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PRODUCTION OF EXOPOLYSACCHARIDE BY LACTOBACILLUS PLANTARUM EB-2 STRAIN

Abstract: *An exopolysaccharide (EPS) in the amount of 250 mg/l was obtained from the culture supernatant of Lactobacillus plantarum EB-2 grown in MRS broth. In the IR spectrum of EPS, intense absorption bands were found, generally characteristic of a class of carbohydrates. The molecular weight of the EPS obtained from L.plantarum EB-2 was 3.16x10^4 Da, the polydispersity index was 1.9. The EPS from L.plantarum EB-2 consisted from a mannose, glucose, galactose and rhamnose in an molare ratio of 21.7:12.4:2:1, respectively. An in vitro study of the antioxidant activity of L.plantarum EB-2 EPS in the diphenylpicrylhydrazil (DPPH) system showed that radical scavenging activity (RSA) has being increased depending on the concentration of the investigated EPS. The maximum RSA was detected at 4 mg/ml being 42%, which is more than the activity of ascorbic acid.*

Key words: *lactobacilli, exopolysaccharide, monosaccharide composition, antioxidant.*
Language: *English*
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Introduction

Lactobacilli represent microorganisms that are wide spread in the environment. High biological and functional activity of strains lactobacilli determines their practical use as probiotics and in food production [1, 2].

Species of the genus *Lactobacillus* and *Bifidobacterium* are widely used as probiotics for humans and animals. According to the act of the American Food and Drug Administration, they have a recognized safety status (GRAS-status), due to their long history of safe use in fermented foods and their

presence in the normal intestinal and urogenital microbiota of people.

Some species, such as *L. plantarum* and *L. fermentum*, have received the qualified presumption of the Safety Status (QPS) assigned by the European Food Safety Authority (EFSA) [3].

One of the most famous biological properties of lactobacilli is a pronounced ability to the production of lactic acid [4]. It is established, that the activity of acid formation depends on the composition of the nutrient medium and cultivation conditions. It is believed that only the L (+) - isomer of lactic acid is a biologically active form, which is quickly and completely utilized by the human body [5]. Lactic acid bacteria antagonism in relation to microorganisms due to the formation of lactic acid, the production of other antimicrobial and antibiotic-like substances: lysozyme, hydrogen peroxide, bacteriocins (lactacins) [6; 7; 8], short chain fatty acids [9; 10], diacetyl [11].

Polysaccharides are used in various industries. Natural food thickeners are of particular interest to the food industry such as guar gum, locust bean gum, pectin, starch (all from plants),gelatin (animals), alginate, carrageenan, agar (all from seaweed), xanthan gum and gellan gum (all from bacteria)[12].

Various researchers have shown that lactobacilli have great potential for the biosynthesis of exopolysaccharides. Most of these supplements, however, are less and less considered desirable.

Lactic acid bacteria produce extracellular polysaccharides (EPS), which provide the texture of fermented milk products [13, 14].

Exopolysaccharides of lactic acid bacteria also positively affect the taste, smell and preservation of the final product [15;16]. Many of the exopolysaccharide-producing strains of lactobacilli belong to the genera *Streptococcus, Lactobacillus, Lactococcus, Leuconostoc, and Pediococcus* [17].

Microbial exopolysaccharides are an object of intensive research due to their importance in the structure and metabolism of microorganisms. Exopolysaccharides of lactic acid bacteria play a crucial role in improving the rheology, texture, taste of fermented foods, have a beneficial physiological effect on human health and have antitumor, immunomodulating and anti-carcinogenic activity [18].

LAB can also produce various functional oligosaccharides. Oligosaccharides have huge industrial applications as prebiotics, nutraceuticals, sweeteners, moisturizers, drugs against colon cancer, immune stimulants, etc. [19].

To justify the use of exopolysaccharides of lactic acid bacteria in biomedical practice, veterinary medicine, the food industry, knowledge of their structure and biological activity is required. In this regard, studies devoted to the allocation of the study of new strains of lactobacilli, producing exopolysaccharides and studying the biological activity of exopolysaccharides isolated by these strains are relevant and can have significant scientific interest and applied value.

