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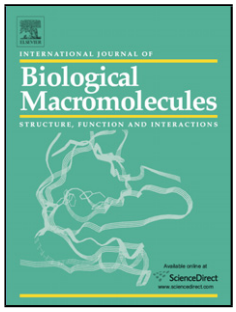

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Brachybacterium sp. CH-KOV3 isolated from an oil-polluted environment – a new producer of levan

Running title: *Brachybacterium* sp. novel levan producer

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Abstract

Various microorganisms isolated from polluted environments, such as *Pseudomonas sp.* and *Micrococcus sp.* can synthesize exopolysaccharides (EPSs) which are natural, non-toxic and biodegradable polymers. EPSs play a key role in protection of microbial cells under various external influences. For humans, these substances have potential use in many industries. EPSs can be applied as a flavor or a fragrance carrier, an emulsifier, a stabilizer, a prebiotic, an antioxidant or an antitumor agent. In this study, we characterized an environmental microorganism that produces EPS, optimized EPS production by this strain and characterized the EPS produced. Isolate CH-KOV3 was identified as *Brachybacterium paraconglomeratum.* The sucrose level in the growth medium greatly influenced EPS production, and the highest yield was when the microorganism was incubated in media with 500 g/L of sucrose. The optimal temperature and pH were 28 ºC and 7.0, respectively. The nuclear magnetic resonance (NMR) results and GC-MS analysis confirmed that the residues were D-fructofuranosyl residues with β-configuration, where fructose units are linked by β-2,6-glycosidic bonds, with β-2,1-linked branches. All these data indicate that the investigated EPS is a levan-type polysaccharide. Thus, it was concluded that *Brachybacterium* sp. CH-KOV3 could constitute a new source for production of the bioactive polysaccharide, levan.

Keywords: polluted environments; exopolysaccharides; *Brachybacterium paraconglomeratum*.

1. Introduction

In the last decades, huge amounts of waste materials from different origins and with varying properties have been released into the environment. The most important sources of industrial waste material are chemical industries and agriculture [**1**, **2**]. Microorganisms isolated from polluted environments are already being used for processes such as bioremediation, in order to remove harmful chemicals from the environment in the most natural way [**3**].

1.1. Exopolysaccharides produced by environmental isolates

Some of the microbial isolates from polluted environments can synthesize a considerable amount of exopolysaccharides (EPSs) [**4**, **5**]. EPSs are natural, non-toxic, and biodegradable polymers. They are synthesized intracellularly, but their polymerization occurs outside the cells [**6, 7**]. Some EPSs, such as xanthan, gellan, alginate, glucans, hyaluronan, succinoglycan, and levan, are long chain, high-molecular-mass water-soluble polymers [**6-9**]. EPSs can form a protective layer for the cells against harsh external environmental conditions; they have a role in protection against desiccation and predation, and many microorganisms produce them as a strategy for growing and adhering to solid surfaces [**9-11**]. Furthermore, these EPSs are synthesized in order to protect microorganisms from harmful effects of toxic chemicals present in the surrounding environment [**8**]. Microbially-produced EPSs have potential utility value for various industrial processes and play many parts in human lives. They can be used in the food and cosmetic industries as thickeners, stabilizers, sweeteners, probiotics, and color and flavor vehicles, as gelling and water-binding agents including viscosifiers, emulsifiers, biosorbents, flocculants, and as heavy metal removal agents in waste water treatment [**1, 7-9, 11-14**]. Furthermore, their biological activities include antitumor, antiviral, immunostimulatory and anti-inflammatory activities, as well as cholesterol-lowering effects [**1, 7-9, 11, 13, 15**].

Several bacterial genera are known for their EPS production: xanthan – *Xanthomonas*; gellan – *Sphingomonas*; alginate – *Azotobacter and Pseudomonas*; glucans and α-glucans – *Lactobacillus, Leuconostoc* and *Streptococcus,* β-glucans – *Achromobacter, Agrobacterium, Aerobacter, Azotobacter, Gluconacetobacter, Rhizobium, Salmonella* and *Sarcina*; hyaluronan – *Pseudomonas aeruginosa*; succinoglycan – *Agrobacterium, Alcaligenes, Rhizobium* and *Pseudomonas* [**6**]. Today, known levan producers are from the following genera: *Aerobacter, Bacillus, Erwinia, Rahnella, Pseudomonas, Streptococcus, Zymomonas* and *Brachybacterium* [**6, 16**].

1.2. Hydrocarbon degrading genus Brachybacterium

Among hydrocarbon degrading microorganisms known for EPS production, Actinobacteria were found in soil polluted with polycyclic aromatic hydrocarbons, pesticides, herbicides and other persistent organic pollutants [**17**]. Recently, members of the genus *Brachybacterium* have been reported to be involved in the degradation of hydrocarbons [**18-20**]. The genus *Brachybacterium* (family Dermabacteraceae, class Actinobacteria), proposed by Collins et al, 1988, includes thirteen species: *B. alimentarium, B. conglomeratum, B. faecium, B. fresconis, B. muris, B. nesterenkovii, B. paraconglomeratum, B. phenoliresistens, B. rhamnosum, B. sacelli, B. saurashtrense, B. tyrofermentans, and B. zhongshanense* [**21, 22**].

1.3. Polluted environment as a source of valuable exopolysaccharide-producing bacteria

The wastewater canal Vojlovica (WWCV), located on the Danube River, collects wastewater from the industrial park in Pančevo, Serbia. WWCV was previously described [**23**]. Briefly, the industrial park consists of a petrochemical factory (HIP Petrohemija), chemical fertilizer factory (HIP Azotara), and oil refinery (NIS Rafinerija Nafte, Pančevo), and it is known for its long term pollution. Furthermore, in 1999 after the NATO air strikes, WWCV was contaminated with

various organic (polar and nonpolar) and inorganic substances: mineral oil, polycyclic aromatic hydrocarbons, ethylene dichloride, benzene, toluene, ethylbenzene, xylene, perfluorinated chemicals and mercury, and therefore, the canal has been proven to be one of the contamination hot spots in Serbia [**23**].

The aim of this study was to characterize environmental isolate CH-KOV3, a bacterium isolated from the sediment of WWCV, and a novel producer of the EPS, levan. Furthermore, optimization of levan production together with structural characterization of this EPS was conducted.

2. Materials and Methods

2.1. Sampling location

Sampling and determination of basic physicochemical and chemical characteristics of the sediment from WWCV was previously described, in which we confirmed the presence of significant amounts of perfluoroalkyl substances (PFASs) [**23**]. Our previous investigations also showed strong bioremediation potential of the microbial consortia composed of bacteria and fungi isolated from sediment of WWCV and sludge taken from the wastewater treatment plant of the industrial zone [**24, 25**]. During analysis of a zymogenous consortium of hydrocarbon-degrading microorganisms isolated from WWCV, some of the isolated bacterial strains were found to synthesize considerable amounts of EPS. The microorganism which exhibited the greatest production of EPS was designated as CH-KOV3.

