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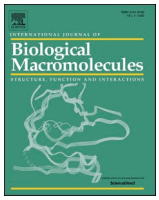

Sustainable production of heavy metal-binding levan by a subarctic permafrost thaw lake *Pseudomonas* strain 2ASCA

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ABSTRACT

Pseudomonas strain 2ASCA isolated in subarctic Québec, Canada, produced a cell membrane bound levan-type exopolymer (yield 1.17 g/L), after incubation in growth media containing 6 % sucrose (w/v) at temperature of 15 °C for 96 h. The objective of this study was to optimize levan production by varying the growth parameters. Moreover, the polymer's chemical characterization has been studied with the aim of increasing knowledge and leading to future applications in many fields, including heavy metal remediation. Higher levan yields (7.37 g/L) were reached by setting up microbial fermentation conditions based on the re-use of the molasses obtained from sugar beet processing. Spectroscopy analyses confirmed the levan-type nature of the exopolymer released by strain 2ASCA, consisting of a β-(2,6)-linked fructose repeating unit. Gel permeation chromatography revealed that the polymer has a molecular weight of 13 MDa. Scanning electron microscopy-energy dispersive X-ray spectroscopy (SEM-EDS) showed that the levan sequestered with a strong affinity Cr(III), which has never been previously reported, highlighting an interesting biosorption potential. In addition, SEM analysis revealed the formation of nanoparticles in acidified water solution.

1. Introduction

Levan-type fructan is essentially a homopolymer of fructosyl residues linked via the β-2,6 carbons. It is synthesized from sucrose by certain plant species and by several microbes including Archaea, Fungi and numerous Bacteria belonging to *Acetobacter, Bacillus, Erwinia, Gluconobacter, Halomonas, Microbacterium, Pseudomonas, Streptococcus* and *Zymomonas* genera. It is a versatile polymer with a large variety of applications [1]. The bacterial conversion of sucrose into polymerized fructans is catalyzed by the multifunctional enzymes grouped as fructosyltransferases. When the product of the polymerization is levan, the enzyme involved is levansucrase (EC 2.4.1.10) [2].

Recently, *Pseudomonas* 2ASCA was isolated from a surface sediment sample from a permafrost thaw pond (thermokarst Lake SAS2A) located in the Sasapimakwananisikw River valley near Kuujjuarapik-Whapmagoostui, in the subarctic Québec, as described by Finore et al. [3]. This environment is rich in organic compounds; carbohydrates are likely produced from the degradation of organic soil and wetland plants and are known to occur at high concentrations [4]. Strain 2ASCA could produce a levan-type fructan that was loosely bound to the cell membrane at a yield of 527.4 mg exopolymer/L of broth when cultivated in R2A medium enriched by sucrose 1 % (w/v) at 10 °C under agitation [3]. This is consistent with the known induction of bacterial metabolic pathways exclusively by sucrose availability in the culture medium. Typically, the microbial levan is released into the culture medium and recovered through alcoholic precipitation of cell-free supernatant [5–10]. The levan produced by strain 2ASCA showed a new attribute in that the polymer was associated with cells and did not dissolve in broth culture. Consequently, polymer recovery does not require the use of conventional alcohols. Instead, the polymer formed a gel layer above the cellular pellet in a single step during the centrifugation of the growth medium, which was then easily removed using a wash buffer. A crucial aspect of the large-scale microbial production of exopolysaccharides (ESPs) is the associated culture and harvesting costs [11]. Numerous

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studies have focused on reducing these costs, including the possible use of alternative and cheaper carbon sources derived from industrial residual materials, agri-food chains, or agricultural waste, to produce higher value materials [12].

The most common alternative sucrose-based source of exopolymers is molasses, a viscous, red-brown liquid that is the primary residue remaining after the sugar recovery from sugarcane or beetroot. It contains non-sugar substances and residual sucrose that can no longer be crystallized in ordinary sugar mill processes. Successful re-use of molasses for the production of levans has been reported for *Brachybacterium phenoliresistens*, *Microbacterium laevaniformans* PTCC 1406, *Bacillus lentus* V8, *Paenibacillus polymyxa* NRRL B-18475, and *Zymomonas mobilis* ATCC 31821 [13–16]. Microbial fermentation of molasses can be preceded by different types of pre-treatments, from chemical to physical, which increase the overall cost and duration of the process. Gojgic-Cvijovic et al. [17] reported that *Bacillus licheniformis* NS032 produced levans from acidified molasses. Various pre-treatments, such as pH adjustment, clarification, and treatment with tricalcium phosphate, sulfuric acid, and activated carbon, as well as their diverse combinations, have been tested for *Halomonas* sp. AAD6 levan synthesis [18]. Investigations of levan have revealed many novel properties that have considerable potential in industrial applications such as preservative of flavor and colour in the food industry, in cosmetics for skin care and whitening, in medicine for the healing of wounds and burns. It also has antimicrobial, antibiofilm, anti-inflammatory, antioxidant and anticancer activities [19–23]. Moreover, literature describes few papers concerning heavy metal chelating capability of levan [24].

The objective of this study was to optimize the microbial production of levan by a new psychrophilic *Pseudomonas* strain 2ASCA using both standard growth medium, enriched by single carbon sources, and by-products derived from the sugar beet processing chain. Moreover, the chemical characterization and technological properties of levans have been studied to increase our knowledge and lead to future applications in many fields, including heavy metal remediation.

