

Biosynthesis of exopolysaccharides by *Weissella confusa* in a new culture medium

Received for publication, December, 18, 2015

Accepted, June, 15, 2016

ANCA ROXANA PETROVICI¹, IRINA ROȘCA^{1*}, IRINA STOICA², MIHAELA SILION¹, ALINA NICOLESCU¹, GIANINA DODI^{3,4}, NATALIA SIMIONESCU¹, CRISTIAN-DRAGOȘ VARGANICI¹, IULIU CRISTIAN IVANOV⁵, MARIANA PINTEALĂ¹

¹ "Petru Poni" Institute of Macromolecular Chemistry of the Romanian Academy, 700487 Iasi, Romania, (phone: +40232-217-454; fax: +40232-211299; e-mail: rosca.irina@icmpp.ro)

² SC.Zeelandia SRL, 707410 Valea Lupului, Iasi, Romania,

³ Faculty of Chemical Engineering and Environmental Protection, "Gheorghe Asachi" Technical University of Iasi, 700050 Iasi, Romania

⁴ SCIENT - Research Center for Instrumental Analysis, 060114, Bucharest, Romania

⁵ TRANSCEND - Regional Oncology Institute, 700483 Iasi, Romania

* Address correspondence to: "Petru Poni" Institute of Macromolecular Chemistry of the Romanian Academy, Centre of Advanced Research in Bionanoconjugates and Biopolymers, 700487 Iasi, Romania, phone: +40232-217-454; fax: +40232-211299, e-mail: rosca.irina@icmpp.ro

Abstract

An exopolysaccharides (EPS) producing strain, isolated from yellow corn flour, was identified as *Weissella confusa* by 16sDNA gene sequencing. The strain was shown to produce the highest reported amount of EPS (26.6 g of freeze-dried EPS/L culture medium) in a novel natural culture medium which mimics the synthetic one. The extracted and purified exopolysaccharides were characterized by FTIR, ¹H-NMR, ¹³C-NMR, 2D-NMR (HSQC), HPLC, GPC and TGA analysis. The HPLC analysis showed only glucose in the exopolysaccharides structure. By NMR and FTIR analysis it was confirmed a dextran structure, with 96.8 % α-(1→6) bonds and 3.2 % α-(1→3) branch linkages. Also, the GPC analysis of purified exopolysaccharides revealed two fractions with a high molecular weight (3.3×10^5 and 1.6×10^7 g mol/L) and two fractions with a low molecular weight (4.4×10^2 and 1.6×10^2 g mol/L). The thermogravimetric analysis showed two steps for exopolysaccharides degradation; the most important degradation peak was registered at 288°C with a weight loss of 65.9%. Due to their characteristics, these exopolysaccharides are suitable candidates as thickeners and gelling agents for the food industry and as hydrophilic matrix for controlled release of drugs pharmaceutical industry.

Keywords: dextran, natural culture medium, FTIR, NMR, HPLC, GPC, TGA.

1. Introduction

Until recently, dextran was the main exopolysaccharide produced by *Leuconostoc* species. Commercial production and structural analysis have focused mainly on dextran from *Leuconostoc mesenteroides* and *Leuconostoc citreum* (MIAO & al. [1]). Crude dextran is used as blood plasma substitute in clinical applications, as standard for size-exclusion chromatography, and as ingredient in cosmetics, bakery, and industrial frozen products (TAO & al. [2]). As compared to other lactic acid bacteria, *Weissella* species have received substantial attention in the recent years due to their comparatively higher dextran production capacity. The polymers produced by *Weissella* strains have been reported to possess very similar structures with those obtained from *Leuconostoc mesenteroides*, involving mainly α-(1→6) linkages and only limited α-(1→3) branch linkages (2.4–4%). The polymer produced by *Weissella* strains contains less branch linkages than the most commercial dextran from

Leuconostoc mesenteroides and, due to its properties, shows very high interest in food applications.

The genus *Weissella* contains 14 bacterial species (FIGUEIREDO & al. [3]) and one of them is *W. confusa* which is one of the most widespread species in beverages and fermented food. *W. confusa* strains have been detected in sugar cane, carrot juice, raw milk, sewage (MEHMETI & al. [4]), and intestines of adults farmed sea bass (*Lates calcarifer*) (SIRIRAT & al. [5]). *W. confusa* strain was successfully used in a multi-species semi-liquid sourdough starter to ferment tomato juice as well as red and yellow peppers. *W. confusa* strain is a suitable alternative to the extensively used *Leuconostoc mesenteroides* in the production of high amounts of linear dextran from sucrose (FUSCO & al. [6]), without strong acid production in fermented culture medium (KATINA & al. [7]).

W. confusa is also an opportunistic human and animal pathogen (FUSCO & al. [6]) being from blood cultures developed infective endocarditis and from peritoneal fluids and abdominal walls isolated (SHIN & al. [8]).

This investigation was aimed at characterizing the EPS, produced by a lactic acid bacteria isolated from yellow corn flour (strain identified as *Weissella confusa*), biosynthesized in a natural culture medium.

