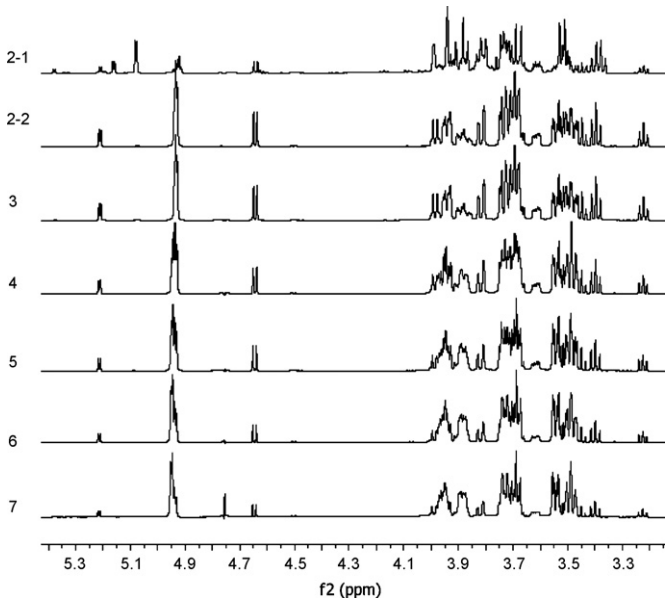

Fig. 7. The ^{1}H NMR spectra of *L. garlicum* PR EPS-derived oligomers.

was identified as αGlc(1→6)Glc. In the ^{1}H NMR spectrum of the dimer fraction 2_1 its signals were still present as a contaminant, however, dominant disaccharides were those with other type of linkages. Thus the comparison of obtained NMR data with those already published for glucose oligosaccharides (Roslund, Tahtinen, Niemitz, & Sjoholm, 2008) was performed. Signal at δ 4.920 was coherent with the chemical shift of the non-reducing Glc unit data due to isomaltose [αGlc(1→6)Glc] disaccharide (~25%). Kojibiose [αGlc(1→2)Glc] presence in the mixture (~45%) was confirmed by the presence of the non-reducing αGlc H1 signal at δ 5.080 together with its corresponding αGlc reducing end unit at δ 5.209. Two signals at δ 5.327 and 5.342 (both in total less than 2%) were characteristic of nigerose [αGlc(1→3)Glc]. The signal at δ 5.386 due to the terminal Glc linked to *O*-4 of reducing Glc (5.20 α reducing end) indicated the presence of maltose [αGlc(1→4)Glc] in the mixture (~5%).

The FT-IR spectrum of *L. garlicum* EPS (Fig. 8) resembled that of EPS produced by *L. dextranicum* (Majumder et al., 2009). Its vibration spectrum showed in the fingerprint region 1200–1000 cm^{-1} the presence of bands characteristic for polysaccharide moiety. The main bands at 1014, 1075, 1107 and 1155 cm^{-1} are due to glucan moiety and the anomeric bands at 847 and 916 cm^{-1} indicate the α-configuration of glucose units. Besides, the band observed at 1650 cm^{-1} is due to associated water content and bands at 2929 and 3396 cm^{-1} arise from stretching vibrations of C-H and OH groups of glucose residues (Kačuráková, Capek, Sasinková, Wellner, & Ebringerová, 2000; Stuart, 1997).

In conclusion, the preliminary studies revealed an interesting type of the extracellular homopolysaccharide – dextran, produced by *L. garlicum* PR, recently proposed species from *Leuconostoc* genera (Kim & Kyung, 2002). Sucrose rich medium was used as the carbon source for cultivation of *L. garlicum* PR and crude extracellular polysaccharide was recovered in the yield of 50 g/L. Its molecular mass was estimated to be higher than 2×10^7. Structural studies showed that the bacterium produces a highly linear α-D-glucan with an α-1,6-linked (~95%) glucopyranose backbone carrying only low content of short side α-1,2, α-1,3-and α-1,4-linked chains (~5%). The main structural features of *L. garlicum* PR

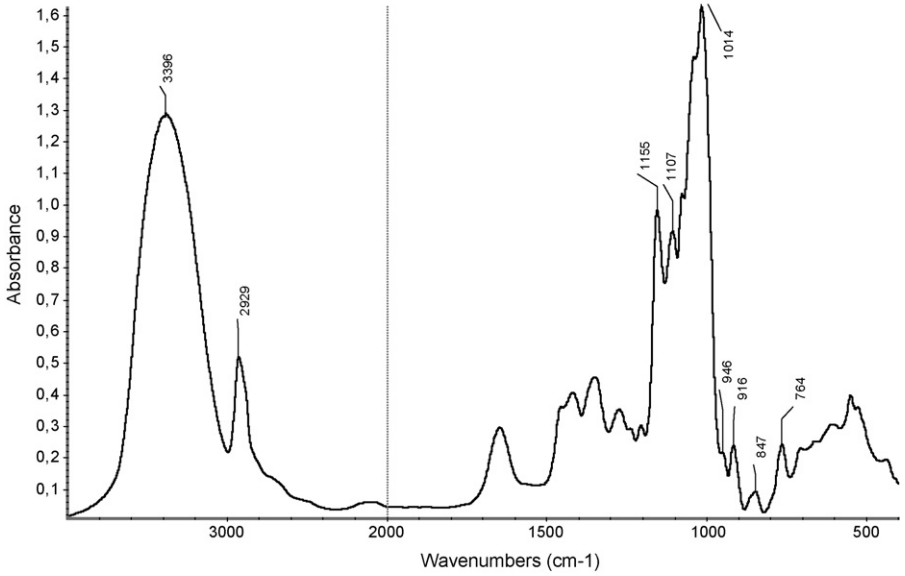

Fig. 8. The FT-IR spectrum of *L. garlicum* PR EPS.

dextran resembled those of dextrans produce by *L. dextranicum* and *L. citreum* E497, i.e. dextrans class 1. Moreover, the *L. garlicum* PR biopolymer was shown to exhibit relatively high degree of purity and forms solutions of relatively low viscosity at higher concentrations which can be an important factor for possible use of this dextran in various industrial applications.

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