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Structural characterization of the uncommon polysaccharides obtained from *Peltigera canina* photobiont *Nostoc muscorum*

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ABSTRACT

Cellular and extracellular polysaccharides of the lichenized fungus *Peltigera canina* photobiont *Nostoc muscorum* (UTEX-2493) were evaluated. From EPS a linear (1 → 4)-linked β-D-xylan was chemically characterized, and because of this and other findings involving lichenized fungi it could be proposed that this polymer may be typical of photobionts isolated from lichen thalli. From cellular biomass extracts a complex polysaccharide formed mainly of L-arabinose (2,3-*O*-Me_2-, 24.8%) and D-xylose (2,3-*O*-Me_2-, 10.6%) both units (1 → 4)-linked was described. A smaller part of this structure has β-L-Ara*p* and β-D-Xyl*p* units branched at O-3 by α-D-Man*p* units, which in turn presents O-3 or O-2 single-unit substitution by α-D-Man*p* or α-L-Fuc*p* as non-reducing ends. Arabinosyl residues were found to be in the β-L-arabinopyranosyl form, different from the most common form described in plant cell wall polysaccharides, α-L-arabinofuranosyl (Ara*f*). Both polymers characterized in this work were not previously described when analyzing *Nostoc* sp. or cyanobacteria in general.

1. Introduction

Like many microorganisms, cyanobacteria also produce polysaccharides (Slodki, 1987; Sutherland, 1987; Zevenhuizen, 1987), which could be cellular or extracellular. While some other microbial polysaccharides have been extensively studied (Sutherland, 1985, 1987; Whitfield, 1988), cyanobacterial polysaccharides are reported in literature mainly for the determination of sugar polymers composition. Among these studies the majority is related to water-soluble released polysaccharides (RPSs), including in this group capsule and slime polysaccharides that may be released as water-soluble material into the surrounding medium, and the properly named exopolysaccharides (EPS). This new field of possible exploitation of cyanobacteria has arisen in the 80's decade by the growing industrial interest towards polysaccharides of microbial origin, that often show advantages over the polysaccharides extracted from plants or marine macroalgae (De Philippis & Vincenzini, 1998).

One of the cyanobacteria studied regarding to its polysaccharides content was the free-living *Nostoc muscorum* (Biswas, 1957). In this study the author determined the monosaccharidic composition of a polysaccharide isolated from *N. muscorum* cell wall, describing a polymer made up of glucose, galactose, arabinose, xylose, ribose, rhamnose and two non-identified sugars (Biswas, 1957). Another study refers to the purification and properties of glycogen isolated from this microorganism (Chao & Bowen, 1971). This same *Nostoc* specie could be isolated as photobiont from the lichen thalli of *Peltigera canina* (Ahmadjian, 1989; Koriem & Ahmadjian, 1986), but its polysaccharide content has not been evaluated.

Due to the fact that the structure and properties of cyanobacteria polysaccharides are described only in a small number of studies, the aim of this work was the structural characterization of *Nostoc muscorum* photobiont polysaccharides either cellular or extracellular. One of the most important prerequisites of a polysaccharide is that it must possess, together an adequate composition, structure (Margaritis & Pace, 1985), and molecular mass (M_w) (Shepherd, Rockey, Sutherland, & Roller, 1995), which could be achieved through the structural characterization of polysaccharides. This knowledge could determine many of the properties generally considered useful for polysaccharides industrial utilization (i.e. high viscosity of its aqueous solutions, capability of forming gels with good tensile strength, stabilising emulsions, etc.).

2. Materials and methods

2.1. Biological material and growth conditions

Nostoc muscorum (UTEX-2493), phycobiont of lichen *Peltigera canina* (Koriem & Ahmadjian, 1986); identified by D. Mollenhauer

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was obtained as pure culture from UTEX the Culture Collection of Algae (University of Texas at Austin).

The strain was cultivated photoautotrophically at 25 °C, illuminated with cool white fluorescent light, in 2.0 L Erlenmeyers flasks containing BG11 medium (1 L) (Rippka, 1988) for 2 weeks. After this time the culture was harvested by filtration through a filter paper and the filtrates were used for EPS analyses. Freshly harvested cells were washed (3×) for total EPS removal of the cells.