2. Materials and methods

2.1. General

The chemical products were purchased from Sigma-Aldrich (St. Louis, MO, USA) and weighed using a Mettler Toledo analytical balance BA-E Series. Dialyses were performed using 6000–8000 MW cut-off dialysis tubes (SpectrPor® - Repligen, Massachusetts, USA). Molasses from sugar beet processing was provided by Co.Pro.B (Cooperativa Produttori Bieticoli, http://www.coprob.com) (Italy) and tested as an alternative and cheaper nutrient source for microbial growth and exopolymer production. Molasses contained (%, w/v) total nitrogen 1.95, alfa-amino nitrogen 0.50, calcium ion 0.14, magnesium ion 0.25, potassium ion 3.15, sodium ion 1.7 (data kindly furnished by Co.Pro.B). The sucrose content of molasses was determined by high-performance anion-exchange chromatography-pulsed amperometric detection (HPAEC-PAD) (Dionex ICS-5000$^+$ DC) with a CARBOPAC PA1 column. Isocratic elution solvent system: 150 mM NaOH (flow 0.25 mL/min). Sucrose standard retention time was 8 min.

2.2. Microorganism and culturing conditions

Pseudomonas 2ASCA was grown at temperature of 10 °C under aerobic conditions and agitation (120 rpm), in 1 L Erlenmeyer flasks containing R2A medium (250 mL) and inoculated with 25 mL of preculture (24 h). Optimal growth conditions in terms of pH, temperature, and NaCl concentration were defined by varying one parameter at time (temperature from 4 to 25 °C; pH from 5.0 to 9.0; NaCl (w/v) from 0 to 5 %) in R2A and measuring the optical density (OD) (Genesys 150, Thermo Fisher) at λ 540 nm after 24 and 48 h of incubation. To study using a single carbon source how to produce an exopolymer material, strain 2ASCA was grown using buffered modified medium R2A, as previously reported [3]. Carbon sources solutions were sterilized by filtration (0.22 μm) and added to growth medium (10 g/L). The following carbon sources were used: maltose, fructose, galactose, glucose, sucrose, lactose, mannose, sodium acetate, glycerol, sodium citrate, and xylose. Growth was followed by determining the optical density at 540 nm until the stationary phase was reached. Growth cultures were centrifuged for 30 min at 10,000 rpm. The presence of exopolymeric material (EP), was determined in three different fractions, namely cell-free supernatant (S), tightly bound (TB), and loosely cell-bound (LB), as previously reported [3], and it was expressed as weight of lyophilized recovered material in one liter of growth medium. The EPs obtained from the S, TB and LB fractions were tested for total carbohydrates, proteins and uronic acids content [25–27]. Since the EP samples were obtained after a dialysis step to exclude low molecular weight material [3], the carbohydrate content, determined by colorimetric assay, coincided exactly with the extracellular polysaccharide substance (EPS).

2.3. Loosely bound levan production optimization and recovery

The optimization of the synthesis of the exopolymer was performed through a step-by-step experiment by changing one variable at a time. Aliquots of bacterial pre-culture (10 %, v/v) were used to inoculate 200 mL of R2A minimal medium. The cultures were incubated for up to 120 h (sucrose 1 % w/v, 15 °C) and the yields at sucrose concentrations of 1, 2, 3, 4, 5, 6, and 7 % w/v (96 h incubation at 15 °C) and the incubation temperatures of 4, 10, 15, 20 and 25 °C (using 6 % sucrose (w/v) and a 96 h incubation) were determined. Molasses was tested as an alternative carbon source for sustainable levan production by substituting commercial sucrose present in the medium. Molasses, provided by Co.Pro.B, contained sucrose 50 % (w/v) (data from present study) and, therefore, it was added 120 mL/L to the fermentation medium and incubated at 15 °C for 96 h. The pH and optical density were monitored at regular growth intervals. Finally, after reaching the stationary phase, all growth was interrupted, and the cells were centrifuged at 50,000 rpm. The polymer loosely bound to cells formed a gelatinous layer which was removed by washing the pellet three times with 0.15 M phosphate buffered saline (PBS) pH 7.2, followed by centrifugation (6000 rpm at 4 °C for 40 min). The supernatants were dialyzed against tap water for three days and then lyophilized for levan yield determination.

2.4. Chemical characterization of exopolysaccharides

Chemical hydrolysis of the lyophilized polysaccharide (3 to 4 mg) was performed using 100 μL of 1 N trifluoracetic acid at 60 °C for 1 h, as previously reported [28]. Subsequently, the separation and identification of sugars were achieved, as reported by Poli et al. [29]. The fructose presence in levan was determined by high-performance anion-exchange chromatography-pulsed amperometric detection (HPAEC-PAD) (Dionex ICS-5000$^+$ DC) with a CARBOPAC PA1 column. Isocratic elution solvent system: 16 mM NaOH (flow 0.25 mL/min). Fructose standard retention time was 5 min.