The purpose of the work is product characterization, isolation, chemical structure and antioxidant potential of the exopolysaccharide from *Lactobacillus plantarum* EB-2 strain.

Materials and methods.

Culture *L.plantarum*EB-2 isolated from domestic Armenian feta cheese according to the generally accepted method identified by morphological, physiological, biochemical properties, as well as 16 sRNA sequencing and deposited in the collection of the Institute of Microbiology of the Academy of Sciences of the Republic of Uzbekistan.

Universal primers of the 16S rRNA gene used in the identification of *L.plantarum* EB-2.

Primer	**Primer nucleotide sequence**
16sRNA2-F	TCG CTA GTA ATC GCG GAT CAG C
16sRNA2-R	GCA TAT CGG TGT TAG TCC CGT CC
16sRNA1-F	TCT CAG TTC GGA TTG TAG GC
16sRNA1-R	ATC GAC TCC TAG TGT CAA GG

BLAST analysis of the obtained nucleotide sequence of 16S rRNA gene of *L.plantarum* EB-2 was performed using the NCBI database. For the compilation of the phylogenetic tree, 15 strains of *Lactobacillus plantarum* were selected according to the Clustal W program.

The phylogenetic tree was compiled using the Simple Phylogeny online program [20].

Isolation of polysaccharide from *L.plantarum* EB-2.

Isolation of polysaccharide from *L.plantarum* EB-2 culture fluid was carried out according to the method described by Cerning, J. et al. [21]. Culture *L.plantarum* EB-2 restored from the lyophilized state 2-3 transfers in MRS-broth and incubated at 37 °C for 48 hours. An inoculum in a volume of 20 ml (2%, w / v) was added to 1 ml of MRS-broth. Under aerobic conditions were incubated at 37 °C for 48 hours. After culture incubation, TCA was added to a final concentration of 4% (w / v) and stirred for 30 minutes at room temperature. Cells and precipitated proteins were removed by centrifugation at 7,000 xg for 30 minutes at 4 °C. Chilled ethanol was added to the supernatant in an equal volume and kept at 4 °C for 48

hours. Precipitated EPS were collected by centrifugation at 7000 xg for 30 minutes at 4 °C. The precipitate was dissolved in distilled water and dialyzed at 4 °C for 48 hours, then dried by lyophilization. Total carbohydrates in lyophilic – dried exopolysaccharides of lactobacilli were determined by the phenol sulfuric acid method [22].The quantitative determination of proteins in the composition of crude exopolysaccharide was carried out by the method described by A. Yermakov et al. [23].

Fourier Transform Infrared (FTIR) Analysis of EPS. FTIR-spectrum of exopolysaccharide *L.plantarum* EB-2, registered Fourier transform infrared spectrometer Vector-22 (Bruker, Germany) in the frequency range 400-4000 cm^{-1}. 2 mg of exopolysaccharide was mixed with 200 mg of potassium bromide (KBr) (1: 100 ratios), then the mixture was pressed into a mold with a diameter of 16 mm and conducted IR spectroscopy for detection of functional groups characteristic of polysaccharides [24].

Analysis of the monosaccharide composition of the exopolysaccharide from *L.plantarum* EB-2. The determination of the monosaccharide composition of the obtained EPS by gas chromatography is described in V.V. Arasimovich [25]. To establish a monosaccharide composition, the polysaccharide was hydrolyzed with 1N concentrated sulfuric acid at a temperature of 100°C for 6 hours. The hydrolyzate was then neutralized with barium carbonate, deionized with KJ-2 (H^+), and 1 ml was evaporated on a rotary evaporator.

The identification and quantitative composition of monosaccharides was determined on a GC Plus 2010 gas chromatograph (Shimadzu, Japan) under the following conditions: T injector -250 °C; total flow - 60 ml / min; flow through the column - 0.89 ml / min; carrier gas is nitrogen; Rxi-624S1MS column; column length 3 m; ID ID 0.25 mm; column temperature - 230 °C.

