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Macromolecular and solution properties of Cepacian: the exopolysaccharide produced by a strain of *Burkholderia cepacia* isolated from a cystic fibrosis patient

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Abstract

Light scattering and viscosity measurements were carried out on the previously chemically characterised exopolysaccharide produced by a strain of *Burkholderia cepacia* isolated from a cystic fibrosis patient. The same exopolysaccharide was also produced by other clinical strains in different laboratories. Therefore, the name Cepacian is now proposed for this exopolysaccharide. Experiments performed as a function of the ionic strength on the native polymer revealed a change in the overall shape of the polymer at low ionic strength. This behaviour was absent in the de-acetylated sample. Potentiometric titrations and light scattering experiments carried out on the acidic form of the native polymer revealed the formation of macromolecular aggregates with a stoichiometry n and $2n$ stabilised by interactions involving the uronic acid residues.

Keywords: Bacterial polysaccharide; Cystic fibrosis; Light scattering; Potentiometric titration; Viscosity

1. Introduction

Cystic fibrosis (CF) is a genetic disease that causes abnormalities affecting the exocrine glands with the consequent malfunction of both respiratory and digestive tracts. The deterioration of lung function favours chronic infections by opportunistic bacterial pathogens such as *Staphylococcus aureus*, *Haemophilus influenzae* and *Pseudomonas aeruginosa*. In the early 1980s, *Burkholderia cepacia* was found in clinical isolates from patients in North American CF Centres and it was immediately clear of the dangerousness of this bacterium.[1] In fact, *B. cepacia* chronic infections could lead to necrotizing pneumonia and subsequent death of the patient, a clinical picture known as '*B. cepacia* syndrome'. In addition, both the patient-to-patient transmission and the characteristic multi-drug resistance of *B. cepacia* left clinicians without an effective antimicrobial therapy.

A number of investigations carried out on *Pseudomonas aeruginosa* and its exopolysaccharide (EPS), alginate, pointed to the role of the polymer in sustaining bacterial infection with different possible mechanisms. Alginate may be an important mediator of cell adhesion. In fact, adherence can be inhibited by addition of monoclonal antibodies specific for bacterial alginate.[2] High production of alginate may be due to a starvation situation experienced by the bacteria in the lung environment. The mucus may then function as a sieve or a trap which concentrates nutrients.[3] Last but not least, alginate may be able to disrupt the normal regulatory immune mechanism accounting for the

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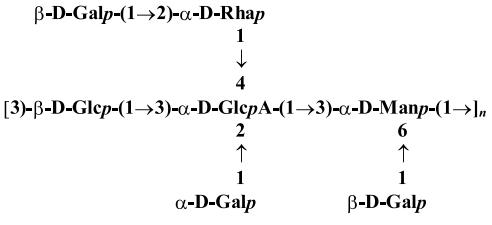
persistence of mucoid *P. aeruginosa* strains and elevated immunoglobulin levels.[4]

All the above evidence is obviously connected with the physico-chemical properties of alginate so that a large number of investigations have been carried out on the structure–function relationships and in particular on the ability of alginate to produce highly viscous solutions and eventually aqueous gels.[5] It has to be remembered that the presence of divalent cations, and particularly calcium ions, results in aggregation of alginate chains that form an extended physically cross-linked network made of both secondary structure and folded chain segments.[6]

Much less is known about the chemistry, biochemistry and the biological activity of EPS's produced by *B. cepacia*. An investigation carried out in our laboratories[7] on the EPS produced by a clinical strain isolated in Portugal[8] defined the primary structure of the polymer, which is depicted in Scheme 1. For this polymer we propose the trivial name '**Cepacian**'. The polymer backbone included acetyl substituents that varied in number depending on the bacterial culture conditions. The EPS investigated in this work had a degree of acetylation equal to one per repeating unit.

An identical structure was found for the EPS isolated from a French[9] and North American CF patients,[10] thus suggesting that *B. cepacia* produces only one type of polysaccharide. However, a screening carried out in the Trieste laboratory on different *B. cepacia* clinical strains pointed out at the capacity of this bacterium to biosynthesize different EPS's.[11] In fact, two strains typed as belonging to genomovars I and IIIA produced a galactan which contains also a 3-deoxy-α-D-*manno*-oct-2-ulopyranosonic acid (Kdo) residue (data from Trieste laboratory), similar, if not identical, to that produced by *Burkholderia pseudomallei*.[12]

This paper reports on the physico-chemical and macromolecular characterisation of the polysaccharide **Cepacian** with the aim to start the elucidation of the structure-function relationships of *B. cepacia* EPS's. To do so, experimental data from viscometric, light scattering and potentiometric measurements were analysed in terms of structure–properties relationships.