2. Material and Methods

Microorganism and chemicals

The lactic acid bacterial strain was isolated from yellow corn flour from Portugal in the laboratories of Biotechnology applied in food industry – Integrated centre for research and education – BIOALIMENT, “Dunarea de Jos” University of Galati, Faculty of Food Science and Engineering, and stored under the code O.03.7.4. – AE, and also in our lactic acid bacteria collection under the code 321. The conservation of the pure strain was made following SIDDIQUI & al. [9] protocols.

The acetonitrile HPLC grade, trichloroacetic acid, ethanol, manganese sulphate, magnesium sulphate, calcium carbonate and dextran from *Leuconostoc* spp., $M_w=40.000$ were purchased from Sigma-Aldrich. The yeast extract was purchased from Bio Springer, and the commercial sugar and milk powder are the industrial compounds used in bakery industry. The D-(+)-glucose and D-(+)-xylose standard were purchase from Dupelco Analytical, and L-(+)-rhamnose, L-(+)-arabinose, D-(+)-mannose, D-(-)-fructose and D-(+)-galactose standards were purchased from Laboratories of Dr. Ehrenstorfer GmbH, Germany. The pullulan standard, Type P-82, Lot 01101, was purchased from Shodex Denko KK, Japan. The deuterated water (99.90% D₂O) and TSP – 98%, sodium 3-(trimethylsilyl)-[2,2,3,3-d₄]-1-propionate were purchased from Euriso-top, France.

Molecular identification

In order to perform a molecular characterization of the lactic acid bacteria, bacterial DNA was extracted from 24 hours culture grown on MRS agar plates at 30°C. DNA purification was made in duplicate with the Genomic DNA Purification Kit (Thermo Scientific) and the elution was made in 100 µL nuclease free water. The spectrophotometric quantification was made in NanoDrop with the following concentrations: 24.2 ng/µL for the first purification and 10.8 ng/µL for the second one.

A 10 ng/µL dilution was made for both samples and 5 µl were used for the PCR reactions. The primer pair: 27F 5' AGAGTTTGATCMTGGCTCAG 3' and 1492R 5' TACGGYTACCTTGTTACGACTT 3' were used for the amplification of 16 S rDNA gene

(FRANK & al. [10]). The PCR reactions were performed in a total volume of 25 μ L (12.5 μ L GoTaq® Master Mix, 2.5 μ L forward primer 10 mM, 2.5 μ L reverse primer 10 mM, 2.5 μ L nuclease free water and 5 μ L DNA). The reaction mixture was first incubated for 10 minutes at 95°C and then cycled for 35 times 30 s at 95°C followed by one cycle of 4 min at 60°C.

In order to verify the reaction and the possible contaminations, the electrophoresis migration of the products was conducted in 2% agarose gel. The PCR products were purified with Wizard SV Gel and PCR Clean-Up Kit (Promega) and a new electrophoresis migration was made with the final products in order to verify the purification and to quantify the quantity to be sequenced. Sequencing reactions were prepared using primers 27F/1492R. DNA sequencing was carried out by using GenomeLab™ Dye Terminator Cycle Sequencing with Quick Start Kit (Beckman Coulter). The sequencing products were purified with glycogen, sodium acetate and Na₂-EDTA, as indicated by the sequencing kit and were migrated in the sequencer GenomeLab™ GeXP Genetic Analysis System with the migration program LFR-a. The sequences were interpreted and exported in program (Chromas Lite, Version 2.01) and examined with deposited sequences using nucleotide BLAST program (NCBI, <http://www.ncbi.nlm.nih.gov>, July 2014) (ALTSCHUL & al. [11]).

Fermentation condition

We used only natural compounds in the culture medium compositions. The culture medium mimics the characteristic culture medium used for lactic acid bacteria growth, with the following composition: 150 g/L commercial sugar, 30 g/L yeast extract, 10 g/L milk powder, 0.05 g/L manganese sulphate and 0.2 g/L magnesium sulphate. The culture medium was sterilized at 120°C for 20 minutes and inoculated with 10% of 48 hours fresh inoculum, with absorbance of 0.5 read at 600 nm. The samples were incubated at 30°C for 72 hours. At the end of the fermentations process, the enzymatic inactivation of culture medium was made after Li & al. [12].

EPS isolation and purification

First, all the proteins and cells were removed by precipitation with 20% trichloroacetic acid (TCA) and centrifugation at 10.000 rpm for 10 min at 4°C using a Beckman Coulter Allegra® X-22 centrifuge equipped with a F0685 rotor. The EPS precipitation and separation from culture medium were made following TAYUAN & al. [13] protocol. The EPS were washed 3 times with chilled ethanol and redissolved in double distilled water (DDW). For the gravimetric quantification, the EPS suspended in DDW were subjected to freeze-dryer process, in an ALPHA 2-4 LD Plus Freeze-Dryer, and the result was expressed as polymer dry mass in one litre of culture medium (PALOMBA & al. [14]).