Molecular weight characteristics were determined on an Agilent 1260 Infinity size exclusion chromatograph (Agilent Technologies, USA).

Antioxidant activity. Antioxidant activity was judged by the binding of the oxide radicals of diphenylpicrylhydrazine (DPPH) [26]. 2.0 ml of an alcohol solution of DPPH (0.4 mM) was added per 1 ml of an aqueous solution of exopolysaccharide L. plantarum EB-2. The mixture was thoroughly mixed and incubated at room temperature in a dark place for 30 minutes. The absorption coefficient of the mixture was measured at 517 nm on a Cary 60 UV-Vis spectrophotometer (Agilent Technologies, USA). Antiradical activity was calculated by the formula: $\%RSA=\frac{(Ao-A1)}{Ao}x100$, where - A_1 is the absorption coefficient of the sample solution; A_o is the absorption coefficient of the DPPH solution without a sample. An aqueous solution of ascorbic acid was used as a control.

Statistical processing. All experiments were carried out in 3 replicates. In statistical processing determined the arithmetic mean, standard deviation, confidence intervals, the significance of differences was determined using Student's criterion. Differences were considered significant at a significance level of $p \leq 0.05$. The results were analyzed using standard packages of licensed programs MS Excel 2003 and STATISTICA 6.0 [27].

Results and discussion.

Identification of the EPS-producing culture of *L. plantarum* EB-2. Cells of *L.plantarum* EB-2 culture are Gram-positive, catalase negative short bacilli, located singly and in pairs, 0.6 μm x 1.2-3.6 μm in size.

The culture actively ferments salicin, mannose, mannitol, melibiosis, ribose, raffinose, trehalose, sucrose, fructose, lactose and galactose, grows with 6.5% NaCl and 0.4% bile.

The *L.plantarum* EB-2 culture was also identified by sequencing and phylogenetic analysis of the 16S rRNA gene. BLAST analysis of the sequence of the 16 S rRNA genes showed high identity (99%) with 15 typical strains of *L. plantarum*. Phylogenetic analysis revealed that the sequencing product from *L.plantarum* EB-2 is very close to the strain *L.plantarum* SRCM103411 (Figure 1).

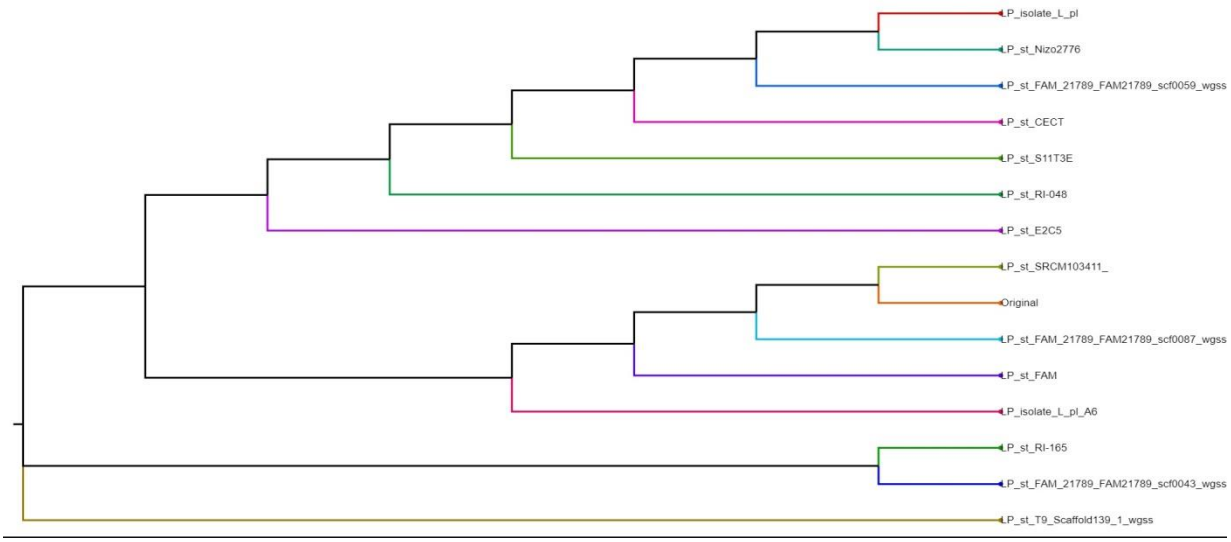


Figure 1. Phylogenetic tree, compiled from sequencing of 16 S rRNA genes of *L. plantarum* strains.