Scheme 1. Repeating unit of **Cepacian**. The structure includes one acetyl group per repeating unit whose position is unknown.

2. Results and discussion

The main feature of the primary structure of **Cepacian** polysaccharide is the presence of a tri-substituted glucuronic acid residue in the repeating unit (see Scheme 1). It is easy to hypothesise that this configuration produces a situation of steric hindrance around the residue that might strongly hamper polymer chain flexibility with effects on both conformational characteristics and viscous properties. In addition, it is reported in the literature[13] that the sequence of (1→3) glycosidic bonds produced a rather elongated polymer backbone as happens in scleroglucan or laminaran.[14] In particular, scleroglucan in aqueous solutions is able to give a triple helix structure.[15]

2.1. Characterisation of the polymer Na^+-salt form

As a first step, the characterisation of the **Cepacian** was carried out on the sodium salt form as obtained from bacterial cultures after purification. Since ^{1}H NMR spectra[7] (Fig. 1) showed the presence of one acetyl group per repeating unit, the macromolecular characterisation was carried out both on the native and on the de-acetylated sample.

The molecular weight (MW) and the intrinsic viscosity ($[\eta]$) data of **Cepacian** are shown in Table 1. The last column of Table 1 contains the stiffness parameter (B) as defined, for polyelectrolytes, by Smidsrød and Haug[16] on the basis of intrinsic viscosity data: the higher the B value, the lower the rigidity of the polymer chain. In Ref. 16, the value of B was defined as $B = S/[\eta]_{0.1}^{\nu}$, where S is the slope of the curve $[\eta]$ versus $1/\sqrt{I}$ (see Fig. 4), I is the ionic strength, $[\eta]_{0.1}$ is the intrinsic viscosity at 0.1 M ionic strength and ν is equal to 1.3. As can be seen from the data reported in Table 1, the de-acetylation procedure did not appreciably decrease the polysaccharide degree of polymerisation. The slightly lower MW values of the de-acetylated sample can be entirely attributed to the removal of the

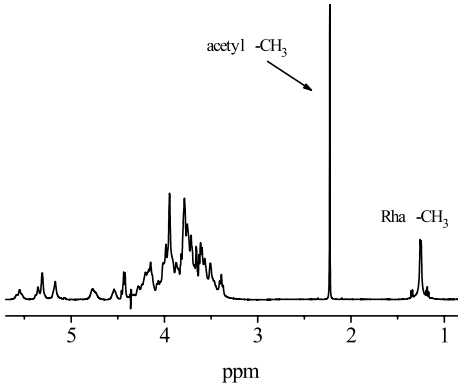


Fig. 1. Five hundred megahertz ^{1}H NMR spectrum of **Cepacian** polysaccharide.

Table 1
Weight average molecular weight ($\langle MW \rangle_w$), intrinsic viscosity at infinite ionic strength ($[\eta]_{I=\infty}$) and chain stiffness parameter[16] of native and de-acetylated Cepacian

Sample	$\langle MW \rangle_w$ (g/mol)	$[\eta]_{I=\infty}$ (mL/g)	Stiffness ***B*** parameter
Native Cepacian	781,000 ± 55,000	513 ± 3	0.059
De-acetylated Cepacian	722,000 ± 46000	447 ± 3	0.065

$-COCH_3$ groups along the polymer chain. However, de-acetylation slightly changed the flexibility of the polysaccharidic chain as indicated by both the intrinsic viscosity and the stiffness parameter. It is interesting to compare the *B* stiffness parameter of **Cepacian** with those reported in the literature for other polysaccharides[16] of similar molecular weight. This is shown in Table 2 where dextran sulfate represents a rather flexible polymer with a high *B* value and DNA represents a typical very stiff polymer exhibiting a low *B* parameter. The EPS from *B. cepacia* exhibited a value included between those of alginate and carboxymethylcellulose that are considered as semiflexible polymers.

