Phisical and chemical properties of exopolysaccharide of the lactic streptococcus

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Abstract. We know that exopolysaccharides of microbial origin are of great practical importance, and have their industrial value because of the biological and physico-chemical characteristics of the producer. We had a goal to study the most important properties of an exopolysaccharide isolated from a bacterial culture of Streptococcus thermophilus. First we got exopolysaccharide. After that we had cultured the bacteria on A. Welman at 38 °C for 48 hours. To do this, 3000 g of culture liquid had been centrifuged for 30 minutes. Next, we removed the biomass sludge, and we evaporated the centrifuge on a rotary evaporator. After that, we precipitated the exopolysaccharide with a double volume of 96% ethyl alcohol. We got a concentrate which we then dissolved in a small amount of distilled water and had centrifuged for 30 minutes. Afterwards my colleges and I precipitated it again. Our further purification of the exopolysaccharide we had to perform using some gel filtration on a column with a Sephadex G-50. It had to be dried on a freeze dryer. Thus, we obtained an exopolysaccharide in the form of a light brown powder, it was odorless, without any foreign impurities and any producer cells. Our further work included following steps: we had to know the molecular weight of the exopolysaccharide. It was possible to make with gel chromatography on a Toyopearl – HW –50F column. We determined the chemical nature of the exopolysaccharide through ion exchange chromatography using a SPS Bio DEA medium with 70 microns. We determined the monosaccharide composition with the help of a thin-layer chromatography on DC-Alufolien Cellulose plates, and the relative viscosity by viscometer. As a result of our research, the exopolysaccharide S. thermophilus were presented by a single neutral fraction, with molecular weight of 20,000 Da, with a small relative viscosity.

1. Introduction
Exopolysaccharides (EPS) of bacterial origin are getting widely distributed [1-2]. Exopolysaccharides are high-molecular compounds with a diverse carbohydrate composition. They are part of a living cell, both locally and in associations with lipids, proteins, and nucleic acids [3]. Bacterial EPS, unlike most chemically synthesized ones, are biodegradable and do not harm the environment [4]. Among them, a special place is occupied by EPS of lactic bacteria [5-7]. Lactic bacteria have a large variation in the production of EPS in terms of a chemical composition, quantity, size of molecules, charge, presence of side chains and rigidity of molecules [8]. They have hydrophilic, wound-healing, and film-forming properties that makes them applicable in the food, petrochemical, and cosmetic industries, medicine, and other fields [9]. To expand the possibility of practical using of EPS of lactic bacteria, knowledge of their structure and physical and chemical properties is necessary. Because of this, the aim of our
work was to study the physical and chemical properties of the exopolysaccharide *Streptococcus thermophilus*.

2. Materials and methods

We would like to study an exopolysaccharide that we had received a bit from the culture of *Streptococcus thermophilus*. We had some information about lactic bacteria from the FSBNU “State scientific research Institute of dairy industry” that is situated in the capital of our country. *Streptococcus thermophilus* refers to lactic bacteria that ferment carbohydrates to form lactic acid. Therefore, it is widely used in the food industry for production various dairy products, and it is also used for lactase deficiency. It has an acidifying effect, providing a bactericidal effect against pathogenic microorganisms, and it is also able to synthesize and secrete polysaccharides [11]. International GRAS status (from Generally Regarded as Safe) allows the use of *S. thermophilus* in the food and pharmaceutical industries and can be taken by children from the first days of life.

Based on the facts, we had already got, we continued to study other properties of our polymer. To do this, we decided to conduct more studies that could give us more detailed characteristics of the substance we obtained. We used such methods that were earlier used by other experienced scientists and had wonderful results. We all were eager to learn what we would find out from our experiments.

**What was done?**

- We received EPS and used the method of J. Cerning in our modification.
- We determined the presence of protein using the Bradford method.
- The content of nucleic acids was determined on the 100 Scan spectrophotometer medium (Varian, USA) at 260 nm [12].
- The molecular weight of the exopolysaccharide was determined by gel chromatography on a column with a Toyopearl – HW –50F medium.
- We determined the presence of EPS fractions by ion exchange chromatography and had to use the SPS Bio DEAE 70mkm medium.
- We determined the monosaccharide composition. We used a thin-layer chromatography (TLC) on plates with a cellulose medium DC - Alufolen Cellulose.
- The relative viscosity we decided to determine with a capillary viscometer vpj-2.

