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Original paper

Isolation and partial characterization of a polysaccharide produced by Klebsiella oxytoca ICCF 419, a newly-isolated strain in Romania

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Abstract

This paper details a preliminary investigation using a newly isolated strain in order to obtain a bacterial exopolysaccharide (EPS) as a source of biomaterials. The 16S rDNA (primers 27F and 1492R) and ARDRA techniques were applied for confirming that the isolated ICCF 419 strain belongs to the *Klebsiella oxytoca* species. Different yields of crude EPS were obtained with the use of glucose and lactose (0.8 and 1 g/100 mL) or sucrose or glycerol (0.6 and 0.43 g/100 mL) as carbon sources. Monosaccharide analysis confirmed the sugar units characteristic for *Klebsiella* [i.e. glucose (16.18%), rhamnose (11.16%) and uronic acids (38.16%)] but also demonstrated the presence of unusual components [such as fructose (6.45%), sucrose (2.54%) and mannose (1.91%)], giving a total sugar content of 78%. Moreover, 2.77 g/L of purified polysaccharide were extracted with the ICCF fermentation medium containing 3% w/v lactose. FTIR analysis revealed typical absorption bands, which can be attributed to the vibrations of the OH, CH, C=O, COO- and COOR functional groups. Based on the chemical shifts in the ^{13}C-NMR spectra, 4 regions were identified: carbonyl carbon, anomeric carbon, ring carbon and alkyl carbon. No cytotoxic effect was recorded and stimulating cell proliferation rates of 49% and 28% were determined.

Keywords Klebsiella oxytoca, exopolysaccharides, chemical characterization of EPS, pharmacological characterization of EPS, cytotoxicity assay.

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Introduction

Increasing attention has been given during recent years to the production of extracellular polysaccharides (EPSs) from microorganisms and a large number of biologically active polymers with different chemical structures have been isolated. These bio-macromolecules have been investigated as antitumor, antioxidant, probiotics and immunostimulants due to their bio-compatibility, biodegradability, and non-toxicity properties, as well as for other health benefits, such as: antibacterian, antiviral, antidiabetic, anticoagulant, cholesterol-lowering, antiulcer, anti-osteorthritic, hepato- and reno-protection activities etc. (AHMAD et al, 2015; MOSCOVICI, 2015; VAMANU et al, 2015; ULLAH et al, 2019). From the medicinal point of view, microbial EPSs are preferred as compared to synthetic similar polymers. In last decades, EPSs have been considered as a novel tool which provided innovative biotechnological solutions and value-added products in many fields, such as: pharmaceuticals, medicine, cosmetics, food and feed, beverages, sustainable agriculture, oil industry, bioremediation, biosorption, wastewater treatment, bioleaching, organic chemistry, metal nanoparticles green synthesis, paint, paper, ink and textile industries, acoustics, oil drilling, cement production and gravel packing, fluid-loss controlling, and anti-icing compositions for airplane surfaces (VU et al, 2009; ROCA et al, 2015; SCHMIDT et al, 2015; LUQUE et al, 2016; HALDER et al, 2017; PIROG et al, 2017; COSTA et al, 2018; RIPARI et al, 2019; SHANMUGAM et al, 2019; SHER et al, 2019; ULLAH et al, 2019).

Beside their capacity to produce polysaccharides, rhizospheric microbes provide many other ecological functions, and bacterial species belonging to *Bacillus, Pseudomonas, Rhizobium, Azotobacter, Klebsiella, Xanthomoas, Chromobacterium, Serratia*, and *Azospirillum* genera are also linked with plant growth promoting activities; these microorganisms are considered key engineers in soils, and most of them are producers of various secondary metabolites. Therefore, microbial diversity which contributes to nitrogen-fixing and root nodules forming (*one gram of soil contains over 4000 different bacterial genomic units*) has a substantial role in host plant specific symbiosis establishment, and offers a vast biotechnological potential for future research development (SINGH, 2019).

EPSs from nitrogen-fixing bacteria (plant growth promoters): *Enteriobacteriaceae* producers (*Enterobacter* and *Raoutella* sp., *Klebsiella oxytoca*), or *Sinorhizobium meliloti* synthesize water-soluble and acidic polymers which are characterized by different structures, containing following main sugar units: galactose and mannose (i.e. *K. oxytoca*); galactose and glucose; galactose, glucose and mannose; fucose, galactose and glucose; fucose, galactose, glucose and mannose; and glucuronic acid, as the charged component. These EPSs are known for their bioemulsifying activity (the presence of uronic acids, and fucose or rhamnose give the ability to emulsify the hydrocarbons or to associate with them), and this fact leads to applications in oil industry and bioremediation field (i.e. hydrocarbons degradation) (PAWLICKI et al, 2010; CASTELLANE et al, 2017).

The genus *Klebsiella* generally develops prominent capsules as complex acid polysaccharides, which cover the bacterial surface and possess antigenic properties (BLEICH et al, 2008; SINGHA, 2012). Among *Klebsiella* species, the first notable research on a polysaccharide producing strain of *K. oxytoca* was published in 1994 (NAKANISHI et al, 2001) and since then, references about these EPSs producing strains, of environmental and pharmaceutical interest, containing a high content of galactose and rhamnose in their EPSs structures, are being continuously given (BAZAKA et al, 2011).

This work refers to fermentation processes using a newly-isolated bacterial strain, from root nodules of bitter cucumber (*Momordica charantia)*, that produced novel EPS structures. A preliminary chemical and pharmacological characterization was employed, to describe a purified EPS as a potential biomaterial. The paper presents the first report on structural characterization of the EPS synthesized by the *K. oxytoca* ICCF 419 strain belonging to the Culture Collection of Industrial Importance Microorganisms (CMII-ICCF-WFCC 232)[1], Romania.

Materials and Methods

Isolation and identification of the exopolysaccharide producing bacterial strain

The strain was isolated from a sample of bitter cucumber root extracted directly from fresh garden soil, which presented nodules; a liquid medium, namely IPS[2], containing (% w/v) 1.0 glucose and 1.5 corn steep liquor (as a waste product from industry) was preferred for the inoculum stage. After cultivation on different agar media (nutrient agar, King's B medium[3], and Yeast Malt agar), various morphological colonies, mucous ones especially, were purified and preliminary screened under fermentation conditions, to select the EPSs producing microorganisms. The screening methodology was initially employed for pseudozan, and curdlan production, using ICCF media (MOSCOVICI et al, 2009; MOCANU et al, 2011), but also other original formulas which were composed of different carbon sources, among them glucose, sucrose, and glycerin, in a concentration of 4.0 %, or lactose 3.0 % (w/v), with supplementary citric acid in a concentration of 0.1 % (w/v).

The pure isolate of a selected bacterial strain was characterized using biochemical tests: Gram staining, cytochrome oxidase and catalase production, and growth on McConkey Agar, MIU (Motility Indole Urea), TSI (Triple Sugar Iron Agar), Lia (Lysine Iron), and Simmons

[1]http://cfarm.ncpri.ro/files/cmii-iccf/CMII-ICCF.pdf

[2]IPS medium - modified formula of an ICCF original medium, IPS broth (SOARE MG et al, 2017).

[3]King's B medium was originally described by King EMS et al, 1954, but the medium used for the *Klebsiella* ICCF 419 strain was modified, according to the formula listed in: http://cfarm.ncpri.ro/files/cmii-iccf/CMII-ICCF.pdf

media. For preliminary identification, conventional tests API Rapid 20E were applied, using the instructions of the manufacturer (BioMeriux, France). The genomic DNA was extracted using an adapted protocol after Vassu et al, 2001. DNA samples separation was performed by agarose gel electrophoresis using agarose 0.8%. 16S-rDNA was amplified using an adapted protocol after Ionescu et al, 2013. PCR was performed in a total volume of 25 µl: PCR master mix (2x), 0.5 µM of each forward and reverse primer (GM3f AGAGTTTGATCCTGGCTCAG, GM4r GGTTACCTTGTTACGACTT) and 10 µg DNA. The reference strain was considered *K. oxytoca* CMGB 479, belonging to the MICROGEN Culture Collection, from the Faculty of Biology, University of Bucharest.