The resulting phylogenetic tree showed that *L. plantarum* strains can be grouped into three separate branches, of which one branch is divided into two branches. Strain *L.plantarum* EB-2 formedas one of the branches alongwith another branch of the strain *L.plantarum* SRCM103411.Within this branch *L.plantarum* EB-2 showed 100% sequence identity with *L. plantarum* FAM 21789 FAM21789 scf0087, *Lactobacillus plantarum* isolate *L. plantarum* A6, *Lactobacillus plantarum* strain Nizo2776 NODE, *L.plantarum* SRCM103411, proving that these five strains are closely related. Sequence identity with the other strains of the cluster was 98%.

Isolation of polysaccharide from *L.plantarum* EB-2 culture supernatant. When growing *L.plantarum* EB-2 culture in MRS-broth yield of lyophilic - dried crude EPS was 250 mg/L. Lyophilized EPS was an amorphous cream-colored powder, it was well dissolved in water, had a smooth fibrous structure. Crude EPS contained 65.59 ± 0.7% of the total polysaccharides and 7.37% of the protein (nitrogen content 1.17%). In the work of Xin Wang et al. (2017) analysis of the raw EPS of *Lactobacillus plantarum* KX041 showed that the content of total sugars in this EPS is 60.59 ± 0.92%. Also, by the Sevage method were determined the protein content equal to 12.88 ± 0.64%. After removal of free EPS proteins by the Sevage method, residual proteins that are covalently linked to the polysaccharide molecule were detected [28]. Proteins contained in exopolysaccharides, play an important role in modifying the physicochemical, thermal properties, and bioactivity of polysaccharides [29].

The yield of heteropolysaccharides is from 0.05 to 0.60 g/l [30] on the contrary, homopolysaccharides are synthesized in large quantities up to a few grams / liter [31].

MRS-broth is the most suitable medium for the growth and synthesis of LAB biopolymers. But for industrial purposes and from an economic point of view waste management other industries as a basis for a nutrient medium is appropriate [32]. Many discussions are ongoing about the formation of EPS under the influence of various conditions. It is generally recognized that cultivation conditions or several other factors (pH, temperature, incubation time and composition of the growing medium) have a significant impact on the yield and composition of EPS. The results of studies by some authors show that strains of lactobacilli, depending on the carbon source of the nutrient medium, can produce EPS with various rheological properties [33].

Fourier Transform Infrared (FTIR) Analysis of exopolysaccharide *L.plantarum* EB-2. Infrared spectroscopic (IR) analysis is an effective technique, on the basis of which is the oscillation communication groups at characteristic frequencies and is used to determine functional groups and to describe covalent bonds. In the IR spectrum of EPS of *L.plantarum* EB-2, intense absorption bands were found that was generally characteristic of the carbohydrate class (Figure 2). Widely located peak at 3412.17 cm^{-1} it depicts the extended vibrations of the hydroxyl group and characterizes the carbohydrate ring [34]. A weak peak at 2935.57 cm^{-1} indicates the presence of aliphatic CH_2 groups, which are contained in proteins and other organic substances. The absorption peak in the region of 1700–1770 cm^{-1} means that glucuronic acid or diacetyl ether is present in the EPS [35]. The peak at 1643.77 cm^{-1} represents the extended vibrations of the C = O group [36]. Short peaks in the region below 1500 cm^{-1}indicate the presence of sulfated groups and that this substance is a polysaccharide [37]. Also, the peak at 1225.24 cm^{-1} belongs to CO bonds in ether or alcohol groups [38]. The peaks in the range of 1051.66 - 920.27 cm^{-1} means vibrations of C-O and C-O-C glycosidic bonds, showing the presence of carbohydrates [39].The

presence of carboxyl groups in the IR spectrum of the polysaccharide can serve as a binding site for bivalent cations [40].

