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Structural elucidation of the EPS of slime producing *Brevundimonas vesicularis* sp. isolated from a paper machine

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Abstract

The slime forming bacteria *Brevundimonas vesicularis* sp. was isolated from a paper mill and its EPS was produced on laboratory scale. After production, the exopolysaccharide (EPS) was purified and analysed for its purity and homogeneity, HPSEC revealed one distinct population with a molecular mass of more than 2,000 kDa. The protein content was around 9 w/w%. The sample was analysed to determine its chemical structure. The EPS was found to consist of rhamnose, glucose, galacturonic acid and glucuronic acid. Due to the presence of uronic acids the molar ratio between the four sugars found varies from 3:5:2:4 by sugar composition analyses after methanolysis to 1:1:1:1 found by NMR. A repeating unit with a molecular mass of 678 Da was confirmed by MALDI-TOF mass spectrometry after mild acid treatment. ^{13}C and ^{1}H hetero- and homonuclear 2D NMR spectroscopy of the native and partial hydrolysed EPS revealed a

→4)-α-L-Glc*p*A-(1→4)-α-D-Gal*p*A-(1→4)-β-L-Rha*p*-(1→4)-β-D-Glc*p*(1→

repeating unit, no non-sugar substituents were present.

Keywords: *Brevundimonas vesicularis*; EPS (slime); Pulp and Paper Industry; ^{1}H and ^{13}C NMR analyses; Structure elucidation

1. Introduction

Slime deposits cause significant operation problems in a paper and board production, such as processing problems and defects in the product quality. Slime is the generic name for deposits of microbial origin in a paper mill.[1] Problematic slimes in the paper and board machines are mixed deposits with thick microbial biofilms as major components. The major structural components of microbial biofilms are polymers excreted by the bacteria present, these polymers largely being heteropolysaccharides. In some cases also proteins and some other substances produced by microbes, e.g., nucleic acids, phospholipids, can be present.[2] The slime deposits may also contain other material derived from the paper manufacture process, such as fibre and organic and inorganic precipitates from process waters.[3]

Paper mills, especially those employing increasingly closed-loop processes and high use of secondary fibers, have high nutrient levels as well as optimal temperature and pH ranges to support serious microbial proliferation. The conditions normally found in the paper machines are pH 5–8, 20–78 °C and biodegradable materials like cellulose, hemicellulose, starch and wood extractives are widely present.[4–7] Slime formation within the paper industry can cause several problems like off-smells, spots and holes in the end product and may cause severe production delay in the case of web breaks. Nowadays the control of slime problems in paper or board mills is mainly carried out using biocides.[4] Because of environmental and legislative reasons, several alternative slime control methods, such as

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enzymes, are currently under development. During the last 20 years there have been several reports on the use of enzymes to degrade the slime structure, in order to decrease the use of biocides.[8,9]

Most information about the chemical structure of paper machine biofilm exopolysaccharides has been limited to identifying the different micro-organisms present and to analyses of the sugar residues of which the secreted exopolysaccharides consist. Several articles describe different slime forming micro-organisms commonly found in paper machines. Mattila-Sandholm and Wirtanen[10] report that the most common group of bacteria found in paper machines belongs to the *Enterobactericae*, *Pseudomonas* sp., *Clavibacter* sp., and *Baccilus* sp. In addition, Vaïsanen et al.[5] identified the same bacterial species in addition to *Klebseilla* sp. to represent frequently found species in a paper machine. Most of the research has been done only by determining the sugar composition of bacterial slime deposits suggesting that the most common sugars found in paper mill slimes are: glucose, rhamnose, galactose, mannose, fucose and glucuronic acid.[3,5,11]

Until now hardly any structures for the EPS in paper machine biofilms or from microbes isolated from paper machine biofilms have been published. It is reported that levan is an EPS secreted by several species of *Bacillus* and *Pseudomonas* bacteria in recirculated water of the paper machine.[1] Levan is a specific fructose-containing polysaccharide that is synthesised from sucrose, which is in short supply in paper machine environment. In addition, bacteria producing levan in paper machine conditions are not very common in the process waters.[9] Thus levan is not considered to be a major constituent of paper machine slimes.

Only few attempts have been reported to solve the problem by elucidation of the complete structure of a defined polysaccharide and to apply enzymes on the basis of these data. An example of this is the use of specific levanase against the occurrence of levan described by Chaudhary et al.[6] As indicated above the first step in a logical approach for finding appropriate enzymes to solve slime problems would be by the structural analysis of a number of exopolysaccharides found in bacterial slimes and attacking the structural components of slime deposits. Within this publication we will describe the structural elucidation of an exopolysaccharide produced by *Brevundimonas vesicularis* VTT-E-981024 isolated from a paper mill.

2. Experimental

Isolation of bacterial species and extraction and purification of the EPS produced.—The bacterial species *B. vesicularis* VTT-E-981048 was isolated from a slime sample obtained from a paper machine. After isolation and purification of the strain the exopolysaccharide was produced in laboratory scale. The EPS was produced using shale flasks at 30 °C in a medium containing glucose (20 g/L), yeast extract (0.5 g/L), $(NH_4)_2SO_4$ (0.6 g/L), KH_2PO_4 (3.18 g/L), K_2HPO_4 (5.2 g/L), $MgSO_4{\cdot}7H_2O$ (0.3 g/L), $CaCl_2$ (0.05 g/L), $ZnSO_4{\cdot}7H_2O$ (0.2 mg/L), $CuSO_4{\cdot}5H_2O$ (0.2 mg/L), $MnSO_4{\cdot}H_2O$ (0.2 mg/L), $FeSO_4{\cdot}7H_2O$ (0.6 mg/L) and $CoCl_2$ (0.2 mg/mL) at pH 7.

After cultivation 0.9% NaCl was added to the medium and the medium was slightly homogenised and centrifuged (14,687*g*, 45 min). Ethanol (75% (v/v)) was added to the supernatant to precipitate the EPS material. Part of the precipitate was solubilised in water and incubated with protease (Neutrase 0,5L, Novozymes, Denmark) for 1 h at 37 °C, re-precipitated, dialysed (Medicell Visking, MWCO 12,000–14,000 Da) and freeze-dried. The other part was directly dialysed and freeze dried without protease treatment and re-precipitation.