2.2. Extraction and purification of EPS

The cell-free supernatant fraction was evaporated to a small volume, dialyzed against tap water in an 8 kDa molecular weight cut-off membrane. The retained material was frozen and then allowed to thaw slowly giving rise to an insoluble material (IENm) were removed by centrifugation at 8000 rpm for 15 min, at 15 °C.

2.3. Extraction and purification of cell biomass polysaccharide

Cells biomass (3 g) was submitted to extraction with H_2O at 100 °C for 6 h (6×, 500 mL) The combined aq. extracts were evaporated to a small volume with the polysaccharide precipitated by addition to excess EtOH (3:1; v/v). The resulting polysaccharide precipitates were dissolved in H_2O, and dialyzed against tap water, giving rise to fraction HW-Nm. This fraction was frozen and then allowed to thaw slowly, resulting an insoluble material that was removed by centrifugation at 8000 rpm for 15 min, at 15 °C. The cold water-soluble fraction (HW-Nm) was purified by successively dialysis through membranes of 100 and 16 kDa M_r cut-off (Spectra/Por® Cellulose Ester) giving rise to eluted and retained (RFS-Nm) material.

2.4. Monosaccharide composition of polysaccharide fractions

Monosaccharide compositions were determined by hydrolysis of the polysaccharide samples (1 mg) with 45% CH_2O_2 (formic acid) at 100 °C for 14 h. The hydrolyzed samples were converted to alditol acetates (GC–MS) by successive $NaBH_4$ reduction, and acetylation with Ac_2O-pyridine (1:1, v/v) following the method described by Sassaki et al. (2008).

The resulting alditol acetates were analyzed by gas chromatography–mass spectrometry (GC–MS) using a Varian model 3300 gas chromatograph linked to a Finnigan Ion-Trap, model 810 R-12 mass spectrometer, using a DB-225 capillary column (30 m × 0.25 mm i.d.), with helium as carrier gas. The analysis was carried out from 50 to 220 °C at 40 °C min^{-1}, maintaining the temperature constant to the end of analysis (30 min). The products were identified by their typical retention times and electron impact profiles.

2.5. Methylation analysis

Per-*O*-methylation of polysaccharide fractions was carried out by the method of Ciucanu and Kerek (1984). Samples (10 mg) were dissolved in dimethyl sulfoxide (1 mL), and powdered NaOH (20 mg) and iodomethane (CH_3I) (1 mL) were added. After 30 min at 25 °C with vigorous stirring, the mixture was maintained overnight at 25 °C. The reaction was interrupted by addition of water, neutralization with HOAc, dialysis against distilled water and freeze-drying. The products were submitted to one more cycle of methylation, and the products were isolated by partition between $CHCl_3$ and water. The per-*O*-methylated derivatives from the lower layer (2 mg) were hydrolyzed with 45% aqueous formic acid (1 mL) for 12 h at 100 °C, followed by NaB^2H_4 reduction and acetylation as above (item 2.4), to give a mixture of partially *O*-methylated alditol acetates, which was analyzed by GC–MS using a DB-225 capillary column (30 m × 0.25 mm i.d.), programmed from 50 to 210 °C at 40 °C min^{-1}, the total analysis time being 36 min.

2.6. Partial acid hydrolysis of heteropolysaccharide (RFS-Nm fraction)

Fraction RFS-Nm (300 mg) was submitted to sequential partial acid hydrolysis with 0.16 M aq. H_2SO_4 (10 mL) at 100 °C for 1 h, residual material of the first hydrolysis was submitted to a new partial hydrolysis with the same conditions cited above, giving rise to fraction which was submitted to the last hydrolysis method either the previously ones. The neutralized solutions were dialyzed against tap water using membranes with a size-exclusion of 2 kDa, and the retained materials were freeze dried giving rise to HP1, HP2 and HP3 fractions, respectively.

