The EuroBiotech Journal
RESEARCH ARTICLE
Food and Feed Biotechnology

Structures of cell-wall glycopolymers of *Lactococcus lactis* BIM B-1024

Evelina Leont'evna Zdorovenko[1], Alexander Stepanovich Shashkov[1], Alexandra Alexeevna Kadykova[1,2], Artem Nikolaevich Fakhrutdinov[1], Elena Pavlovna Kiseleva[3], Danuta Tofilevna Plotnikava[4], Galina Ivanovna Novik[4] and Yuriy Alexandrovich Knirel[1]

Abstract

Glycopolymers of two types were isolated from the cell wall of *Lactococcus lactis* BIM B-1024 by stepwise extraction with cold and hot 10% CCl_3CO_2H and separated by anion-exchange gel chromatography. The following structures of the glycopolymers were established by sugar analysis, dephosphorylation with 48% HF, 1D and 2D NMR spectroscopy, and ESI-MS:

β-D-Gal*p*-(1→4)-β-D-Glc*p*-(1→6)┐
→4)-β-D-Gal*p*-(1→4)-β-D-Glc*p*-(1→4)-α-D-Glc*p*-(1→ PSI

β-D-Glc*p*-(1→3)┐
→6)-α-D-Gal*p*NAc-(1→3)-β-D-Gal*p*NAc-(1→5)-Rib-ol-1-*P*-(O→ PSII
β-D-Glc*p*-(1→4)┘

Polysaccharides with the same or similar structures to PSI have been found earlier in various *Lactobacillus* species, whereas, to our knowledge, the structure of PSII is new.

Introduction

Lactic acid bacteria possesses probiotic properties and are beneficial to human health including the following activities: production of antimicrobial substances (bacteriocins, organic acids) and adherence to gut mucosa, enhancement of mucosal and host immunity; prevention of diarrhea, colon cancer, hypercholesterolemia and general improvement of the gastrointestinal microflora (1-2). The *Lactococcus* genus includes 5 species relating to a group of mesophilic lactic acid bacteria. *Lactococcus lactis* is the only-begotten one which has industrial importance. Lactococci are gram-positive, non-motile, catalase-negative, microaerophilic, non-spore forming bacteria and are able to excreting extracellular polysaccharide substances (3-4). Green plants are considered as the natural habitat for *L. lactis*, but frequently these microorganisms are also isolated from milk and dairy products. Lactococci and other lactic acid bacteria are currently used in biotechnology for production of a wide variety of fermented foods, especially meat and vegetables. *L. lactis* strains are also used in manufacturing of fermented dairy products, such as cream, butter, cheese (5-6). The problem of bacteriophage infection in factories is therefore important because of great economic damage (7).

The probiotic properties of different lactic acid bacteria are closely related to their biologically active glycoconjugates, which are structural molecules of the bacterial cell envelope. These substances are represented by polysaccharides, glycoproteins, teichoic and lipoteichoic acids, along with and in complexes with proteins (8-9). Some strains of *L. lactis* produce thermostable proteins bacteriocins with bactericidal properties

[1]N.D. Zelinsky Institute of Organic Chemistry, Russian Academy of Sciences, Moscow, Russia
[2]Higher Chemical College of the Russian Academy of Sciences, D. I. Mendeleev University of Chemical Technology of Russia, Moscow, Russia
[3]Institute of Bioorganic chemistry, National Academy of Sciences of Belarus, Minsk, Belarus
[4]Institute of Microbiology, National Academy of Sciences of Belarus, Minsk, Belarus

Corresponding author: Evelina Leont'evna Zdorovenko
E-mail: zdorovenkoe@mail.ru

Published online: 27 January 2017
doi:10.24190/ISSN2564-615X/2017/01.07

against closely related bacteria (10). These compounds prevent the growth of pathogens that makes them promising for use as probiotics (11-12). Furthermore, *L. lactis* is able to reduce the level of cholesterol (13). Another practical application of lactococci is live vaccines (14-15). Exopolysaccharides producing by lactic acid bacteria stimulate the immunity of host (16-18).