Protein content.—Protein content was measured using the combustion (Dumas) method on a Thermo Quest NA 2100 nitrogen and protein analyzer (Inter Sciences, The Netherlands) according to the instructions of the manufacturer. The sample (5–6 mg) was weighed in to a sample cup and directly analysed using D-methionine as an external standard. The protein content was calculated using 6.25 as nitrogen to protein conversion factor.

HPSEC of the native EPS.—The EPS (2 mg) was dissolved in 1mL of distilled water and analysed by high-performance size exclusion chromatography (HPSEC) using pullulan for calibration. HPSEC was performed on a ThermoQuest HPLC using three Bio-Gel TSK columns in series (60 XL, 40 XL, 30 XL) preceded by an TSK XL guard column (40 × 6 mm). Elution took place at 30 °C using 0.8 mL/min 0.2 M $NaNO_3$ as eluent. Detection was performed using a Shodex RI 71 refractive index detector and a Viscotec viscosity and Right Angle Laser Light Scatter (RALLS) detector.

Sugar composition.—The EPS sugar composition was determined using two different methods: Sulphuric acid hydrolysis and methanolysis as described by De Ruiter et al.[12]

For methanolysis, the EPS was treated with 2N HCl in dry methanol for 16 h at 80 °C, followed by 1 h of 2N CF_3CO_2H (TFA) at 121 °C.

For sulphuric acid hydrolysis the EPS was pretreated with 72% H_2SO_4 for 1 h at 30 °C, followed by hydrolysis with 1 M H_2SO_4 for 3 h at 100 °C.

The released sugars were analysed using high-performance anion exchange chromatography (HPAEC) with pulsed amperometric detection (PAD). HPAEC was performed using a Dionex system containing a ThermoQuest HPLC system, a Dionex EDM Helium degas

unit, a Carbopac PA1 column (4 × 250 mm) with a Carbopac PA100 guard column and a Dionex ED40 PAD detector. Separation was performed at flow rate 1 mL/min using gradient elution by mixing distilled water, 100 mM NaOH and 1,000 mM NaOAc in 100 mM NaOH. Sample (20 μL) was injected and separated by the following elution program: 26 min isocratically with 15 mM NaOH followed by a linear gradient to 100 mM NaOH in 7 min. Then a linear gradient was started to 60 mM NaOAc within 12 min followed by a linear gradient to 300 mM NaOAc in another 33 min, keeping the NaOH concentration at 100 mM. The column was washed for 5 min with 1,000 mM NaOAc followed by a 15 min wash step with 100 mM NaOH and equilibrated again for 15 min with 15 mM NaOH.

Absolute configuration.—The absolute configurations of the monosaccharides present in the EPS were determined as described by Gerwig et al.[13] The GC-FID analyses of the trimethylsilated (−)-2-butyl glycosides was performed using a Carlo Erba Mega 5160 GC, equipped with a CP-Sil 5 CB column (25 m × 0.32 mm, Chrompack). The temperature programme was: 80 → 135 °C at 20 °C/min; 135 → 220 °C at 2 °C/min. The injection port and detector temperatures were 200 and 250 °C, respectively. The Helium flow rate was 3 mL/min and the samples (ca. 0.05 μL) were injected directly on the column without a stream splitter.

Sugar linkage analyses.—The EPS sample was methylated according to Hakomori[14] and subsequently dialysed against water and evaporated in a stream of dry air. The methylated samples were hydrolysed using 2 M CF_3CO_2H (1 h, 121 °C). After evaporation in a stream of air ($T < 20$ °C), the partially methylated sample was converted to alditol acetates and analysed by GC-FID. Identification of the compound was performed using GC–MS as described by van Casteren et al.[15]

Partial hydrolyses of the native EPS.—EPS (1 mg) was hydrolysed using 0.5 ml 0.05N TFA for 1 h at 100 °C. After cooling, TFA was evaporated in a stream of dry air at 40 °C and the released oligomers were dissolved in 0.5 ml distilled water. HPAEC-PAD was used to analyse the released oligomers using the same Dionex system as described above only with a different gradient. The sample (20 μL) was injected with the following elution program: separation was started by applying a linear gradient to 500 mM NaOAc in 100 mM NaOH within 30 min, followed by a linear gradient to 1,000 mM NaOAc in 100 mM NaOH within 40 min. Finally the column was equilibrated with 100 mM NaOH for 15 min.

Part of the sample was desalted using Dowex AG 50W-X8 (Biorad) and analysed by matrix-assisted laser desorption/ionisation–time of flight (MALDI-TOF) mass spectrometry. MALDI-TOF MS was performed by mixing 1 μL sample with 1 μL matrix on a plate. The matrix was made by mixing isocarbostyryl (3 mg), 2,5-dihydroxybenzoic acid (9 mg), acetonitril (0.3 mL) and distilled water (0.7 mL). Spectra were recorded using a Voyager-DE RP Biospectrometry Workstation (Applied Biosystems, Framingham, USA) in the positive mode. The laser intensity was set at 2,294, which equals 8.6 μJ per pulse. The pulse delay time was 200 ns, the acceleration voltage was 12,000 V, the grid voltage was 7,200 V and the guide wire voltage was 9.6 V. The instrument was used in the reflector mode and calibrated with a mixture of maltodextrines.

^{13}C and ^{1}H NMR.—Prior to NMR analyses, the samples were exchanged in 99.96% D_2O (Cambridge Isotope Laboratories, USA) and after freeze drying dissolved in 99.996% D_2O (Cambridge Isotope Laboratories, USA). NMR spectra were recorded at a probe temperature of 70 °C on a Bruker AMX-500 spectrometer located at the Wageningen NMR Centre. Chemical shifts were expressed in ppm relative to internal acetone: δ 2.225 ppm for ^{1}H and δ 31.55 ppm for ^{13}C.