2.2. Isolate CH-KOV3, an exopolysaccharide producer

Isolate CH-KOV3 originally belonged to a consortium of zymogenous hydrocarbon-degrading microorganisms isolated from the sediment of WWCV, and is capable of using oil hydrocarbons as the sole source of carbon during growth on mineral base medium containing 2 g

of standard D2 diesel fuel in 1 L of medium. Medium was prepared according to the procedure described earlier [**26**]. Enrichment was performed by three successive inoculations in liquid mineral base medium with D2 diesel, incubated at 28 ºC and 200 rpm, and then the consortium was inoculated onto an agar plate. For further study, microorganisms were selected according to their morphological characteristics. Many isolates synthesized EPSs during growth on mineral media supplemented with D2 diesel; however, the microbial isolate CH-KOV3, as the best EPS producer, was selected for further, more detailed analysis.

2.2.1. Characterization of isolate CH-KOV3

2.2.1.1. Fatty acid methyl ester profile

Fatty acid methyl esters of isolate CH-KOV3 were determined according to the modified procedure given by Minnikin et al, 1975 [**27**]. Wet biomass (0.2 g) was refluxed for 3 hours with a mixture of toluene:methanol:sulfuric acid (5:5:0.2 V/V/V) in three replicates. After cooling in a round-bottomed reflux flask, 10 mL of saturated sodium chloride was added, and the whole solution was extracted twice with a mixture of chloroform:hexane (1:4 V/V, 2 x 10 mL). Extracts were washed with distilled water, dried with anhydrous sodium sulfate, and evaporated to dryness. Fatty acid composition was determined by comprehensive two dimensional gas chromatography–mass spectrometry (2D GC×GC-MS) performed with a GCMS (QP2010 Ultra, Shimadzu, Kyoto, Japan) and a 2D GC×GC thermal modulator (Zoex Corp). An Rtx®-1 (RESTEK, Crossbond® 100 % dimethyl polysiloxane, 30 m × 0.25 mm I.D., 0.25-μm film thickness) and a BPX50 (SGE Analytical Science, 2.6 m×0.1 mm I.D., d_f=0.1 μm) column were connected through the 2D GC×GC modulator as the first and second capillary columns, respectively. Helium was used as the carrier gas. Injector temperature was constant: 200 ºC. The temperature program used was: initial temperature 40 ºC for 1 min, then 4 ºC per min until 300

ºC, and isothermal at 300 ºC for 5 min. Thermal modulation was 6 sec. The 2D GC×GC-MS data was collected and analyzed (automated mass spectral deconvolution and identification) with GCMS Solution software (Shimadzu). Spectrum analysis was performed using NIST11 and Wiley8 database libraries, and relative ratios of components were calculated from the corresponding peak areas. The 2D GC×GC-MS data were analyzed using ChromSquare Ver.2 (Shimadzu), capable of directly reading 2D GC×GC data obtained with GCMS Solution, converting it to a 2-dimensional image.

2.2.1.2. Gram-staining, catalase and oxidase tests

Gram-staining of isolate **CH-KOV3,** as well as catalase and oxidase tests, were performed using standard methods [**28, 29**].

2.2.1.3. Physiological-biochemical characterization

Physiological-biochemical characterization of isolate CH-KOV3 was conducted using Analytical Profile Index (API) (BioMérieux, France) kits: 20 NE, 20 E, CORYNE, and API ZYM. All API tests were performed according to the manufacturer's recommendations.

2.2.1.4. MALDI-TOF MS characterization

Isolate CH-KOV3 was characterized using a Bruker Autoflex II MALDI-TOF MS (Bremen, Germany) equipped with a UV nitrogen laser (337 nm) and a dual microchannel microplate detector. Together with whole (intact) bacteria, crude cell extracts were prepared accordingly [**30**] and also analyzed as previously described [**31**]. Spectra were recorded by Flex Control software (Bruker Daltonics, Bremen, Germany), and samples were analyzed in six replicates using Flex Analysis (Bruker Daltonics, Bremen, Germany). For each measurement, at least 3,000 individual spectra (300 shots at laser power from 10 different points of a dried sample spot) were collected and averaged to obtain MALDI-TOF MS spectrum. External calibration was

performed with protein standards (Bruker Protein Test Standard, Bruker Daltonics, Bremen, Germany). MALDI BioTyper db 6903 software was used for the protein profile analysis by pattern matching with the libraries.

2.2.1.5. Molecular characterization of isolate CH-KOV3

Genomic DNA was extracted using the DNeasy Blood & Tissue Kit (Germany). The 16S rRNA gene was amplified by PCR using 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-CGGCTACCTTGTTACGACTT-3') primers. The amplified fragments were purified using QIAquick PCR Purification Kit, and sequencing was performed by Macrogen in the Netherlands. The obtained sequence was deposited in the NCBI Genbank database under the accession number KC881303.1.

2.3. Production and purification of EPS

For EPS production, basal medium (BM) (100 g sucrose, 1 g beef extract, 0.6 g yeast extract, 3 g $K_2HPO_4 \cdot 3H_2O$, 3 g KH_2PO_4, 1 g NaCl, 0.2 g $MgSO_4 \cdot 7H_2O$, 0.001 g $FeSO_4 \cdot 7H_2O$ in 1000 mL of water, pH 7.0) was used [**15**]. Isolate CH-KOV3 was cultured in BM for 5 days at 28 ºC and 200 rpm. After centrifugation at 10,000g for 10 min, the supernatant was mixed with two volumes of cold ethanol and left at 4 °C overnight. The precipitate was collected by centrifugation at 10,000g for 10 min and the pellet was dissolved in distilled water. The precipitation procedure with ethanol was repeated three times. Subsequently, the dissolved pellet was dialyzed against tap water and then against distilled water, concentrated by rotary evaporation, and finally lyophilized. EPS obtained by triple ethanol precipitation and dialysis is labeled as purified EPS hereinafter.

2.4. Optimization of EPS production

Optimization of EPS production was conducted in BM. The influence of the incubation temperature (20, 28, 37, and 45 ºC in BM with 100 g/L sucrose, pH 7.0, 5 days incubation and 200 rpm), pH (pH of 5.0, 6.0, 7.0 and 8.0 in BM with 100 g/L sucrose, 5 days at 28 ºC and 200 rpm) and concentration of sucrose (60, 100, 140, 200, 300, 500 and 600 g/L in BM at pH 7.0, 5 days at 28 ºC and 200 rpm) on the production of levan were analyzed. All experiments were carried out in 500 mL Erlenmeyer flasks containing 200 mL of cultivation medium. Inoculation was performed by the addition of 1 mL of fresh overnight culture of CH-KOV3 containing 10^6 CFU/mL. All experiments were replicated independently five times.

2.5. Characterization of purified EPS

Every batch of produced and purified EPS was analyzed in triplicate and mean values were calculated. For the structural instrumental characterization of purified EPS, the following methods were applied: determination of molecular weight, planar chromatography (PC), thin layer chromatography (TLC), elementary organic microanalysis, Fourier transform infrared spectroscopy (FTIR), and nuclear magnetic resonance (NMR – 1D and 2D). Furthermore, purified EPS was analyzed for optical rotation, measurement of refractive index, and kinematic and dynamic viscosity.

2.5.1. Molecular weight of purified EPS

The average molecular weight (MW) of the investigated EPS was determined using gel permeation chromatography (GPC) on Sepharose CL-4B, as previously described [**32**].