In this study, we isolated and studied structures of exopolysaccharides from *Lactococcus lactis* 1024-BIM, which appears antibiotic susceptible according to the European Food Safety Authority (EFSA) requirements and is perspective for application as probiotic and live vaccines.

Materials and Methods

Isolation of polysaccharides

A cell wall preparation was obtained by cell disintegration using a UZDN_1 ultrasonic disintegrator as described (19). Polysaccharides were extracted from the cell walls with 10 % TCA (1:10 w/v) at 4 °C for 48 h. The mixture was centrifuged, the supernatant was dialyzed against distilled water and lyophilized to give CE. The sediment was extracted with 10 % TCA at 100 °C for 5 min, the supernatant was dialyzed against distilled water and lyophilized to give HE. Both polysaccharide preparations obtained were dissolved in pyridinium acetate buffer and applied to a column (80 ´ 1.6 cm) of TSK HW-40 (S) using 1% AcOH as eluent and monitoring with Knauer differential refractometer.

The polysaccharide preparations (22 mg) from cold and hot extracts were combined and fractionated by anion-exchange chromatography on a column (20 × 1 cm) of DEAE-Trisacryl M using a stepwise gradient of 0.005, 0.01, 0.1, 0.25, and 0.5 M hydrogen sodium phosphate pH 6.3. As result, two polysaccharides were obtained: PSI eluted in 0.005 M buffer (6.1 mg) and PSII eluted in 0.1 M buffer (10.4 mg).

Chemical methods

Hydrolysis was performed with 2 M CF_3CO_2H (120 °C, 2 h), the monosaccharides were analyzed by GLC as the alditol acetates on an Ultra 2 capillary column using a Hewlett–Packard 5880 instrument and a temperature gradient of 180 °C (1 min) to 290 °C at 10 °C min^{-1}. The absolute configurations of the monosaccharides were determined by GLC of the acetylated glycosides with (*S*)-2-octanol as described (20).

PSII was treated with 48 % HF at 4 °C for 16 h, and the products were fractionated on a TSK HW-40 column as described above.

NMR spectroscopy

NMR spectra were recorded for solutions in 99.95% D_2O after deuterium-exchange by freeze-drying from 99.9% D_2O. Spectra were measured on Bruker DRX-500 and Bruker Avance II 600 spectrometers with 5 mm broadband inverse probehead at 30 °C. Sodium 3-(trimethylsilyl)propanoate-2,2,3,3-d_4 (δ_H 0.0, δ_C -1.6) was used as internal standard for calibration. 2D NMR experiments were performed using standard Bruker pulse programs. The 2D TOCSY and ROESY spectra were recorded with a 60 ms duration of MLEV-17 spin-lock and a 150 ms mixing time, respectively. The gradient-selected $^1H,^{13}C$ HMBC spectrum was recorded with a 60 ms delay for evolution of long-range spin couplings.

Mass spectrometry

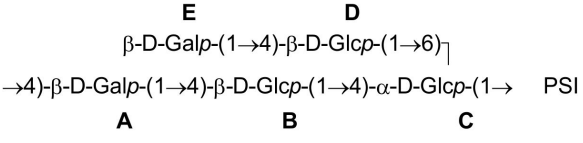
High-resolution ESI mass spectra were measured on a Bruker micrOTOF II instrument (21). The measurements were done in positive or negative ion modes (interface capillary voltage –4500 V or 3200 V, respectively); mass range from *m*/*z* 50 to *m*/*z* 3000 Da. Internal calibration was done with Electrospray Calibrant Solution (Fluka). A syringe injection was used for 1:1 acetonitrile/water solutions (flow rate 3 mL min^{-1}). Nitrogen was applied as a dry gas; interface temperature was set at 180 °C.