The 1D ^{1}H proton spectra were recorded at 500.13 MHz using 8–200 scans of 8,192 data points and a sweep width of 3,000 Hz. The 1D ^{13}C proton decoupled carbon spectra were recorded at 125.77 Hz using 100,000 scans of 32,768 data points and a sweep width of 31,250 Hz.

The 2D COSY spectrum was acquired using the double quantum filtered (DQF) method with a standard pulse sequence delivered by Bruker.

2D TOCSY, 2D NOESY and 2D ROESY spectra were acquired using standard Bruker pulse sequences with 110, 200, 200 ms mixing time, respectively. For all homonuclear 2D spectra 512 experiments of 2,048 data points were recorded using 16–64 scans per increment.

For the 2D HMBC spectrum[16] a standard gradient enhanced 2D HMQC pulse sequence delivered Bruker was changed into a HMBC sequence by setting the delay between the first proton and carbon pulse to 50 ms. For the HMBC experiment 1,024 experiments of 2,048 data points were performed with 128 scans per increment.

Time domain data were multiplied by phase-shifted (squared-) sine-bell functions.

3. Results and discussion

Isolation and purification.—*B. vesicularis* VTT-E-981024 was isolated from paper machine slime and grown on laboratory scale to produce extracellular polysaccharide. The EPS was precipitated from the culture filtrate by ethanol, treated with protease, re-precipitated, dialysed and freeze-dried. After the extraction and purification it was subjected to further structure elucidation. Part of the sample was not treated with protease in order to detect possible modification of the

polymer by side-activities which might be present in the protease preparation.

Prior to the experiments needed for structure elucidation the EPS was analysed for protein content and molecular size distribution. Protein content of the sample without protease and with protease was 9 (w/w%) and 8 (w/w%), respectively, which indicates that protease treatment did not have notable effect. On the other hand this result also indicates that the crude EPS seems not to contain major amounts of proteins that would be readily hydrolysable by the protease preparation used, and consequently that the polysaccharide was considerably pure from contaminating proteins.

The molecular size distribution was measured using HPSEC with RI-, RALLS- and viscosity-detection. The EPS was found to consist of only one distinct population with a molecular weigh of $2–4 \times 10^6$ kg/mol and an intrinsic viscosity of around 6.5 dL/g.

On the basis of these data it was considered that the polysaccharide was adequately pure for further analysis, and the experiments needed for the elucidation of the EPS structure were carried out without further purification.

Sugar composition and absolute configuration.—The monomer composition of the novel EPS of *B. vesicularis* was determined using HPAEC-PAD ($pH > 12$) after H_2SO_4 hydrolysis and methanolysis and a specific longer gradient was used to detect oligomeric fragments due to incomplete hydrolyses (Fig. 1).

Comparing the results found for both hydrolyses methods as shown in Table 1 and Fig. 1 we could see a significant difference. Hydrolysis with H_2SO_4 yields a considerably lower amount of rhamnose, galacturonic acid and glucuronic acid than found using the methanolyses method (Table 1). This is obviously due to incomplete hydrolysis by sulphuric acid resulting in some remaining oligosaccharides (Fig. 1). Incomplete hydrolyses can be explained by the presence of galacturonic acid and glucuronic acid involved in very stable aldobiuronic acid linkages, which are incompletely hydrolysed by H_2SO_4, a weaker hydrolysing agent than HCl in methanol. It can be concluded that for this kind of EPS containing uronic acids the use of methanolysis would give more reliable results. The higher yield of monosaccharides by methanolysis from the protease treated polysaccharide as compared to the non-treated EPS (Table 1) possibly reflects the higher purity of this preparation, due to the two precipitation steps in its isolation procedure of the EPS. Although the sugar compositions of the protease- and non-protease treated EPS are not completely similar, it can be concluded that the former treatment resulted in a rather pure EPS, which was further characterised without further purification.

As shown in Table 1 methanolysis revealed the presence of five sugar residues in a molar ratio of Rha:Gal:Glc:GalA:GlcA 3:1:5:2:4. NMR analysis suggests the presence of four hexoses in a molar ratio of GlcA:GalA:Glc:Rha 1:1:1:1. This rises the question why there was a small amount (6.7%) of galactose and an undisproportional higher amount of glucose. These observations will be explained by the NMR data below. The galacturonic acid to rhamnose ratio by methanolysis was lower then we would expected on the basis of the NMR results; this deviation can be explained by the occurrence of an oligomeric fragment even resistant to methanolysis.

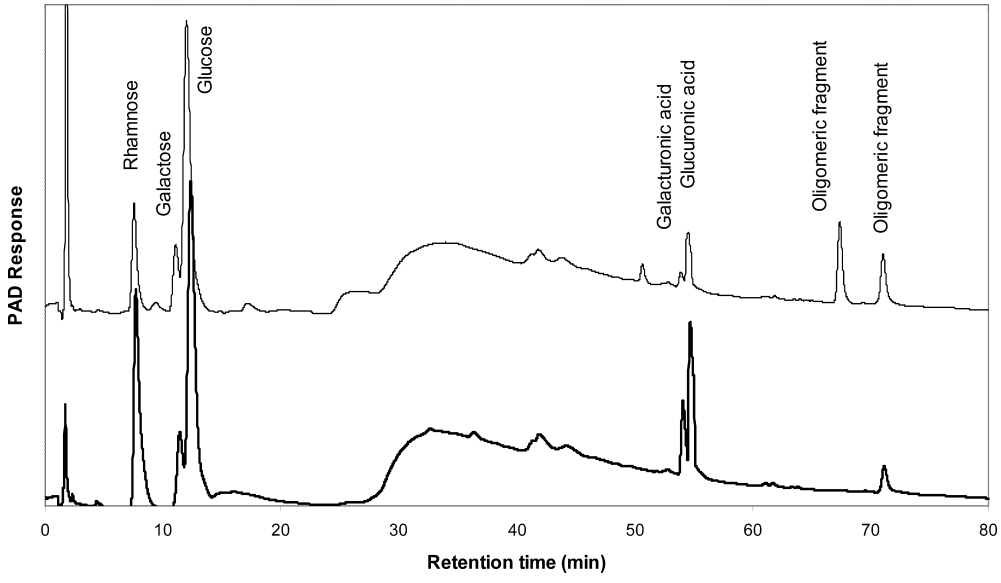

Fig. 1. HPAEC-PAD elution patterns of the protease treated EPS of *B. vesicularis* after H_2SO_4 hydrolysis and methanolysis followed by TFA hydrolyses, respectively. H_2SO_4 hydrolysis = upper trace, methanolysis = lower trace.