Since HPAEC-PAD data suggested the presence of fructose residues, the samples for gas chromatography–mass spectrometry (GC–MS) analysis were prepared following the protocol for ketoses [30]. Briefly, the samples were hydrolyzed (1 % CH_3COOH at 100 °C for 4 h). After drying, it was resuspended in 0.1 mL of methanol and reduced by adding $NaBH_4$ while stirring at 25 °C for 1 h. After neutralization with a few drops of 1 M HCl, the sample was acetylated with Ac_2O at 100 °C for 30 min. The derivatives obtained were analyzed using GC–MS (Agilent technologies GC-7820A MS-5977B), equipped with an Agilent HP-5 capillary column (30 m × 0.25 mm i.d., flow rate 1 mL/min, He as carrier gas), by using the following temperature program: 150 °C for 3 min, and 150 °C → 330 °C at 3 °C/min. The linkage positions of fructose units were determined using methylation. Extracellular polysaccharides

(0.5 mg) were treated with 0.1 mL of CH_3I in dimethyl sulfoxide (DMSO) and NaOH for 2.5 h [31]. The methylated sample was hydrolyzed with 2 M TFA, reduced with $NaBD_4$, and acetylated. Partially methylated and acetylated alditols (PMAAs) were analyzed using GC-MS as previously reported [32].

Fourier-transform infrared spectroscopy (FT-IR) measurements of the EPS were performed using a NICOLET spectrometer (Cary 630 Agilent) with a light source in the mid-infrared (range 650–4000 cm^{-1}). Samples were prepared as mixtures with KBr (0.1 % wt of sample).

The molecular weight distributions of the samples were measured using gel permeation chromatography (GPC). Analysis was performed using a GPC Max Viscotek system equipped with a TDA 305 triple detector array (refractive index, low-angle light scattering, right-angle light scattering, and viscometer) and a UV detector. The column set comprised a TSK pre-column (Tosoh Corporation) and two columns: PolySep-GFC-P5000 and PolySep-GFC-P3000 (Phenomenex). Temperature of columns and detectors was set at 40 °C. Samples were dissolved and eluted in H_2O (0.2 % wt NaN_3 and 0.1 M $NaNO_3$). After complete dissolution, the samples (5 mg/mL) were filtered through nylon membranes (0.22 μm). The injection volume was 100 μL, and the flow rate was set at 0.6 mL/min. The samples were evaluated using the triple-point calibration method based on the polyethylene oxide (PEO) standard with a narrow molecular weight distribution provided by Viscotek. All measurements were performed in duplicate for 50 min.

Thermogravimetric analysis (TGA) of the levan was performed using a TGA/DTA Perkin-Elmer PyrisDiamond instrument with a gas station. Approximately 4 mg of sample (kept under vacuum at 60 °C for 24 h prior to analysis) were placed in a ceramic crucible and heated from room temperature up to 600 °C at a rate of 10 °C/min, under nitrogen flux at 30 mL/min.

ZetasizerNano Z (Malvern Instruments Ltd) was used to evaluate the hydrodynamic diameter (DH) and polydispersity index (*Đ*).

Samples were dissolved in D_2O, and the NMR spectra were measured using a Bruker DRX-600 spectrometer (600.13 MHz) equipped with a TCI Cryo ProbeTM, fitted with a gradient along the Z axis, and on Bruker 400 MHz and 400 MHz Prodigy instruments.

2.5. Heavy metal chelating behavior of EPS

The heavy metal chelating capability of the exopolymer released by strain 2ASCA was evaluated by testing one of the following metals one at a time (990 ppm final concentration in distilled water solution pH 5.6): Cu(II), Zn(II), Pb(II), Fe(III), Cr(III), Cd(II), and at the same time: Fe(III) plus Cr(III). Aliquots (5 mL) of the EPS water solution (1 mg/mL) were placed in small dialysis tubes (12 kDa cut-off) and dipped in 495 mL of 1000 ppm metal solutions for 24 h. Another set of experiments was conducted by using aliquots (5 mL) of EPS in 1 % (v/v) acetic acid solutions (pH 2.8), placed in small dialysis tubes (12 kDa cut-off) and dipped for 24 h in 495 mL of 1000 ppm metal solutions. Subsequently, the tubes were dipped in 495 mL of water or an acidified water solution for 24 h to remove unbound metals. A control solution of EPSs was prepared. EPS samples, before and after the washing step, were recovered and freeze-dried for further analysis using scanning electron microscopy (SEM) (Quanta 200 FEG, 338 FEI, Eindhoven, The Netherlands). The latter were placed on a stub and coated with a homogeneous layer (18 ± 0.2 nm) of Au and Pd alloy using a sputtering device (MED 020, Bal-Tec AG, Tucson, AZ, USA). Images were recorded at room temperature in a high-vacuum environment. During SEM acquisition, chemical analysis of selected microscopic regions was performed using energy-dispersive X-ray spectroscopy (EDS). EDS was performed using an Oxford Inca Energy 250 System equipped with an INCAx-act LN2-free detector at an accelerating voltage of 20 kV under low-vacuum conditions.

3. Results and discussion

3.1. Utilization of carbon sources

The optimal growth conditions for *Pseudomonas* 2ASCA were 15 °C, 3 % (w/v) NaCl, and neutral pH (data not shown). The strain could utilize all tested carbon sources for growth. The highest optical density was reached with growth in the presence of sucrose, while the lowest growth was on lactose and maltose (Table 1). This ability to utilize many sugars as the sole carbon source motivated our investigation of exopolysaccharide production and transfer into the cell-free supernatant (S) and into loosely and tightly bound fractions (LB and TB, respectively). The yield of the exoproduct (EP) was expressed as weight of lyophilized recovered material in one liter of growth medium. The EPs obtained from S, TB and LB fractions were tested for extracellular polysaccharide substance (EPS), proteins and uronic acids content as previously reported [3].