Results and Discussion

Polysaccharides were isolated from disintegrated cells of *L. lactis* BIM B-1024 by stepwise extraction with 10% CCl_3CO_2H first at 4 °C for 48 h and then at 100 °C for 5 min. The cold extract and hot extract were separately dialyzed, lyophilized, and purified by GPC on TSK HW-40 (S). Sugar analysis by GLC of the alditol acetates revealed similar composition of both extracts, which contained ribitol (Rib-ol), glucose, galactose, and GalNAc. The D configuration of the monosaccharides was determined by GLC of the acetylated (*S*)-2-octyl glycosides (22).

The NMR spectra suggested that both extracts are mixtures of polysaccharides, which were separated by anion-exchange chromatography on DEAE-Trisacryl M using a stepwise gradient of 0.005 to 0.5 M hydrogen sodium phosphate pH 6.3. As result, two polymers were obtained: a neutral polysaccharide PSI and an acidic polysaccharide PSII, which were studied by 1D and 2D NMR spectroscopy.

The ^1H and ^{13}C NMR spectra of PSI showed signals for anomeric atoms at δ_H 4.46-4.92 and δ_C 101.1-104.6 of five monosaccharide residues designated units **A**-**E**. Tracing connectivities in the 2D $^1H,^1H$ COSY, 1D and 2D $^1H,^1H$ TOCSY, and $^1H,^{13}C$ HSQC spectra enabled assignment of the ^1H and ^{13}C NMR signals of PSI (**Table 1**). The ^1H and ^{13}C NMR chemical shift data combined with data of linkage and sequence analyses by 1D NOE, 2D ROESY, and 2D $^1H,^{13}C$ HMBC experiments (not shown) revealed the following structure of PSI:



This structure has been established earlier for an exopolysaccharide of *Lactobacillus delbrueckii* subsp *bulgaricus* 291 (23). Also, exopolysaccharides with similar structures differing in O-acetylation of one of the glucose residue or

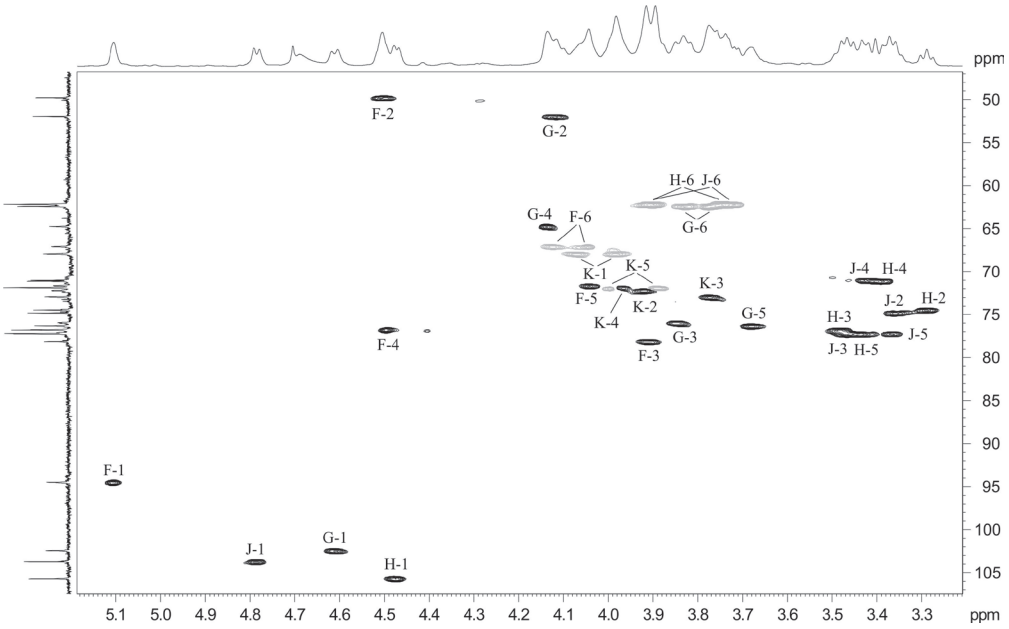


Figure 1. Parts of a 2D $^{1}H,^{13}C$ edHSQC spectrum of PSII. The corresponding parts of the ^{1}H NMR spectrum are shown along the axes. Cross-peaks of transglycosidic protons are annotated in bold face. Numbers refer to protons of sugar units designated as shown in Table 1.