Table 1
Sugar composition (mol%) of the protease treated and non-protease treated preparations of novel EPS from *B. vesicularis*

Hydrolysis conditions	EPS	L-Rhamnose	D-Galactose	D-Glucose	D-Galacturonic acid	L-Glucuronic acid	Total sugar content (w/w%)
Hydrolysis 72% H_2SO_4 + 3 h 1 M H_2SO_4	Protease treated	15.4 ± 2.1	11.0 ± 1.0	58.1 ± 2.8	4.5 ± 0.8	10.9 ± 1.5	31.7 ± 1.6
Methanolysis 16 h 2N HCl in MeOH + 1 h 2N TFA	Protease treated	20.4 ± 4.3	6.7 ± 1.71	32.5 ± 2.9	15.0 ± 1.5	25.4 ± 2.7	79.1 ± 6.8
	Non-protease treated	30.5 ± 3.7	8.2 ± 1.0	29.5 ± 3.6	12.6 ± 1.5	19.3 ± 2.3	57.8 ± 5.5

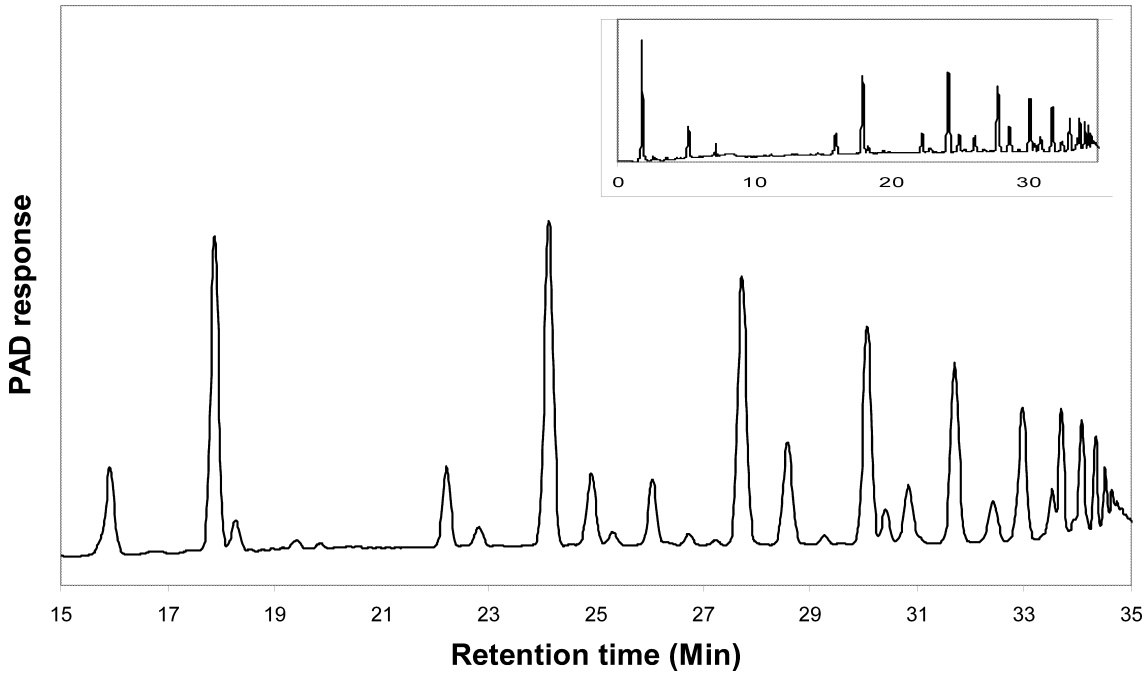

Fig. 2. HPAEC-PAD elution profile of the mild acid treated (0.05N TFA) EPS of *B. vesicularis*.

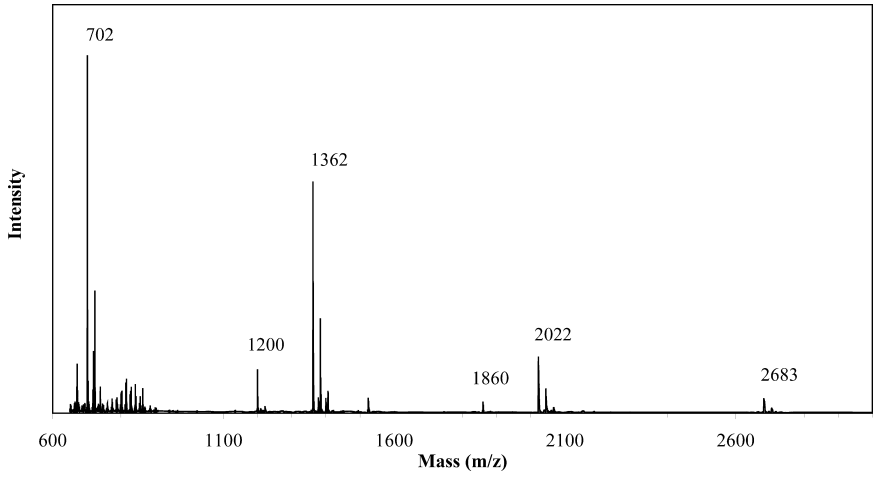

Fig. 3. MALDI-TOF mass spectrum of the mild acid treated (0.05N TFA) EPS of *B. vesicularis*.

After methanolysis the sugar residues released were also converted in trimethylsilylated (−)-2-butyl glycosides and analysed by GLC resulting in recognition of D-glucose, D-galacturonic acid, L-rhamnose L-glucuronic acid and D-galactose.