Evaluation of molecular weight averages and molecular weight distribution

The gel permeation chromatography (GPC) measurements were carried out on a device from Polymer Laboratories System (PL-GPC 120, Varian) instrument equipped with refractive index detector. The eluent (0.2M NaNO₃ 0.01M NaH₂PO₄ pH=7) was filtered, degassed by sonication and pumped at 1 mL/min. The system of columns consists of three PL-aqua gel packed columns filled with porous gel composed of vinyl copolymers (cross-linked) beads with polymeric hydroxyl functionality (8 μ m particle size and 20, 40 and 60Å pore type), connected in series and placed in the column oven at 30°C. **Sample preparation:** EPS sample (0.1 \pm 0.001g) was placed in a 10 mL volumetric flask filled with the eluent and stirred at room temperature overnight. One millilitre of this solution was filtered on 450 nm

pore size cellulose filter and 100 μL were used for injection. The calibration of the system was performed with Pullulan standards of the following molecular weight: 0.6×10^4 , 1×10^4 , 2.17×10^4 , 4.88×10^4 and 11.3×10^4 , 21×10^4 , 36.6×10^4 , 80.5×10^4 g/mol. Data collection and processing were performed using Cirrus GPC software.

Monosaccharide composition analysis

For quantitative monosaccharide analysis, hydrolysis of the purified EPS was needed. The 0.5 g of crude EPS was dissolved in 5 mL of DDW and 1.5 mL of 5 M trichloroacetic acid (TCA) was added (TAYUAN & al. [13]). The suspension was heated at 100°C for 6 h for polysaccharide hydrolysis. Final solution was neutralized with 0.1 M NaOH (pH 7 ± 7.5) after removal of the TCA by steam evaporation (MOZZI & al. [15]). The stock solution of hydrolysed EPS was made in HPLC mobile phase and 20 μL were injected in HPLC system.

The HPLC analyses were carried out using a Perkin Elmer HPLC system with a Flexar Refractive Index LC Detector. It was used an amino column ($3\mu\text{m}$, 150 mm x 4.5mm), the temperature was kept at 35°C , and as mobile phase was used a mixt of 75:25 v/v acetonitrile HPLC grade and DDW with a flow of 0.98 mL/min. All the dilutions of the samples were made in the mobile phase. EPS content of individual sugars was made on a calibration curve using glucose, fructose, galactose, xylose, rhamnose, mannose and arabinose as standard samples. In order to achieve the standard curve there were prepared stock solutions of each type of sugar with a concentration of 10 mg/mL. Based on the stock solutions, dilutions of 8, 6, 4, 2, 1, 0.8, 0.6, 0.4, 0.2 and 0.1 mg/mL were prepared, using the same injection volume as the sample.

NMR spectroscopy analysis

For the NMR analysis the EPS sample was dissolved in deuterated water with TSP used as internal standard. The spectra have been recorded at room temperature (aprox. 24°C). Chemical shifts are reported in ppm and referred to TSP (ref. ^1H 0.00 ppm and ^{13}C 0.00 ppm). The NMR spectra had been recorded on a Bruker Avance III 400 MHz Spectrometer, equipped with a 5 mm inverse detection z-gradient probe, operating at 400.1 and 100.6 MHz for ^1H and ^{13}C nuclei. Heteronuclear single quantum coherence (HSQC) experiment was recorded using standard pulse sequences in the version with z-gradients, as delivered by Bruker with TopSpin 2.1 PL6 spectrometer control and processing software.

Fourier-transform infrared spectroscopy (FTIR)

Fourier transform infrared spectroscopy (FTIR) spectra for EPS and dextran sample were recorded in KBr pellet, using a Bruker Vertex 70 spectrometer. The absorption was measured in a wavenumber range from 4000 to 400 cm^{-1} , with a resolution of 4 cm^{-1} and the numbers of 64 scans were taken, with a baseline correction. FTIR spectra were processed using a specialized program from *SpectraManager* series.

Thermal analysis measurements - thermogravimetry (TGA) and differential scanning calorimetry (DSC)

The thermogravimetric (TG) and differential thermogravimetric (DTG) analysis of samples were performed on STA 449F1 Jupiter NETZSCH equipment. DSC measurements were performed on a Maia F3 200 DSC device (Netzsch, Germany). Approximately 10 mg of freeze-dryer sample was used for all the analysis. Measurements were carried out in the 30–

700°C temperature range, applying a heating rate of 10°C/min. Nitrogen purge gas was used as inert atmosphere at a flow rate of 50 mL/min. Samples were heated in open Al_2O_3 crucibles. For TG and DTG analysis, the device was calibrated for temperature and sensitivity with indium, according to standard procedure. For DSC measurements, its device was calibrated with five different metals according to standard procedures.

3. Results and Conclusions

Identification of the lactic acid bacterial strain no. 321

Strain no. 321, with high capability to produce EPS, was selected for this study. The data base researches of the sequencing results showed 97% identities with those available in the nucleotide BLAST data base and led to the identification of the *Weissella confusa* bacterial species (see experimental section) (ALTSCHUL & al. [11]).

Fourier-transform infrared spectroscopy (FTIR) of purified EPS produced by *W. confusa* in natural culture medium

The freeze-dryer EPS extracted from natural culture medium were subjected to FTIR analyze and the resulted spectrum was compared with that of pure dextran (Figure. 1).