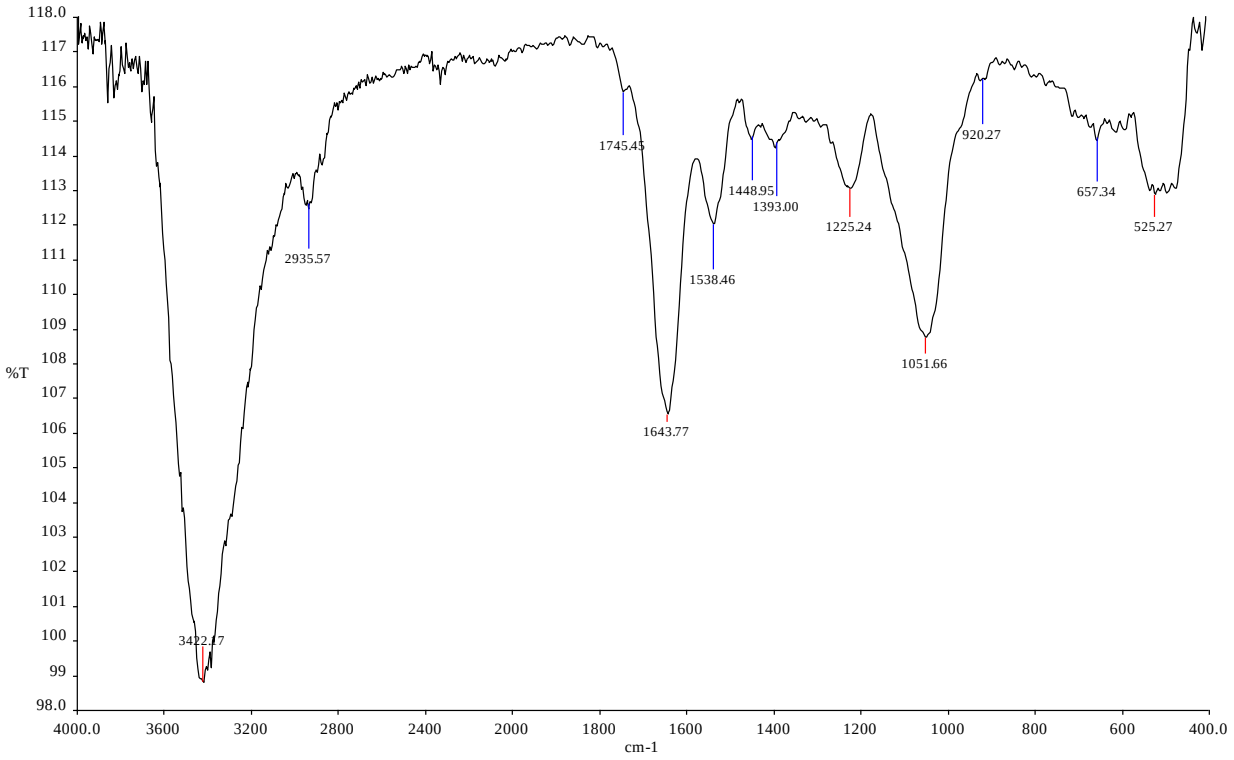


Figure 2. FTIR-spectrum of EPS from *L.plantarum* EB-2

Monosaccharide composition of exopolysaccharide *L.plantarum* EB-2.

GC analysis of the monosaccharide composition of EPS *L.plantarum* EB-2 showed that this EPS consists of mannose, glucose, galactose and ramnose in a molar ratio of 21.7: 12.4: 2: 1, respectively (figure 2).