2.5.2. Hydrolysis of purified EPS

Lyophilized purified EPS (2 mg) was hydrolyzed with 3 M trifluoroacetic acid – TFA (2 mL) in a sealed tube for 12 h at 121 ºC, followed by evaporation under reduced pressure at 45 ºC to dryness. Residual TFA was removed by three evaporation cycles by addition of 0.4 mL of

isopropanol. The final residue was dissolved in 0.02 mL of distilled water and used for further analysis. Hydrolyzate was examined by PC on Whatman No.1 chromatography paper (descending method) in the solvent system ethyl-acetate:pyridine:water (10:4:3 V/V/V). Components were detected with alkaline silver nitrate [**33**].

2.5.3. Partial acid hydrolysis of purified EPS

Partial acid hydrolysis of purified EPS was performed by the modified method given by Dahech et al, 2012 [**34**] to determine the monosaccharide composition of the studied EPS. Aqueous solutions containing purified EPS (10 mg/mL, 8.0 mL) and 0.125 % oxalic acid (8.0 mL) were separately heated to 80 ºC and then were mixed in a 1:1 ratio. Hydrolysis was performed at 80 ºC for 20 min. At regular intervals, every two minutes, aliquots (2.0 mL) were taken, neutralized with $BaCO_3$, and products of hydrolysis were examined by TLC in the solvent system chloroform:acetic acid:water (6:7:1 V/V/V). The spots were visualized by spraying with 50 % sulfuric acid solution and heating at 100 ºC for 10 min. Fructose, glucose and sucrose were used as standards.

2.5.4. Methylation and reductive cleavage

Methylation was performed by the Ciucanu and Kerek method [**35**] as modified by Needs and Selvendran [**36**]. In brief, lyophilized purified EPS was dried at 50 °C for 24 h prior to use. A sample of EPS (10 mg) was dissolved in anhydrous DMSO (1.0 mL) under argon and then methylated by treatment with powdery NaOH (0.15g) and iodomethane (0.25 mL). The permethylated EPS was extracted with $CHCl_3$, and the FTIR spectrum of the extracted EPS was examined. The absence of the characteristic frequencies corresponding to hydroxyl groups in the range 3200 – 3500 cm^{-1} was an indication of complete methylation.

The permethylated EPS was subjected to reductive cleavage as described by Rolf and Gray (1984) [**37**]. The reducing agent was prepared by mixing the following compounds: boron trifluoride etherate (1.55 mL), triethylsilane (2.0 mL), trifluoroacetic acid (0.32 mL), and dichloromethane (1.13 mL). The reducing mixture (2.75 mL) was added to the methylated EPS (5 mg) and the reaction was peformed for 24 h at 0 ^{o}C to produce corresponding anhydroalditols. These were then acetylated with acetic anhydride (0.25 mL) at room temperature for 1 h. The acetylated, methylated anhydroalditols were dissolved in CH_2Cl_2, the solution was washed with water three times, and the organic layer was evaporated under a stream of nitrogen, and analyzed by GC-MS.

2.5.5. GC-MS analysis

GC-MS analyses were performed on a GC×GC-MS (Shimadzu, Kyoto, Japan) with the thermal modulator turned off. Samples were injected in splitless mode. The injection volume was 1 µl and the injector temperature was 250 °C. The carrier gas (He) flow rate was 2.07 mL/min at 40 °C (constant pressure mode). The column temperature was programmed linearly in a range 40–310 °C at a rate of 10 °C/min with an initial 1 min and final 7 min hold. Mass spectra were acquired in the electron ionization mode (EI) with ion source temperature of 220 °C and the scan range 40–550 *m/z*.

2.5.6. Elementary organic microanalysis

The content of carbon, hydrogen, nitrogen and sulfur in purified EPS was analyzed using an automated analyzer (Vario EL III CHNS/O, Elementar, Hanau, Germany). Combustion temperature was 1150 °C, detector: TCD. Dynamic working range: C: 0.03-20 mg; H: 0.03-3

mg; N: 0.03-2 mg; S:0.03-6 mg. Precision/standard deviation < 0.1 % abs. Recovery rate: > 99.5 %.

2.5.7. FTIR spectroscopy

FTIR spectra of purified EPS were recorded on a FTIR spectrometer (a Nicolet 6700 from Thermo Nicolet Corp., Madison, WI) in the Attenuated Total Reflectance-Fourier Transform Infrared Spectroscopy (ATR-FTIR) mode.

2.5.8. NMR spectroscopy

All NMR spectra of the investigated EPS were measured on a Bruker AVANCE III 500 spectrometer at 500.26 MHz (^{1}H) and 125.8 MHz (^{13}C), using a 5 mm broad-band probehead. Spectra were obtained at 298 K, in deuterium oxide (D_2O), with trimethylsilyl propionate (TSP) as the internal reference standard. Chemical shifts are expressed in ppm. Standard homonuclear and heteronuclear correlated two-dimensional (2D) techniques were used: correlation spectroscopy (COSY), distortionless enhancement by polarization transfer (DEPT 135) and heteronuclear single quantum coherence (HSQC).

2.5.9. Optical rotation

Optical rotation of purified EPS dissolved in Milli-Q water was determined using a polarimeter (Polartronic MH8, Schmidt and Haensch, Germany), in a 100 mm long cuvette, with a volume of 4.4 cm^3, at polarized light wavelength 589 nm and 25 ºC.

2.5.10. Refractive index

Measurement of the refractive index of purified EPS dissolved in Milli-Q water was performed using a refractometer (Abbemat 300, Anton Paar, Germany), at polarized light wavelength of 589 nm and 20 ºC.

2.5.11. Dynamic and kinematic viscosity

Measurement of dynamic and kinematic viscosity of purified EPS dissolved in Milli-Q water was performed using a capillary viscometer (Ostwald, Witteg, Germany), at 25 ºC.

3. Results and Discussion

3.1. Characterization of isolate CH-KOV3

In order to characterize isolate CH-KOV3, the following methods were applied: analysis of fatty acid methyl ester profile, API tests, MALDI-TOF MS characterization, and molecular characterization by sequencing of 16S rRNA gene followed by construction of a phylogenetic tree.

3.1.1. Fatty acid methyl ester profile

Figures 1A and 1B show GC-MS and comprehensive 2D GCxGC-MS results of fatty acid methyl ester analysis of isolate CH-KOV3, respectively. The spectrum consists of straight-chain, saturated, iso and anteiso methyl-branched fatty acids. The relative ratios of fatty acid methyl esters was: *ai*-C_{15}, 42.63 %; *i*-C_{15}, 19.93 %; *ai*-C_{17}, 16.18 %; *i*-C_{17}, 9.06 %; *i*-C_{16}, 5.97 %; *n*-C_{16}, 3.91 %; *n*-C_{18}, 1.71 %; $C_{18:2}$, 0.61 % (*n* - normal fatty acid methyl esters; *i* – iso methyl-branched fatty acids; *ai* - anteiso methyl-branched fatty acids). The fatty acid composition of isolate CH-KOV3 is similar to data from the literature for *Brachybacterium (B.) paraconglomeratum* NCIB9861^T and DSM46341 ^{T}, but the relative ratio differed in relation to these previously published data. This may be due to differences among the strains or the stress conditions in which *Brachybacterium* sp. CH-KOV3 lived [**38-40]**. Based on the literature, *B. paraconglomeratum* is a Gram-positive, nonmotile, non-sporulating, aerobic or facultatively anaerobic, catalase positive and oxidase negative bacterium. Cells are small, single, oval or rod-shaped, colonies are smooth and yellow [**38**]. The characteristics of our isolate, *Brachybacterium* sp. CH-KOV3, corresponded to this literature description of *B. paraconglomeratum.* Thus, based

on fatty acid methyl ester profile, it was confirmed that the EPS-producing isolate CH-KOV3 belongs to the genus *Brachybacterium*.

