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New exopolysaccharides produced by *Aureobasidium pullulans* grown on glucosamine

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Abstract

The polysaccharides produced by *Aureobasidium pullulans*, grown using glucosamine as the carbon source, were investigated by means of methylation analysis, affinity chromatography and NMR spectroscopy. The results indicated that, besides a small amount of pullulan, this micro-organism was capable of producing—in low yields—mixtures of at least two different complex polysaccharides containing mainly mannose and galactose. ^{1}H NMR spectra of two fractions obtained by lectin affinity chromatography indicated that one polymer was constituted exclusively of mannose residues while the other contained both galactofuranosyl and mannopyranosyl residues.

Keywords: *Aureobasidium pullulans*; Polysaccharides; Structure; NMR spectroscopy; Lectin affinity chromatography

1. Introduction

The possibility of producing structurally different exopolysaccharides from the same microbial source, in a controlled fashion and in good yields, by simply changing the nutrient composition, is of obvious potential. This would open the way to obtaining tailor-made polysaccharides without having to resort to complex genetic engineering procedures. In this context, we acted upon the findings reported by others in the recent literature[1,2] dealing with the production from *Aureobasidium pullulans* cultures of linear glucomannans with a mannose-to-glucose ratio predetermined on the basis of the amount of glucosamine introduced in the culture medium.

Our studies aimed at the upgrading of vegetal glucomannans (e.g. glucomannan from *Amorphophallus konjac* and from *Orchis mascula*) via their C-6 regioselective oxidation and subsequent C-5 partial epimerization in order to obtain novel, 'alginate-like', polysaccharides[3,4] of industrial potential. Glucomannans from microbial sources could offer additional samples with an alleged easily controllable main chain glucose/mannose composition.[1,2]

The results reported here show that, contrary to expectations, cultures of *A. pullulans* (a commercial ATCC strain used independently in two of our laboratories) produced in low yields at least two types of complex, branched polysaccharides quite different from the expected linear glucomannans. The structures of these branched polymers are the subject of the present investigation.

2. Results and discussion

The growth of *A. pullulans* on glucosamine, according to the procedure of Lee et al.,[1] yielded two samples named **APU1** and **APU3**. The sample **APU1** was fractionated by means of non-solvent precipitation to give two fractions, **APU1a** and **APU1b**. The sample **APU2** was produced by growing *A. pullulans* first on glucose and then on glucosamine. Separation of this sample by

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Table 1
Composition analysis

Sample	Man	Gal	Glc
APU1	2.6	1.0	0.3
APU2	1.9	1.0	0.6
APU1a	2.4	1.0	0.3
APU1b	1.3	1.0	0.1
APU3	6.3	1.0	1.4

Data are reported as sugar molar ratio relative to Gal.

means of gel filtration chromatography yielded fractions **APU2a** and **APU2b**.

Composition analysis.—All samples investigated showed a similar sugar composition comprising mannose, galactose and glucose (Table 1). In many samples, glucose was the minor component, attributed to the presence of pullulan, by NMR spectroscopy. The Man-to-Gal ratio ranged from 1.3 to 2.6 in all samples, except the **APU3** fraction, which was particularly rich in mannose.

The results of methylation analysis (Table 2) revealed the presence of up to 13 different sugar derivatives. The relative molar ratios calculated for each derivative suggested the co-existence of at least two different polymers in each sample, the presence of hexoses in the furanose form and di-substituted and non-reducing terminal residues indicating the occurrence of branched chains. Comparison between the alditol acetate and methylation analysis data was used to assign the furanosyl residues. The presence of a rather high amount of galactose in the polymers, as obtained from alditol acetate analysis, and the indication, given in the literature, that micro-organisms can synthesise galactans exhibiting Gal*f* residues,[5,6] suggested that the Hex*f* residues were galactose.

NMR spectroscopy.—All the samples exhibited very similar ^{1}H NMR spectra. The main difference between the spectra obtained for the various samples investigated was due to changes in the relative intensity of the main resonance peaks. In particular, all the ^{1}H NMR spectra obtained can be classified in two groups, which are well represented by the spectra obtained for fractions **APU1a** and **APU1b**. The anomeric regions of the two spectra are shown in Fig. 1. The main difference between the two spectra is the intensity of the resonance at 5.08 ppm with respect to those at 5.21, 4.97 and 4.93 ppm.