EPS isolation and purification

Fermentation media were inoculated with 10% (v/v) 24 hrs inoculum cultures grown in IPS. Supplementary, an original medium with lactose (30 g/L) and corn step liqueur (11.5 g/L) was prepared, to check the strain potential to metabolize the carbon source, while maximizing the polysaccharide production. Batch cultures were incubated on a rotary shaker, 220 rpm/min, at 33-34°C, for 72 hrs. Cell growth was monitored by measuring the OD at $\lambda = 570$ nm; the cell biomass was determined from the bacterial suspensions samples after centrifugation at 8000 rpm/30 min, and by measuring the dry weight of pellets after heating 10 hrs, at 105°C. Measurement of the apparent viscosity corresponding to the fermentation broth was achieved by using a rheometer - Rheotest 2 (MLW).

Downstream processing followed an adapted procedure (MOSCOVICI et al, 2009; MOCANU et al, 2010). The EPS was isolated by centrifugation at 8000 rpm/ 30 min/4°C (in some cases, for highly viscous cultures, samples were first diluted with water, 1:1) and filtration, using a celite precoat. From the cell-free solution, the crude polysaccharide was precipitated with ethanol (≥99.5%), 1:3 (v/v); the alcoholic supernatant was centrifuged at 3000 rpm/min for 15 min/4°C. The precipitate was dried to constant weight, in a vacuum oven (Memmert) at 85°C, for 8-10 hrs.

The rate of the carbon source consumption was determined by measuring free reducing sugars in the fermentation broth, at the final stage of bioprocesses (72 hrs), using Luff-Schoorl method. The results were expressed in terms of glucose. For the lactose containing fermentation medium, a preliminary hydrolysis with a solution of HCl (20%), at 67°-70°C, for 10 min, was carried out.

The crude polysaccharide was purified by ultrafiltration and diafiltration, as a 3% (w/v) solution, and by using a standard Pellicon module (Merck-Millipore), equipped with a cassette containing 10 polysulfone membranes (PTGC, 10 kDa), with a filtration area of 0.465 m^2. The EPS retentate solution was additionally concentrated at 45°C in a rotary evaporator under reduced pressure. The concentrate was precipitated with three volumes of ethanol p. a.

The total sugar content of the product was estimated by the phenol-sulfuric acid assay using glucose as standard and a UV-VIS spectrophotometer Helios (Gamma); the absorbance of the solutions was measured at 490 nm (DUBOIS et al, 2002).

Preliminary chemical composition of the EPS

An elemental analyzer PRECISELY 2400 Series II CHNS/O, PERKIN ELMER, was used to estimate the composition of the EPS in carbon, hydrogen, nitrogen, and sulfur. Impurities were assayed measuring the ash content by a technique based on sulfated mineral residue determination (European Pharmacopoeia, 9th Ed.).

Chromatography analysis (HPLC) was employed after an adapted protocol from European Pharmacopoeia (9th Ed.).

A complete hydrolysis to determine the sugar components of the purified EPS was performed, and the results were compared with those from some available sugar standards; the monomer composition of EPS was quantified using ELITE LaChrom Merck equipment, with a RI L-2490 detector and a steel column (Kromasil 100-5NH2, 250 x 4.6 mm), containing aminopropylsilane filler (L8). The injected volume was 50 µL. The sample was eluted, at a flow rate of 1.0 mL/min, at 38°C.

Sample processing: 250 mg of the purified sample was hydrolyzed with 10 mL of 23% trifluoroacetic acid solution at 120°C, 60 min (European Pharmacopoeia 9th Ed.). After centrifugation, the supernatant was brought to a 10 mL flask, in methanol.

Chromatography conditions: reagents – sodium dihydrogen phosphate dihydrate (R), acetonitrile (HPLC); ultrapure water; mobile phase – 0.253 g of sodium dihydrogen phosphate (R) dissolved in 220 mL of water and 780 mL acetonitrile; test solution – 250 mg of hydrolyzed sample diluted up to 10 mL with methanol; standard solutions – 40 mg of each standard sugar, such as rhamnose, arabinose, mannose, sucrose, and cellobiose were dissolved, in 10 mL flasks, then 5 mL acetonitrile was added and the solution was diluted with water up to 10 mL.

Uronic acid content was determined by HPLC, using glucuronic acid as standard, and the ELITE LaChrom HPLC (Merck) equipment, with a RI L-2490 detector and a steel column (Nucleogel Sugar 810 H 300 x 7.8 mm), containing a polystyrene/divinylbenzene (PS/DVB) filling. The sample was eluted at a flow rate of 1.0 mL/min, at 40°C.

FTIR and NMR spectroscopic analysis

Infrared spectroscopy was employed for a further understanding of the EPS structure, using a FTIR-ATR spectrometer (Perkin Elmer). A mixture of 2 mg sample with 200 mg of dry potassium bromide (KBr) was analyzed in the frequency range of 4000-400 cm^{-1}.

^{1}H and ^{13}C-NMR spectra were recorded using a Bruker Advance III Ultrashield Plus 500 MHz spectrometer, operating at 11.74 T, corresponding to the resonance frequency of 500.13 MHz for the ^{1}H nucleus, equipped with a direct detection four nuclei probe head and field gradients on z-axis, and a NMR Bruker Advance DRX400 spectrophotometer. Typical parameters for ^{1}H-NMR spectra were:

45º pulse, 8.3 s acquisition times, 8.01 kHz spectral window, 64 scans, 20 K data points, delay time 1 s. The average acquisition time of the ^{1}H-NMR spectra was approximately 5 min. The resonance frequency for the ^{13}C nucleus was 125.77 MHz, 45º pulse, 1.67 s acquisition times, 39.68 kHz spectral window, 4096 scans, 64 K data points, delay time 1 s. The average acquisition time of the ^{13}C-NMR spectra was approximately 2 hrs and 40 min. For NMR measurements of the EPS samples (originated from both glucose and lactose substrates), 10 to 30 mg were dissolved in deuterated water (D_2O). The ^{1}H and ^{13}C-NMR spectra were recorded at 25°C and 50°C.

Biological activity of the EPS

In vitro cytotoxicity assay was conducted by using an animal cell line – the murine fibroblast line – line L929 (ATCC CRL-6364). The medium used for the cell cultures was Eagle's Minimum Essential Medium, containing: horse serum, penicillin/streptomycin/neomycin solution in 0.9% NaCl (10,000 µg/mL/10,000 U/mL) (PSN), 0.25% trypsin solution, saline phosphate buffer (PBS). The cells were incubated in the presence of the two samples of polysaccharide (Pz 1 and Pz 2 from glucose and lactose, respectively) for 20 hrs, at 37°C, in 5% CO_2 atmosphere, at concentrations of 400 µg/mL, 200 µg/mL, 100 µg/mL and 50 µg/mL, and the cell viability was determined by a colorimetric method using the CellTiter 96® Aqueous Non-Radioactive Cell Proliferation Assay Kit (Promega, USA).

To evaluate the cytotoxic potential, the "MTS Test" was chosen (based on the use of a tetrazolic compound, MTS: [3-(4, 5-dimethylthiazol – 2 – yl) – 5 – (3 – carboxymethoxyphenyl) -2- (4-sulfophenyl) - 2H-tetrazole]). The MTS test is a colorimetric method for determining the number of viable cells in proliferation and cytotoxicity assays. The quantitative evaluation of possible cytotoxic effects was then determined by cell viability, replacing the medium with 300 µl of MTS reagent, diluted 1:10 with fresh medium. The cells were incubated for 3 hrs in the dark, in the incubator, with 5% CO_2, and then optical densities were measured at 490 nm, using a Microplate Reader (Chameleon V Plate Reader, LKB Instruments). Optical densities were recorded and reported values of the control samples were considered to be the maximum cell viability values.