Partial hydrolysis of the novel EPS of B. vesicularis.—Partial hydrolysis was performed with 0.05N TFA for 1 h at 100 °C. This modification of the EPS was performed because rhamnose was present in the repeating unit of the EPS and the first NMR results indicated that rhamnose could be located in the backbone of the polymer. It is assumed that rhamnosyl linkages are weak linkages within the polymeric structure and would make it possible to release oligomeric fractions on the repeating unit level with rhamnosyl residues at the reducing end.

Partial hydrolyses was also carried out to decrease the viscosity, which results in the improvement of the resolution in the later shown NMR spectrums. The elution profile of the partially hydrolysed EPS is shown in Fig. 2. On the basis of the retention time of monogalacturonic acid (12.9 min) in the conditions used it could be concluded that the mild acid treatment of the EPS did not release that many monomeric residues. Taking into account the elution time of monogalacturonic acid it was suggested that the major peaks in the HPAEC elution profile represent a series of oligomeric substances with an increasing number of repeating units.

The same mild acid treated EPS was subjected to MALDI-TOF mass spectrometry, Fig. 3 shows the MALDI-TOF mass spectrum also with a series of main peaks. This series would start with the sodium adduct of one tetrameric repeating unit (UA_2, Rha_1, Hex_1) eluting at 17.9 min representing m/z 702 in the MALDI-TOF mass spectrum (Fig. 3), followed by two repeating units (UA_2, Rha_1, $Hex_1)_2$ eluting at 24.1 min representing m/z 1,362 and three repeating units eluting at 27.7 min representing m/z 2,022, etc. Combining these results with the results found by sugar composition analyses using hydrochloric acid in methanol we could already suggest that the repeating unit of the EPS is a tetramer consisting of glucose, rhamnose, galacturonic acid and glucuronic acid.

Apart from the oligomeric series of repeating units also another series of minor importance could be found [(UA_2, Rha_1, $Hex_1)_n + (UA_2$, $Rha_1)$]. This series starts with an heptamer (UA_2, Rha_1, $Hex_1) + (UA_2$, Rha_1) eluting at 22.2 min representing two repeating units minus one hexose residue m/z 1,200 and an undecamer (UA_2, Rha_1, $Hex_1)_2 + (UA_2$, Rha_1) eluting at 26.1 min representing three repeating units minus one hexose m/z 1,860, etc.

This indicates that the mild TFA treatment results in partial hydrolysis of the linkage between rhamnose and glucose and that only in a few cases it was also possible to hydrolyse the linkage between glucose and glucuronic acid.

$^1H-^{13}C$ NMR analysis of the native EPS of B. vesicularis.—The signals at δ 5.17, 4.66 and 4.44 ppm of **A** (Table 2) belong to the same sugar residue according to the COSY and TOCSY spectra (Figs. 4 and 5). Based on their chemical shifts they can be assigned as the H-1, H-5 and H-4 of α-Gal*p*A, respectively.[17–20]

Similarly the signals at δ 5.07 and 4.48 ppm of **B** are assigned as the H-1 and H-5 of α-Glc*p*A, respectively.[17,18]

The signal at δ 1.42 ppm, connected in the TOCSY spectrum with the H-1 at δ 4.48 ppm indicates that residue **C** can be assigned to β-Rha.[17,18,20]

Finally the chemical shifts found for residue **D** (Table 2) are typical for a β-glucose residue.

The total of four anomeric signals confirms the presence of a repeating unit of four sugar residues, as suggested by the sugar composition and results from the mild hydrolysis experiment.

The ^{13}C NMR spectrum of the native EPS also showed four anomeric signals at δ 107.54, 105.6, 104.74 and 103.96 ppm, respectively. The whole spectrum gave 22 signals where a repeating unit with the four sugar residues mentioned above would normally contain 24 ^{13}C signals. In this case the two expected downfield (around 180 ppm) signals for the ^{13}C of the carboxyl- or carboxylate group were not visible; it is known that these signals can have a very low sensitivity.[21] This information also proves the presence of two uronic acid residues.

$^1H-^{13}C$ NMR analysis of EPS of B. vesicularis *after mild acid treatment*.—The 0.05N TFA partially hydrolysed sample could also be used for NMR experiments. The very low amount of monomeric residues present in the hydrolysate might give rise to additional signals in the spectrum due to the rhamnose units at the reducing end and the glucose units at the non-reducing ends of these fragments. However, the partial hydrolysis with 0.05N TFA resulted in only some small fragments and no visible interference with the other residues was observed in the NMR spectra. On the other hand mild acid treatment made it possible to increase the EPS concentration in the sample improving the resolution in the NMR spectrums. By increasing the EPS concentration in the sample it became possible to record a HMBC 2D NMR spectrum (Fig. 6) to assign the glycoside linkage between the different sugar residues and to assign the signals in the ^{13}C NMR spectrum (Table 3).

Using the data obtained by heteronuclear HMBC experiment in Fig. 6 it was possible to assign al the signals in the ^{13}C NMR spectrum. This time it was possible to detect the two downfield signals for the carboxylate groups at the GalA and GlcA residues at δ 174.15 and 174.35 ppm, respectively. A clear glycosidation effect could be seen by the approximately 10 ppm downfield shift of the carbon atoms involved in the glycoside linkages between the different residues (C-4

Table 2
500 MHz ^1H NMR chemical shifts (δ) in ppm of the residues A, B, C and D or α-Gal*p*A, α-Glc*p*A, β-Rha and β-Glc*p* of the native EPS

Residue	Proton						
	H-1	H-2	H-3	H-4	H-5	H-6	H-6′
(A) α-D-Gal*p*A	5.17	3.96	4.01	4.44	4.66	–	–
(B) α-L-Glc*p*A	5.07	3.58	3.83	3.66	4.48	–	–
(C) β-L-Rha	4.86	4.07	3.65	3.55	3.52	1.42	–
(D) β-D-Glc*p*	4.5	3.35	3.68	3.65	3.57	3.96	3.88