In order to assign the resonances of the ^{1}H NMR spectra and correlate them with the methylation analysis data, a detailed investigation was carried out on the **APU1** sample by means of both 1D and 2D ^{1}H and ^{13}C NMR experiments. The anomeric regions of ^{1}H and ^{13}C NMR spectra of the **APU1** sample are shown in Fig. 2. In the following, according to the previous discussion, Hex*f* was assigned to galactose residues. From the methylation analysis data, only the sugar derivatives present in quantity higher than 10% were considered for the assignment of the NMR resonances, accounting for about 90% of the total sugar residues. As already mentioned, the residues 6-Glc and 4-Glc, that were always present in low amount, came almost entirely from pullulan.

In the ^{13}C NMR spectrum, the resonance at 108.7 ppm confirmed the presence of furanosyl sugar rings.[6] The area ratio between this resonance and all the others in the region 104–98 ppm, which were attributed to

Table 2
Methylation analysis of the samples **APU1**, **APU1a**, **APU2a**, **APU1b**, **APU2b**, **APU3**

Glycosyl residues	Molar ratios[a]					
	APU1	**APU1a**	**APU2a**	**APU1b**	**APU2b**	**APU3**
t-Man	1.00 (18%)	1.00 (18%)	1.00 (10%)	1.00 (10%)	1.00 (12%)	1.00 (26%)
t-Glc	0.23 (4%)	0.24 (4%)	0.63 (6%)	0.19 (2%)	0.17 (2%)	0.14 (4%)
t-Hex-*f*	0.41 (7.5%)	0.35 (6%)	0.79 (8%)	1.40 (14%)	1.33 (16%)	0.08 (2%)
t-Gal	–	0.05 (1%)	0.24 (2%)	–	0.09 (1%)	–
3-Glc	–	0.06 (1%)	–	0.15 (2%)	–	–
2-Man	0.94 (17%)	0.92 (16%)	0.82 (8%)	1.75 (17%)	1.31 (16%)	0.77 (20%)
6-Man	0.61 (11%)	0.71 (13%)	1.40 (14%)	0.88 (9%)	0.71 (8%)	0.36 (9%)
6-Glc	0.11 (2%)	0.08 (1.5%)	0.39 (4%)	0.08 (1%)	0.22 (3%)	0.11 (3%)
4-Gal	0.21 (4%)	0.28 (5%)	0.60 (6%)	0.23 (2%)	0.15 (2%)	0.05 (1%)
4-Glc	–	0.06 (1%)	0.36 (3%)	0.09 (1%)	0.48 (6%)	0.22 (6%)
6-Hex-*f*	0.64 (12%)	0.64 (11%)	1.55 (15%)	2.22 (22%)	1.43 (17%)	0.14 (4%)
3,6-Man	0.67 (12%)	0.92 (16%)	2.24 (22%)	0.68 (7%)	0.53 (6%)	0.20 (5%)
2,6-Man	0.67 (12%)	0.35 (6%)	0.30 (3%)	1.27 (13%)	1.02 (12%)	0.81 (21%)

[a] Molar ratios are reported relative to t-Man. Molar percentages are in parenthesis.

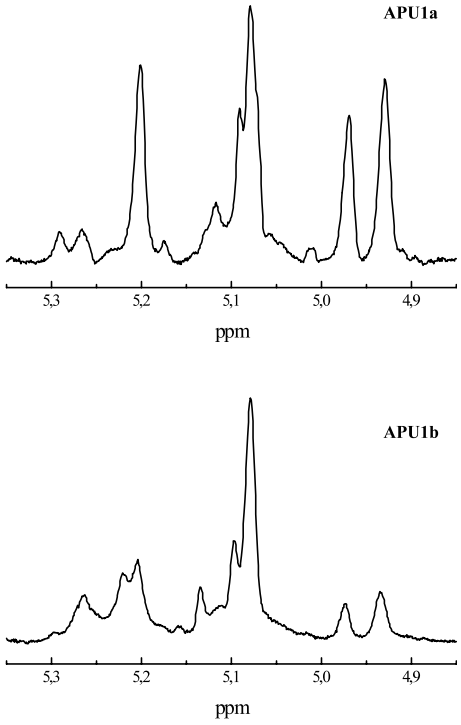


Figure 1. Anomeric regions of the 500 MHz ^{1}H NMR spectra of the fractions **APU1a** and **APU1b**.