2.7. Determination of homogeneity and molar mass (M_w) of purified fraction

Determination of homogeneity and molar mass (M_w) were performed on a Waters high-performance size-exclusion chromatography (HPSEC) apparatus coupled to a differential refractometer (RI) and a Wyatt Technology Dawn-F Multi-Angle Laser Light Scattering detector (MALLS). Waters ultrahydrogel columns (2000, 500, 250 and 120) were connected in series and coupled with multidetection equipment, using a $NaNO_2$ solution (0.1 M) as eluent, containing 0.5 g/L NaN_3. The polysaccharide solutions (1 mg/mL) were dissolved in the same solvent and filtered through a nitrocellulose membrane (Millipore), with pores of 0.22 or 0.45 μm. HPSEC data were collected and analyzed by the Wyatt Technology ASTRA program. The specific refractive index increment (dn/dc) was determined using a Waters 2410 detector. All experiments were carried out at 25 °C.

2.8. Nuclear magnetic resonance spectroscopy

NMR spectra (^1H, ^{13}C, DEPT, HMQC) were obtained using a 400 MHz Bruker model DRX Avance spectrometer incorporating Fourier transform. ^{13}C NMR (100.6 MHz) analyzes were performed at 70 °C, with the samples being dissolved in D_2O or Me_2SO-d_6 according to fraction solubility. The chemical shifts are expressed in ppm (δ) relative to acetone at δ 30.20 and δ 2.22, or to Me_2SO-d_6 at δ 39.70 and δ 2.40, for ^{13}C and ^1H signals, respectively.

3. Results

3.1. Structural characterization of the xylan obtained from EPS fraction

The EPS (1 g) obtained after culture media dialysis against tap water through an 8 kDa molecular weight cut-off membrane was submitted to freeze/thawing purification process, giving rise to an insoluble fraction (IENm, 46.3 g%) containing only xylose as monosaccharide component, according to derived GC–MS alditol acetates. ^{13}C NMR experiment showed the presence of five ^{13}C signals, consistent with a linear pentose homopolymer (Fig. 1). The anomeric region showed one signal at δ 101.8, which suggests D-Xyl*p* units having a β-glycosidic configuration and one at δ 76.6 corresponding to *O*-substituted C-4 (Gorin, 1973). The β-glycosidic configuration was suggested by the presence of a high-field H-1 signal at δ 4.27 when analyzed in Me_2SO-d_6 (Gorin, Spencer, & Bhattacharjee, 1969), present in its HMQC spectrum (Table 1), being confirmed by values of the coupling constants $J_{C\text{-}1,H\text{-}1}$ = 163.7 Hz found in $^1H/^{13}C$ coupled HMQC spectra. The others ^{13}C signals at δ 73.9, 72.9 and 63.2 could be attributed to C-3, C-2 and C-5, respectively (Fig. 1). The DEPT NMR spectrum of this xylan

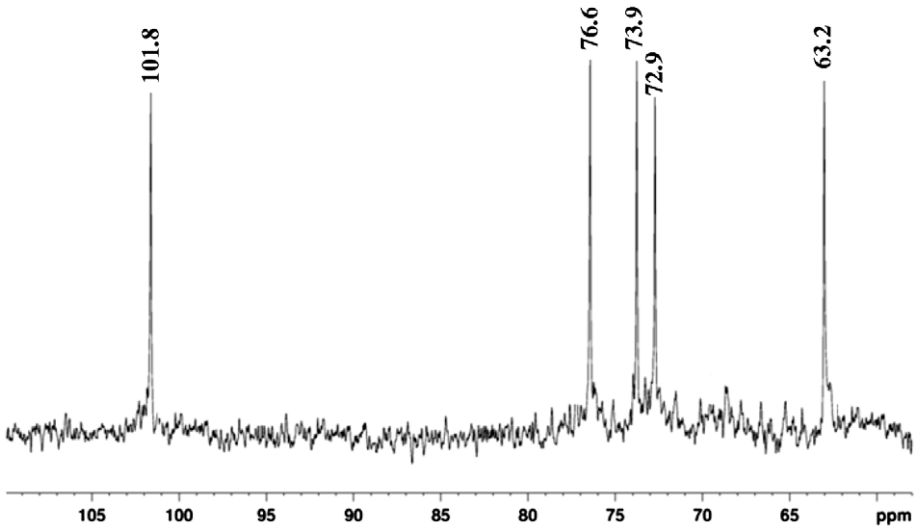

Fig. 1. ^{13}C NMR spectra of IENm fraction obtained at 70 °C in Me_2SO-d_6 (chemical shifts are expressed in δ, ppm).

contained an inverted $-CH_2$ signal at δ 63.2, arising from unsubstituted C-5.