Figure 1.

3.1.2. Physiological-biochemical characterization of isolate CH-KOV3

API test results are available in the Supplementary data (Table S-1). Based on the literature data, *B. paraconglomeratum* produced urease, indole, and H_2S, hydrolyzed starch and esculin, but not gelatine or Tweens 20, 40, 60, or 80, and reduced nitrate [**38-40**]. Based on the API test software, it was not possible to obtain positive identification to a high percentage of agreement with the database; however, valuable data was collected. Our isolate, *Brachybacterium* sp. CH-KOV3, hydrolyzes esculin, but not urea or gelatine, it produces acid and acetoin (3-hydroxybutanone), but does not produce H_2S or indole. Reduction of nitrate to nitrite was confirmed. Clearly, the isolate we studied produces a range of different enzymes. There is evidence that *B. paraconglomeratum* performs assimilation and fermentation of many carbon sources [**38-40**], but *Brachybacterium* sp. CH-KOV3 has far less scope for substrate assimilation and fermentation. The relatively different biochemical profile of *Brachybacterium* sp. CH-KOV3 compared to other strains of this genus, particularly *B. paraconglomeratum*, can be interpreted as its adaptation to the habitat in which it previously lived.

3.1.3. MALDI-TOF MS

This technique was used to characterize intact whole cells of isolate CH-KOV3, and also to examine an ethanol extract of this microorganism. There was no significant difference between MALDI-TOF MS spectra of whole cells and an ethanol extract of CH-KOV3, and thus, only the spectrum of intact whole cells of isolate CH-KOV3 is shown in Figure 2. In the field of prokaryotic taxonomy, MALDI-TOF MS has been increasingly gaining in importance over the

last few years [**21, 31, 41]**. Ribosomal proteins, being dominant in the cell, are the target of this analysis, as well as other proteins copied in high numbers [**42**]. The mass spectrum obtained can be considered as a specific fingerprint of the bacterium analyzed, because each particular protein has a unique pattern of *m/z* values. The *Brachybacterium* strains included in the MALDI Biotyper db 6903 are the following: *Brachybacterium faecium* DSM 4810T DSM, *Brachybacterium muris* 7 RLT, *Brachybacterium nesterenkovii* DSM 9573T DSM and *Brachybacterium nesterenkovii* DSM 9574 DSM. However, our MALDI-TOF MS did not reliably identify environmental isolate CH-KOV3. This is not surprising because MALDI Biotyper db 6903 software was developed primarily for routine identification of clinical bacterial isolates. Also, other authors have stated that *Brachybacterium sacelli* is not present in the MALDI-TOF database from Brüker [**43**]. However, the given protein profile of *Brachybacterium* sp. CH-KOV3 can be considered as a characteristic of the isolated microorganism, and according to available literature, this is the first MALDI-TOF MS spectrum of *B. paraconglomeratum* published.

Figure 2.

3.1.4. Molecular characterization of isolate CH-KOV3

The gene for 16S rRNA was isolated and analyzed, and the sequence of base pairs was deposited in the database GenBank, accession number KC881303.1. Based on this analysis, CH-KOV3 belongs to the genus *Brachybacterium* and contains 56.7 % of GC pairs. When compared to the National Center for Biotechnology Information (NCBI) sequence database, the 16S rRNA sequence of CH-KOV3 displayed the greatest level of homology to *B. paraconglomeratum* (99 % similarity with *B. paraconglomeratum* JCM 17781 (NR_113401, 31/08/2016, Actinobacteria). However, *Brachybacterium* sp. CH-KOV3 also showed 98 % sequence similarity to *B.*

conglomeratum J 1015 (NR_104686) and 98 % similarity to *B. saurashtrense* (NR_116516.1). As already stated, Actinobacteria are found in soil polluted by pesticides, herbicides and other persistent organic pollutants (POPs), and due to their ability to use pollutants as carbon sources, these bacteria have considerable potential for biodegradation. In addition to biodegradation, the removal process of POPs involves mechanisms of absorption, adsorption, and desorption [**44-46**]. In response to microbial growth on hydrocarbons in contaminated soil, Actinobacteria can produce biosurfactants and bioemulsifiers. These structurally diverse substances reduce surface tension at the air-water interface, solid-liquid interface or between immiscible liquids [**47**]. In the case of *Brachybacterium* sp. CH-KOV3, EPSs may act as bioemulsifiers [**4**].

3.1.5. Phylogenetic tree of the genus Brachybacterium

A phylogenetic tree for genus *Brachybacterium* was constructed in MEGA 7 software using the Maximum Likelihood method. The 16S rRNA gene sequence-based tree showing the position of *Brachybacterium* sp. CH-KOV3 within the genus *Brachybacterium* is given in Supplementary data (Figure S-1).

3.2. Production of EPS

3.2.1. Optimization of EPS production

3.2.1.1. Growth curve of Brachybacterium sp. CH-KOV3

A typical growth curve of *Brachybacterium* sp. CH-KOV3 is presented in Figure S-2A. The highest number of microorganisms was obtained after 48 h ($1.2x10^6$ CFU/mL), followed by stationary phase of growth. After 84 h, a decrease in the number of microorganisms occurs. Also, concentration of levan produced is given in the same figure. The highest concentration of levan was detected in 72 h.

3.2.1.2. Effect of temperature on EPS production

The effect of temperature on EPS production by *Brachybacterium* sp. CH-KOV3 in BM with 100 g/L sucrose at different times (24, 72, 120 h) is presented in Figure S-2B. Based on our results, the temperature affected EPS production to a minor extent. *Brachybacterium* sp. CH-KOV3 is an environmental isolate exhibiting maximum growth rate in laboratory conditions at 28 ºC. Production of EPS was the highest at this temperature, and ranged from 2.13 to 2.63 g/L at the tested time intervals. The same production level was confirmed at 37 ºC after 24 h of incubation (2.13 g/L), but after 72 and 120 h, the yield first increased then decreased (2.5, 1.43 g/L respectively). The lowest production rate and growth was confirmed when the incubation temperature was 45 ºC (24 h - 1.63 g/L; 72 h - 1.26 g/L; 120 h - 1.76 g/L).

3.2.1.3. Effect of pH on EPS production

The culture medium pH also has an important role at the start of the production of EPS [**48**]. The effect of pH on production of EPS by *Brachybacterium* sp. CH-KOV3 in BM with 100 g/L sucrose, together with changes in the number of bacteria is given in Supplementary data (Figure S-2C). Maximum growth and maximum EPS production was obtained at pH 7.0, which is not surprising, since the sediment from which *Brachybacterium* sp. CH-KOV3 was isolated was pH 7.3 [**23**].