Viable cells reduced the MTS reagent to a soluble formazan in the culture medium; after the 20 hours of exposure to the tested concentrations of the substances, a qualitative assessment of possible cytotoxic effects was first carried out, microscopic examination of cell morphology, degree of exposure, vacuolization and detachment, cell lysis and membrane integrity.

Results

Bacterial identification

The classical tests showed the new isolate and the reference strain as gram-negative rods, catalase-positive, and oxidase negative. Preliminary identification of the ICCF 419 strain was performed using API Rapid 20E; the results showed a high similarity to the *K. oxytoca* CMGB 479, and with specific literature data (AL-AGHA et al, 2017). After DNA isolation, the 16S rDNA gene was amplified by PCR using universal primers (27F and 1492R). The amplicons were at the expected size (1500 bp). A restriction to the 16S rDNA genes was applied using *Alu* I and *Hind* III, the most common enzyme used in the Amplified Ribozomal DNA Restriction Analysis technique (ARDRA). Polyacrylamide gel 7% was used for the digestion products. The restriction profiles obtained were identical for both strains. These results were in line with other previous study for *Klebsiella* sp. (ABDELMONEM et al, 2009). According to the conventional taxonomy data and the molecular genetics results (Fig. 1), the strain ICCF 419 can be classified as belonging to the *K. oxytoca* species.

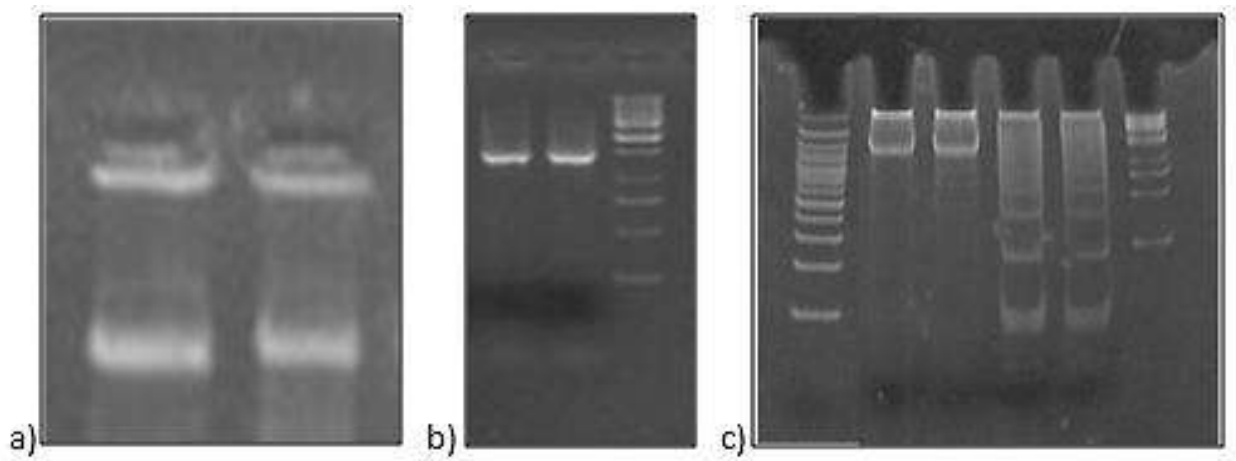

Figure 1. Agarose gel electrophoresis of **a)** DNA samples: 1- *K. oxytoca* and 2 - ICCF 419 strain; **b)** PCR products: 1- *K. oxytoca*, 2 - ICCF 419, 3 - marker 1kb (Promega); **c)** ARDRA patterns: 1 - marker 100 pb (Promega), 2 - *K. oxytoca* (Hind III), 3 - ICCF 419 strain (*Hind* III), 4 - *K. oxytoca* (*Alu*I), 5 - ICCF 419 strain (*Alu*I), 6 - marker 1 kb (Promega)

EPS isolation and purification

The determined apparent viscosities of the fermentation broth, after 72 hrs were $\eta = 93.49$ mPas at 1280 s^{-1} when lactose was used as carbon source, and $\eta = 23.54$ mPas, when glucose was used, which indicates a superior molecular weight resulted from the first case.

The total content of sugars measured for the produced EPS was approx. 78%.

After a preliminary screening performed to evaluate the most appropriate carbon source, results showed that better yields of polysaccharide were obtained when glucose and lactose were used as carbon sources (0.8 and 1.0 g/100 mL of crude EPS). In the case of sucrose and glycerol, the EPS yields were 0.6 and 0.43 g/100 mL. When lactose was used, 2.77 g/L of purified polysaccharide were obtained after 72 hrs bioprocess.

Preliminary chemical characterization of the EPS

The elemental analysis showed the following composition (%): C - 31.16, H - 5.50, O - 46.16, N - 0.69 and S - 0.49. These results led to a calculated empirical formula of CH_2O, nitrogen and sulfur appeared only as impurities. The C/H/O ratios correspond with a possible molecular formula of a hexose unit ($C_6H_{12}O_6$).

The HPLC analysis was performed for the purified EPS originated from lactose; the polysaccharide was previously hydrolyzed with trifluoroacetic acid, primarily to identify the monomeric sugars. The revealed characteristic values for the retention times (Rf), according to the standards used, are shown in Table 1 and Fig. 2.

Table 1. Polysaccharide composition

EPS content expressed in glucose	%
Rhamnose	11,16
Fructose	6,45
Mannose	1,91
Sucrose	2,54
Glucose	16,18
Maltose	0,82
Other sugars expressed as glucose	1,17
Uronic acids expressed as glucuronic acid	38,16
Ash content (residue after calcination)	7,93
Total	**86,3**

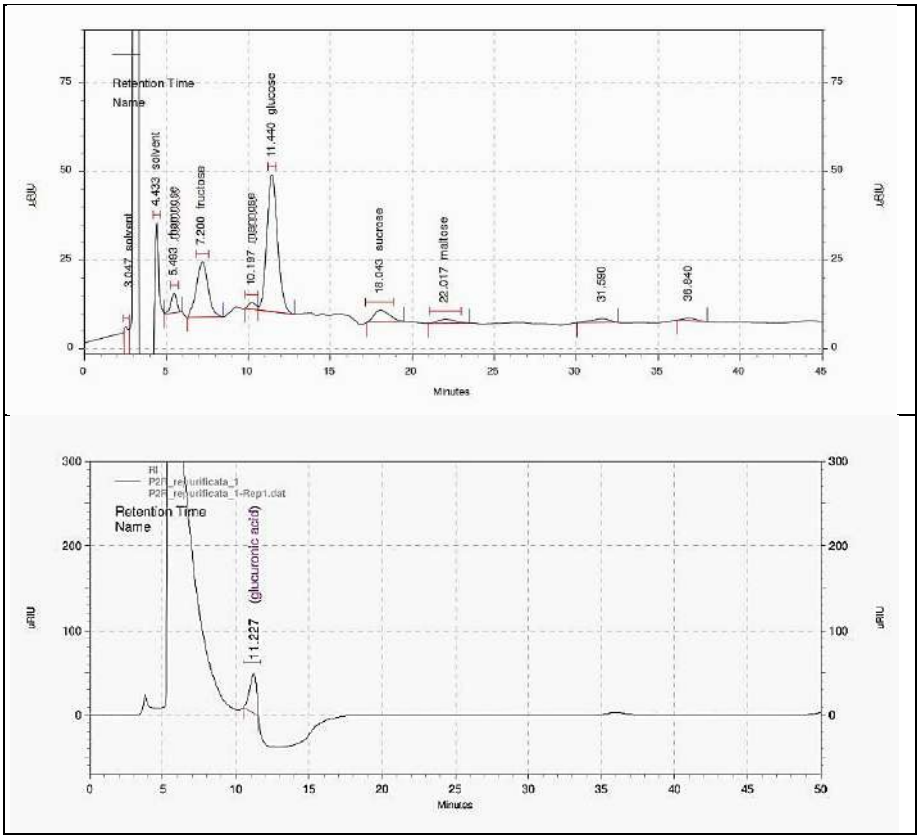

Figure 2. HPLC profile and retention times corresponding to: **a)** EPS and **b)** Glucuronic acid