Among the tested substrates, sucrose supported the best cell growth and was the most suitable carbon source for polysaccharide pathway induction, resulting in the exoproduct release of 613.2 mg/L, of which 86.0 % were carbohydrates and a low percentage of proteins and uronic acids, into the loosely bound fraction. The yield of the exopolymer was studied using incubation times of 24, 48, 72, 96, and 120 h to determine the maximum production. As shown in Fig. 1A, the optimal production was at 96 h, with a yield of 827 mg/L of which 83.0 % were carbohydrates. The full-time course for incubation of strain 2ASCA in the presence of 1 % sucrose revealed that although optical density reached the maximum value of 1424 mg after 120 h, the highest percentage of polysaccharide production occurred after 96 h, with a good balance between EPS purity and yield at that time.

The exopolymer production was evaluated after 96 h of incubation at 15 °C while increasing the sucrose percentage from 1 to 7 %. The results showed the highest yield of EPS production of 1170 mg/L under incubation conditions with 6 % sucrose (Fig. 1B). The polymeric product obtained was mostly composed of carbohydrates, with proteins or uronic acids not detected (>0.01 %, w/v). The influence of the incubation temperature on the production of the exopolysaccharide was tested at 4, 10, 15, 20 and 25 °C under the optimized conditions previously established (i.e., sucrose 6 % (w/v) and 96 h incubation). The EPS yield decreased by 11 % at 20 °C, nearly 55 % at 10 and 25 °C, and 87 % at 4 °C, and therefore 15 °C resulted to be the most suitable temperature for EPS synthesis.

The procedure proposed to recover levans from *Pseudomonas* strain 2ASCA is based on rinsing with buffer followed by centrifugation. It is solvent-free, underlining its green and sustainable potential, described only for this bacterium for the recovery of levans of microbial origin [3]. EPS was produced by substituting pure sugar with agro-industrial waste to address the sustainability and circular economy objectives. Bacterial fermentation with molasses from sugar beet processing as the sole sucrose source resulted in a greatly amplified production of EPS, with a yield of 7.37 g/L. The increased EPS yield was, most probably due to the presence of nitrogen and mineral components in molasses, with respect to the R2A minimal medium containing sucrose added 6 % (w/v). Moreover, molasses was used without any pre-treatment in this study, further reducing the environmental impact, overall cost, and duration of the whole process. This contradicts what is reported in the literature, in which many chemical and physical treatments of molasses were needed before microbial fermentation [17,18].

3.2. Sugar composition and chemical characterization of EPS

The sugar composition and chemical characterization of the crude EPSs obtained from microbial growth on sucrose and sugar beet molasses as carbon sources were determined using thin layer chromatography (TLC), HPAEC-PAD, GC–MS, FT-IR, GPC, TGA, SEM, and NMR. The similarity of the results for both crude polysaccharides suggests that

Table 1
Pseudomonas strain 2ASCA yields of exoproduct material (EP) determined in cell-free supernatant (S), tightly (TB) and loosely cell bound (LB) fractions, obtained from different carbon sources (1 %, w/w) after 96 h of incubation at 15 °C in minimal medium R2A. The growth reached at the end of the incubation is shown as optical density (OD) measured at λ 540 nm. Extracellular polysaccharide substance (EPS), proteins and uronic acids content present in EP were measured. The highest values are shown in bold.

Carbon source	OD $_{\lambda\ 540\ nm}$	Fraction	EP (mg/L)	EPS (%)	EPS (mg/L)	Proteins (%)	Proteins (mg/L)	Uronic acids (%)	Uronic acids (mg/L)
Fructose	0.993	LB	33.6	4.9	1.7	2.9	1.0	0.7	0.2
		TB	150.4	20.6	30.9	3.5	5.3	0.9	1.3
		S	178.8	0.5	0.8	0.3	0.5	0.3	0.5
Galactose[a]	1.073	LB	28.8	2.7	0.8	0.8	0.2	0.5	0.1
		TB	47.2	26.6	12.5	3.3	1.5	1.3	0.6
		S	167.6	2.0	3.4	0.2	0.4	0.1	0.2
Glycerol	1.083	LB	37.2	4.9	1.8	1.2	0.4	0.7	0.2
		TB	138.4	21.6	29.8	5.1	7.1	1.2	1.6
		S	138.0	2.8	3.8	0.3	0.3	0.5	0.6
Glucose[a]	1.209	LB	51.2	6.1	3.1	0.6	0.3	0.4	0.2
		TB	77.6	12.1	9.4	3.2	2.5	1.1	0.8
		S	275.2	1.1	3.1	0.5	1.4	0.2	0.6
Lactose	0.555	LB	8.8	3.4	0.3	0.8	0.1	0.5	0.0
		TB	97.2	12.3	11.9	5.4	5.3	0.9	0.8
		S	119.2	1.0	1.2	0.7	0.8	0.3	0.4
Maltose	0.586	LB	30.8	3.4	1.0	0.8	0.2	0.4	0.1
		TB	117.6	24.2	28.4	6.4	7.5	0.8	0.9
		S	264.8	2.5	6.6	0.5	1.4	0.5	1.2
Mannose[a]	1.016	LB	27.6	2.9	0.8	0.7	0.2	0.7	0.2
		TB	21.2	25.0	5.3	3.4	0.7	1.2	0.2
		S	135.2	3.3	4.5	0.6	0.8	0.5	0.6
Na acetate	1.003	LB	17.6	6.4	1.1	0.6	0.1	0.7	0.1
		TB	126.8	17.7	22.4	6.1	7.7	1.2	1.5
		S	212.0	1.6	3.3	0.7	1.4	0.5	1.0
Na citrate	1.128	LB	45.6	5.7	2.6	0.6	0.3	0.4	0.2
		TB	68.4	27.1	18.6	3.4	2.3	1.0	0.7
		S	175.2	3.9	6.9	0.6	1.1	0.4	0.7
Sucrose[a]	**1.325**	**LB**	**613.2**	**86.0**	**527.5**	0.2	0.9	4.2	25.6
		TB	34.8	18.7	6.5	3.5	1.2	1.3	0.5
		S	158.4	4.0	6.3	0.6	1.0	0.9	1.4
Xylose	1.099	LB	29.2	3.2	0.9	1.4	0.4	0.9	0.3
		TB	146.0	28.4	41.5	6.3	9.2	1.0	1.4
		S	77.2	1.3	0.965	1.0	0.8	0.5	0.4