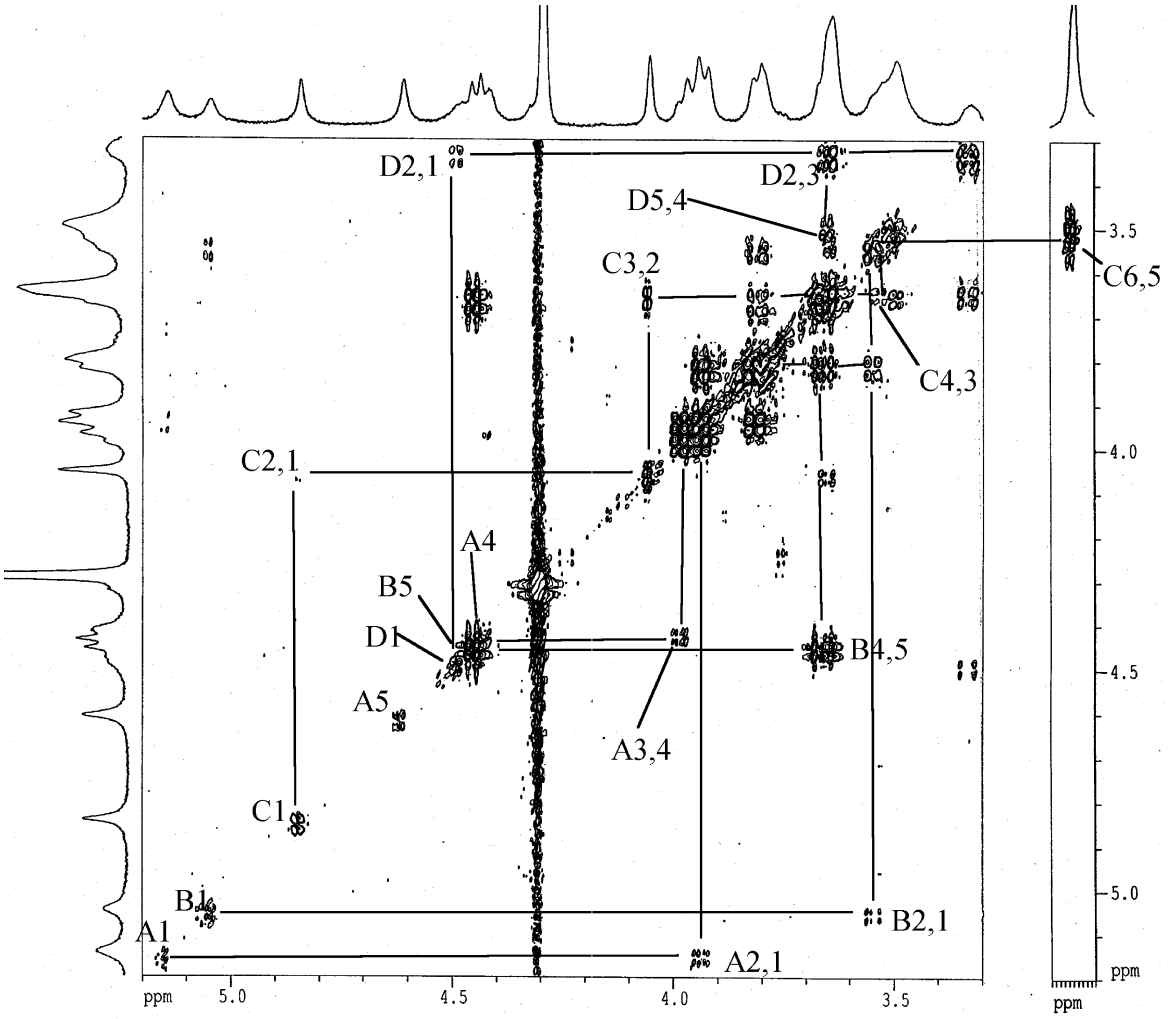

Fig. 4. 500-MHz 2D COSY spectrum of the native EPS of *B. vesicularis* recorded in D_2O at 70 °C. The code A1 stands for the diagonal peak belonging to **A** H-1; A 2,1 indicates the cross peak between **A** H2 and H 1, etc.

α-Gal*p*A δ 80.04 ppm, C-4 α-Glc*p*A δ 81.13 ppm, C-4 β-Rha δ 81.86 ppm and C-4 β-Glc*p* δ 78.09 ppm).

Looking at the proton chemical shifts within Table 3 and comparing these with the proton chemical shifts found for the native polysaccharide, one could see a clear difference in especially the values found for H-4 and H-5 of the uronic acid residues. It is known that a lower pD results in a downfield shift of the protons of uronic acids[22,23] and the use of mild acid treatment could have resulted in a lower pD value.

The assignment of the glycosidic linkages between the different residues is based on the HMBC experiment and a homonuclear NOESY experiment (results not shown). Starting with residues A and B which show a **B** C-1, **A** H-4 and a **B** H-1, **A** C-4 cross-peak supported by a NOESY cross-peak between **B** H-1 and **A** H-4 typical for a (1→4)-glycosidic linkage between GluA and GalA. The inter residual cross-peaks between **A** H-1 and **C** H-5 and **C** H-6 found in the NOESY spectrum and the heteronuclear cross-peak between **A** C-1 and **C** H-4, suggest a GalA-(1→4)-Rha linkage.