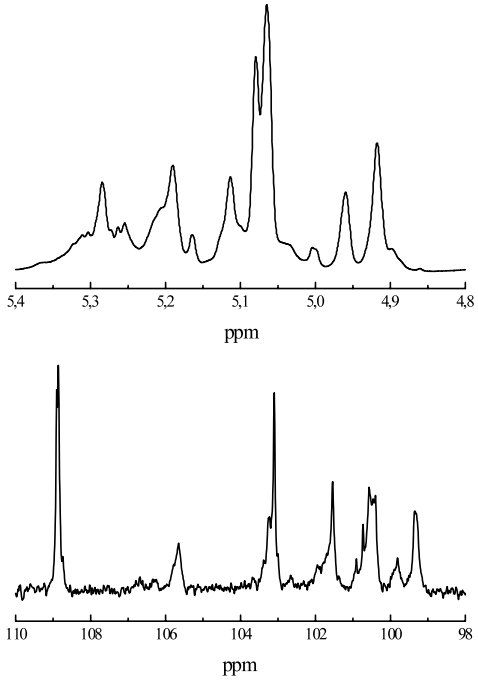


Figure 2. Anomeric regions of the 500 MHz ^{1}H (top) and ^{13}C NMR (bottom) spectra of the sample **APU1**.

Man residues, gave a value of 0.24. This number is in good agreement with the ratio obtained from methylation analysis data (0.28). The assignment of ^{13}C mannose resonances was partially achieved by using literature data and it was straightforward to assign the resonance at 103.1 ppm to t-Man.[7,8] The peak area ratio between the resonance at 103.1 ppm and the sum of the resonances in the range 99–102 ppm was 0.33, close to the molar ratio between t-Man and the remaining Man residues obtained from methylation analysis data (0.37). Therefore, it was deduced that almost only Man residues contributed to these peaks. The resonance at 101.5 ppm was assigned to 2-Man, and the resonance at 99.3 ppm to 2,6-Man.[8] The assignment of the resonances in the cluster at 100.9–100.4 ppm was more difficult due to the clear overlapping of different signals. According to the literature, the 6-Man anomeric carbon was certainly in the cluster.[8] However, to match the methylation analysis data, it was assumed that this cluster also included part of 2-Man and 3,6-Man resonances. The assumption that each of these residues gives rise to two resonance peaks can be justified either with the possible presence of both α and β anomers or with the presence of these residues in different electronic environments.

The ^{13}C NMR assignments were used as a starting point to assign the ^{1}H NMR resonances through the interpretation of the HSQC spectrum of **APU1** (Fig. 3). The carbon resonances of both Gal*f* and t-Man (108.7 and 103.1 ppm, respectively) correlated with the resonance at 5.08 ppm. The resonance of 2-Man at 101.5 ppm correlated with that at 5.28 ppm and the cluster of resonances in the ppm range 101–100 correlated with the proton resonances at 5.226, 5.206, 4.978 and 4.934. They were assigned, in the order, to 3,6-Man, 6-Man, 3,6-Man and 2-Man on the basis of the comparison between the peak area integrals and the molar percentages obtained from methylation analysis data. The resonance of 2,6-Man at 99.3 ppm correlated with the two proton signals, at 5.14 and 5.11 ppm, thus suggesting either the presence of both anomers, also for this residue or the occurrence of some structural irregulari-