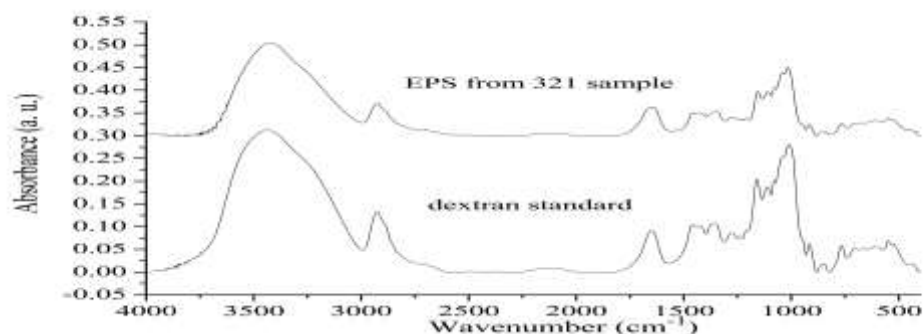


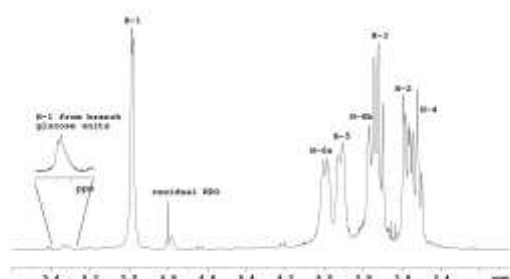
Figure 1. FTIR spectra of: pure dextran, $M_w=40000$ and purified EPS produced by *W. confusa* in natural culture medium.

The high intensity peaks at 3391 cm^{-1} were assigned to the hydroxyl groups stretching vibrations and are responsible for the presence of large amount of water in the both samples (Figure. 1) (Q. XIAO & al. [16]). Also, the other characteristics bands at 2926 cm^{-1} for EPS sample and 2925 cm^{-1} for dextran were detected and represent the stretching and bending vibrations of C-H bond (XIAO & al. [16]), corresponding to methyl and methylene groups from carboxymethylates groups of the sugar ring (AHMED & al. [17]). The presence of the medium signals at 1668 and 1655 cm^{-1} for EPS and dextran, respectively, represent the bending vibration of C-OH groups of glucopyranosyl unit (AHMED & al. [17]) which suggests that the samples are saccharides. The peak of 1419 cm^{-1} is attributed to the symmetric stretching vibration of $>\text{C}=\text{O}$ and C-O bonds of the $-\text{COO}^-$ groups which is characteristic for polymers. Peaks around 1458 and 1342 cm^{-1} were attributed to the asymmetric deformation of C-H bond, confirming the polysaccharide nature of the compounds (SAMAL & al. [18]). Also, peaks at 1239 cm^{-1} are specific for in-plane vibrations of hydroxyl groups and the bands of 1271 and 1280 cm^{-1} from EPS and dextran samples, respectively, were assigned to C-O-C stretching from polysaccharide structure (SHENG & al.

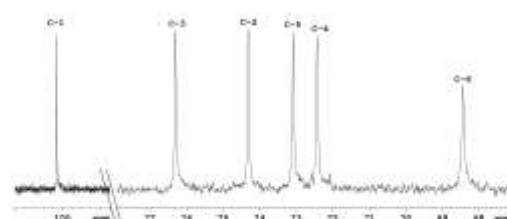
[19]). It should be mentioned that each particular polysaccharide has a specific band in the 1200–1000 cm^{-1} region. This region is dominated by ring vibrations overlapped with stretching vibrations of (C–OH) side groups at around 1041 cm^{-1} (AHMED & al. [17]) and the (C–O–C) glycosidic band vibration at 1155 cm^{-1} for EPS sample and 1160 cm^{-1} for dextran, indicating the presence of pyranosic form and glycosidic bridge (SHENG & al. [19]). The shoulder bands at 1108 and 1110 cm^{-1} for EPS sample and dextran, respectively, can be assigned to the vibration of the C–O bond at C (4) position of a glucose residue (Figure. 3d) (XIAO & al. [16]). Meanwhile, the very strong signals at 1013 and 1011 cm^{-1} of EPS sample and dextran, respectively, can be attributed to the intense C–O stretching vibration characteristic for polysaccharides (SHENG & al. [19]). The bands at 916 cm^{-1} for EPS sample and at 915 cm^{-1} for dextran are attributed to the asymmetric telescopic vibration of glucopyranosic ring (CHEN & al. [20]) and are characteristic for β -D-glucans and (1 \rightarrow 3)- α -D-glucans (GOO & al. [21]). The presence of α -anomeric configuration and chair conformation of the glucopyranose units were confirmed by the presence of bands at 843 cm^{-1} for EPS sample and 845 cm^{-1} for dextran (CHEN & al. [20]). Taken into account of all the similarities between the two spectra (EPS sample and dextran with $M_w=40.000$), we can assign a dextran structure for EPS sample biosynthesized by *W. confusa* in a natural culture medium.