The polyelectrolyte nature of **Cepacian** suggested a possible role of the ionic strength in driving conformational transitions of the disorder-to-order type. Circular dichroism experiments carried out on **Cepacian** aqueous solutions in an ionic strength range from 0 to 1.0 M $NaClO_4$ did not show any variation of the dichroic band in the 190–230 nm range (Fig. 2), attributed to the presence of both carboxylate and acetyl groups. More information about the conformation assumed by the polysaccharidic chain was obtained analysing the radius of gyration of both the native and the de-acetylated polymers as a function of the ionic strength (see Fig. 3). The radius of gyration of the native polymer did not change significantly upon decreasing the ionic strength to a value lower than 0.1 M. Beyond that value, the radius of gyration increased abruptly. These experimental findings indicated an expansion of the hydrodynamic radius of the polymer chain conformation triggered by the screening of the charged carboxylate groups exerted by the salt cations. The expansion may be due to a loss

Table 2
Stiffness *B* parameters of different polysaccharides[16]

Sample	Stiffness ***B*** parameter
Dextran sulfate	0.230
Carboxymethylcellulose (DS = 1) [a]	0.065
Alginate [b]	0.040
DNA	0.006

[a] DS = Degree of substitution.
[b] The value refers to a seaweed alginate produced by *Laminaria digitata* (Mannuronic acid to Guluronic acid ratio = 1.6).

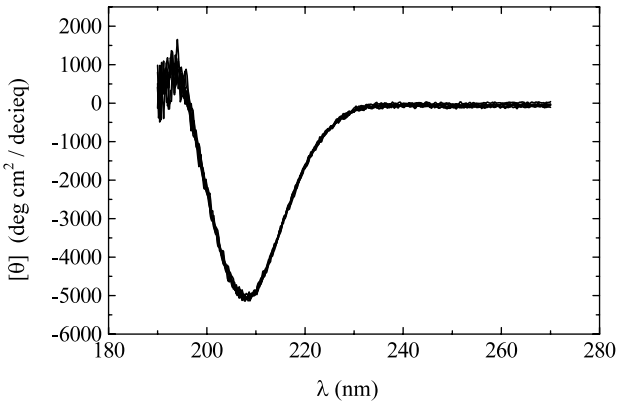


Fig. 2. Circular dichroism spectra of the native **Cepacian** as a function of the ionic strength in the range 0–1.0 M $NaClO_4$.

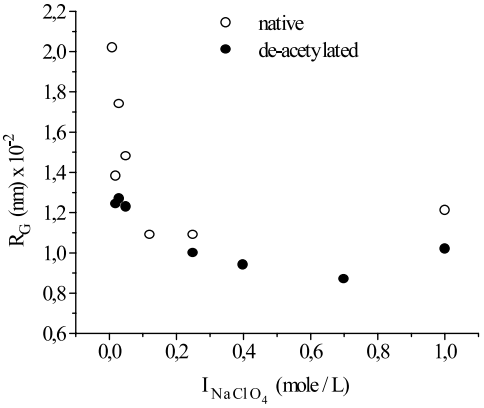


Fig. 3. Radius of gyration of **Cepacian** as a function of the ionic strength for the native and the de-acetylated sample.

of freedom of rotational degrees attributable to some of the glycosidic bonds. This change does not necessarily imply the gain of a defined secondary structure, thus accounting for the lack of spectral variations in the circular dichroism investigations. Alternatively, a defined secondary structure actually might exist, but the distance between two consecutive carboxylate groups is large enough to inhibit the set up of exciton effects. Consequently, the dichroic effect is entirely due to the local mean electronic environment experienced by the chromophores. In this respect, conformational changes of the backbone caused by variation of ϕ and ψ conformational angles far enough from the chromophore, might not affect its electronic environment.

Contrary to the native polymer, the de-acetylated EPS exhibited low variations of its radius of gyration as a function of the ionic strength (Fig. 3).