According to the literature, EPS from various strains of *Lactobacillus plantarum* are heteropolysaccharides and mainly consist of glucose, galactose, mannose, arabinose, and rarely xylose or fructose. When analyzing the monosaccharide composition of *L. plantarum* NTMI05 and NTMI20 by HPLC methods the presence of a single peak belonging to galactose was revealed [41]. Yanjun Tang et al. as part of the polysaccharide *L. plantarum* revealed a wide range of monosaccharides, as ribose, ramnose, arabinose, xylose, mannose, glucose and galactose in a molar ratio of 2: 1: 1: 10: 4: 205: 215. Also, this polysaccharide hada relatively high molecular weight of 2.4×10^6 Da [42]. The exopolysaccharide produced by *L. plantarum* YW32 consisted of mannose, fructose, galactose, and glucose in an approximate mass fraction of 8.2: 1: 4.1: 4.2, respectively [43].The exopolysaccharide from *L. plantarum* KX041 contained arabinose, mannose, glucose and galactose in a ratio of 0.95: 12.94: 7.26: 3.31. The presence of galactose and arabinose in the molecule is associated with the antioxidant activity of the polysaccharide [44].

Molecular weight of exopolysaccharide *L.plantarum* EB-2.

The molecular weight, XPS of *L.plantarum* EB-2 was $3.16x10^4$Da, the polydispersity index was 1.9 (Figure 4).

The molecular mass of exopolysaccharides of lactobacilli ranges from 40 to 6000 kDa [15]. The molecular weight of EPS obtained from *L. plantarum* YW32 is determined to be 1.03×10^5 Da. The polydispersity index was 1.255, which means the presence of a homogeneous EPS material in the test sample [43].

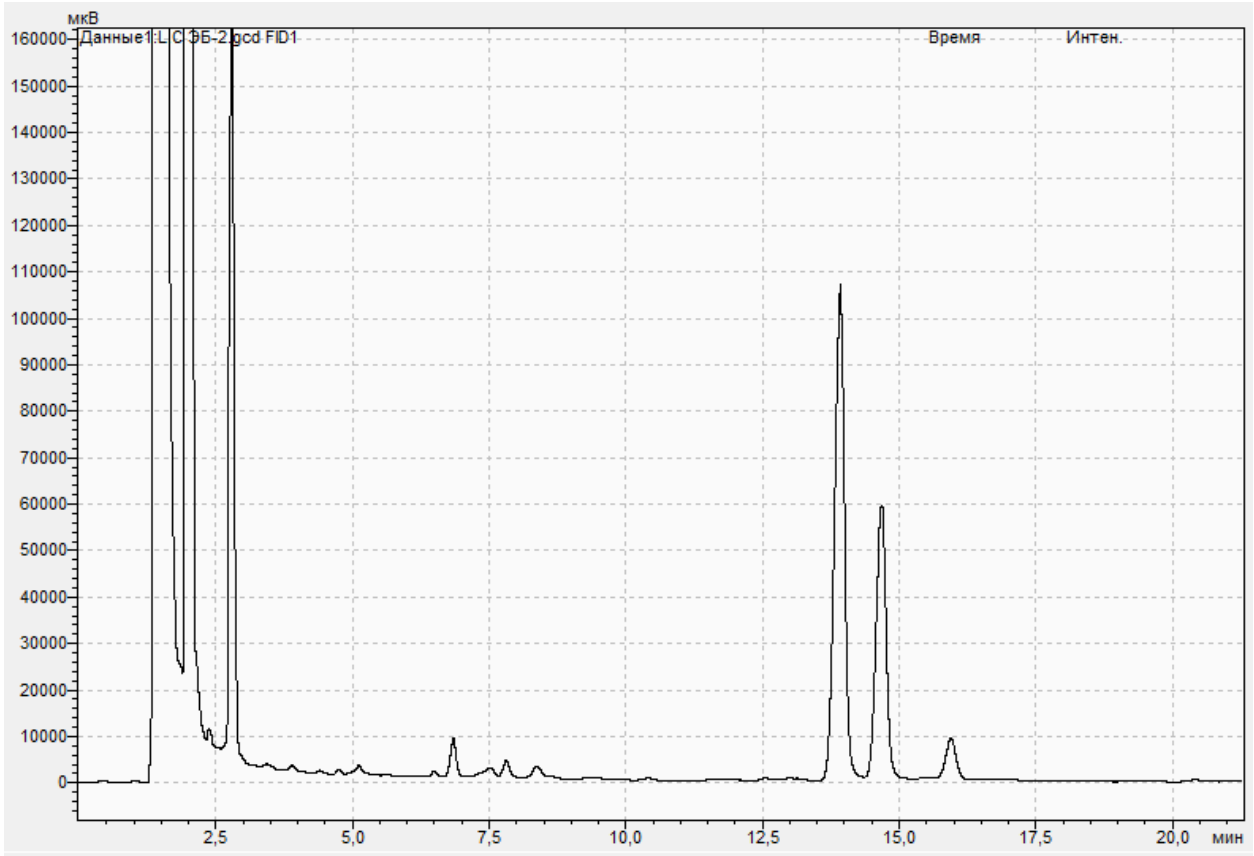
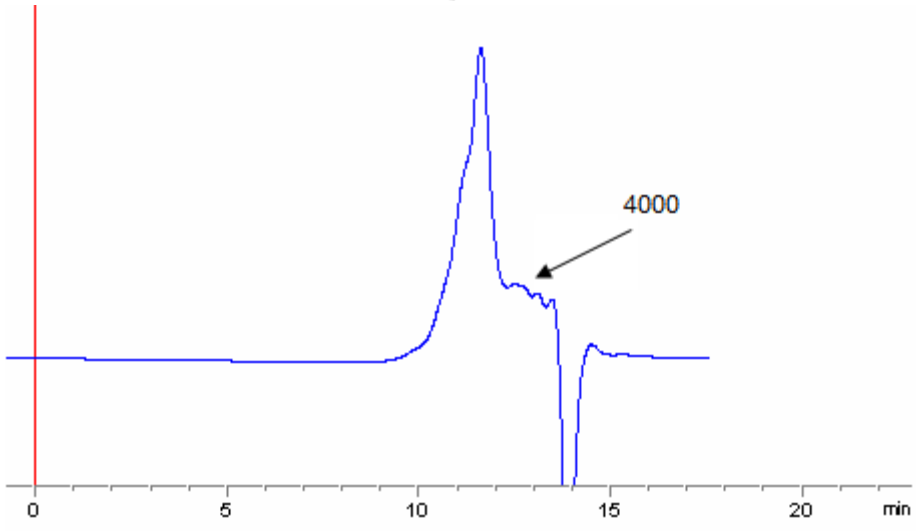