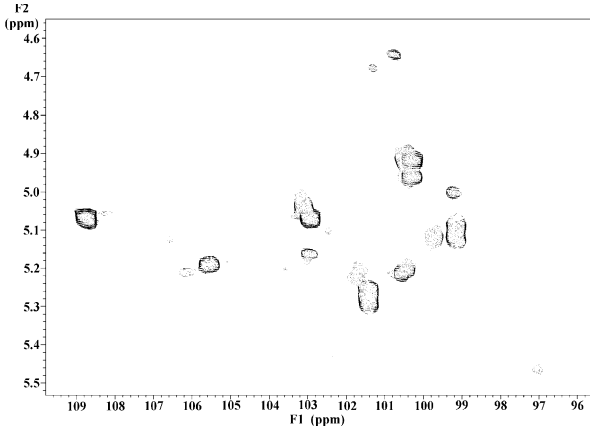


Figure 3. Expansion of the anomeric region of the HSQC plot for the sample **APU1** recorded at 50 °C.

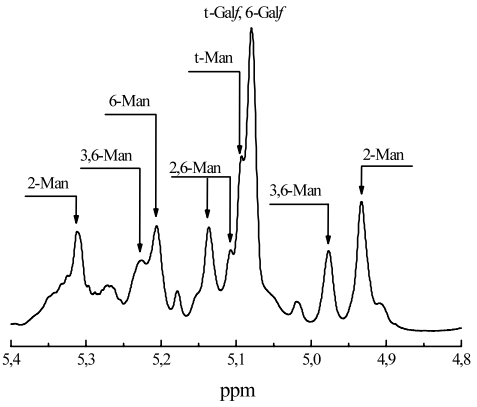


Figure 4. Anomeric region of the 500 MHz ^{1}H NMR spectrum of the sample **APU1** and peaks assignment.

Table 3
Comparison of NMR peak integrals and methylation analysis data for the sample **APU1**

Sugar residues	NMR (%)	Methylation analysis (%)
t-Man + 6-Gal*f* + t-Gal*f*	41	41
2-Man	24	19
6-Man	14	12
2,6-Man	13	13
3,6-Man	8	12

ties. The resonance assignment, reported on the proton spectrum of **APU1** (Fig. 4), is in agreement with literature data for different mannans.[7–9]

The comparison of the NMR data with those from methylation analysis data is given in Table 3, where different sugar percentages were grouped together according to peak superimposition present in the ^{1}H NMR spectrum of **APU1**. The percentages reported in Table 3 were referred to the sum of the sugar residues recognised, and hence surely assigned in the NMR spectrum. Therefore, the values are not coincident with the percentages given in the Table 2, which were normalised taking into account the sugar residues occurring in minor amount. The agreement between NMR and methylation analysis data is very good, therefore confirming the assignment of ^{1}H and ^{13}C NMR resonances.

Considering the ^{1}H NMR assignment obtained for the sample **APU1**, the comparison of the NMR and methylation analysis data for the samples **APU1a**, **APU1b** and **APU3** was also carried out. The results are reported in Table 4. The overall agreement of the two sets of values calculated independently was very good, further confirming the general validity of the NMR assignments.

Affinity chromatography.—The results of NMR experiments together with those of methylation analysis strongly indicated the presence of at least two different polymers in each sample examined. Moreover, these polymers were not successfully separated by either solvent fractionation (**APU1a** and **APU1b**) or gel-filtration chromatography (**APU2a** and **APU2b**). Therefore, the **APU1** sample was subjected to affinity chromatography using Concanavalin A (Con A) immobilized on Sepharose 4B.[10] The separation afforded two fractions: **CON1**, that was not retained by Con A, and **CON2**, that bound to Con A and was eluted using a solution containing α methyl-mannoside. The ^{1}H NMR spectra of the two fractions are shown in Fig. 5. The signal-to-noise ratio of the **CON1** spectrum was low because of the small amount of sample recovered after affinity chromatography.