Table 1. ^{1}H and ^{13}C NMR chemical shifts (δ, ppm) of the PSI, PSII and oligosaccharide obtained after dephosphorylation of PSII (OS1)

Sugar residue		H-1 *C-1*	H-2 *C-2*	H-3 *C-3*	H-4 *C-4*	H-5 *C-5*	H-6 *C-6*
PSI							
→4)-β-D-Gal*p*-(1→	(**A**)	4.52 104.6	3.59 72.0	3.77 73.2	4.03 78.6	3.80 76.7	3.94/3.84 61.4[c]
→4)-β-D-Glc*p*-(1→	(**B**)	4.63 103.4	3.37 74.2	3.68 75.5	3.68 79.7	3.61 76.1	3.84/3.99 61.2[c]
→4,6)-α-D-Glc*p*-(1→	(**C**)	4.92 101.1	3.61 72.8	3.86 72.5	3.80 79.1	4.40 70.8	4.16/4.00 68.4
→4)-β-D-Glc*p*-(1	(**D**)	4.55 103.5	3.38 74.0	3.68 75.6	3.68 79.7	3.61 76.0	3.84/3.99 61.3[c]
β-D-Gal*p*-(1	(**E**)	4.46 104.2	3.56 72.2	3.67 73.8	3.94 69.8	3.74 76.7	3.76/3.79 62.3
PSII							
→3,4,6)-α-D-Gal*p*NAc-(1→	(**F**)	5.11 94.4	4.50 49.9	3.91 78.2	4.49 76.8	4.04 71.7	4.05/4.12 67.1
→3)-β-D-Gal*p*NAc-(1→	(**G**)	4.62 102.5	4.12 52.1	3.84 76.0	4.13 64.7	3.68 76.3	3.77/3.82 62.4
β-D-Glc*p*-(1→	(**H**)	4.47 105.7	3.29 74.5	3.48 76.9	3.39 71.1	3.42 77.3	3.73/3.90 62.1
β-D-Glc*p*-(1→	(**J**)	4.78 103.8	3.36 74.9	3.48 77.2	3.42 71.1	3.37 77.3	3.75/3.90 62.1
→5)-Rib-ol-1-*P*-(O→	(**K**)	3.98/4.06 68.0	3.94 72.3	3.77 73.0	3.92 72.2	3.89/4.00 72.0	
Oligosaccharide 1							
→3,4)-α-D-Gal*p*NAc-(1→		5.09 95.1	4.50 49.8	3.93 78.0	4.49 76.7	4.08 71.7	3.79 62.0
→3)-β-D-Gal*p*NAc-(1→		4.59 102.5	4.37 49.5	3.82 76.3	4.13 64.9	3.66 76.4	3.84 61.8
β-D-Glc*p*-(1→		4.49 105.8	3.32 74.3	3.49 76.8	3.40 71.1	3.44 77.1	3.73/3.92 62.1
β-D-Glc*p*-(1→		4.83 103.3	3.37 74.7	3.50 77.2	3.43 71.1	3.41 77.0	3.73/3.92 62.1
→5)-Rib-ol		3.89/3.99 71.9	3.89 72.1	3.72 73.2	3.81 73.2	3.89/4.00 72.0	

containing an additional galactose residue have been found in *Lactobacillus lactis* subsp. *cremoris* B891 (24) and *Lactobacillus helveticus* K16 (25), respectively.