FTIR analysis was employed to study the EPS structure derived from lactose as substrate. The assigned absorption bands revealed a typical polymeric structure (as shown in Table 2 and Fig. 3). In the IR spectrum, the absorption bands for the EPS produced by the ICCF 419 strain were attributed to the stretching and deformation vibrations characteristic for the OH, CH, C = O, COO-, COOR functional groups, belonging to the typical saccharides found in EPSs.

Table 2. Characteristic infrared bands shown by various groups present in the spectrum of the EPS produced by the *K. oxytoca* ICCF 419

Bands (cm^{-1})	Type of vibration and group
3410	υ OH
2924	υ_{as} CH (CH_2,CH_3)
2854	υ_s CH (CH_2,CH_3)
1725	υ COOR)
1615	υ C=O
1407	$\upsilon_{as}COO^-$
1259	υ_s COO-
1073	δ CH
1038	υ_sC-O
* υ = streching; δ = bending; s = symmetric; as = asymmetric	

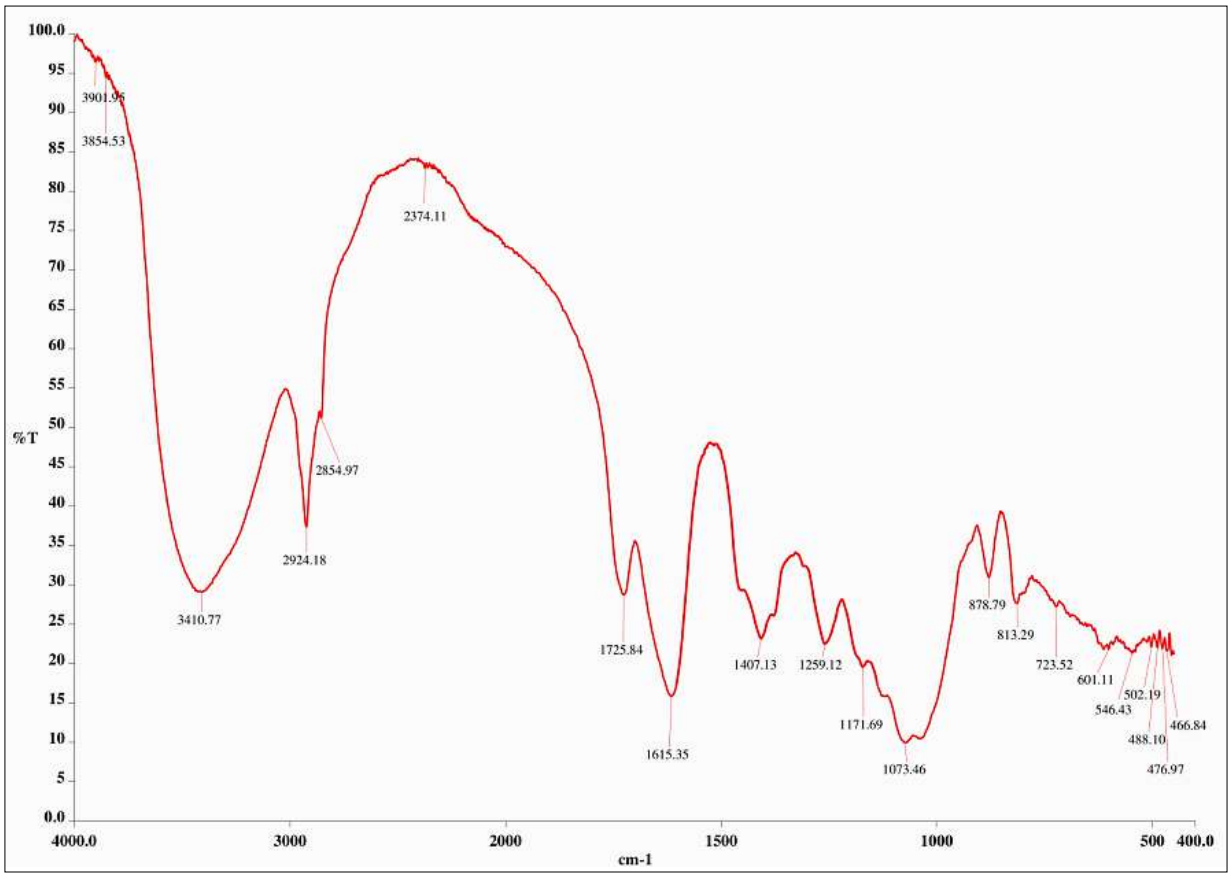

Figure 3. Infrared spectrum of purified EPS derived from *K. oxytoca* ICCF 419 strain

Techniques such as water suppression, COSY and HMQC were utilized to confirm and a preliminary structure for the polysaccharide obtained from media with lactose and glucose, respectively. The results are presented in Fig. 4 and 5.