3.2.1.4. Effect of sucrose concentration on EPS production

The effect of sucrose concentration on the production of EPS by *Brachybacterium* sp. CH-KOV3 after 24, 48 and 72 hours is presented in Figure S-3. It is known that in laboratory conditions, sucrose concentration has a great influence on EPS production. For example, Őner et al, 2016 [**49**] compared levan production by various bacteria exposed to sucrose-containing medium (60-400 g/L sucrose) over various time intervals, and found greater levan yields in higher sucrose

media. Furthermore, the level of levan production by *Bacillus licheniformis* NS032 was 47.8 g/L when sucrose was low (sucrose 196.8 g/L), while in a high sucrose system, the levan yield was 99.2 g/L (sucrose 397.6 g/L) [**48**]. In the case of *Brachybacterium* sp. CH-KOV3, the sucrose level also had great influence on EPS production, and the highest yield, 45.23 g/L, was obtained when *Brachybacterium* sp. CH-KOV3 was incubated in media with 500 g/L of sucrose. It was concluded that *Brachybacterium* sp. CH-KOV3, in optimized conditions, produces close to 50 g/L of EPS. As far as we are aware, this is one of the rare studies on levan-producing *Brachybacterium* sp., and the level of levan produced was five times higher than was described in another very recently published study [**16**].

3.2.2. Characterization of EPS

3.2.2.1. Molecular weight of the purified EPS

The molecular weight of the investigated EPS produced by *Brachybacterium* sp. CH-KOV3 was determined by GPC. EPS was eluted at the column void volume, indicating that the molecular weight was higher than separating range of the Sepharose CL-4B column used (> 10^6 Da). This was consistent with the absence of α-D-Glc signal in the ^{1}HNMR spectrum (section 3.2.2.7).

3.2.2.2. Hydrolysis of purified EPS

After total acid hydrolysis with 2 M TFA, the pure EPS produced just one monosaccharide component, fructose. *Brachybacterium* sp. CH-KOV3 produces EPS belonging to the group of levan-type fructans.

3.2.2.3. Partial acid hydrolysis of purified EPS

The results of partial hydrolysis of EPS with 0.0625 % oxalic acid in TLC are shown in Figure S-4. With increasing time of partial acid hydrolysis (up to 16 min), fructo-oligosaccharide fragments were obtained that had a degree of polymerization of three or more, along with

decreasing basic polysaccharide, which is in accordance with literature data for other levan-type polysaccharides [**50, 51**]. In the presence of 0.0625 % oxalic acid, the EPS was completely hydrolyzed in 10 min, unlike some levan-type polysaccharides which took one or more hours to completely hydrolyze with stronger oxalic acid **[34, 51]**. It can be assumed that the differences in hydrolysis patterns are the result of differences in molecular weight and/or branching degree of the various EPSs.

3.2.2.4. GC-MS analysis of methylation products

The purified EPS was permethylated and subsequently subjected to reductive-cleavage and acetylation of the cleaved monomer units, which were further analyzed by GC-MS. According to the peak areas on the total ion chromatogram (Figure 3), three sets of peaks were identified by their retention times and typical breakdown patterns [**37, 52**]. Two dominant peaks (peaks 3 and 4) occurred at retention times 18.34 min and 18.54 min, the fragmentation patterns of which corresponded to 6-O-acetyl-2,5-anhydro-1,3,4-tri-*O*-methyl-D-mannitol and 6-*O*-acetyl-2,5-anhydro-1,3,4-tri-*O*-methyl-D-glucitol respectively, and represented (2,6)-linkages of fructofuranoses. The summation of these peaks had a peak area that corresponded to 77.5% and referred to the main chain. Two peaks eluted at 16.21 min and 16.38 min were identified as 2,5-anhydro-1,3,4,6-tetra-O-methyl-D-mannitol and 2,5-anhydro-1,3,4,6-tetra-O-methyl-D-glucitol, respectively (peaks 1 and 2), and corresponded to the nonreducing terminal units of the glycan molecules. The fructosyl residues that indicate the branching points of the polysaccharide chain, i.e. (2,1) branching, corresponded to peaks at retention times 20.24 min and 20.54 min, which were identified by fragmentograms (peaks 5 and 6) as 1,6-di-O-acetyl-2,5-anhydro3,4-di-O-methyl-D-mannitol and 1,6-di-O-acetyl-2,5-anhydro-3,4-di-O-methyl-D-glucitol, respectively. The sums of the areas of peaks relating to the terminal units (11.2%) and areas of peaks

corresponding to the branching points (11.3%) had a ratio very close to 1:1, indicating that at each branching point, one nonreducing fructofuranosyl residue was attached. Analysis of methlation results shown by GC-MS suggested that the main structural feature of the EPS produced is a basic chain of (2,6)-linked fructofuranoses. The presence of small quantities (~11%) of di-O-methyl-fructoses indicated the moderate branching of the polysaccharide. The main chain was substituted at position O-1 with single D-fructofuranoses (~11%). Mass spectra of partially methylated anhydroalditol acetates are given in Figure 4.

Figure 3.

Figure 4.

3.2.2.5. Structural characterization of elementary organic constituents of EPS

The EPS consisted of carbon, hydrogen, oxygen, and based on microanalysis, the relative ratio was 39.04:6.74:54.22. After purification, the EPS did not contain any nitrogen or sulfur, while the content of C and H determined corresponded to polysaccharide molecules. In fact, the obtained values were close to the theoretical values for a neutral polysaccharide, purified from protein, and nucleic acids (C:H:O=38.86:7.02:54.12) [**15**].

3.2.2.6. FTIR spectroscopy

The FTIR spectrum of purified EPS is given in Figure S-5. The spectrum contained absorption bands, characteristic for a carbohydrate structure, in the range of 3500-807 cm^{-1}. The FTIR spectrum contained a characteristic broad absorption band in the range 3500 - 3000 cm^{-1}, corresponding to the valence vibrations of -OH groups. The band at 2946.8 cm^{-1} came from C–H stretching vibration, and the band at 1647.9 cm^{-1} was due to bound water molecules. The bands in the region of 1500 and 1200 cm^{-1} were assigned to C–H deformation vibration. The bands between 1127.4 and 1059.0 cm^{-1} corresponded to C–O–C and C–O–H stretching vibration.

Characteristic absorption at 925.2 cm^{-1} and 807.1 cm^{-1} resulted from the stretching vibration of the furan ring. All absorption bands were identical with bands from literature for levan-type polysaccharide [**15, 53**].

3.2.2.7. NMR spectroscopy

The ^{1}H NMR spectrum of the investigated EPS (Figure 5A) shows characteristic chemical shifts for carbohydrate ring protons, in the range of 3.4 to 4.3 ppm. There is no signal in the anomeric proton region, which suggests the absence or low abundance of glycosidic protons. The ^{1}H NMR spectrum of EPS showed seven main proton signals between 3.4 and 4.3 ppm. These values were due to β-Fruf units and suggested the EPS had a levan type structure. Similar chemical shifts for proton signals were observed with the levan produced by *Bacillus* or *Zymomonas* [**54, 55**]. The ^{13}C NMR spectrum of the investigated EPS, given in Figure 5B, contained chemical shifts in the range of 62.6 to 106.9 ppm, which are typical for carbons in the carbohydrate rings of polysaccharides. In the ^{13}C NMR spectrum of the investigated EPS, six intense peaks from low to high magnetic field occurred: 106.9 ppm (C2), 82.98 ppm (C5), 78.99 ppm (C3), 77.89 ppm (C4), 66.08 ppm (C6), and 62.60 ppm (C1). The resonances of carbons were ascribed to β-fructofuranose units by comparison with the ^{13}C NMR data of the standard methylglycoside (Bock and Pedersen, 1983) [**56**]. The relative positions of the signals were in accordance with levan type fructan [**15, 57**].