Figure 3. GC chromatogram of the monosaccharide composition of the polysaccharide from *L.plantarum* EB-2



M_w/M_n=1,9 **31600 Da**

Figure 4. Chromatogram of the molecular weight of EPS from *L.plantarum* EB-2

Many authors claim that molecular weight has a great importance in the manifestation of the biological activity of EPS. It was shown that the high antioxidant activity of EPS from *Bifidobacterium animalis* PH is due to its low molecular weight [45]. EPS with a high molecular weight possess antitumor activity than EPS with a low molecular weight [46].

Antioxidant activity of EPS from *L.plantarum* EB-2.The antioxidant activity of EPS from *L. plantarum* EB-2 was judged by the activity of binding of free oxide radicals of DPPH. DPPH free radicals are stable radicals with an unpaired valence electron of one atom of the nitrogen bridge, which significantly decrease under the influence of protons of the radical acceptor [47]. In our work, the antiradical activity of

EPS of *L.plantarum* EB-2 was determined using a colorimetric method at concentrations of 2, 3, and 4 mg / ml, ascorbic acid served as a positive control. As can be seen from figure 5, the antiradical activity of EPS from *L.plantarum* EB-2 increases, depending on the concentration of the investigated EPS and % RSA were at an EPS concentration of 2 mg / ml 14.6%; at 3 mg / ml 24% and at 4 mg / ml 42%.