NMR spectroscopy analysis

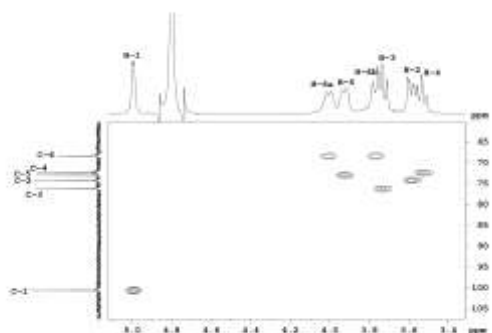
The obtained ^1H and ^{13}C NMR spectra show the characteristic signals of an exopolysaccharide. Based on previously reported data (MAINA & al. [2]; CHEN & al. [20]) it was deduced that the exopolysaccharide produced by *W. confusa* was dextran.



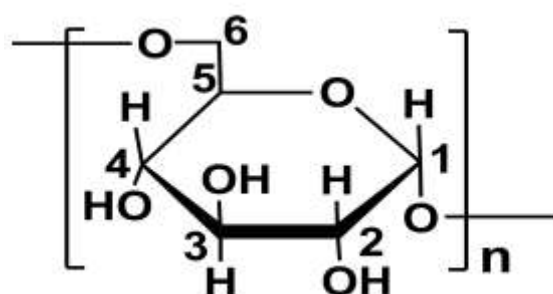
A)



B)



C)



D)

Figure 2. NMR spectra of EPS sample produced by *W. confusa*, recorded in D₂O. **A)** ¹H NMR spectrum, **B)** ¹³C NMR spectrum, **C)** H,C HSQC spectrum, **D)** Numerations of the carbons from glucose structure.

In the ¹H NMR spectrum (Figure 2a) several signals are observed in the region 3-6 ppm. The most intense signal in the anomeric region, at $\delta = 4.98$ ppm (d, $J = 2.97$ Hz), belongs to the protons from position 1 of the α -(1 \rightarrow 6)-linked glucose units, in the main chain. The rest of the glucose units signals appear in the region 3.3-4.1 ppm and were assigned as it follows: $\delta = 3.52$ (H-4, t, $J = 9.4$ Hz), 3.58 (H-2, dd, $J = 9.84, 3.24$ Hz), 3.72 (H-3, t, $J = 9.36$ Hz), 3.74-3.77 and 3.98-4.00 (H-6a and H-6b, m), 3.90-3.92 (H-5, m).

The presence of α -(1 \rightarrow 3)-linked glucose units, as branches, is proved by the less intense anomeric signal at $\delta = 5.33$ ppm. The rest of the signals for the branch glucose units could not be assigned because of their low intensity and overlap with the signals from the main chain units.

Based on the two anomeric signals and the ratio of their integrals, the percentage of glycosidic linkages was established as being 96.8% for α -(1 \rightarrow 6) and 3.2% for α -(1 \rightarrow 3). This data are in concordance with literature, where the per cent for α -(1 \rightarrow 3) bonds is 3% (SHUKLA & al. [22]). The water-solubility of dextran is affected by their chemical structure, for example, dextran with more α -(1 \rightarrow 6) linkages are more water-soluble than dextran with less α -(1 \rightarrow 6) linkages (KATINA & al. [7]).

In the ¹³C NMR spectrum (Figure 2b), six signals are observed in the interval 68-101 ppm. Starting from previously assigned proton signals and using the information from 2D H,C HSQC spectrum (Figure 2c), these six ¹³C signals were assigned as it follows: $\delta = 68.4$ (C-6), 72.4 (C-4), 73.1 (C-5), 74.3 (C-2), 76.3 (C-3), 100.6 (C-1) (Figure 2d).

Monosaccharide composition analysis of the purified EPS

Monosaccharide composition of EPS hydrolyzate samples with trichloroacetic acid was determined by using HPLC technique as described by *Tayuan* and co-workers [13] (see experimental section).

Taking into consideration that the culture medium used for EPS biosynthesis contained a commercial sugar as carbon source, which is a disaccharide formed by fructose and glucose with a usual name of sucrose, the results (Figure 3) indicated that the glucose was the only monosaccharide of the EPS polymer. The EPS produced by *W. confusa* had glucose in a high amount as previously reported by literature (KATINA & al. [7]).

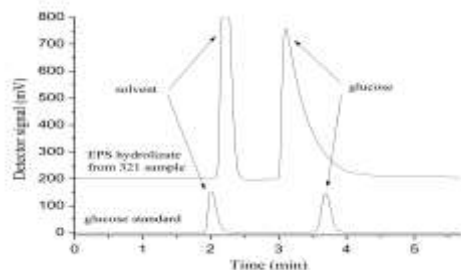


Figure 3. HPLC chromatograms for glucose standard and hydrolyzed EPS sample produced by *W. confusa* strain fermentation in natural culture medium.