DEPT 135 analysis (the relevent section of the spectrum is given in Figure 5C) was used to determine the degree of hydrogenation of each carbon, considering that DEPT signals of CH and CH_3 carbons have positive and opposite amplitudes to CH_2 carbons. The DEPT spectrum showed intense signals at δ 82.99, δ 79.00, and δ 77.90 due to CH protons of C-5, C-3 and C-4, respectively. The signals at δ 66.09 and δ 62.61 were attributed to CH_2 protons of C-6 and C-1,

respectively. The results obtained are consistent with the data reported by Dahech et al, 2013 [**58**].

The part of the COSY spectrum of the investigated EPS, shown in Figure 5D, unequivocally confirmed cross peaks H6a/H6b at δ 3.88/3.54, H5/H6b at δ 3.94/3.53, H4/H5 at δ 4.09/3.94 and H3/H4 at δ 4.17/4.08. These characteristic chemical shift correlations, and the absence of any correlation peaks with the other protons in the region 3.6-3.8 ppm, confirmed their assignation as H1a and H1b, which is in accordance with the literature data for levan [**59**].

The 2D hetero-correlated HSQC spectrum of the investigated EPS, shown in Figure 5E, indicates direct correlations between skeletal protons and carbons of the sugar units that constitute the EPS. Diagnostic cross peaks H5/C5 at δ 3.94/82.98, and H6a, H6b/C6 at δ 3.89, 3.55/66.08 were detected, which are similar to the values of another fructan with levan structure [**54**]. Other cross peaks observed in the HSQC spectrum (H1a, H1b/C1 at δ 3.76;3,66/62.60, H3/C3 at δ 4.17/78.99, H4/C4 at δ 4.09/77.89) were also in accordance with data for fructan obtained by Matulova et al, 2011.

Figure 5.

3.2.2.8. Optical rotation, refractive index, and dynamic and kinematic viscosity

The measured refractive index, dynamic and kinetic viscosities are given in Table 1. Total EPS hydrolysis showed the presence of fructose only, and the negative value of specific optical rotation obtained in the current study **(**-38.5 º**)** indicates the presence of beta glycoside bonds. Both results suggest that the produced EPS is fructan type [**48, 60**]. The measured refractive index, dynamic and kinetic viscosities can be considered as characteristic of EPS produced by *Brachybacterium* sp. CH-KOV3.

Based on all collected instrumental data, the chemical structure of EPS produced by *Brachybacterium* sp. CH-KOV3 is given in Figure 6.

Figure 6.

4. Conclusions

In conclusion, isolate CH-KOV3 belongs to the genus *Brachybacterium*. The EPS produced by *Brachybacterium* sp. CH-KOV3 is a levan-type polysaccharide. This is one of the rare studies on levan-producing *Brachybacterium* sp., but the level of levan produced was five times higher than that produced by another *Brachybacterium* isolate. All data indicate that *Brachybacterium* sp. CH-KOV3 could be a new source for the production of levan.

Conflict of interest

The authors declare no competing financial interest.

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Figure captions

Figure 1. The fatty acid methyl ester profile of *Brachybacterium* sp. CH-KOV3 a) GC-MS; b) 2D GCxGC-MS. For identification, NIST11 and Wiley8 databases were used. The spectrum consists of straight-chain, saturated, iso and anteiso methyl-branched fatty acids (*n*- normal fatty acids methyl esters; *i* - iso; *ai* - anteiso methyl-branched fatty acids).

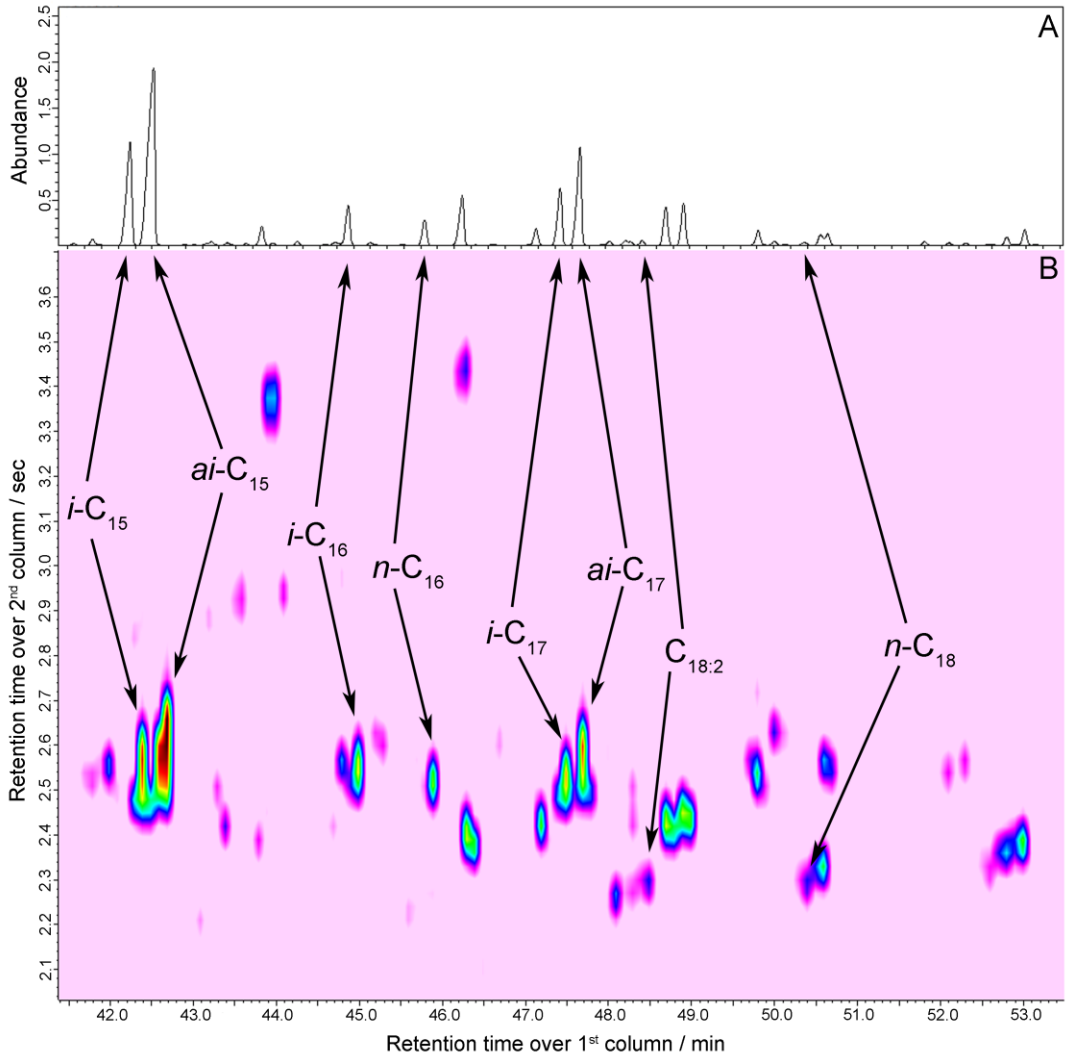
Figure 2. MALDI-TOF MS spectrum of intact whole cells of *Brachybacterium* sp. CH-KOV3. This spectrum was analyzed with MALDI Biotyper db 6903 software.