3. Results

To get *S. thermophilus* EPS, the bacteria had been cultured on a temperature-controlled shaker incubator ES-20 (Lithuania) at 38 °C for 48 hours on A. Welman medium. Then we made centrifuge the 3000 g of culture liquid. It took us 30 minutes. Then we tried to remove the resulting biomass. Our crew evaporate the culture liquid released from the producer cells on a rotary evaporator N-1100VWD made in Japan. We could take under control the absence of bacteria at this stage with the Gram microscopy. After that we deposited EPS with a double volume of 96% ethyl alcohol. We dissolved the received concentrate in a small amount of distilled water and had centrifuged it for 30 minutes. Then we precipitated it. Subsequent purification was performed by gel filtration on a column using Sephadex G-50 using it as a medium (figure 1). For the eluent, a 1M solution of acetic acid (CH₃COOH) with a pH of 5.5 was used. After drying (freeze-drying, COOLSAF 55-4 SISTEM, ScanLaf, Denmark), the isolated EPS looked like light brown, odorless powder that did not contain protein, nucleic acids or any producer cells.

It should be mentioned that previously we studied the production of EPS according to the carbon source. We all know from different articles that the biosynthesis of EPS is influenced by different sources of carbon in the culture medium [13]. Glucose, sucrose, lactose, and combinations of different kinds of sugars are the main sources for lactic acid bacteria. For this purpose, the maximum yield of exopolysaccharide was determined when *S. thermophilus* was cultured in a. Welman medium alternately with glucose, sucrose, and lactose. It was shown that when growing Streptococcus on A. Welman medium with glucose, the yield of EPS was 1.5 g / l, lactose-1.7 g/l, but when adding sucrose, the production of EPS increased to 2.3 g/l (table.1). There is evidence that sucrose was also the best
carbon source for EPS production for other bacteria, such as leuconostocci. We got amazing results that made us get more sure about what is the real carbon source for further production of this substance. Sucrose is a very good nutrient substrate, which is available for bacterial growth. We could say we have taken the next step on the path of new discovery.

Table 1. Effect of carbohydrates on EPS production S. thermophilus.

| Carbohydrates | EPS products, g / l |
|---------------|---------------------|
| Glucose       | 1.5±0.4             |
| Lactose       | 1.7±0.2             |
| Sucrose       | 2.3±0.2             |

The effect of cultivation time on EPS production was also studied. For this purpose, the growth curve of S. thermophilus was made. The bacteria themselves were grown on A. Welman medium with sucrose. At the same time we determined the latest amount of EPS and used the phenol-sulfur method. We could observe that the maximum production of EPS was just the same as the biggest growth of Streptococcus culture and it occurred in the stationary growth phase (figure 2), which is proved by the literature data [14].

Figure 1. Chromatograms of exopolysaccharide S. thermophilus (1.490 nm), pigment (2), impurities, (3) on a column with Sephadex G-50.

Figure 2. The growth curve (1) and production of EPS (2) S. thermophilus.
When determining the molecular weight of *S. thermophilus* exopolysaccharide, an analytical column with a Toyopearl – HW–50F medium was used. A buffer of 1 M acetic acid solution (H$_3$COOH) was used for the eluent. The temperature inside the column was maintained at 30 °C, and the elution rate was 1 ml/min. During gel chromatography, the exopolysaccharide was detected on a flow spectrophotometer LKB Bromma 2238 UVICORDSII. The analytical column was calibrated with dextrans (Fluka Switzerland, Merck Germany) with known molecular weights (180, 6000, 20000 da). For calibration, the dependence of elution volumes on the molecular weights of dextrans was graphically displayed. The molecular weight of the exopolysaccharide *S. thermophilus* was found using data on the elution volumes of received EPS and a calibration graph.

The molecular weight was 20000 da (figure 3).

![Figure 3. Determination of the molecular weight of *S. thermophilus* EPS on a Toyopearl – HW–50F column.](image)

For example, Vaningelgem (2004) has data showing a wide variety of molecular weights, in particular in *Streptococcus thermophilus*, in the range from 10 to > 2000 kDa.

To separate the received *S. thermophilus* EPS into fractions, the method of ion exchange chromatography was used on a column with an anion exchanger SPS Bio DEAE 70nm. Elution was performed in two stages: initially with a buffer of 0.04% NaN$_3$, 0.05 M KH$_2$PO$_4$. After separation of the neutral fractions, the elution was carried out with a NaCl solution in the same buffer with a concentration gradient of 1M. The fractions from the column were collected using a collector in test tubes, and then examined for carbohydrate content by the phenol-sulfur method. As can be seen from the chromatogram in figure 4, the samples were released in one fraction corresponding to neutral substances. The presence of one neutral fraction was also characteristic of some EPS of lactic acid bacteria such as *Lactobacillus delbrueckii* subsp. *delbrueckii* B-1596, *L. delbrueckii* B-1936, and *L. delbrueckii* ssp. bulgaricus [15].