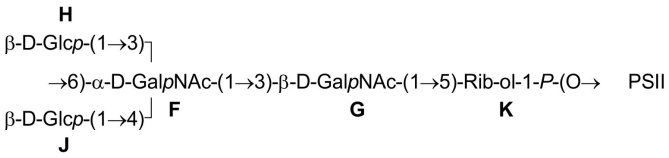
The ^{1}H NMR spectrum of PSII showed signals for four anomeric protons at δ 4.47-5.11, other sugar ring protons at δ 3.29-4.50, and two *N*-acetyl group at δ 2.02-2.09. The ^{13}C NMR spectrum of PSII contained signals for four anomeric carbons at δ 94.4-105.7, a number of OCH_2 groups (C-6 of hexoses and GalNAc, C-1 and C-5 of Rib-ol) at δ 62.1-72.0, nitrogen-bearing carbons at δ 49.8-51.9, other sugar ring and ribitol carbons at δ 64.7-78.1, and two *N*-acetyl groups δ 23.4-23.8 (CH_3) and 176.0 (CO). The ^{31}P NMR spectrum of PSII showed signal for a phosphate group at δ 1.0.

The NMR spectra of PSII were assigned using ^{1}H,^{1}H COSY, TOCSY, ROESY, H-detected ^{1}H,^{13}C HSQC, HSQC-TOCSY, and ^{1}H,^{31}P HMBC experiments (**Table 1**), and it was found that PSII consists of repeating units contaning two glucose and two GalNAc residues and ribitol designated units **F-K**. The a-linkage of GalpNAc (**F**) and b-linkages of Gal*p*NAc (**G**) and both Glc*p* (**H** and **J**) were established by characteristic H-1 and C-5 chemical shifts (**Table 1**) and the presence of H-1/H-2 cross-peaks for unit **F** and H-1/H-5 cross-peaks for units **G-J** in the ROESY spectrum (**Table 2**).

The C-1-C-5 chemical shifts of Glc*p* (**H**) and Glc*p* (**J**) (**Table 1**) were similar to those of non-substituted b-Glc*p* (22) and, hence, both glucose residues occupy the terminal positions in side chains. Downfield displacements were observed for the signals of C-3, C-4 and C-6 of GalpNAc (**F**), C-3 of GalpNAc (**G**), C-1 and C-5 of ribitol (**K**), as compared with their positions in the corresponding non-substituted compounds (22). These shifts defined the positions of substitution of the sugars and ribitol in the repeating unit.

The sequence of the monosaccharides was determined by correlations between the anomeric protons and protons at the linkage carbons or the linkage carbons revealed by the ROESY and HMBC experiments, respectively (**Table 2**). The ^{1}H,^{31}P HMBC spectrum showed correlations between a phosphate group and H-1 of ribitol (**K**) at δ 1.0/3.98, 4.06 and H-6 of GalNAc (**G**) at δ 1.0/4.05 and 1.0/4.12. Phosphorylation of ribitol (**K**) at O-1 was confirmed by a low-field positions of the signal for C-1 at δ 68.0 as compared with its position at δ 63.8 in non-substituted ribitol (26).

Therefore, the repeating unit of PSII is a branched tetrasaccharide-ribitol phosphate having the following structure:



In addition to the major signals tabulated in **Table 1**, the NMR spectra showed a number of minor signals, which were not assigned owing to their low intensities. These signals may belong to monosaccharides from terminal oligosaccharide units of PSII.

Treatment of PSII with 48% HF afforded dephosphorylated oligosaccharides, which were fractionated by GPC on TSK HW-40 (S) and studied by 1D and 2D NMR spectroscopy (for assigned ^{1}H and ^{13}C NMR chemical shifts see **Table 2**) and high-resolution ESI MS. Two major compounds were identified as tetrasaccharide-ribitol (**1**) ($Hex_2HexNAc_2Rib\text{-}ol_1$, $(M+Na)^+$ ion peak at *m/z* 905.3201, calculated *m/z* 905.3221) and tetrasaccharide (**2**) ($Hex_2HexNAc_2$, $(M+Na)^+$ ion peak at *m/z* 771.2617, calculated *m/z* 771.2642). Oligosaccharide **2** resulted evidently from overhydrolysis of a glycosidic linkage during dephosphorylation. Structures of compounds **1** and **2**