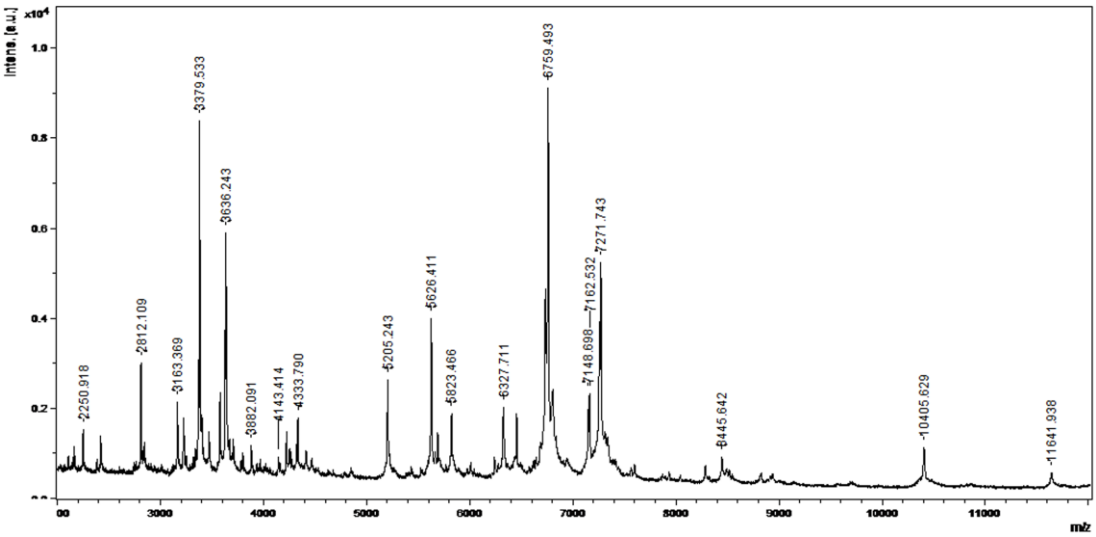
Figure 3. Gas chromatogram of partially methylated anhydroalditol acetates of EPS. Numbered peaks: 1) 2,5-anhydro-1,3,4,6-tetra-O-methyl-D-mannitol**;** 2) 2,5-anhydro-1,3,4,6-tetra-O-methyl-D-glucitol; 3) 6-O-acetyl-2,5-anhydro-1,3,4-tri-O-methyl-D-mannitol; 4) 6-O-acetyl-2,5-anhydro-1,3,4-tri0-methyl-D-glucitol; 5) 1,6-di-O-acetyl-2,5-anhydro-3,4-di-O-methyl-D-mannitol; 6) 1,6-di-0-acetyl-2,5-anhydro-3,4-di-O-methyl-D-glucitol.

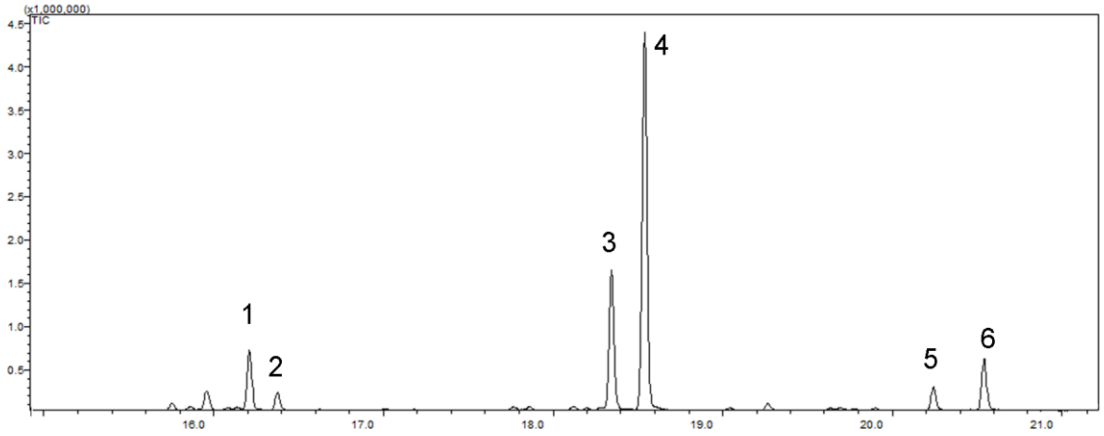
Figure 4. Mass spectra of partially methylated anhydroalditol acetates. (A) 2,5-anhydro-1,3,4,6-tetra-O-methyl-D-mannitol**; (**B) 2,5-anhydro-1,3,4,6-tetra-O-methyl-D-glucitol; (C) 6-O-acetyl-2,5-anhydro-1,3,4-tri-O-methyl-D-mannitol; (D) 6-O-acetyl-2,5-anhydro-1,3,4-tri-methyl-D-glucitol; (E) 1,6-di-O-acetyl-2,5-anhydro-3,4-di-O-methyl-D-mannitol; (F) 1,6-di-O-acetyl-2,5-anhydro-3,4-di-O-methyl-D-glucitol.