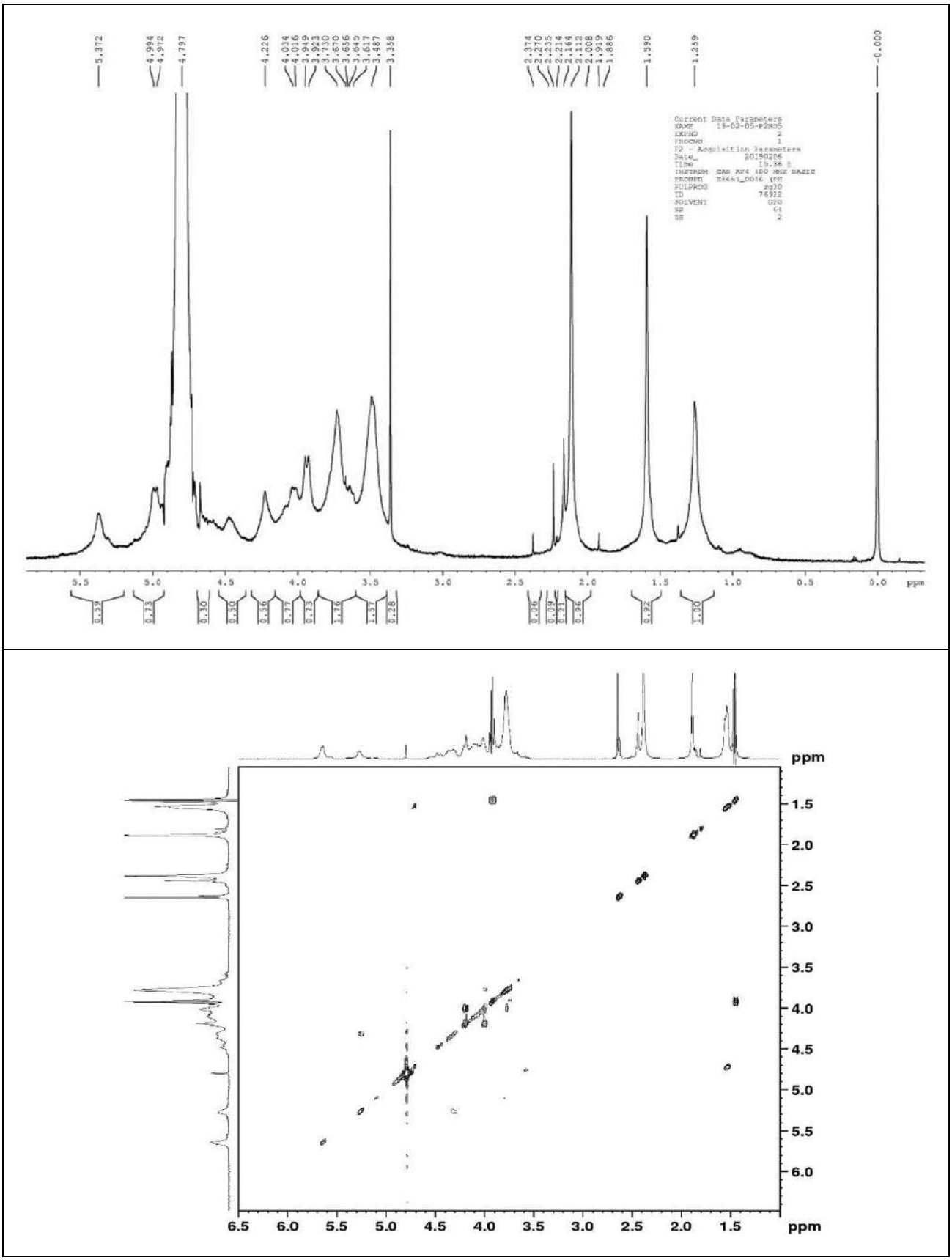

Figure 4. NMR for the EPS samples obtained from lactose: **a)** ^{1}H-NMR spectrum (Bruker Advance DRX400); **b)** Correlation spectroscopy: 2D COSY-NMR spectrum (Bruker Advance III Ultrashield Plus 500 MHz)

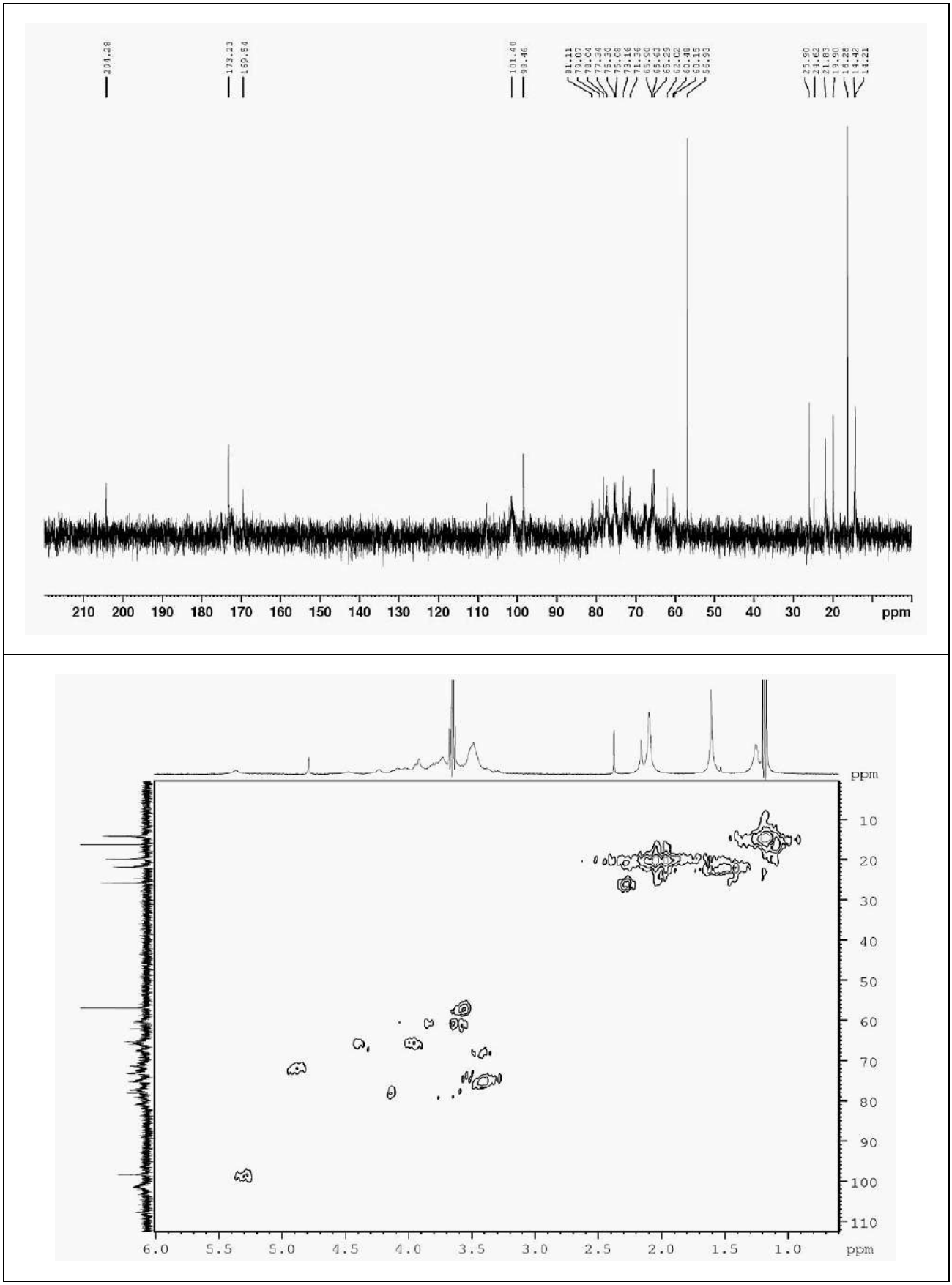

Figure 5. NMR for the EPS sample obtained from lactose: **a)** ^{13}C-NMR spectrum and **b)** 2D HMQC-NMR spectrum (Bruker Advance III Ultrashield Plus 500 MHz)

In addition, Fig. 6 presents spectra recorded for the EPS originated from glucose as substrate, in order to be compared with those obtained using lactose as the main carbon source.