Methylation analysis showed the presence of non-reducing ends (0.3%) and 4-*O*-substituted (99.7%) Xyl*p* units, which confirmed the homopolymer structure suggested by ^{13}C NMR spectrum as a (1 → 4)-linked β-D-xylan.

3.2. Structural characterization of the heteropolysaccharide obtained from cell biomass fraction

Cells biomass resulting aqueous extract was submitted to polysaccharide precipitation by addition of excess EtOH (3:1; v/v) giving rise to fraction HW-Nm (43.5 g% yield), which was subjected to freezing, followed by gentle thawing was further purified by closed dialysis. First through a 100 kDa M_r cut-off membrane (Spectrapor®), giving rise to an eluted and a retained material. The latter was submitted to a new dialysis, through a 16 kDa M_r cut-off membrane (Spectrapor®), resulting in an eluted and a final retained fraction, named RFS-Nm (10.7 g% yield).

RFS-Nm fraction was analyzed by HPSEC-MALLS showing the presence of a symmetrical peak in its elution profile, indicative of a homogeneous fraction, which molar mass was determined to be 59 kDa (d*n*/d*c* 0.143). Moreover this fraction contains arabinose (57%), mannose (16%), xylose (14%) and fucose (13%) as monosaccharide constituents.

^{13}C NMR spectrum of RFS-Nm fraction show at least five ^{13}C signals at anomeric region at δ 101.0, 100.9, 100.1, 99.0 and 98.5 (Fig. 2). The absence of ^{13}C signals at lower field than δ 101.0 suggests that L-arabinose units were in β-configuration and in pyranosidic conformation (Gorin & Mazurek, 1975). The presence of L-fucose could be confirmed because of a signal at δ 15.4, characteristic of $-\underline{C}H_3$ group.

Table 1
1H and ^{13}C chemical shifts (δ) of RFS-Nm fraction.[a]

H and C nuclei	Chemical shifts (ppm)[a]	
	^1H (δ)	^{13}C (δ)
H-1/C-1	4.25	101.6
H-2/C-2	3.15	72.7
H-3/C-3	3.27	73.7
H-4/C-4	3.55	76.4
H-5 and H-5′/C-5	3.18/3.86	63.0

[a] Data obtained from ^1H (sample dissolved in Me_2SO-d_6), ^{13}C and HMQC experiments.

The DEPT NMR spectrum of RFS-Nm fraction contained only inverted $-CH_2$ signals at δ 62.6, 62.2 and 61.2 arising from unsubstituted C-5/C-6, suggesting the absence of substitution in these positions.

Based on methylation analysis (Table 2) it could be suggested that the RFS-Nm heteropolysaccharide is mainly formed of L-arabinose (2,3-*O*-Me_2-, 24.8%) and D-xylose (2,3-*O*-Me_2-, 10.6%) both units (1 → 4)-linked. A smaller part of this structure is formed either by β-L-Ara*p* and β-D-Xyl*p* units substituted at O-3 (2-*O*-Me-Ara*p* and 2-*O*-Me-Xyl*p* 14.5% and 6.5%, respectively) by α-D-Man*p* units (2,4,6-*O*-, 3,4,6-*O*- and 2,3,6-*O*-Me_3-) having mainly as non-reducing end α-L-Fuc*p* units (2,3,4-*O*-Me_3-, 23.8%) or α-D-Man*p* units (2,3,4,6-*O*-Me_4-, 2.3%) in minor proportions.

In order to elucidate the structure of the heteropolysaccharide, partial acid hydrolysis were carried out, giving rise to polysaccharides HP1, HP2 and HP3 regarding to the sequence of successive hydrolysis of 1 h each.

The residual products (HP1, HP2 and HP3) were analyzed by GC–MS as alditol acetates and present a gradual increase of L-arabinose, and simultaneous decrease of L-fucose and D-manose.