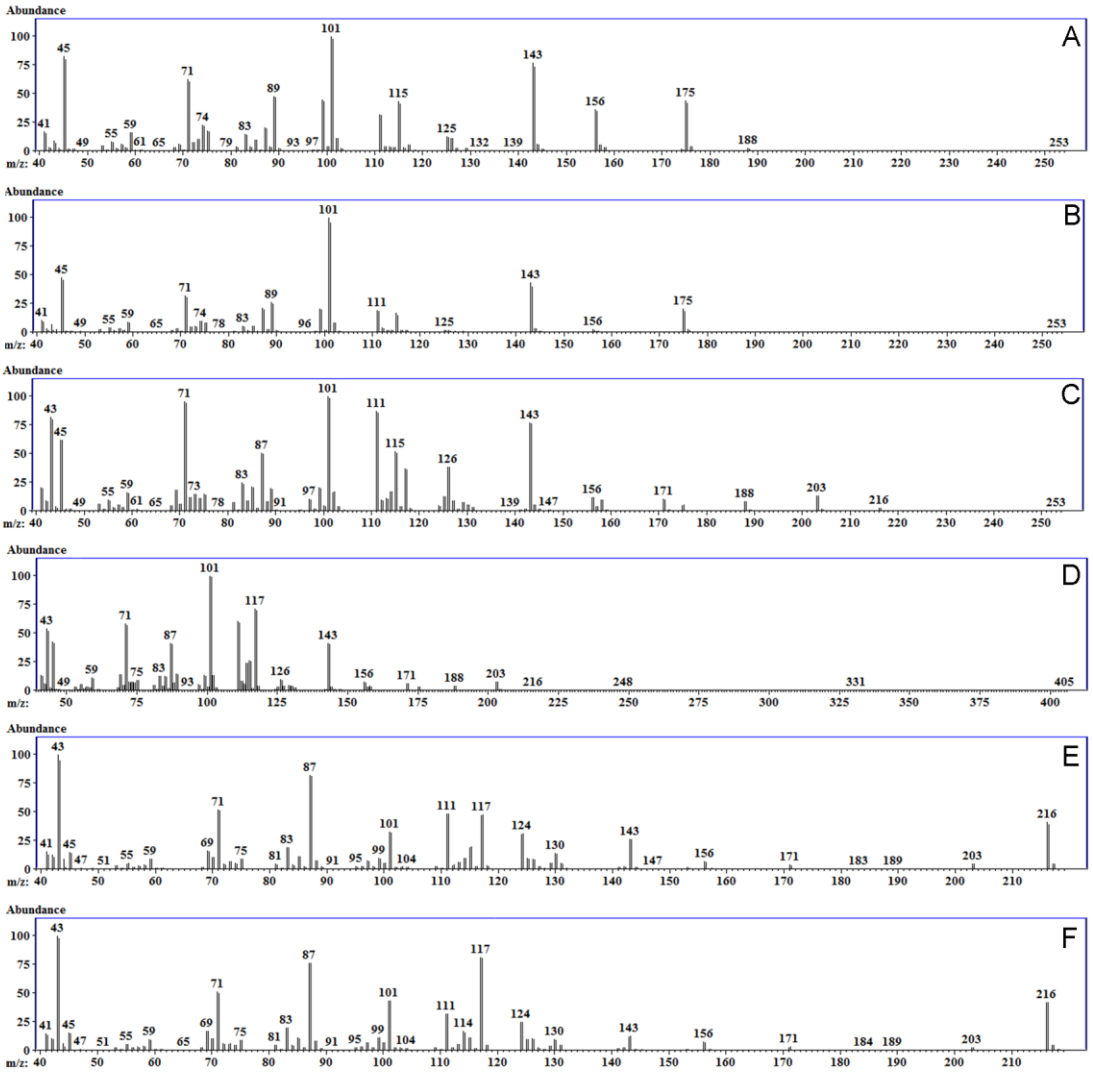
Figure 5. (A) The ^{1}H NMR spectrum of purified EPS produced by *Brachybacterium* sp. CH-KOV3; (B) The ^{13}C NMR spectrum of purified EPS produced by *Brachybacterium* sp. CH-KOV3; (C) Section of the DEPT 135 spectrum of purified EPS produced by *Brachybacterium* sp. CH-KOV3; (D) Part of the COSY spectrum of purified EPS produced by *Brachybacterium* sp. CH-KOV3; (E) Fragment of the HSQC spectrum of purified EPS produced by *Brachybacterium* sp. CH-KOV3

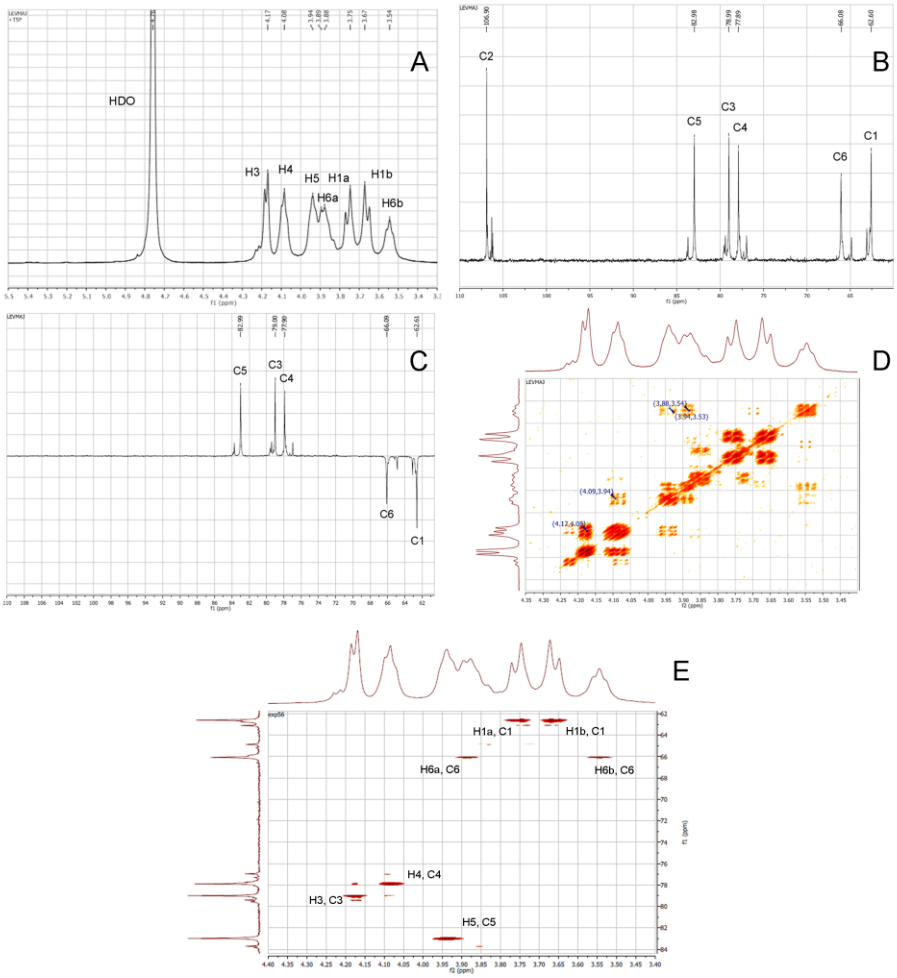
Figure 6. Chemical structure of EPS produced by *Brachybacterium* sp. CH-KOV3. This EPS is a levan-type polysaccharide, with fructose units linked by β-2,6-glycosidic bonds (the main chain), and with β-2,1-linked branches.

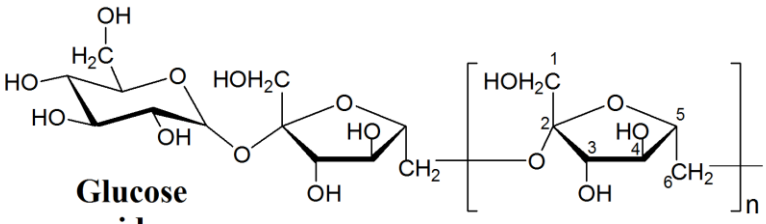












Glucose residue

Fructofuranosyl repeating unit

β-2,6-linkages

Table 1. Optical rotation, refractive index, kinematic and dynamic viscosity of purified polysaccharide solution.

Specific rotation $[\alpha]^{25}_{D}$ (^{o})	-38.5 **(c=0.2 g / 100 g water)**
Refractive index	1.33452±0.00002
Density of solution (g / cm^3)	1.0018
Dynamic viscosity (mPa x s)	1.1602
Kinematic viscosity (10^{-6} m^2 / s)	1.1581