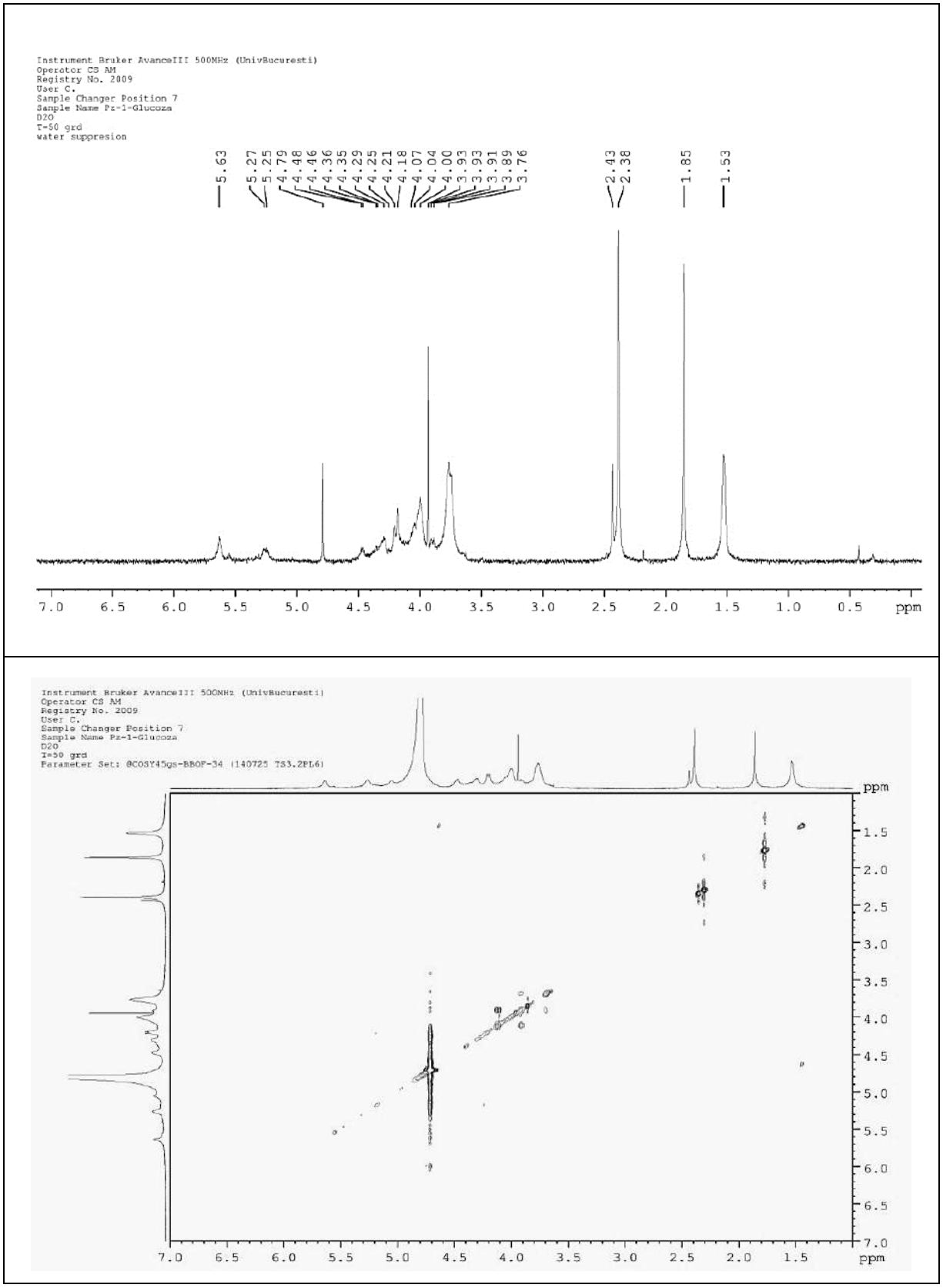

Figure 6. NMR for the EPS sample obtained from glucose (Bruker Advance III Ultrashield Plus 500 MHz): **a)** ^{1}H-NMR spectrum; **b)** 2D COSY-NMR spectrum

Evaluation of cytotoxicity – in vitro assay

A preliminary pharmacological study to characterize the EPS produced by the ICCF 419 strain was employed, aiming to evaluate the cytotoxicity effects on fibroblasts. The cell viability/proliferation chart for the tested products is illustrated in Fig. 7.

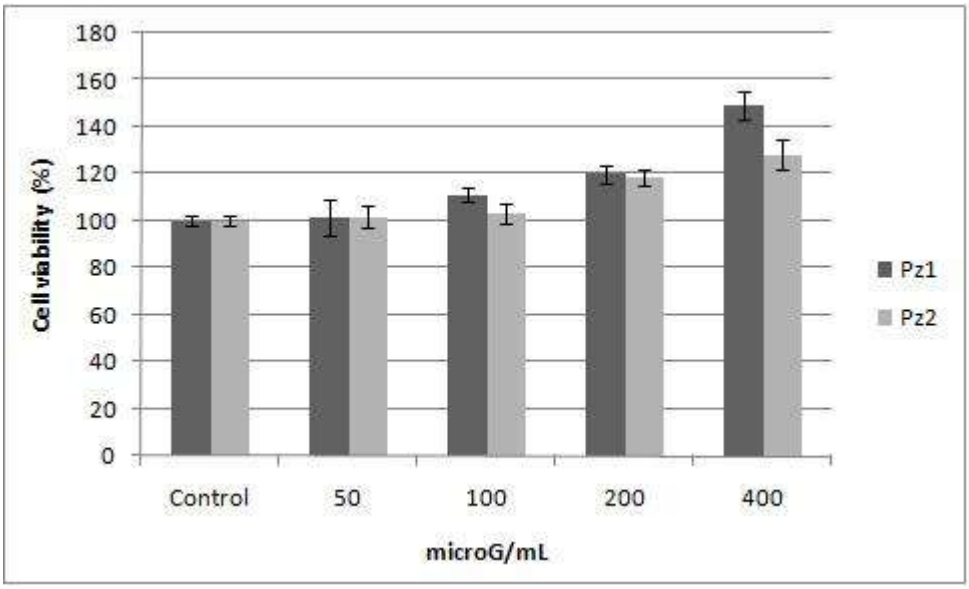

Figure 7. The effect of Pz1 and Pz2 samples on the viability and proliferation of L929 murine fibroblasts (exposure time – 20 hrs)

Discussions

Assignment of EPS composition and structure

The structural characterization of EPSs is the most important factor to be considered when determining their functions and applications. These polymers are commonly formed by *monosaccharides* (e.g. pentoses: D-arabinose, D-ribose, D-xylose, hexoses: D-glucose, D-galactose, D-mannose, D-allose, L-rhamnose, L-fucose, amino sugars: D-glucosamine, D-galactosamine), *uronic acids* (D-glucuronic acids, D-galacturonic acids, D-mannuronic acid) and *other non-carbohydrate compounds* (e.g. organic substituents: acetate, succinate, pyruvate, glycerate or inorganic: sulphate, phosphate).

Klebsiella spp. are slime forming bacteria and produce EPSs of related structures, consisting of 3-5 repeating sugar monomers and uronic acid, mainly as glucuronic acid, while O-acetyl is a hydrophobic group, and pyruvate ketals are the most commonly found as organic substituents (the acetyl group determines the small differences between *Klebsiella* polysaccharides) (VERHOEF, 2005; PAULO et al, 2012; MISHRA and JHA, 2013). Furthermore, the heteropolysaccharides secreted by soil microbes, which produce nodules attached to the plant roots, are commonly formed by repeating units of sugars, such as glucose, galactose, mannose, and rhamnose, and they usually present uronic acids with substituents like pyruvate, acetate or succinate (BOYLE and READE, 1983).

The preliminary chemical analysis (HPLC) employed to check the monosaccharides in the EPS, confirmed the expected sugar units characteristic for the *Klebsiella* genus, such as glucose (16.18%) and rhamnose (11.16%), both in a significant percent (ROCA et al, 2015), as well as a high content of glucuronic acid (38.16%), but also demonstrated the presence of other unusual components in small concentrations, such as fructose (6.45%), sucrose (2.54%), and maltose (0.82%). Therefore, the results could indicate a possible new structure, but further studies to elucidate the chemical composition are needed.

As literature data shows, the range of 1200-900 cm^{-1} corresponds to carbohydrates and their derivatives, and it is generally attributed, mainly to the stretching and bending vibrations of C-C, C-O-C and C-O-P groups of polysaccharides (including bands of -C-O- of esters, ethers, and hydroxyl groups), which form the so-called "fingerprint region" (VERHOEF, 2005; KAUR et al, 2013; SAJNA et al, 2013); other authors reported the 1350-950 cm^{-1} region as a characteristic of vibrations associated with polysaccharides, for which the presence of sugar monomers like glucose, galactose, and mannose was signalized (OMOIKE and CHOROVER, 2004).