As reported for the stiffness B parameter, the comparison of the radius of gyration of different polymers may help to understand the solution behaviour of the **Cepacian**. This should be done by comparing the characteristic ratio of the different polymers using its relationship with the mean radius of gyration: ($C_x = 6\langle R_g^2\rangle/\langle N\rangle L^2$). However, the previous relationship holds for unperturbed random coils. As suggested by Urbani and Brant,[21] in the absence of this condition it is more appropriate the comparison of the experimental parameter $\langle R_g^2\rangle/\langle N\rangle_w$, where $\langle N\rangle_w$ is the weight average mean number of sugar residues in the polymer chain which is obtained from the $\langle M\rangle_w$ value. For the de-acetylated **Cepacian** the $\langle R_g^2\rangle/\langle N\rangle_w$ ratio was 5.3 nm^2. The literature[17] reports the values 7.5 nm^2 for the rather stiff polymer Xanthan and 0.9 nm^2 for the more flexible soluble barley glucan thus confirming the rigid nature of the **Cepacian** backbone.

Intrinsic viscosity measurements of the native polymer solutions as a function of the ionic strength showed a behaviour similar to that described for the radius of gyration. In fact, the plot of the intrinsic viscosity as a function of the inverse of the square root of the ionic strength (Fig. 4) showed a neat departure from linearity for the native sample evidenced as a sigmoid shape around $1/\sqrt{I} = 7$ ($I = 0.02$). The linear behaviour of the intrinsic viscosity of a polyelectrolyte as a function of the ionic strength and in the absence of conformational changes was described.[18] Any deviation from linearity can be interpreted as a change in the effective charge density. This may have its source in conformational changes.[19]

Fig. 4 also shows the intrinsic viscosity data for the de-acetylated sample. According to the findings relative to the radius of gyration, no sigmoid trend was detected indicating a regular and continuous expansion of the polymer coil when decreasing the ionic strength.

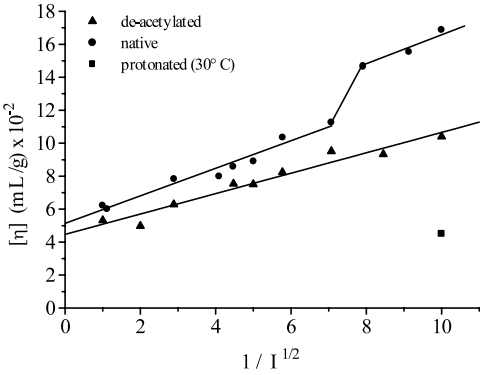


Fig. 4. Plot of the intrinsic viscosity ([η]) as a function of the inverse of the square root of the ionic strength (I) for native and de-acetylated **Cepacian**. (■) Intrinsic viscosity value relative to the protonated **Cepacian** sample as obtained after dialysis at 30 °C (see text).

2.2. Characterisation of the polymer as function of pH

A variation of the polymer chain charge density can be obtained also lowering the solution pH thus leading to protonation of the carboxylate groups present in the polymer repeating unit. Potentiometric titrations can give information on possible pH-induced conformational transitions. In fact, the charge density variation during the titration causes a continuous change of the pK value. A pH-induced conformational transition leads to an additional variation of the polymer charge density, resulting in 'bumps' in the curve of pK plotted against the degree of protonation.[20] Therefore, the native **Cepacian** was exchanged into the acidic form. Although the aim of the experiments was the detection of possible conformational transitions, the data obtained drove us to a different and unexpected direction.

The proton exchange procedure was carried out at 5 °C in order to avoid polymer degradation and the potentiometric titration was carried out adding 0.1 M NaOH solution at 25 °C. The titration curve is shown in Fig. 5. The evaluation of the equivalents of added NaOH indicated a molar mass for the repeating unit, reported as mass per equivalent, considerably higher than that indicated by the formula in Scheme 1: 1537 g per equiv instead of 1175 g per equiv, that corresponds to the theoretical one. This evidence prompted us to carry out further potentiometric measurements on samples exchanged into the acidic form at 15 and 30 °C. The titration curves of these samples are also shown in Fig. 5. As it can be easily seen, the sample exchanged at 15 °C showed a titration curve very similar to that one exchanged at 5 °C, whilst the sample exchanged at 30 °C required a higher amount of NaOH to reach the equivalence point. The calculation of the molar mass for the repeating unit of the sample

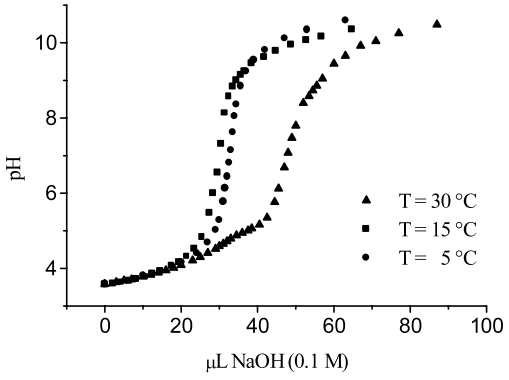


Fig. 5. Titration curves obtained for native **Cepacian** after proton exchange carried out at different dialysis temperatures.

exchanged at 30 °C gave a value in very good agreement with the formula in Scheme 1: 1157 g per equiv.