Methylation analysis of HP3 residual product showed a substantial decrease of non-reducing L-Fuc*p* units (3.5%) and 3,4-di-*O*-substituted L-Ara*p* units, besides the absence of 4-*O*-substituted D-Man*p* units, with concomitant increase of 4-*O*-substituted L-Ara*p* and D-Xyl*p* units, suggesting the removal of the non-reducing end-groups of α-L-Fuc*p* and 4-*O*-substituted α-D-Man*p* units, which can possibly be substituting the (1 → 4)-linked L-Ara*p* or D-Xyl*p* units in O-3. HMQC examination (Fig. 3) of the polymeric product (HP3 fraction) contained signals in the anomeric region which correspond to the C-1/H-1 from 4-*O*-substituted β-D-Xyl*p* units (δ 101.0/4.56) and β-L-Ara*p* units (δ 100.9/4.79), and also a signal related to the α-D-Man*p* units (δ 99.2/5.42). The high-field H-1 signals (Fig. 3) indicated that the units of L-Ara*p* and D-Xyl*p* had an β-configuration, being confirmed by values of the coupling constants $J_{C\text{-}1,H\text{-}1}$ 160.1 and 161.7 Hz, respectively, found in $^1H/^{13}C$ coupled HMQC spectra (Perlin & Casu, 1969), while that of D-Man*p* had α-configuration, consistent with $J_{C\text{-}1,H\text{-}1}$ 172.8 Hz.

The 4-*O*- and 3-*O*-substituted signals of β-L-Ara*p* and β-D-Xyl*p* units were present at δ 77.9 and 77.2, and δ 80.0, respectively (Fig. 3). These data were confirmed by methylation analysis due

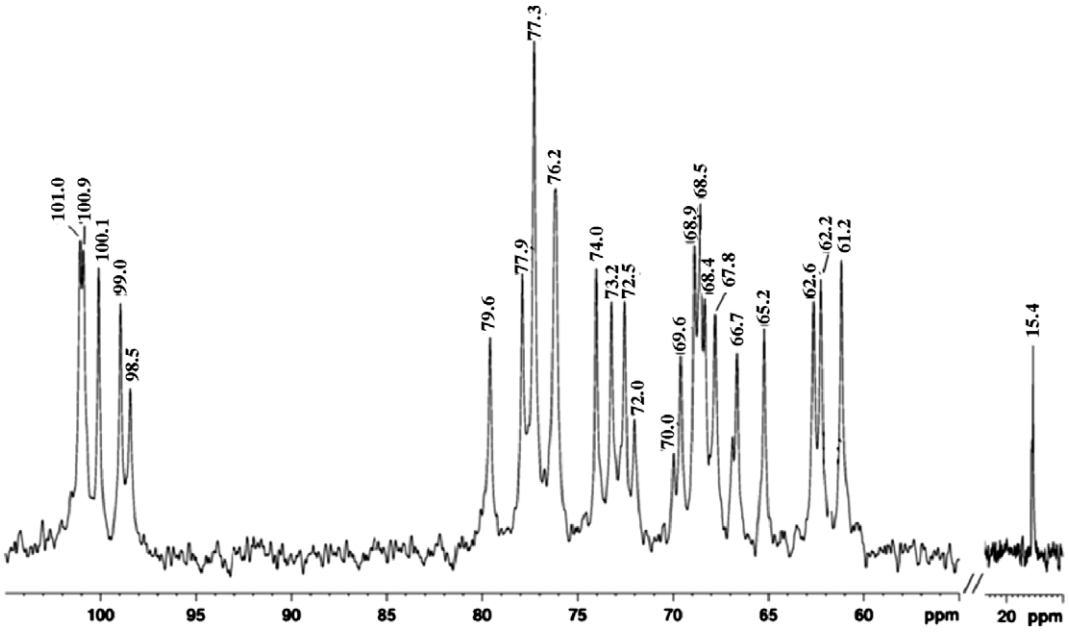

Fig. 2. ^{13}C NMR spectra of RFS-Nm fraction obtained at 70 °C in D_2O (chemical shifts are expressed in δ, ppm).

Table 2
Partially *O*-methylalditol acetates formed on methylation analysis of a heteropolysaccharide and these obtained on partial hydrolysis (HP3 fraction).