As can be seen, the spectra were very similar to those discussed above. However, it is worth noting that the signals at 5.08, 5.11 and 5.28 ppm (marked with asterisks in Fig. 5) exhibited high intensity in the **CON2** sample, while they were very low or absent in the **CON1** sample. The resonance at 5.08 ppm was part of

Table 4
Comparison of NMR peak integrals and methylation analysis data for the samples **APU1a**, **APU1b** and **APU3**

	APU1a	
Sugar residues	NMR (%)	Methylation analysis (%)
t-Man + 6-Gal*f* + t-Gal*f*	39	40
2-Man	20	19
6-Man	19	15
2,6-Man	9	7
3,6-Man	12	19
	APU1b	
t-Man + 6-Gal*f* + t-Gal*f*	51	51
2-Man	19	19
6-Man	12	8
2,6-Man	8	14
3,6-Man	5	8
	APU3	
t-Man + 6-Gal*f* + t-Gal*f*	40	40
2-Man	20	20
6-Man	7	9
2,6-Man	13	13
3,6-Man	5	5

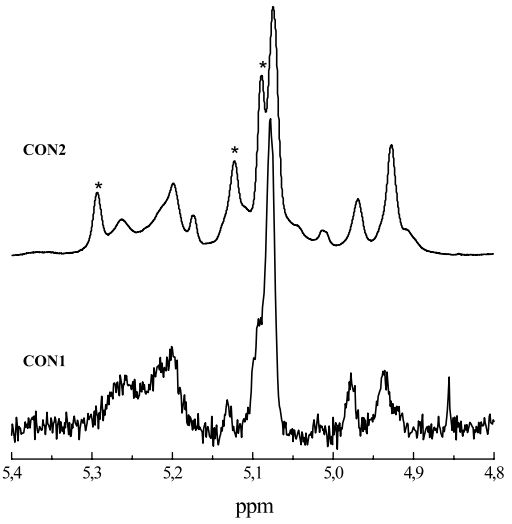
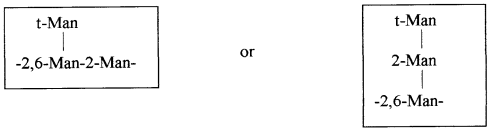


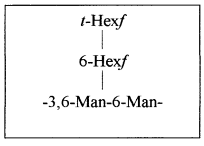
Figure 5. Anomeric regions of the 500 MHz ^{1}H NMR spectra of the fractions **CON1** and **CON2**.



Scheme 1.

Table 5
Comparison of molar percentages of sugar residues in the sample **APU1** after subtraction of values relative to the sugar residues of the **CON2** fraction

Sugar residue	Methylation analysis (%)	^{1}H NMR (%)
t-Gal*f* +6-Gal*f*	46	43
6-Man +3,6-Man	54	57



Scheme 2.

the cluster containing the t-Man signal. Since t-Man can be safely assumed as the major lectin-binding sugar residue, the signal at 5.08 ppm was assigned to t-Man, while the signal at 5.06 ppm took into account the Gal*f* contributions. According to previous assignments, the signals at 5.11 and 5.28 ppm were assigned to 2,6-Man and 2-Man, respectively. Therefore, it can be concluded that the polymeric fraction bound to Con A (**CON2**), and retained in the affinity chromatography column, contained a mannan characterised by the possible structures depicted in Scheme 1. It is worth mentioning that, considering the methylation analysis data, a similar repeating unit could be assumed for the **APU3** sample, where the t-Man, 2-Man and 2,6-Man residues constituted the 67% of all the sugar residues and their relative molar ratios were about 1:1:1.

To further pursue the determination of the structure of the polymers produced by *A. pullulans* grown on glucosamine, only the sugar present in the highest percentages were considered, after eliminating those pertaining to the repeating units depicted in Scheme 1. To do so, the sugar percentages found for **APU1** by means of methylation analysis (Table 2) were re-normalized after elimination of t-Man, 2-Man and 2,6-Man. The results are shown in Table 5, where the comparison with the percentages obtained by peak integration of the proton NMR spectrum of **CON1** is also reported.