[a] Data from Finore et al. [3].

these polymers were levan-type polysaccharides.

3.2.1. TLC and HPAEC-PAD

TLC analysis of the hydrolyzed sample revealed the presence of a spot that was identified as fructose when compared with the standards. The composition of EPS was confirmed using HPAEC-PAD analysis. The chromatogram indicated that EPS had a monomer composed of fructose residues (Supplementary Material, Fig. S1).

3.2.2. GC–MS

The glycosyl composition was obtained after the hydrolysis, reduction, and acetylation of the EPS. The GC–MS chromatogram of the EPS revealed the presence of fructose. The linkage positions of the monosaccharides were determined using derivatization to PMAAs. The chromatogram revealed the presence of t-Fruf, 6-substituted Fruf, and 1,6-di-substituted Fruf at a molar ratio of 1:12:0.6 Reduction with $NaBD_4$ enabled the distinction between 6-Fruf and 1-Fruf (Supplementary Fig. S2). These results were comparable to those of other levan-type EPSs previously isolated [33].

3.2.3. FT-IR

The FT-IR spectrum showed signals around 3300 cm^{-1}, ascribed to hydroxyl (-OH) stretching vibration, and 2950 and 2900 cm^{-1} due to C—H stretching vibration of CH_2 and CH groups, respectively [28]. The C═O stretching of bound water was observed around 1644 cm^{-1} [34]. The bands in the region of 1420 and 1218 cm^{-1} were assigned to C—H plane deformation combined with skeletal vibrations [35,36]. The band at 1020 cm^{-1} was assigned to the stretching vibrations of the glycosidic linkage contributions of C–O–C and C–O–H. Characteristic absorption at 926 cm^{-1} and 815 cm^{-1} was also observed, indicating the presence of a furanose ring [35] (Supplementary Material, Fig. S3). These results combined with that of the chromatography studies and the high level of resemblance with the FT-IR observed in the other studies, suggested that the polysaccharide polymers produced by strain 2ASCA were levan-type fructan polysaccharides [18,29,37].

3.2.4. GPC

Fig. 2A shows the GPC chromatogram of the EPS of 2ASCA. The green and red lines represent Right-angle laser light scattering (RALS) and refractive index (RI) signals, respectively. We detected a weak viscometer signal (not shown) typical of branched polymer chains (Fig. 2B), which was ascribed to its compact and spherical molecular conformation [38].

An intense double peak eluted in the range between 10 and 16 mL in RI and RALS signals, approximately 25 % of the overall sample, indicated a molecular weight of 13 MDa and 5 MDa, respectively. The RI signal also presented several peaks (15–21 mL) before the last peak (22.2 mL) related to the salts present in the eluent, i.e., sodium nitrate, and sodium azide. The intermediate peaks, which represent 75 % of the overall injected sample by weight, can be attributed to oligomers and other species with low molecular weights (such as salts and monomers).

Bacterial levans with different molecular weights can be produced, and their applications may vary based on this [10]. High molecular weight levans are produced by *Bacillus licheniformis NS032* (5.8 MDa), *Bacillus subtilis* AF17 (20 MDa), and *Bacillus paralicheniformis* LB1-1 A (50 MDa) [6,10,17]. Levans with high molecular weights have been utilized as stabilizing, encapsulating, and emulsifying agents [10], suggesting that levans produced by *Pseudomonas* strain 2ASCA (13 MDa and 5 MDa) could have different industrial uses.