As shown in Fig. 3, peaks at 1259 cm^{-1}, 1073 cm^{-1}, and 1038 cm^{-1} indicate the presence of COO-, CH, and

C-O groups, similar results to other findings in the field (SHENG et al, 2006; SUDHESH and SUMATHY, 2016), while peaks at 1078 cm^{-1}, 1070 cm^{-1}, and 1035 cm^{-1} correspond to galactose, mannose, and glucose according to Sajna et al, 2013. The widest and intensive adsorption bands identified here, at frequencies of 3410 cm^{-1}, 2924 cm^{-1}, and 2854 cm^{-1}, and 1725 cm^{-1} were attributed to the characteristic vibrations of the hydroxyl bonds of carbohydrate rings of polysaccharides, C-H streching of methyl group and C=O stretching of carbonyl groups (VERHOEF, 2005; SHUKLA et al, 2011; KAUR et al, 2013; MISHRA and JHa, 2013; SAJNA et al, 2013; SUDHESH and SUMATHY, 2016).

Therefore, the fingerprint absorption region for carbohydrate identification (1200-900 cm^{-1}) showed the following: four different absorption bands (cm^{-1}) at 1259, 1171.69, 1073.46 and 1038.03, which were assigned to the stretching vibrations of COO- and CH groups, specific to polysaccharides; a broad stretching at 3410.77 cm^{-1} was assigned to the hydroxyl group; two relevant peaks in the protein region (1700-1500 cm^{-1}), characteristic for carbonyl group and indicating the C=O streching, whereas peaks (cm^{-1}) at 1407, 2924 and 2854 were attributed to the COO- group, C-H streching of methyl (-CH3) and methylene (-CH2-) groups.

Following the results obtained from FT-IR and HPLC analysis, the polysaccharide is an anionic polymer, a property which makes it very interesting for further pharmaceutical applications research. Literature mentions that these types of compounds have been successfully used as biomaterials, nanocarriers in drug delivery, and as agents in the new generation of anticancer therapy, especially. The presence of carboxyl groups (COO^-) contributes to the negative charge of the polymers, along with uronic acids. Also, the functional group of carboxyl facilitates electrostatic connections with proteins and growth factors (fibroblast or vascular endothelial growth factors), which makes the polysaccharides that contain these moieties, suitable for tissue regeneration and control release for growth factors delivery (MARTINEZ et al, 2016). Furthermore, the presence of some other negative charged groups in the vicinity of the carboxyl lead to an increased electric field intensity, which could make the polysaccharide applicable as polyelectrolyte in food industry (thickeners, emulsifiers), cosmetics, biomedicine (polyelectrolyte complexes for tissue engineering), water treatment, and oil recovery (BERILLO et al, 2012; ZHIVKOV, 2013).

Proton NMR spectroscopy has been successfully used in the structural analysis of *Klebsiella* polysaccharides, and the technique of ^{13}C-NMR has been extensively applied to mono- and higher-saccharides (FOLKMAN, 1979; CHENG and NEISS, 2012).

^{1}H and ^{13}C-NMR spectra of EPSs obtained in this study were quite complex and they were analyzed extrapolating some information; the assignment of determined resonances was corroborated by comparisons of the ^{1}H and ^{13}C chemical shifts with literature data.

Fig. 4 a) presents the ^{1}H-NMR spectrum of the EPS, which is formed of three regions: anomeric region of the proton in the range of 4.5-5.7 ppm (Bruker Advance III Ultrashield Plus 500 MHz) and 4.9-5.4 ppm (Bruker Advance DRX400), the segment of 3.5-4.3 ppm and 3.6-4.5 ppm, related to the protons region of the saccharide cycle (the ring proton) and the alkyl region between 1.4-2.6 ppm and 1-2.4 ppm, where the presence of some protons characteristic for the CH_3 in an acetoxy or another group of the structure is indicated. The 2D COSY-NMR spectrum (Fig. 4 b) indicates the coupling between protons that are directly coupled to each other.

The ^{13}C-NMR spectrum of EPS can be also divided into different regions. Based on chemical shifts in ^{13}C-NMR, in Fig. 5 a) four regions could be identified: the carbonyl carbon (170-204 ppm), the anomeric carbon (95-101 ppm), the ring carbon (50-81 ppm) and the alkyl carbon (14-20 ppm). The 2D HMQC-NMR spectrum of the EPS (Fig. 5 b) shows some correlations between the directly attached carbons and protons.

The ^{1}H-NMR spectra for the *Klebsiella*'s EPS was compared with one corresponding to pseudozan (spectra not published), a polysaccharide synthesized by *Pseudomonas* sp. ICCF 400, which has a known structure composed of galactose, glucose, fucose, rhamnose, and acyl groups (MOCANU et al, 2011). In addition, a pseudozan produced by *Pseudomonas oleovorans* was identified to contain mannose, and acyl groups such as succinyl, piruvyl and acetyl, which are responsible for the anionic character, according to Freitas et al (2009). Even so, the EPS produced by the ICCF 419 strain has a structure which seems to be more complex than the above mentioned. A carbohydrate fingerprint was obtained through a method which overlays UV and MS - liquid chromatography coupled with ultra violet and electrospray ionization ion trap detection (Rühmann et al, 2014) (data not published), to evaluate the exopolysaccharide synthesised by *K. oxytoca* ICCF 419, which confirmed the presence of fucose and galactose, and more than that, xylose may be present.

^{1}H and ^{13}C NMR peak assignments for the EPS derived from lactose as substrate are presented in Table 3, according to cited literature. Comparative with spectra obtained for the EPS derived from glucose (Fig. 6) it was observed only a small difference in structure, represented by the absence of the peak at 204.28 ppm, which is the signal of a carbonyl compound (possible from fructose).

As shown in Table 3, a summary of the possible assignments for the main sugars in the EPS structure was evaluated.