The comparison of the equivalent weight values obtained on the samples dialysed at 5 and 30 °C, respectively, suggested that one third of the carboxylic acid groups showed a p*K* different from that of the remaining ones. In addition, it was impossible to titrate that part of the carboxylic group when analysing the polymer dialysed at 5 and 15 °C.

The above experiments indicated that the proton exchange at a temperature lower than 30 °C either rendered some of the carboxylate groups not accessible to protons or caused the protonated carboxylic groups to become part of a structure not reachable by the titrating OH^- species. We have no evidence to select one of the two hypotheses, although the very small radius of protons could play in favour of the second one. A situation where protonated groups are not accessible to basic titrating groups is rather common in protein tertiary structures where such groups can be deeply buried in internal cavities or in protected active sites. However, the phenomenon is not common at all in polysaccharidic systems.

In order to elucidate possible structures assumed by the **Cepacian** polysaccharide, a light scattering investigation was carried out on the protonated samples prepared at different temperatures. The results, shown in Table 3, clearly indicated that a polymer aggregation was obtained at the lowest temperature of proton exchange and the ratio of the different molecular weight obtained were 2 and approx 4 passing from 30 to 15 °C and from 30 to 5 °C, respectively. A confirmation of the aggregation behaviour was attained measuring the intrinsic viscosity of a solution of the protonated form of the polymer obtained at 30 °C. As shown in Fig. 4, the experimental value was markedly lower than those obtained for the Na^+ salt form at the same ionic strength. Assuming that the form stable at 30 °C was a single chain one, the forms stable at 15 and 5 °C must be a dimer and a tetramer, respectively. It is interesting to note that the molecular weight value found for the sodium salt form of the native polymer (see Table 1) was in agreement with the dimeric form, thus indicating that also sodium cations may induce polymer aggregation.

Table 3
Weight average molecular weights ($\langle MW \rangle_w$) and gyration radii (R_g) of protonated samples obtained at different temperatures

Temperature (°C)	$\langle MW \rangle_w$ (g/mol)	R_g (nm)
5	1,550,000 ± 150,000	150
15	674,000 ± 67,000	120
30	337,000 ± 33,000	70

In conclusion, it is interesting to evaluate the data reported with respect to the possible role of **Cepacian** in *B. cepacia* infections. The experimental results strongly indicated the formation of polymer aggregation resulting in production of rather highly viscous solutions. This property may create an environment around the bacterial cells capable of influencing the infection maintenance by providing a protected niche suitable for bacterial survival in the CF patient lungs.

3. Experimental

3.1. Polysaccharide production, purification and characterisation

B. cepacia IST408 was cultivated in the S liquid medium described before.[8] After 3 days of growth at 30 °C with orbital agitation (250 rev/min), cells were removed by centrifugation and the EPS produced was precipitated from the cell-free supernatant by the addition of 2.5 volumes of cold ethanol. The ethanol-precipitable material was air-dried, dissolved in de-ionised water, dialysed (membrane cut-off 12,000 Da) against de-ionised water and freeze-dried.

This material was later precipitated in four volumes of isopropanol and subsequently dissolved in tri-distilled water. This procedure was repeated three times. The solution of EPS was then dialysed first against NaCl (0.1 M) and then against de-ionised water, the pH adjusted to neutrality and the polymer was recovered by freeze-drying. No proteins and nucleic acids were present, as revealed by the UV spectrum of the EPS solution. The final yield was 33% with respect to the carbon source added to bacteria culture (glucose).