O-Me-alditol acetate[a]	t_R[b]	Fraction (%[c])		Linkage type[d]
		RFS-Nm	HP3	
2,3,4-Me_3-fucose	7.473	23.8	3.5	Fucose-(1→
2,3,4,6-Me_4-mannose	8.713	2.3	3.7	Mannose-(1→
2,3-Me_2-arabinose	9.129	24.8	35.7	→4)-Arabinose-(1→
2,3-Me_2-xylose	9.493	10.6	34.8	→4)-Xylose-(1→
2,4,6-Me_3-mannose	11.363	3.3	6.4	→3)-Mannose-(1→
3,4,6-Me_3-mannose	12.045	2.6	5.0	→2)-Mannose-(1→
2-Me-arabinose	12.127	14.5	4.5	→3,4)-Arabinose-(1→
2,3,6-Me_3-mannose	12.270	8.5	–	→4)-Mannose-(1→
2-Me-xylose	13.278	6.5	6.3	→3,4)-Xylose-(1→
3-Me-manose	17.649	3.1	–	→2,4,6)-Mannose-(1→

[a] *O*-Me-alditol acetates obtained by methylation analysis, followed by successive acid hydrolysis, reduction with NaB^2H_4 and acetylation, analyzed by GC–MS (column DB-225).
[b] Retention time in minutes.
[c] Percentage of peak area relative to total peak area.
[d] Based on derived *O*-methylalditol acetate.

to the presence of the derivatives 2,3-di-*O*-Me_2-Ara and -Xyl (35.7% and 34.8%, respectively) and 2-*O*-Me-Ara and -Xyl (4.5% and 6.3%, respectively). The non substituted C-6 of α-D-Man*p* units appears as a doublet at δ 61.2 (δ 3.90, 3.75).

The heteropolysaccharide could be suggested to have a complex structure mainly formed of β-L-Ara*p* and β-D-Xyl*p*-(1 → 4)-linked units, partially substituted at O-3 by α-D-Man*p* units. The latter appeared to be substituted at O-3 or O-2 by single-unit α-D-Man*p* or α-L-Fuc*p* as non-reducing ends.

4. Discussion

Most EPS synthesized by cyanobacteria (about 80% of the polymers) are quite complex, being composed of six or more monosaccharides, differing then to those polymers synthesized by other bacteria or macroalgae, in which the number of monomers is usually less than four (Sutherland, 1990).

Most of the studies on cyanobacterium exo- and polysaccharides have been devoted to the determination of the sugar composition of these polymers. The monosaccharide most frequently found in cyanobacterium EPS is glucose (in more than 90% of the polymers), followed by galactose, mannose and rhamnose (80–85% of the polymers) (De Philippis & Vincenzini, 1998). In a large number of EPS, glucose is also the most abundant monosaccharide, but there are also polymers where other sugars, like arabinose, galactose or fucose, are present at higher concentrations than glucose (De Philippis & Vincenzini, 1998).

Differing from that a polysaccharide containing only xylose has not been previously described for cyanobacteria either as cellular or extracellular polymer. Xylose-containing polysaccharides, in the form of hemicelluloses, are one of the most abundant biopolymers, after cellulose, synthesized in the biosphere (Joseleau, Comtat, & Ruel, 1992).

In this study we found a linear (1 → 4)-linked β-D-xylan as EPS produced by *Nostoc muscorum* (UTEX-2493) isolated as *Peltigera canina* photobiont, this structure is commonly found in higher plants and some algae (Ahmadjian, 1993). It was further described for the cultured *Trebouxia* sp. photobionts isolated from *Ramalina celastri* (Cordeiro et al., 2003) and has been detected for the first time in intact lichen *Dyctionema glabratum* (Carbonero, Sassaki, Gorin, & Iacomini, 2002). The authors suggested that this polymer could be produced by the cyanobacterium *Scytonema* sp. found as *D. glabratum* photobiont (Carbonero et al., 2002). This suggestion may now be reinforced due to our finding, and could be proposed that this polymer may be typical of photobionts isolated from lichen thalli.

Besides the linear β-D-xylan found as EPS, the other structure related in this study was a heteropolysaccharide with a complex structure mainly formed of β-L-Ara*p* and β-D-Xyl*p*-(1 → 4)-linked units, partially branched units at O-3 by α-D-Man*p*, which appeared to be substituted at O-3 or O-2 by single-unit α-D-Man*p* or α-L-Fuc*p* as non-reducing ends.