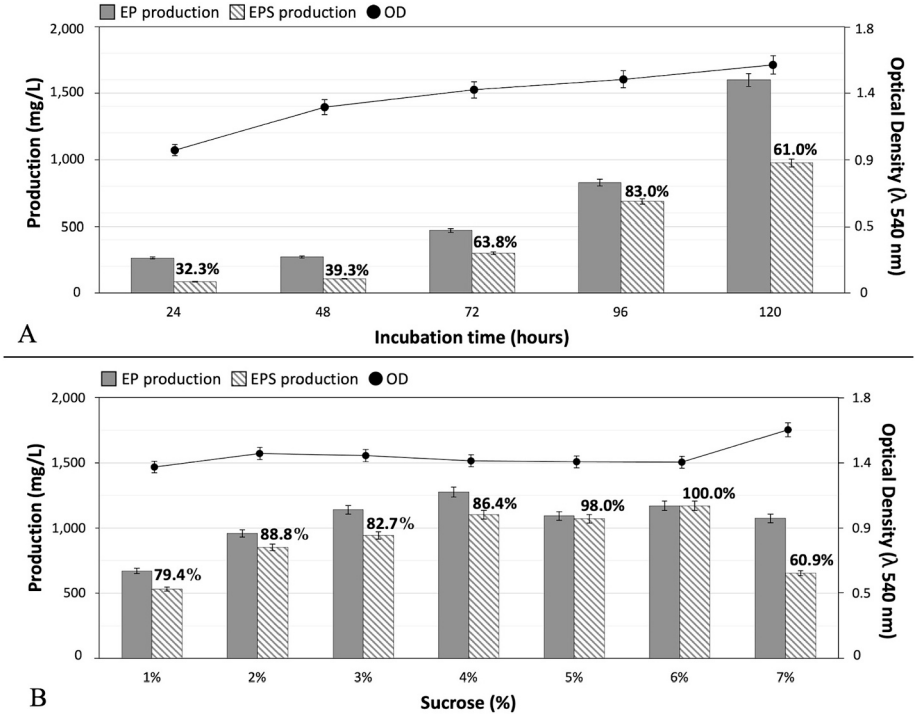

Fig. 1. Production of exoproduct (EP, mg/L) and exopolysaccharide fraction (EPS, mg/L) by *Pseudomonas* strain 2ASCA under different conditions. A: production using different incubation times (using 1 % sucrose (w/v) and 15 °C incubation temperature). The percentage of EPS (w/w) of the total EP is shown as hatched bars, and biomass reached at each time (optical density OD, measured at 540 nm) is shown as the line and dots. Inoculum ranged between OD 0.090 and 0.100. Values are means of triplicates ± SD. B: production with increasing sucrose in the medium (%, w/v) after 96 h of incubation at 15 °C. The cell density reached at the end of the incubation is shown as optical density (OD) measured at λ 540 nm.

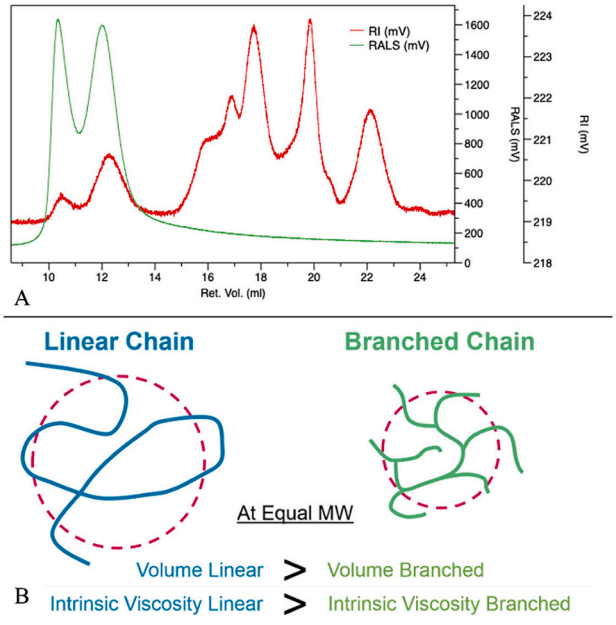

Fig. 2. A: Overlap chromatogram between RALS (green) and RI (red) analysis EPS from *Pseudomonas* strain 2ASCA using GPC. B: comparison of linear versus branched polymer.

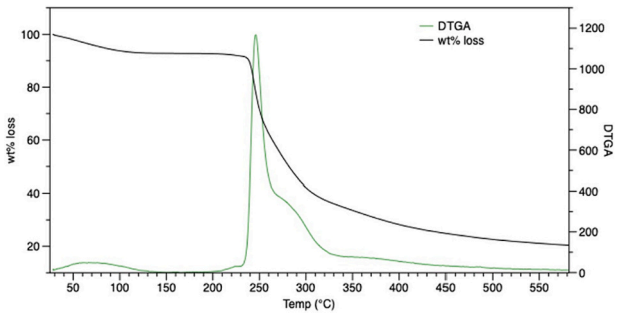

Fig. 3. Thermogravimetric analysis of EPS from *Pseudomonas* strain 2ASCA.

3.2.5. TGA

The TGA analysis indicated a loss relative to the removal of water up to 100 °C (Fig. 3). The first degradation step, with a degradation temperature (Td) of 260 °C and a weight loss of about 50 % was associated with β(2 → 1) bond degradation, which is related to ramification in polymeric structures. This degradation step is in accordance with GPC evaluation [39]. The two shoulders around 300 °C and 350 °C are consistent with main chain β(2 → 6) bond degradation.

The levan produced by *Pseudomonas* strain 2ASCA showed an elevated level of thermal stability, suggesting its possible use in all devices and processes that require extreme temperatures and sterility, thereby eliminating the risk of microbial contamination. A similar finding was reported for the levan from *Leuconostoc mesenteroides* S81, which had a degradation temperature between 200 and 250 °C [40]. The levan produced by *Chromohalobacter salexigens* 3EQS1 had a lower thermal stability of up to 210 °C [41].