Thin-layer chromatography was performed using a plate with a cellulose carrier DC-Alufolien Cellulose with a layer thickness of 0.1 mm. Alcoholic solutions of monosaccharides (Fluca) such as D-glucose, D-rhamnose, and gluconic acid were used as standards. The EPS was hydrolyzed with a 4 n solution of trifluoroacetic acid at 98°C for 4 hours in a water bath with a reverse refrigerator. A solution consisting of ethylacetate, pyridine, acetic acid, and water in a ratio of 5:5:1:3 was used as an eluent. A 1% alcohol solution of aniline phthalate was used as a developer. It was shown that the composition of EPS includes rhamnose and glucose (figure 5). The results are the same as in the literature data. Glucose and rhamnose, along with mannose, xylose, and galactose, can be the main structural monosaccharides.
Figure 4. Thermophilus EPS elution in ion exchange chromatography (for the detection of carbohydrates).

Figure 4. The monosaccharide composition of the EPS of S. thermophilus by thin-layer chromatography. Note: 1, 2, 4 – carbohydrates-witnesses, 3 – EPS.

Relative viscosity was determined using a capillary viscosimeter VPZH-2 (Russia). To do this, we installed a viscosimeter in a thermostat (25°C). We poured 10 ml of a solution of the studied EPS of a certain concentration (10%) and we measured its expiration time. After measuring the expiration time of the solution with EPS, we determined the expiration time of the solvent (distilled water). We also had to measure the relative viscosity and we did it with the formula. During studies on the viscosimeter, we found that the exopolysaccharide S. thermophilus had a low viscosity. We determined the relative viscosity using a capillary viscosimeter and it was 1.23 mm²/s.

4. Conclusion

Thus, the exopolysaccharide received from Streptococcus thermophilus is presented by a single neutral fraction with a molecular weight of 20,000 Da, which has a small relative viscosity. We can make a conclusion that the work we had done was not useless at all. The data that we got were not different from those that were received by other microbiologists. There is not any doubt that today, a great number of scientists show their big interest to this topic. New laboratories are built and old ones are improved to conduct research work and experiments to obtain new data. We believe that the study, application and production of this biological cultures is one of the most promising areas of modern biotechnology. We hope that we will have the opportunities to carry out new researches and get great results in future.
Acknowledgments
The authors express their gratitude to the employee of LLC "VIC - animal health" candidate of Biology S. V. Semenov for his assistance in carrying out gel filtration.

References
[1] Garcia-Ochoa F 2000 Biotechnol. Adv. 18 549-579
[2] Kichemazova N V, Bukharova E N, Selivanov N Yu, Bukharova I A and Karpunina L V 2017 Applied Biochemistry and Microbiology 53(3) 285–290
[3] Flemming H C and Wingender J 2010 Nature Reviews Microbiology 8 623-633
[4] Muhammadi N Ahmed 2008 Iranian Polymer Journal 17(5) 315-323
[5] Paulo E M, Vasconcelos M P and Oliveira I S 2012 Food Science and Technology 32(4) 710-714
[6] Vaningelgem F, Zamfir M, Mozzi F T, Adriany M, Vancanneyt J, Swings L and Vuyst De 2004 Appl. Env. Microbiol. 70(2) 900-912
[7] Zeidan A A, Kuzina P V, Janzen T, Buldo P D, Patrick M F, Oregaard G and Rute Neves A 2017 FEMS Microbiol. Rev. 41 168-200
[8] Zannini E, Waters D M, Coffey A and Arendt E K 2016 Applied Microbiology and Biotechnology 100(3) 1121-1135
[9] Khusainov I A 2014 Technological University Bulletin 17(5) 167-172
[10] Fokina N A, Uryadova G T and Karpunina L V 2016 Agrarian scientific journal 12 40-42
[11] Pachekrlepapol U, Lucey J A, Gong Y, Naran R and Azadi P 2017 Journal of Dairy Science 100(5) 3424-3435
[12] Osterman L A 1985 Moscow: Nauka 536
[13] Yuksekdag Z N and Aslim B 2008 Braz. arch. biol. technol. 51(3) 581-585
[14] Deveau H, van Calsteren M and Moineau S J 2002 Applied and Environmental Microbiology. 68 4364-4369
[15] Ruas-Madiedo P, Hugenholtz J and Zoon P 2002 Int. Dairy J. 12 163-171