The overlap between H-3 and H-4 of the β-glucose residue (**D**) made it difficult to assign the right glycosidic linkage between rhamnose and glucose. But since it is known that the carbon atoms involved in a glycosidic linkage shift around 10 ppm downfield there could only be a (1→4)-linkage present because **D** C-4 was the only carbon atom that shows a significant downfield shift.[24] A Rha-(1→4)-Glc linkage could be proven by the **D** H-4, **C** C-1 and **C** H-1, **D** C-4 cross-peaks shown in the HMBC spectrum. In addition the 2D NOESY spectrum showed a **C** H-1 and **D** H-3, 4 crosspeak. The glycosidic linkage between residue D and B can be revealed by a **D** C-1, **B** H-4 and a **D** H-1, **B** C-4 cross peak in the HMBC spectrum. From the results found by ^{13}C and ^{1}H NMR analysis it could be concluded that the structure of the repeating unit of the novel EPS of *B. vesicularis* is as follows:

→4)-α-L-Glc*p*A-(1→4)-α-D-Gal*p*A-(1→4)-β-L-Rha*p*-(1→4)-β-D-Glc*p*(1→

(**B**) (**A**) (**C**) (**D**)

Sugar linkage composition.—Sugar linkage analysis combined with mass spectrometry detection confirmed the interpretation of the NMR spectra, but was mainly qualitative. No quantitative assignment could be achieved, because of the presence of unmethylated material and the presence of uronic acids, which were not reduced. Linkage analysis also revealed the presence a (1→4)-glucose and a (1→4)-linked rhamnose and of minor amounts of terminal galactose, (1→3)-linked glucose and 1,3,4-branched glucose. No evidence is present that these residues are connected to the EPS. If this would be the case, this would mean that there is a (3→1)-Glc-(3→1)-Gal side chain present on the glucose residue in one out of five repeating units, but this is not seen by NMR. During the purification steps no residues were lost due to glycosidase activity in the protease used, because no significant differences could be found in the proton NMR spectra of both EPS's. Biochemically, a suggestion of a side chain on one out of five repeating units would be surprising, because it is rather rare to find heteropolysaccharides with an irregular composition with respect to the repeating unit backbone and side chain structure.[25]

4. Conclusions

From the data found by chemical, mass spectrometry and NMR experiments it can be concluded that *B. vesicularis* sp. produces a linear exopolysaccharide without non-sugar substituents containing a tetrasaccharide-repeating unit with the following structure:

→4)-α-L-Glc*p*A-(1→4)-α-D-Gal*p*A-(1→4)-β-L-Rha*p*-(1→4)-β-D-Glc*p*(1→

The novel EPS consists of only one distinct homologue population with a molecular weight distributed around 2,000–4,000 kDa and an intrinsic viscosity of around 6.5 dL/g.

It also became clear that for our EPS methanolysis with 2N HCl in dry methanol followed by TFA hydro-

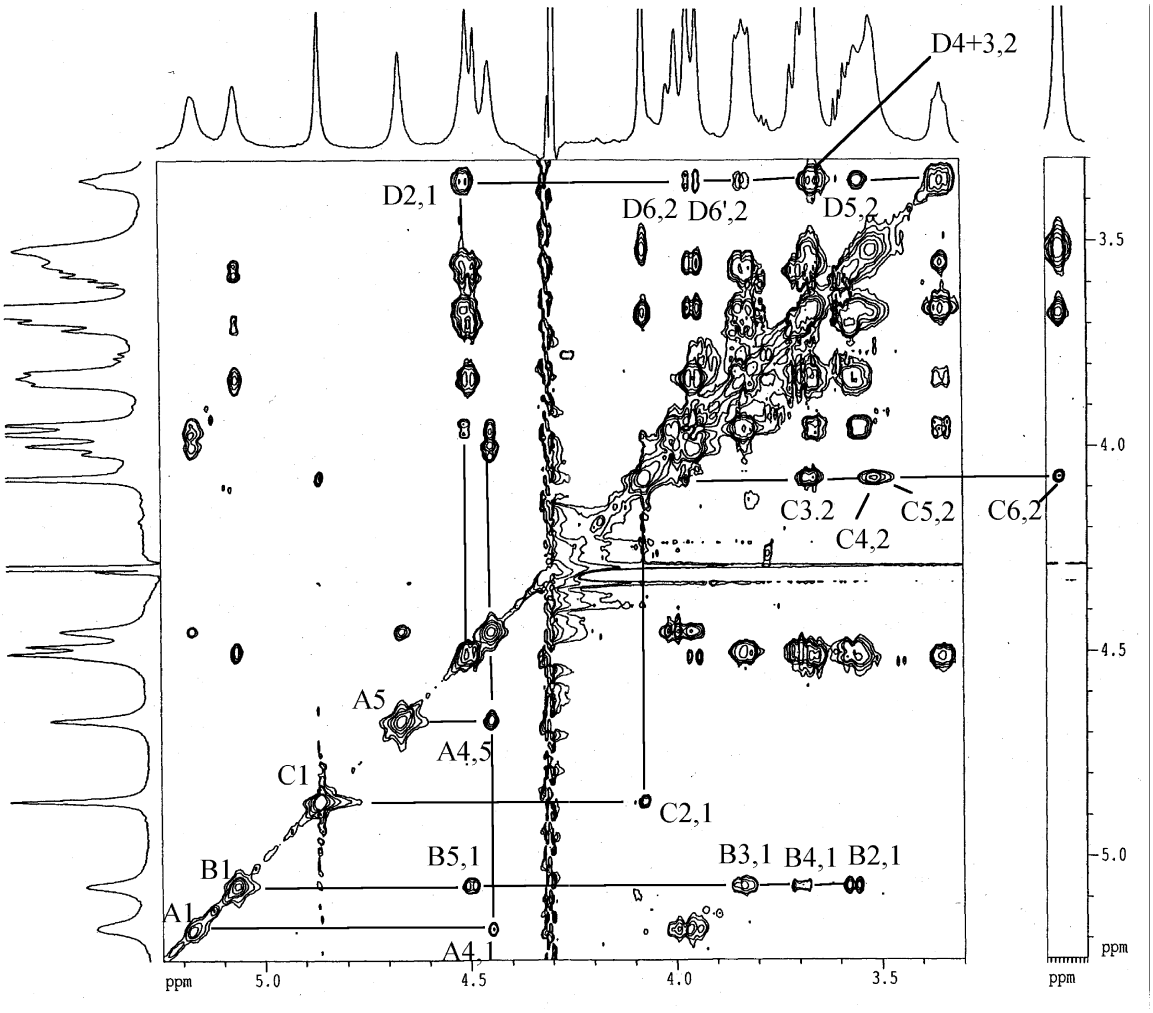

Fig. 5. 500-MHz 2D TOCSY spectrum (110 ms mixing time) of the native EPS of *B. vesicularis* recorded in D_2O at 70 °C. The code A1 stands for the diagonal peak belonging to **A** H-1; A 4,1 indicates the cross peak between **A** H4 and H 1, etc.