Table 2. Correlations for anomeric proton in the 2D ROESY and ^{1}H,^{13}C HMBC spectra of PSII

H-1 in sugar residue (d)	Correlations to atoms in sugar residue (d)	
	ROESY	HMBC
F H-1 (5.11)	**F** H-2 (4.50), **G** H-4 (4.13), H-3 (3.84)	**G** C-3 (76.0), **F** C-5 (71.7), C-3 (78.2)
C-1 (94.4)		**G** H-3 (3.84), **F** H-5 (4.04), H-4 (4.49), H-1 (5.11)
G H-1 (4.62)		**K** C-5 (72.0)
C-1 (102.5)		**K** H-5 (3.89/4.00), **G** H-1 (4.62), H-2 (4.12), H-4 (4.13), H-5 (3.68)
H H-1 (4.47)	**H** H-3 (3.48), H-5 (3.42), H-2 (3.29), H-4 (3.39), **F** H-3 (3.91)	**F** C-3 (78.2)
C-1 (105.7)		**F** H-3 (3.91), **H** H-1 (4.47), H-3 (3.48), H-5 (3.42), H-2 (3.29)
J H-1 (4.78)	**F** H-4 (4.49), **J** H-3 (3.48), H-4 (3.42), H-5 (3.37)	**F** C-4 (76.8), **J** C-2 (74.9)
C-1 (103.8)		**F** H-4 (4.49), **J** H-1 (4.78), H-3 (3.48), H-5 (3.37)

confirmed the structure of PSII.

To the best of our knowledge, the PSII structure is unique among known bacterial polysaccharides. Earlier, a cell wall polysaccharide having a similar structure of the main chain but with only one side chain of a single galactose residue has been reported in *Lactococcus lactis* subsp. cremoris H414 (27).

Acknowledgement

This work was supported by the Russian Foundation for Basic Research (grant 16-54-00081-Bel_a). High-resolution ESI mass spectra were recorded in the Department of Structural Studies of the N. D. Zelinsky Institute of Organic Chemistry, Moscow.

Conflict of interest statement

The authors declare no conflict of interest.