Table 3. Peak assignments for H-1 and C-13 NMR

^{13}C-NMR (ppm)		^{1}H-NMR (ppm)		References
204.28	Carbonyl compound (RC(=O)-H in aldehydes and ketones)	**5.66, 5.64**	Doublet – extension of the anomeric proton (H-1) from glucuronic acid, but also from a sugar in the furanose form	www.chemguide.co.uk; https://sites.science.oregonstate.edu/; Abraham and Mobli, 2008; Lundqvist, 2015
173.23	carboxyl groups (RCO_2H) of glucuronic acid	**5.28**	H-1: glucuronic acid	https://hmdb.ca/; https://sites.science.oregonstate.edu/; Bush, 2010; Jia et al, 2012; Lundqvist, 2015
169.54		**4.8**	H-1: glucose	https://www.drugbank.ca; Cheng and Neiss, 2012; Jia et al, 2012; Pomin, 2012
101.40	C-1: mannose	**4.48**	H-1: *galactose*	http://bmrb.wisc.edu/; Hounsell et al, 1986; Corsaro et al, 2005; Abraham and Mobli, 2008; Kubota et al, 2012; Jia et al, 2012
98.46	C-1: Glucuronic acid	**4.37, 4.34, 4.32**	Triplet - H-2: glucuronic acid	https://hmdb.ca/; https://www.drugbank.ca; Leone et al, 2007; Jia et al, 2012.
81.11	C-2: mannose	**4.21, 4.18**	H-1: *fucose*	www.chemicalbook.com; http://bmrb.wisc.edu/; Kubota et al, 2012
79.07	C-3: mannose/sucrose	**4.10, 4.07, 4.05**	H-3: sucrose; H-2: *galactose*	http://bmrb.wisc.edu/; https://www.drugbank.ca; https://hmdb.ca/; Jia et al, 2012
78.04	C-2: *galactose*	**4.01**	H-2: mannose	Richards et al, 1988; Jia et al, 2012; Kubota et al, 2012
77.34	C-3: maltose	**3.95, 3.94, 3.93, 3.93, 3.92, 3.92**	Multiplet – H-3: *galactose*, H-2/H-3: glucose, H-3/H-4: mannose	http://bmrb.wisc.edu/; https://hmdb.ca/; Hounsell et al, 1986; Bubb, 2003; Cheng and Neiss, 2012; Kubota et al, 2012; Pomin, 2012
75.30, 75.08	Doublet – C-3: glucose; C-4: sucrose	**3.90**	H-5: glucose	http://bmrb.wisc.edu/; https://hmdb.ca/; Cheng and Neiss, 2012; Jia et al, 2012; Pomin, 2012
73.16	C-3: fucose; C-5: mannose	**3.84**	H-3: fucose/rhamnose; H-5: mannose	http://bmrb.wisc.edu/; www.chemicalbook.com; Prestegard et al, 1983; Jia et al, 2012
71.36	C-5: glucose; C-3: rhamnose	**3.78**	H-6: galactose	www.chemicalbook.com; http://bmrb.wisc.edu/; Hounsell et al, 1986; Cheng and Neiss, 2012; Jia et al, 2012; Pomin, 2012
65.90, 65.63, 65.29	C-5: sucrose	**3.69, 3.68, 3.66**	Triplet – H-6: glucose/maltose; H-3: mannose	http://bmrb.wisc.edu/; https://hmdb.ca/; Prestegard et al,1983; Bekiroglu et al, 2003; Cheng and Neiss, 2012; Pomin, 2012
62.02	C-5: fructose; C-6: sucrose	**2.64, 2.63, 2.63, 2.62**	Multiplet – acyl groups	www.chemicalbook.com; Matulova et al, 1994; Guillén and Ruiz, 2003; Richards et al, 2014
60.48, 60.15	C-6: fructose	**2.45, 2.43**	Doublet – methyl group: 6-deoxy sugars (rhamnose/fucose) or from O-acetyl groups	www.chemicalbook.com; Lundqvist, 2015
56.93	RCOOR', carboxylic acid (ester)	**2.38**	methyl group: 6-deoxy sugars (rhamnose/fucose) or from O-acetyl groups	Lundqvist, 2015; Obarska-Pempkowiak et al, 2015
25.90	Alkyl groups – i.e CH_3CO (acetyl)	**1.88**	CH_3CO- acetyl group	Kirby and Moody, 1995; Lundqvist, 2015
24.62		**1.54, 1.53**	H-6: fucose	http://bmrb.wisc.edu/; www.chemicalbook.com; Kirby and Moody, 1995
21.83		**1.47, 1.45, 1.44**	Triplet – H-6: rhamnose	http://bmrb.wisc.edu/; www.chemicalbook.com; Kirby and Moody, 1995; Jia et al, 2012
19.90	C-6: CH_3-C in rhamnose			Jia et al, 2012
16.28	C-6: fucose			http://bmrb.wisc.edu/
14.42, 14.21	Doublet – methyl group			Lundqvist, 2015

The EPS produced by the *K. oxytoca* ICCF 419 strain has a high content of glucuronic acid (38.16%), and this could give some indications regarding its possible applications.

Among the acidic polysaccharides, which are composed of similar sugar units (such as glucose, rhamnose, mannose or galactose) like those from our studied polymer it is worth mentioning some of the comparable ones in terms of major chemical components.

Thereby, *mauran* is an anionic EPS containing glucuronic acid (21.9%), glucose, mannose and galactose, and this structure gives pseudoplasticity, emulsifying activity, immunomodulatory effect, lead binding capacity, with applications in drug delivery, bio-imaging, cancer therapy, bioremediation, wastewater treatment, bionanofusion, bioabsorption, cosmetics, and in food industry (ARIAS et al, 2003; LLAMAS et al, 2006; MATA et al, 2006; RAVEENDRAN et al, 2015; AHMED and SOUNDARARAJAN, 2018); *gellan* is another well-known anionic EPS, made of glucose, rhamnose or mannose and glucuronic acid (18.9% in the case of a polymer derived from lactose as substrate) (FIALHO et al, 1999) which can be used as thickening, film-forming, and stabilizing agent in food and feed industry, wastewater treatment, removal of gasoline hydrocarbons, or for enzymes and bifidobacteria immobilization, in drug delivery and wound healing, construction chemistry and many others. In these case, acetyl groups have an important role in gel forming capacity (BAJAJ et al, 2007; SCHULTEIS et al, 2008; AHMAD et al, 2015; OZCAN and ONER, 2015; ROCA et al, 2015; SCHMIDT et al, 2015; HUI LI et al, 2016; CASTELLANE et al, 2017); *rhamsan*, having a similar backbone of gellan, composed of glucuronic acid, glucose, and rhamnose, but also *welan* and *diutan* or *sanxan*, which

have in addition mannose, show viscoelasticity and thickening capacity, which makes them good candidates in the construction field, oil drilling, food industry, personal care products and pharmaceuticals, plastic surgery, and agriculture (ROCA et al, 2015; RUHMAN et al, 2014; SCHMIDT et al, 2015; HUANG et al, 2016; HUI LI et al, 2016; CASTELLANE et al, 2017; IVANOV and STABNIKOV, 2017; WU et al, 2017; GONZALES et al, 2019), and *xanthan*, the most common polysaccharide due to many industrial uses, which is formed by glucose, mannose and glucuronic acid, components which made it applicable in pharmaceutical field (tissue engineering), food and beverages industries, textile and oil industry, and others (GONDIM et al, 2019; MURAD et al, 2019).

Besides these major components – glucose, mannose, rhamnose, glucuronic acid, the studied EPS contains fructose, which is mentioned in literature as a common sugar found in the polysaccharides produced by *Paenibacillus* spp., among glucose, galactose, fucose, glucuronic acid, mannose or xylose, structures responsible of demonstrated properties, such as antioxidant and antitumor activities, superoxide scavenging, inhibition of in vitro gastric cancer cells, bioflocculation and metal chelating capacity, and with applications in pharmaceuticals (drug delivery, tissue engineering, wound healing, skin rejuvenation), bioremediation (cadmium removal), wastewater treatment, soil aggregation, food and feed (LIANG et al, 2015; GRADY et al, 2016; COSTA et al, 2018).

EPS Pharmacological properties

The pharmacological assay revealed that on the L929 fibroblast line (ATCC CRL-6364), samples Pz 1 and Pz 2 had not determined any cytotoxic effect. Furthermore, both EPS samples stimulated cell proliferation, and a promising fact was that the rates of 49% (Pz 1) and 28% (Pz 2) were higher than the one recorded for the control (the L929 fibroblast line).

Determination of cytotoxicity was based on incubating the cells in the usual growth medium, over which the test products were added. Exposure of cells to test substances was done using "semiconfluent" cultures (about 70%), and effects were measured at a shorter time than a doubling time.

The increased cell viability of cells treated with Pz1 and Pz2 samples indicated that the polysaccharide from the *K. oxytoca* ICCF 419 strain provides a high level of cytoprotection; taking into consideration the dose levels used, the EPS does not present cytotoxicity. Therefore, this fact, along with the fibroblasts proliferation stimulation activity leads to a first assumption that the polysaccharide could be a suitable biomaterial recommended for pharmaceutical applications, within the products with healing effect of the wounds (i.e inclusion in medical devices such as scarring plasters).

Conclusions

Based on the presented results and considering the research limitations, a newly-bacterial isolate, identified as *K. oxytoca* and included in the CMII-WFCC 232 Culture Collection of ICCF Bucharest, was selected as a promising polysaccharide producer, following a screening conducted in liquid fermentation media, and with possible applications in the biomedical and pharmaceutical fields. A higher content in crude EPS was obtained when lactose was used as main substrate. The chemical analyses confirmed the primary structure of the polymer, for which the HPLC analysis showed a high content of glucuronic acid (38.16%), with glucose and rhamnose as main monomers, and small amounts of fructose, mannose, sucrose and maltose, or some other sugars. As shown in the pharmacological study, this biopolymer has no cytotoxicity, and it has the capacity to enhance cell proliferation, results which encourage its possible use as a biomaterial. More knowledge on the EPS chemical structure is further needed to elucidate if this could be a possible new compound.

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