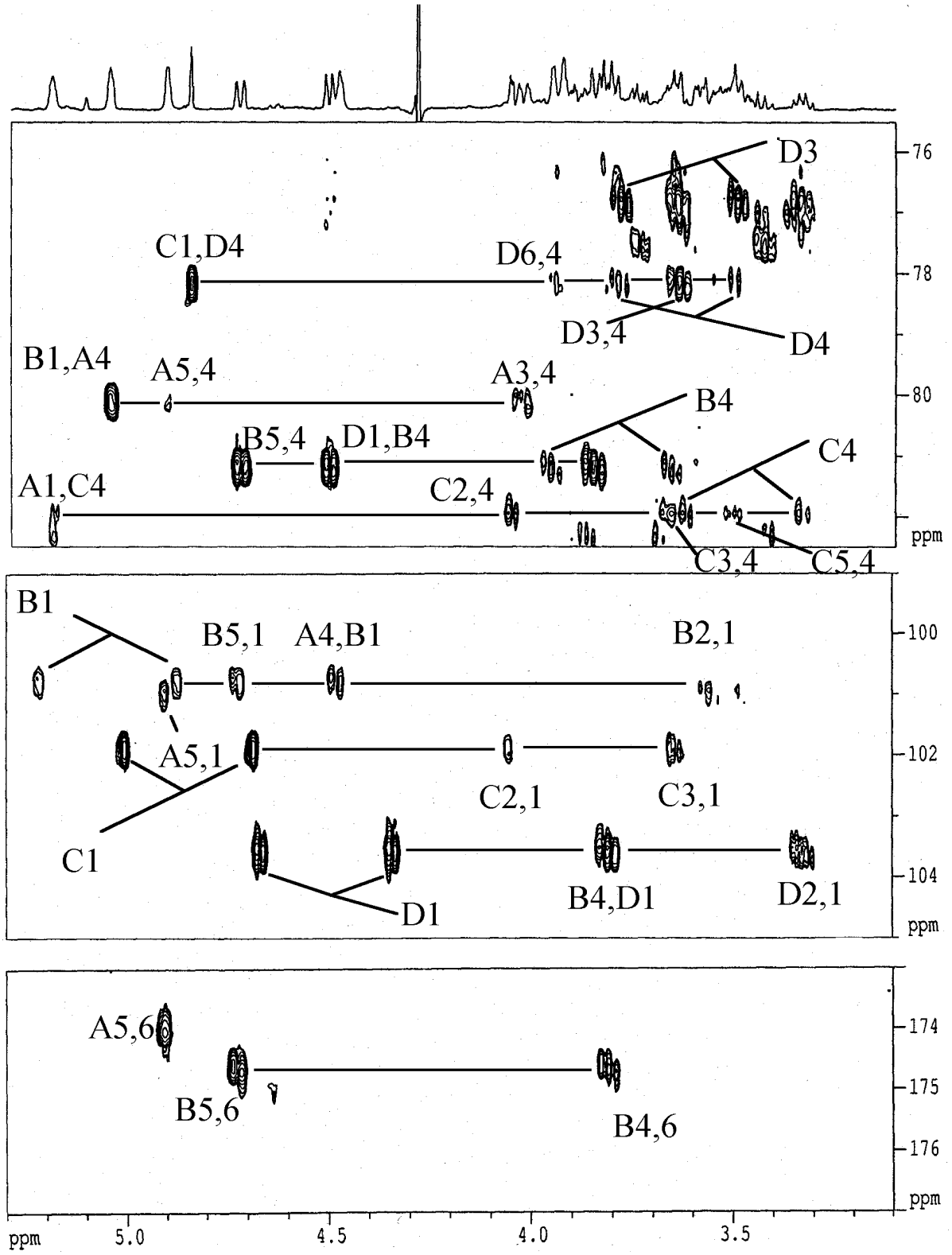

Fig. 6. 500-MHz 2D ^{1}H ^{13}C undecoupled HMBC spectrum of the partial hydrolysed EPS of *B. vesicularis* recorded in D_2O at 70 °C. The code C1 stands for the coupling between **C** H-1 and C-1, C2,1 stands for the coupling between **C** H-2 and C-1 and A4, B1 stands for the long range coupling between **A** H-4 and **B** C1, etc.

Table 3
^{1}H and ^{13}C chemical shifts (δ) in ppm of the residues A, B, C and D or α-Gal*p*A, α-Glc*p*A, β-Rha and β-Glc*p* of the hydrolysed EPS

Residue	H-1/(C-1)	H-2/(C-2)	H-3/(C-3)	H-4/(C-4)	H-5/(C-5)	H-6/(C-6)	H-6′/(C-6)
(A) α-D-Gal*p*A	5.20 (100.88)	3.95 (69.58)	4.02 (69.72)	4.48 (80.04)	4.91 (71.65)	– (174.15)	–
(B) α-L-Glc*p*A	5.11 (100.68)	3.60 (72.34)	3.86 (72.87)	3.79 (81.04)	4.72 (71.78)	– (174.35)	–
(C) β-L-Rha	4.85 (101.81)	4.06 (72.65)	3.67 (72.65)	3.49 (81.86)	3.49 (73.10)	1.40 (18.29)	–
(D) β-D-Glc*p*	4.51 (103.51)	3.33 (74.53)	3.65 (77.01)	3.64 (78.09)	3.54 (70.98)	3.90 (62.17)	3.75 (62.17)

lysis proved to be more useful than sulphuric acid hydrolysis in determining the sugar composition of the EPS.

The novel EPS found contains a linear backbone consisting of four sugar residues. It is obvious that enzymatic degradation may be rather limited due to the variation in sugars, conformation and linkage type present. Further research will be directed towards screening of such enzyme activity in crude enzyme preparations and possibly by screening of cDNA libraries.

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