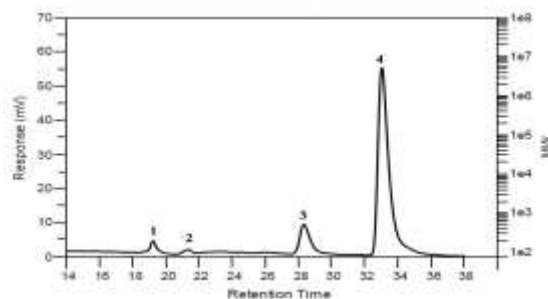


Figure 4. GPC curve of EPS produced by *W. confusa* strain in a natural culture medium.

Evaluation of molecular weight averages and molecular weight distribution

The majority natural polymers (excluding proteins) and synthetic polymers show a molecular weight distribution which drastically affects polymer properties. In this regard, it is very important to characterize the polymers in terms of molecular weight averages (the number-average molecular weight, denoted by M_n , and weight-average molecular weight, denoted by M_w) and molecular weight distribution which can be characterized by the polydispersity index (M_w/M_n). It should be mentioned that the molecular weight of the molecules is dependent upon the ionic strength, and hence, on its own concentration in solution. Within this context, the gel permeation chromatography (GPC) is by far the most widely used method for determining the average molecular weight of polymers and their polydispersity index. Separation is accomplished on a set of chromatographic columns filled with beads of a rigid porous material characterized by a certain range of pore sizes that separates the polymer molecules according to size, a phenomenon often referred to as molecular sieving (FEKETE & al. [23]).

A typical gel permeation chromatogram plots detector response against the volume of dilute polymer solution that passes through the column. To obtain molecular weights at a given retention volume, the chromatograms were compared with a reference chromatogram obtained with fractions of known average molecular weight in the same solvent and at the same temperature. In Figure 4 are presented the GPC plot of EPS sample produced by *W. confusa* in a natural culture medium and of pullulan, as standard. The weight-average molecular weight (M_w) and the number-average molecular weight (M_n) were estimated through comparison with pullulan standard curve.

As expected, four fractions, ranging from 10^2 and up to 10^6 , were obtained after gel permeation chromatography of EPS sample (Figure 4). High molecular weight (HMW) material $M_w=1.6 \times 10^7$ and 3.3×10^5 g/mol, respectively include fraction 1 and 2, while low molecular weight (LMW 4.4×10^2 and 1.6×10^2 g/mol, respectively) consist of fraction 3 and 4. The use of commercially available pullulan standards did not allow an exact estimation of the molecular weight of the EPS sample, as the sample eluted earlier than the highest pullulan standard available (80.5×10^4 Da), indicating that the molecular mass of EPS sample exceeded 80.5×10^4 Da for the first fraction. The same issue occurred with the third and four fraction, as the sample eluted later than the lowest pullulan standard (0.6×10^4 Da), indicating lower molecular mass. From Figure 4 a small polydispersity index of each fraction can be observed, meaning that each fraction is nearly monodisperse (fraction 1 - 1.29 PD; fraction 2 - 1.0 PD, fraction 3 - 1.18 PD). It is well known that the best controlled synthetic polymers have PD of 1.02 to 1.10 and step polymerization reactions yield a PD of around 2.0.

The obtained molecular weights are in good accordance with the literature data, since microbial exopolysaccharides are well known to be high molecular weight carbohydrate polymers consisting of monosaccharide like glucose, fructose etc. (VAN HIJUM & al. [24]). The differences in exopolysaccharide molecular mass could be, therefore, attributed to strain-specific growth conditions. By comparing the obtained molecular weights of EPS with those of a standard (of known molecular weight) relative (not absolute) values are given.

Thermal analysis measurements - thermogravimetry (TGA) and differential scanning calorimetry (DSC)

TGA is an analytical technique used for measuring the dynamic weight loss of a material as a function of temperature (MISHRA & al. [25]). By analysing the TGA and corresponding first derivative DTG curve in Figure 5a, it can be noticed that the degradation

of purified EPS sample obtained by the fermentation of *W. confusa* strain in a natural culture medium occurs in two stages.

The first thermal decomposition stage occurs in the range 49°C–123°C with a maximum degradation rate at 91°C and a weight loss of 5%. This stage is attributed to the loss of moisture and alcohol content trapped in the exopolysaccharide structure (AHMED & al. [17]) and probably of some crystallization water related with phosphate components. This weight loss shows that some residual water is present in components due to the increased level of hydroxyl carboxyl groups, making the EPS sample to have affinity towards water. The second thermal degradation stage with a maximum degradation rate at 288°C and a weight loss of 65.9% can be attributed to polysaccharide depolymerisation and degradation by random chain scission. Therefore, by comparing the degradation profile of EPS sample biosynthesized by *W. confusa* strain with that of pure dextran (Figure 5a) it can be concluded that the EPS sample degradation presented a similar behaviour to that of dextran (MAIA & al. [26]).

It can be noticed from the second DSC heating curves of EPS sample and dextran (Figure 5b), that the dextran exhibits a glass transition temperature domain (T_g) at 220°C conform to literature data (STANCIU and NICHIFOR [27]).