The good agreement between the corrected methylation analysis data and the percentages obtained by integration of the resonance peaks of **CON1** might indicate that the second polymer produced by *A. pullulans* was a galactomannan, whose possible primary structure could contain the fragment reported in Scheme 2. In this scheme, the usual structure of plant galactomannans was assumed. This galactomannan is also partly retained by Con A since its NMR signals are present in the proton spectrum of the **CON2** fraction (See Fig. 5). This might be due to the lack of high selectivity of Con A towards t-Man and t-Gal*f*.

The NMR data relative to the two fractions, **CON1** and **CON2**, were further confirmed by methylation analysis data. The results are shown in Table 6, where the percentages of the different sugar residues are indicated. The relative amount of t-Man, 2-Man and 2,6-

Table 6
Methylation analysis of the two fractions obtained by affinity chromatography on Concanavalin A

Sugar residue	**CON1**	**CON2**
t-Man	4	24
t-Glc	14	4
t-Gal*f*	12	8
t-Gal	4	1.7
2-Man	5.5	19
6-Man	10	10
6-Glc	5	–
4-Gal	6	–
6-Gal*f*	19	9
3,6-Man	12	11
2,6-Man	8.5	13

The data are reported as molar percentages of the total sugar residues.

Man were decreased in **CON2** with respect to **CON1**, in agreement with NMR data and further confirming the resonance peaks assignment previously discussed. In addition, the methylation analysis data were consistent with the polysaccharide repeating units shown in Schemes 1 and 2.

In conclusion, although the *A. pullulans* strain was able to produce pullulan under 'standard' culture conditions, our data unequivocally demonstrated that feeding commercial strains with glucosamine led essentially to complex, branched polysaccharides and not of linear glucomannans. In addition, the presence of glucosamine in the culture medium of *A. pullulans*, and other fungal strains normally producing pullulan (data from our laboratories), caused stressful growth conditions and low yields of production of complex polysaccharides.

3. Experimental

The fungal strain *A. pullulans* ATCC 42023 was employed in this study. The micro-organisms were supplied as a lyophilised culture and was re-hydrated and cultivated according to the instructions provided by ATCC. The micro-organisms were grown on glucosamine according to the procedure given by Lee et al.[1]

Samples.—**APU1**: sample obtained at the University of Rome and purified by ethanol precipitation and pullulanase treatment. **APU1a** and **APU1b**: fractions obtained by ethanol fractionation from the sample **APU1**. **APU2**: sample obtained by growing *A. pullulans* first on glucose and subsequently on glucosamine. The sample **APU2** was dissolved in 0.05 M $NaNO_3$ and subjected to gel filtration chromatography on a Sephacryl S-400 column at a flow rate of 7 mL/h. The eluent used was 0.05 M $NaNO_3$. Only a partial separation was obtained and according to the elution profile, the fractions 38–46 and 55–65 were pooled together to give the samples named **APU2a** and **APU2b**, respectively. **APU3**: sample obtained at the University of Bari.

General methods.—Analytical GLC was performed on a Hewlett–Packard 5890 gas chromatograph equipped with a flame ionisation detector and an SP2330 capillary column (Supelco, 30 m), using He as the carrier gas. The following temperature programs were used: for alditol acetates, 200–245 °C at 4 °C/min; for methylated alditol acetates, 150–250 °C at 4 °C/min. GLC–MS analyses were carried out on a Hewlett–Packard 5890 gas chromatograph coupled to a Hewlett–Packard 5971 mass selective detector.

Composition analysis. Hydrolysis of the polymers was carried out with 2 M trifluoroacetic acid at 125 °C for 1 h. Alditol acetates were prepared as previously described.[11] Methylations were performed according to the modified Hakomori method[12] using potassium methylsulfinyl–methanide.[13] The partially methylated alditol acetates obtained from these reactions were analysed by GLC and GLC–MS. Molar ratio values were corrected by use of effective carbon-response factors.[14]