3.2.6. SEM

SEM analyses of the control samples showed a matrix texture, revealing the tendency of the polymer to develop particles in water (Fig. 4A), especially when acidified (Fig. 4B). Representative SEM images of the polymer-metal samples are shown, particularly for Fe(III), under both conditions, before and after the washing cycle. In the unwashed sample, the matrix structure was difficult to recognize (Fig. 4C), whereas the washing step made the aggregated structures more visible

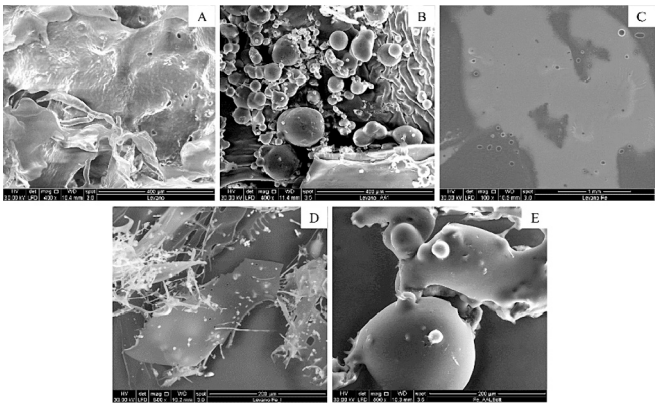

Fig. 4. SEM analyses. A: EPS in water (400X). B: EPS in acidified water (400X). C: Iron/EPS in water (100X). D: Iron/EPS in water after the washing cycle (800X). E: Iron/EPS in acidified water after the washing cycle (100X).

and complete (Fig. 4D and E).

This behavior was also studied using dynamic light scattering (DLS) by suspending the EPS (2 mg/mL) in distilled and acidified water (1 % acetic acid) after stirring for 1 h. As shown in Fig. 5, a single peak with a broad distribution (DH 217 nm, Đ 0.9) was detected for the distilled water sample, while the acidified water sample (in green) showed two peaks (DH 218 nm and 44 nm), probably due to partial levan degradation as suggested from literature [42].

3.3. Heavy metal adsorption behavior of EPS

Zn(II), Cu(II), Fe(III), Cd(II), Pb(II), and Cr(III) were used to test the capability of EPS produced by *Pseudomonas* strain 2ASCA to adsorb metals commonly found in polluted water [43]. All metals were initially adsorbed onto the EPS; however, only Fe(III) and Cr(III) were retained after the washing step (Table 2).

Cr(III) and Fe(III) were highly adsorbed both before (line A) and after washing (line B), indicating a tendency to preferentially bind to metal species (III). Therefore, another test was conducted by placing the EPS in a solution containing Cr(III) and Fe(III). In this case, Cr(III) showed a lower percentage than in the previous test, but a higher percentage of Cr (III) was retained after washing. To the best of our knowledge, this absorption property of Cr(III) has never been reported in scientific literature. Moreover, literature is pretty poor in this field in terms of levan seizing capability and metal ions employed [44]. Levan from *Pseudomonas* strain 2ASCA showed an higher Cu(II) and Zn(II) adsorption percentage with respect to levan from recombinant *Escherichia coli* BL2, engineered with levansucrase encoding gene from *Bacillus licheniformis* BK1, 20 and 35 (wt% metal), respectively [44]. Given the DLS results obtained under acidic conditions (HAc), adsorption tests were conducted on Fe(III) and Cr(III) under identical conditions. The percentage retention of both metals was lower than under unacidified conditions

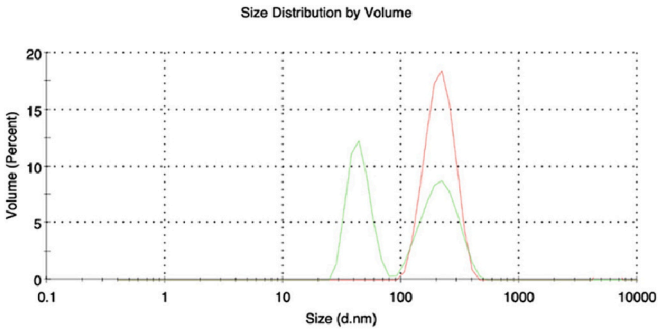

Fig. 5. DLS of EPS dissolved in distilled water (red) and acidified water (green).

Table 2
Heavy metal adsorption by levan from *Pseudomonas* strain 2ASCA. Weight (wt%) of metals detected using EDX. A: amount of metals detected in levan after 24 h of exposition; B: amount of metals detected in levan samples (A) after a washing cycle. Each value is the mean (± SD) of four replicates.

Metal	A Wt% metal	B Wt% metal
Fe(III)[a]	21.64 ± 1.97	10.76 ± 2.11
Cr(III)[a]	52.5 ± 2.34	31.0 ± 1.25
Pb(II)[a]	15.20 ± 6.73	1.66 ± 0.73
Cu(II)[a]	26.03 ± 1.83	0.21 ± 0.09
Zn(II)[a]	49.80 ± 14.68	0.85 ± 0.53
Cd(II)[a]	41.43 ± 3.16	–
Cr(III)/Fe(III)[a]	Fe(III) 4.3 ± 1.22 Cr(III) 29.7 ± 2.45	Fe(III) 4.25 ± 0.27 Cr(III) 21.4 ± 0.54
Cr(III)/Fe(III)[b]	Fe(III) 7.3 ± 2.24 Cr(III) 44.1 ± 1.43	Fe(III) 5.1 ± 0.32 Cr(III) 23.2 ± 1.15
Cr(III)[b]	30.4 ± 2.24	8.0 ± 0.24

[a] Distilled water metal solution (pH 5.6).
[b] 1 % acetic acid metal solution (pH 2.8).