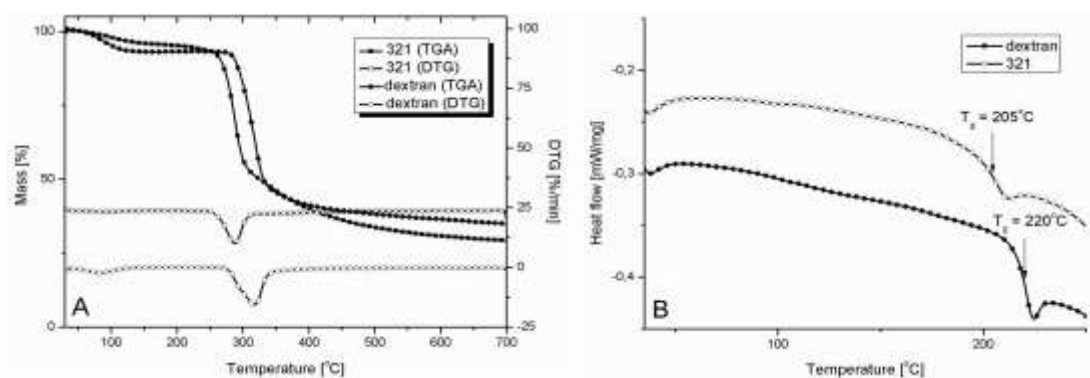


Figure 5. A) TGA and B) DSC analysis for EPS sample extracted from the natural culture medium inoculated with *W. confusa* lactic acid bacteria and pure dextran (M_n 40000).

The EPS produced by *W. confusa* strain exhibited a lower T_g domain at 205°C. This aspect may be explained by *W. confusa* strain producing a dextran with higher segmental chain mobility probably due to a more amorphous structure.

In the present study it was identified an efficient EPS producing strain, *W. confusa*, by fermentation using a natural culture medium. Taking into account the results from FTIR, $^1\text{H-NMR}$, $^{13}\text{C-NMR}$, HSQC, HPLC, TG/DTA and GPC analysis, EPS have a dextran structure, with 100% glucose composition and high molecular weight. These characteristics classify the pure EPS produced by *W. confusa* as a suitable ecological candidate product for the pharmaceutical and alimentary field.

4. Acknowledgments

This research was funded by Romanian National Authority for Scientific Research, CNCS – UEFISCDI, project number PN-II-ID-PCCE-2011-2-0028 and H2020 ERA Chairs Project no.667387: SupraChem Lab Laboratory of Supramolecular Chemistry for Adaptive Delivery Systems ERA Chair initiative.