The de-acetylated polysaccharide was prepared by stirring an aqueous solution of the native polymer (0.1%) under nitrogen atmosphere for 5 h in NaOH (0.01 M) at room temperature.[21] After dialysis and neutralisation, the de-acetylated EPS was recovered by freeze-drying. The efficiency of the acetyl groups removal was checked by NMR analysis. An average content of 11% water of hydration was estimated in the lyophilised samples, as detected by means of thermal analysis. All polymer solutions were prepared by weighing the sample and subtracting the hydration water.

3.2. Macromolecular characterisation

Viscometric measurements were carried out by using automatic Schott-Geräte equipment with a Cannon-Ubbelohde suspended level capillary viscometer (diameter 0.53 mm) immersed in a Schott-Geräte water thermostat (20.0 ± 0.1 °C). Solutions of both the native and the de-acetylated EPS were prepared by stirring the

polymers in milli-Q water for at least 3 days. A certain volume of a concentrated $NaClO_4$ solution was added in order to have the final desired ionic strength. The salt solutions of the polymers were equilibrated in a constant-volume cell by dialysing against salt solutions at the same concentration, using 12,000 Da cut-off membranes. In all cases the outer dialysis solutions were used as solvents in the dilution procedures. Intrinsic viscosity data were obtained by linear regression of reduced specific viscosities, η_{sp}/C (mL/g), as a function of polymer concentration. The Hagenbach–Coutte correction for kinetic effects was always performed. The rather high intrinsic viscosity of **Cepacian** solutions (Fig. 4) might rise the question about possible shear dependence effects on experimental data. However, the low polymer concentration (0.3 g/L in the starting solution; 0.09 g/L after the final dilution) and the constancy of the capillary diameter for all the measurements safely assured that these possible effect influenced all the data in a similar way.

Light scattering measurements were carried out using a Brookhaven Instruments BI200SM photogoniometer with an Innova-70 Argon ion laser as incident source (tuned to $\lambda = 488$ nm). Total counts were recorded over the angular range 30–150° with an EMI-9865A photomultiplier and a Brookhaven BI-2030 correlator. The mean number of counts averaged over 5 successive counting periods was always used at each angle and polymer concentration. Toluene was used as calibration liquid and the differential refractive index increment was approximated as 0.141 mL/g. Simultaneous linear least squares fits to both the angular and concentration dependence of scattering data were employed in the Zimm plot analysis. Solutions were prepared as described above for viscometric experiments. The samples were then filtrated two times through 0.45 μm Millipore filters directly in carefully cleaned borosilicate cylindrical scattering cells in order to avoid extraneous scattering due to dust contamination. Filled cells were suspended in the goniometer with decalin (decahydronaphtalene) as the thermostatic liquid. The polymer concentration range was usually 0.5–0.05 g/L and the least concentrated solutions displayed scattering intensities 5–10 times higher than that of solvent. The Zimm plots did not show any anomalous behaviour and the second virial coefficients were positive in the range 0–0.8×10^{-6} cm^3 mol g^{-2}.

Potentiometric titrations were carried out using a Radiometer PHM240 pH-meter and a glass electrode (Metrohm) calibrated with standard buffers at pH 4.01 and 7.00. Aqueous solutions of the native EPS in the free acid form were prepared by dissolving the Na^+ salt form of the polymer in water for a minimum of 3 days. The solutions were subsequently dialysed against acetic acid (0.1 M) and then against distilled water. Proton exchange dialyses were carried out, separately, at 5, 15 and 30 °C. Potentiometric titrations of the EPS solutions in the free acid form were performed at 25 °C with NaOH (0.1 M).

Circular dichroism measurements were performed using a JASCO J-710 spectropolarimeter in the temperature range 20–80 °C. The solutions to be analysed were prepared by mixing a 2×10^{-3} equiv/L EPS solution (the polymer concentration is given in terms of molarity of carboxylate groups) with a $NaClO_4$ solution (2 M) in order to obtain solutions with the desired ionic strength. The molar ellipticity $[\theta]$ values were calculated on the basis of the polymer concentration expressed in equiv/L.

^{1}H NMR spectra were run on a 500 MHz Varian UNITY INOVA spectrometer at a probe temperature of 80 °C. Before recording the NMR spectra, the EPS samples (1 g/L) were sonicated in an ice bath with 5 bursts of 1 min each, separated by 1 min intervals using a Branson sonifier equipped with a microtip at 2.8 A, in order to decrease the molecular weight of the polysaccharides.

Acknowledgements

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