References

1. Marco ML, Pavan S, Kleerebezem M. Towards understanding molecular modes of probiotic action. Curr Opin Biotechnol 2006; 17:204-10.
2. McNaught CE, MacFie J. Probiotics in clinical practice: a critical review of the evidence. Nutr Res 2001; 21: 343–53.
3. Samarzija D, Antunac N, Havranek JL. Taxonomy, physiology and growth of *Lactococcus lactis*: a review. Mjekarstvo 2001; 51(1): 35-48.
4. De Vuyst L, Degeest B. Heteropolysaccharides from lactic acid bacteria. FEMS Microbiol Rev 1999: 23: 153-77.
5. Kelleher P, Murphy J, Mahony J, Van Sinderen D. Next-generation sequencing as an approach to dairy starter selection. Dairy Sci Technol 2015; 95: 545-68.
6. Deveau H, Labrie SJ, Chopin MC, Moineau S. Biodiversity and classification of lactococcal phages. Appl Environ Microbiol 2006; 72(6): 4338-46.
6. Leroy F, De Vuyst L. Lactic acid bacteria as functional starter cultures for the food fermentation industry.
7. Perdigo G, Galdeano CM, Valdez JC, Medici M. Interaction of lactic acid bacteria with the gut immune system. Eur J Clin Nutr 2002; 56(4): 21-6.
8. Novik G, Sidarenka A, Kiseleva E, Kolomiets E, Szwajcer Dey E. Probiotics. in: Biotransformation of waste biomass into high value biochemicals / S.K. Brar *et al*. (eds.) – New York: Springer Science+Business Media 2014: 187–235.
9. Rakhuba D, Novik G, Dey ES. Application of supercritical carbon dioxide ($scCO_2$) for the extraction of glycolipids from *Lactobacillus plantarum* B-01. J Supercrit Fluids 2009; 49(1): 45-51.
10. Holo H, Nilssen O, Nes IF. Lactococcin A, a new bacteriocin from *Lactococcus lactis* subsp. *cremoris*: isolation and characterization of the protein and its gene. J Bacteriol 1991; 173(12): 3879-87.
11. Vinderola CG, Mocchiutti P, Reinheimer JA. Interactions among lactic acid starter and probiotic bacteria used for fermented dairy products. J Dairy Sci 2002; 85: 721-9.
12. Masood MI, Qadir MI, Shirazi JH, Khan IU. Beneficial effects of lactic acid bacteria on human beings. Crit Rev Microbiol 2011; 37(1): 91-8.
13. Kimoto H, Ohmomo S, Okamoto T. Cholesterol removal from media by lactococci. J Dairy Sci 2002; 85(12): 3182–8.
14. Klijn N, Weerkamp HA, de Vos WM. Genetic marking of *Lactococcus lactis* shows its survival in the human gastrointestinal tract. Appl Environ Microbiol 1995; 61(7): 2771-4.
15. Chopin A, Bolotin A, Sorokin A, Dusko Ehrlich S, Chopin MC. Analysis of six prophages in *Lactococcus lactis* IL1403: different genetic structure of temperate and virulent phage populations. Nucleic Acids Res. 2001; 29(3): 644-51.
16. Ruas-Madiedo P, Tuinier R, Kanning M, Zoon P. Role of exopolysaccharides produced by *Lactococcus lactis* subsp. *cremoris* on the viscosity of fermented milks. Int Dairy J 2002; 12: 689-95.
18. Looijesteijn PJ , Trapet L , De Vries E , Abee T, Hugenholtz J. Physiological function of exopolysaccharides produced by *Lactococcus lactis*. Int J Food Microbiol 2001; 64: 71-80.
19. Streshinskaya GM, Naumova IB, Panina LI. Chemical-composition of the cell-wall of streptomyces-chrysomallus, forming the antibiotic aurantin. Microbiology 1979; 48: 814-9 (in Russian).
20. Leontein K, Lönngren J. Determination of the absolute configuration of sugars by gas-liquid chromatography of their acetylated 2-octyl glycosides. Methods Carbohydr Chem 1993; 9: 87-9.
21. Belyakov PA, Kadentsev VI, Chizhov AO, Kolotyrkina NG, Shashkov AS, Ananikov VP. Mechanistic insight into organic and catalytic reactions by joint studies using mass spectrometry and NMR spectroscopy. Mendeleev Commun 2010; 20: 125–31.
22. Bock K, Pedersen C. Carbon-13 Nuclear magnetic resonance spectroscopy of monosaccharides. Adv Carbohydr Chem Biochem 1983; 41: 27–66.
23. Faber EJ, Kamerling JP, Vliegenthart JFG. Structure of the extracellular polysaccharide produced by *Lactobacillus delbrueckii* subsp *bulgaricus* 291. Carbohydr Res 2001; 331(2): 183-94
24. Van Casteren WHM, de Waard P, Dijkema C, Schols HA, Voragen AGJ. Structural characterisation and enzymic modification of the exopolysaccharide produced by *Lactobacillus lactis* subsp. *cremoris* B891. Carbohydr Res 2000; 327(4): 411-42
25. Gorska S, Grycko P, Rybka J, Gamian A. Exopolysaccharides of lactic acid bacteria: structure and biosynthesis. Postepy Higieny i Medycyny Doświadczalnej (Polish) 2007; 61: 805-18
26. Shashkov AS, Potekhina NV, Senchenkova SN, Kudryashova EB. Anionic polymers of the cell wall of *Bacillus subtilis* subsp. *subtilis* VKM B-501. Biochemistry (Moscow) 2009; 74; 543-8.
27. Gruter M, Billy D, de Waard P, Kuiper J, Kamerling JP, Vliegenthart JFG. Structural studies on a cell wall polysaccharide preparation of *Lactococcus lactis* subspecies *cremoris* H414. J Carbohydr Chem 1994; 13(3): 363-82.