References

1. M. MIAO, A. BAI, B. JIANG, Y. SONG, S.W. CUI, T. ZHANG. Characterization of a novel water-soluble polysaccharide from *Leuconostoc citreum* SK24.002. *Food Hydrocolloid*, 36: 265 (2014).
2. Y. TAO, R. ZHANG, W. XU, Z. BAI, Y. ZHOU, S. ZHAO, Y. XU, D. YU. Rheological behavior and microstructure of release-controlled hydrogels based on xanthan gum crosslinked with sodium trimetaphosphate. *Food Hydrocolloid*, 52: 923 (2016).
3. H.C.P. FIGUEIREDO, F.A.A. COSTA, C.A.G. LEAL, G.A. CARVALHO-CASTRO, R.C. LEITE. *Weissella* sp. outbreaks in commercial rainbow trout (*Oncorhynchus mykiss*) farms in Brazil. *Vet. Microbiol*, 156, 359, 366 (2012).
4. J. MEHMETI, S. MUJI, D.B. DIEP, J.F. NES. High frequency of the potential pathogen *Lactococcus garvieae* in raw milk from Kosovo. *Food Control*, 53: 194 (2015).
5. R. SIRIRAT, R. THOSAPORN, P. SOMKIAT. Evaluation of lactic acid bacteria as probiotics for juvenile *Lates calcarifer*. *Aquat. Resour*, 39: 134 (2008).
6. V. FUSCO, G.M. QUERO, G. STEA, M. MOREA, A. VISCONTI. Novel PCR-based identification of *Weissella confusa* using an AFLP-derived marker. *Int. J. Food Microbiol*, 145: 437 (2011).
7. K. KATINA, N.H. MAINA, R. JUVONEN, L. FLANDER, L. JOHANSSON, L. VIRKKI, M. TENKANEN, A. LAITILA. *In situ* production and analysis of *Weissella confusa* dextran in wheat sourdough. *Food Microbiol*, 26: 734 (2009).
8. J.H. SHIN, D.I. KIM, H.R. KIM, D.S. KIM, J.-K. KOOK, J.N. LEE. Severe infective endocarditis of native valves caused by *Weissella confusa* detected incidentally on echocardiography. *J. Infection*, 54: 149 (2007).
9. N.N. SIDDIQUI, A. AMAN, A. SILIPO, S.A.U. QADER, A. MOLINARO. Structural analysis and characterization of dextran produced by wild and mutant strains of *Leuconostoc mesenteroides*. *Carbohydr. Polym*, 99: 331 (2014).
10. J.A. FRANK, C.J. REICH, S. SHARMA, J.S. WEISBAUM, B.A. WILSON, G.J. OLSEN. Critical Evaluation of two primers commonly used for the amplification of bacterial 16 rRNA genes. *Appl. Environ. Microb*, 74 (8): 2461 (2008).
11. S.F. ALTSCHUL, T.L. MADDEN, A.A. SCHAFFER, J. ZHANG, Z. ZHANG, W. MILLER, D.J. LIPMAN. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res*, 25: 3389 (1997).
12. W. Li, M. MUTUVULLA, X. CHEN, M. JIANG, M. DONG. Isolation and identification of high viscosity-producing lactic acid bacteria from a traditional fermented milk in Xinjiang and its role in fermentation process. *Eur. Food. Res. Technol*, 235: 497 (2012).
13. A. TAYUAN, G.W. TANNOCK, S. RODTONG. Growth and exopolysaccharide production by *Weissella* sp. from low-cost substitutes for sucrose. *Afr. J. Microbiol. Res*, 5 (22): 3693 (2011).
14. S. PALOMBA, S. CAVELLA, E. TORRIERI, A. PICCOLO, P. MAZZEI, G. BLAIOTTA, V. VENTORINO, O. PEPE. Wheat Sourdough from *Leuconostoc lactis* and *Lactobacillus curvatus* Exopolysaccharide-producing Starter Culture: Polyphasic Screening, Homopolysaccharide Composition and Viscoelastic Behavior. *Appl. Environ. Microb*, 78 (8): 2737 (2012).
15. F. MOZZI, G. ROLLAN, G.S. DE GIORI, G. FONT DE VALDEZ. Effect of galactose and glucose on the exopolysaccharide production and the activities of biosynthetic enzymes in *Lactobacillus casei* CRL 87. *J. Appl. Microbiol*, 91(1): 160 (2001).
16. Q. XIAO, Q. TONG, L.T. LIM. Drying process of pullulan edible films forming solutions studied by ATR-FTIR with two-dimensional correlation spectroscopy. *Food Chem*, 150: 267 (2014).
17. Z. AHMED, Y. WANG, N. ANJUM, H. AHMAD, A. AHMAD, M. RAZA. Characterization of new exopolysaccharides produced by coculturing of *L. kefirifaciens* with yoghurt strains. *Int. J. Biol. Macromol*, 59: 377 (2013).
18. P.K. SAMAL, J.S. DANGI. Isolation, preliminary characterization and hepatoprotective activity of polysaccharides from *Tamarindus indica* L. *Carbohydr. Polym*, 102: 1 (2014).
19. L. SHENG, G. TANG, P. SU, J. ZHANG, Q. XIAO, Q. TONG, M. MA. Understanding the influence of Tween 80 on pullulan fermentation by *Aureobasidium pullulans* CGMCC1234. *Carbohydr. Polym*, 136: 1332 (2016).
20. Y. CHEN, W. MAO, Y. GAO, X. TENG, W. ZHU, Y. CHEN, C. ZHAO, N. LI, C. WANG, M. YAN, J. SHAN, C. LIN, T. GUO. Structural elucidation of an extracellular polysaccharide produced by the marine fungus *Aspergillus versicolor*. *Carbohydr. Polym*, 93:478 (2013).

21. B.G. GOO, G. BAEK, D.J. CHOI, Y.I. PARK, A. SYNITSYA, R. BLEHA, D.H. SEONG, C.-G. LEE, J.K. PARK. Characterization of a renewable extracellular polysaccharide from defatted microalgae *Dunaliella tertiolecta*. *Bioresource Technol*, 129: 343 (2013).
22. S. SHUKLA, Q. SHI, N.H. MAINA, M. JUVONEN, M. TENKANEN, A. GOYAL. *Weissella confusa* Cab3 dextransucrase: Properties and *in vitro* synthesis of dextran and glucooligosaccharides. *Carbohydr. Polym*, 101: 554 (2014).
23. S. FEKETE, A. BECK, J.-L. VEUTHEY, D. GUILLARME. Theory and practice of size exclusion chromatography for the analysis of protein aggregates. *J. Pharmaceut. Biomed*, 101: 161 (2014).
24. S. VAN HIJUM, S. KRALJ, L.K. OZIMEK, L. DIJKHUIZEN, I.G.H. VAN GEEL-SCHUTTEN. Structure-function relationships of glucansucrase and fructansucrase enzymes from lactic acid bacteria. *Microbiol. Mol. Biol. R*, 70: 157 (2006).
25. A. MISHRA, K. KAVITA, B. JHA. Characterization of extracellular polymeric substances produced by micro-algae *Dunaliella salina*. *Carbohydr. Polym*, 83: 852 (2011).
26. J. MAIA, R.A. CARVALHO, J.F.J. COELHO, P.N. SIMOES, M.H. GIL. Insight on the periodate oxidation of dextran and its structural vicissitudes. *Polymer*, 52: 258 (2011).
27. M.C. STANCIU, M. NICHIFOR. New biocompatible amphiphilic diblock copolymer based on dextran. *Eur. Polym. J*, 71: 352 (2015)