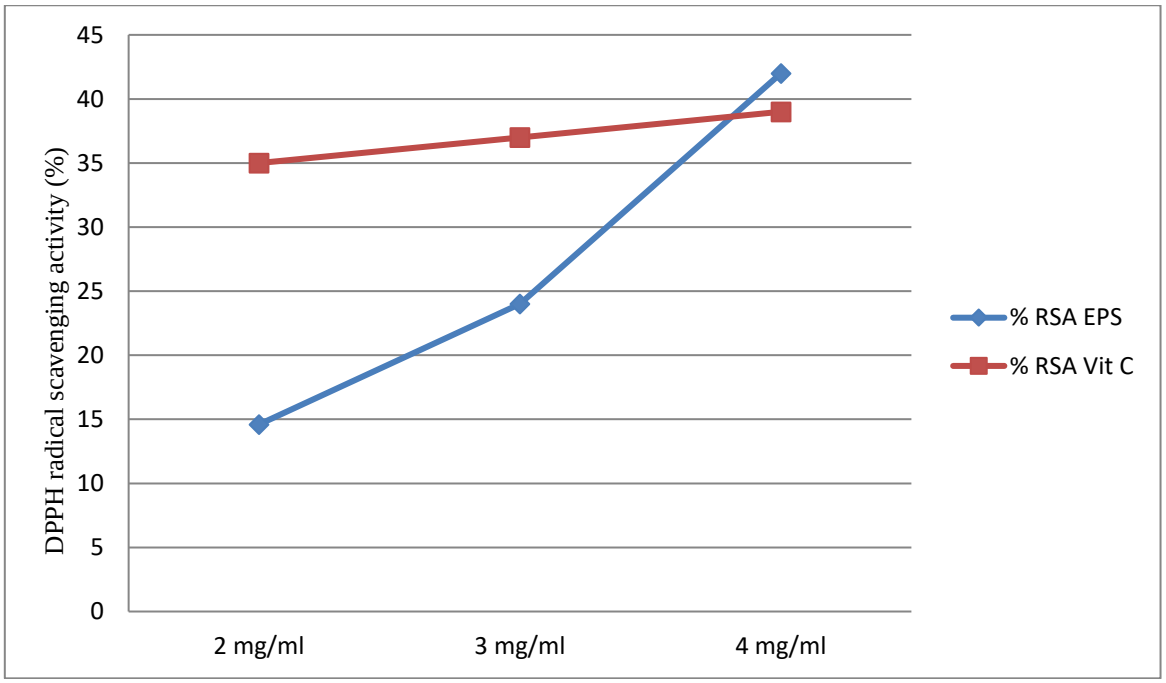


Figure 5. Antioxidant activity of EPS *L.plantarum* EB-2 and ascorbic acid.

Wei Li, et al. report that crude EPS has greater antioxidant activity than purified. This is due to the presence in the raw EPS of other antioxidant substances, such as proteins, peptides and trace elements. Moreover, they act as synergists and interact with the binding of free radicals [48]. Tsiapali et al. stated that antiradical activity depends on the monosaccharide composition and glycosidic bonds [49].

Excess free radical production leads to oxidative damage to biomolecules (lipids, proteins, DNA), leading to the emergence of many chronic diseases [50]. In the work of Zouaoui Benattouche et al. data on that when studying the activity of EPS from *S. thermophilus*, the highest antioxidant activity - 55.83% was observed at a concentration of EPS of 1000 mg/ml [51]. Although the EPS from *L. plantarum*YW32 comparatively lower results were obtained than ascorbic acid, but at a dose of 5 mg / ml, EPS shows a promising antioxidant activity with 30% DPPH free radical binding activity [43].

EPS bioactivity may depend on many factors, such as chemical composition, molecular weight, structure, configuration, extraction and purification conditions. The molecular mass of EPS plays an important role in antioxidant activity [52].

Conclusions.

EPS isolated from *L.plantarum* EB-2 has a molecular weight of $3.16x10^4$Da and consists of mannose, glucose, galactose and ramnose in a molar ratio of 21.7: 12.4: 2: 1, respectively. Also, this EPS has a high anti-radical activity and can be used as an alternative to chemical antioxidants in the food and pharmaceutical industries.

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