(Table 2). This biosorption capacity may be related to the dimensions of the aggregates of the polymer in solution and indicates a potential application of the levan for bioremediation purposes. It should also be noted that the sampling and collection of this strain were from a lake in the Cree First Nation territory (Canada), and any commercialization would need to address indigenous rights and benefit-sharing [3].

3.3.1. NMR

NMR analysis of levans derived from both sources indicated small differences but consistent patterns. ^{1}H NMR spectra of EPS and EPS treated with the previously mentioned metals were recorded. Fig. 6 shows the superimposed spectra of EPS and washed EPS_Cr(III), this being the sample with better %wt metal in EDX analysis, in orange and blue, respectively. The signal pattern of EPS confirmed its composition as a levan-type polysaccharide. Moreover, slight differences in chemical shift and peak appearance in the 4.08–4.16 ppm region could be attributed to Cr(III) complex, since the signal in this region is assigned to H-4 bearing -OH groups that are capable of metal complexing [45]. The full-size ^{1}H NMR spectra of EPS is provided in the Supplementary Material (Fig. S4).

4. Conclusions

In this study, high-level production of a levan-type exopolysaccharide, described for the first time as being located in the loosely bound cell fraction, was achieved in a psychrophilic bacterium isolated from subarctic Québec, belonging to the genus *Pseudomonas*. It has a high molecular weight of 13 MDa and a degradation temperature of 260 °C, making it an attractive molecule because of its stability. This work is significant because it investigated the technological properties of this exopolysaccharide, particularly its metal adsorption capability, revealing a strong affinity for Cr(III), which has never been reported before for microbial levans, suggesting a new application in the bioremediation sector. The accepted techniques for heavy metal remediation include mainly physicochemical precipitation that require suitable places and are high energy consumption, beside huge amounts of toxic compounds. Therefore, biotechnological approaches are preferable for their safe, sensitive, eco-compatible and renewable properties, and the use of EPSs has high potentialities because still unexplored, due to their unlimited chemical diversity. Furthermore, EPS can be produced by exploiting waste raw material, so reducing fermentation costs.

It is worth noting that the recovery of levan requires a washing buffer step that eliminates the conventional alcohol precipitation procedure, making the entire process greener and more sustainable. Notably, a six-fold increase in polymer yield was attained when untreated molasses was used as an alternative and cheaper carbon source for *Pseudomonas*

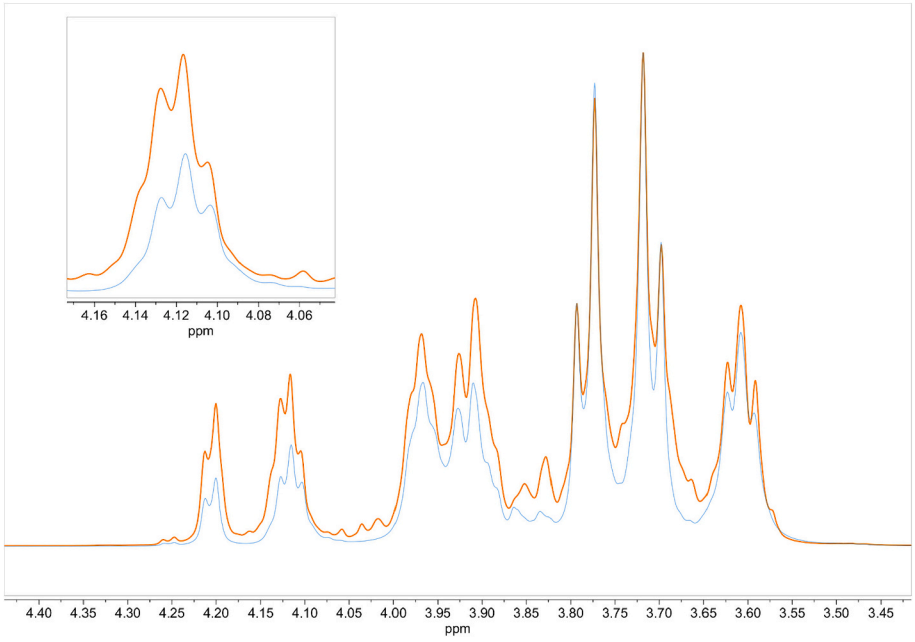

Fig. 6. ^{1}H NMR spectra of EPS (orange) and EPS_Cr(III) (blue).

fermentation. This goal allows for reducing costs associated with natural polymer production with high industrial interest, starting from the valorization of byproducts derived from the sugar beet processing chain, according to sustainability and circular economy approaches.

CRediT authorship contribution statement

Ilaria Finore: Writing – review & editing, Writing – original draft, Visualization, Validation, Investigation, Data curation, Conceptualization, Methodology. **Giovanni Dal Poggetto:** Writing – original draft, Visualization, Validation, Methodology, Investigation, Data curation, Conceptualization. **Luigi Leone:** Methodology, Investigation, Validation. **Andrea Cattaneo:** Writing – original draft, Visualization, Validation. **Barbara Immirzi:** Validation, Methodology, Investigation. **Maria Michela Corsaro:** Investigation, Methodology, Validation. **Angela Casillo:** Investigation, Methodology, Validation. **Annarita Poli:** Writing – original draft, Methodology, Conceptualization, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijbiomac.2024.131664.

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