The presence of β-L-arabinopyranosyl as related in this study is uncommon in these polymers and few authors have mentioned its presence. L-Arabinose is a predominant glycosyl residue of plant cell wall polysaccharides (Ishii et al., 2005), like arabinoxylan or present as side-chains that are linked to the backbone of the pectic polysaccharide rhamnogalacturonan-I (RG-I) (Ridley, O'Neill, & Mohnen, 2001). In these polysaccharides the arabinosyl residues exist predominantly in the α-L-arabinofuranosyl (Ara*f*) form

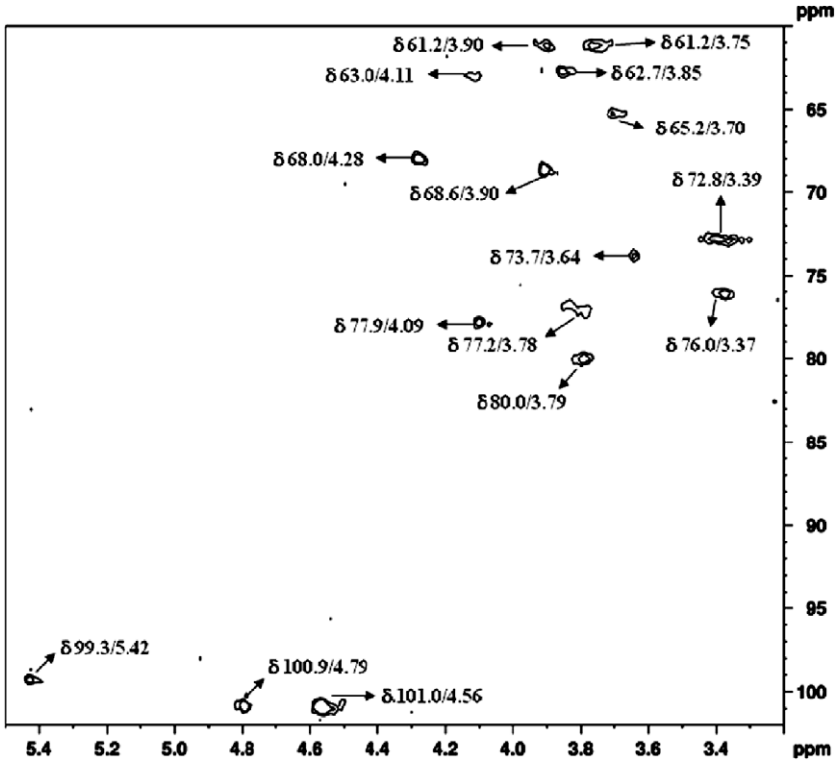

Fig. 3. ^{1}H (obs.), ^{13}C HMQC spectrum of HP3 fraction from *N. muscorum* in D_2O at 70 °C (chemical shifts are expressed in δ, ppm).

(Carpita & Gibeaut, 1993). α-L-Arabinopyranosyl (Ara*p*) residues are quantitatively minor components of arabinans isolated from several plants (Aspinall, Fairweather, & Wood, 1968; Capek, Toman, Kardosova, & Rosik, 1983; Kiyohara, Yamada, & Otsuka, 1987; Swamy & Salimath, 1991). 3-*O*-β-L-Arabinopyranosyl-L-arabinose was isolated from partial acid hydrolyzates of larch wood arabinogalactan (Odonmažig, Ebringerová, Machová, & Alföldi, 1994) and from gum exudate of the trunk of *Prunus persica* (Andrews, Ball, & Jones, 1953). β-L-Ara*p* (4-*O*- and 2,4-di-*O*-substituted) was also described as part of a polysaccharide isolated from gum exudate of the trunk and fruit of *P. persica* and from trunk of nectarine (*Prunus persica* var. *nucipersica* Schneid.) (Simas et al., 2008; Simas-Tosin et al., 2009), and in general arabinopyranose has also been found in the non-reducing end of pectic galactan (Huisman et al., 2001).

Both polymers characterized in this work were not previously described when analyzing *Nostoc* sp. or cyanobacteria in general. Therefore this study may contribute to the knowledge of polysaccharides produced by cyanobacteria, more specifically of the genus *Nostoc* principally due to the fact that the structure and properties of cyanobacterial polysaccharides are described only in some rare cases because of the great structurally diversity and complexity of the produced polymers.

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