Affinity chromatography. A glass column was filled with 14 mL of the resin ConA-Sepharose (SIGMA), which was washed with 0.01 M Tris–HCl buffer, pH 7.5, 0.001 M $MnCl_2$, 0.001 M $CaCl_2$ and 0.5 M NaCl and subsequently equilibrated with the elution buffer 0.01 M Tris–HCl, pH 7.5, 0.001 M $MnCl_2$, 0.001 M $CaCl_2$ and 0.15 M NaCl.[10] The sample **APU1** (13.2 mg) was dissolved in 0.7 mL of the elution buffer and loaded on the column; the flow was stopped for 1 h in order to allow the sample to interact with Concanavalin A. The elution was then started with a flow rate of 7 mL/h and it was monitored with a Differential Refractometer (WGE Dr. Bures, LabService, Analytica). The fraction which did not interact with the resin was named **CON1**. In order to displace the fraction which interacted with Concanavalin A, 0.25 M α-methyl mannoside was added to the elution buffer. Although this changed the refractive index of the solution, it resulted only in a drift of the baseline and therefore, the elution could still be monitored using the Differential Refractometer. The fraction, which was retained by the Concanavalin A, was named **CON2**. The samples **CON1** and **CON2** were desalted by means of dialysis (cut off 3500 MW) before performing chemical derivatisation and NMR spectroscopy experiments.

NMR experiments. NMR experiments were performed on a 500 MHz Varian UNITY INOVA spectrometer. Proton spectra were run at a probe temperature of 70 °C, while carbon spectra were obtained at 50 °C. The samples were dissolved in D_2O at a final concentration of about 5 mg/mL, for ^1H NMR experiments and of 10 mg/mL for ^{13}C NMR experiments. The HSQC spectrum was recorded at 50 °C using the standard Varian pulse sequence. HSQC correlations were investigated using 1D proton and carbon spectra obtained at 50 °C.

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References

1. (a) Lee J. W.; Yeomans W. G.; Allen A. L.; Deng F.; Gross R. A.; Kaplan D. L. *Appl. Environ. Microbiol.* **1999**, *65*, 5265–5271;
(b) Lee J. W.; Deng F.; Gross R. A.; Kaplan D. L. *Polymer Preprints* **1998**, *39*, 110–111.
2. Kim J. H.; Kim M. R.; Lee J. H.; Lee J. W.; Kim S. K. *Biotechnol. Lett*. **2000**, *22*, 987–990.
3. Crescenzi V.; Skjak-Braek G.; de Nooy A. E. J.; Rori V.; Masci G. *Biomacromolecules* **2000**, *1*, 360–364.
4. Crescenzi V.; Dentini M.; Bernalda M. S.; Masci G.; Rori V.; Skjak-Braek G. *Biomacromolecules* **2001**, *2*, 958–964.
5. Faber E. J.; van den Haak M. J.; Kamerling J. P.; Vliegenthart F. G. *Carbohydr. Res*. **2001**, *331*, 173–182.
6. Corsaro M. M.; De Castro C.; Evidente A.; Lanzetta R.; Molinaro A.; Mugnai L.; Parrilli M.; Surico G. *Carbohydr. Res*. **1998**, *308*, 349–357.
7. Kobayashi H.; Watanabe M.; Komido M.; Matsuda K.; Ikeda-Hasebe T.; Suzuki M.; Shibata N.; Hisamichi K.; Suzuki S. *Carbohydr. Res*. **1995**, *267*, 299–306.
8. Corsaro M. M.; Evidente A.; Lanzetta R.; Lavermicocca P.; Molinaro A. *Carbohydr. Res*. **2001**, *330*, 271–277.
9. Vinogradov E.; Petersen B.; Bock K. *Carbohydr. Res*. **1998**, *307*, 177–183.
10. Ellsworth J. H.; Reiss E.; Bradley R. L.; Chmel H.; Armstrong D. *J. Clin. Microbiol*. **1977**, *5*, 91–99.
11. Albersheim P.; Nevins D. J.; English P. D.; Karr A. *Carbohydr. Res*. **1967**, *5*, 340–345.
12. Hakomori S. *J. Biochem. (Tokyo)* **1964**, *55*, 205–208.
13. Phillips L. R.; Fraser B. A. *Carbohydr. Res*. **1981**, *90*, 149–152.
14. Sweet D. P.; Shapiro R. H.; Albersheim P. *Carbohydr. Res*. **1975**, *40*, 217–225.