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Methylobacterium sp. isolated from a Finnish paper machine produces highly pyruvated galactan exopolysaccharide

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Abstract

The slime-forming bacterium *Methylobacterium* sp. was isolated from a Finnish paper machine and its exopolysaccharide (EPS) was produced on laboratory scale. Sugar compositional analysis revealed a 100% galactan (EPS). However, FT-IR showed a very strong peak at 1611 cm^{-1} showing the presence of pyruvate. Analysis of the pyruvate content revealed that, based on the sugar composition, the EPS consists of a trisaccharide repeating unit consisting of D-galactopyranose and [4,6-*O*-(1-carboxyethylidene)]-D-galactopyranose with a molar ratio of 1:2, respectively. Both linkage analysis and 2D homo- and heteronuclear ^{1}H and ^{13}C NMR spectroscopy revealed the following repeating unit: →3)-[4,6-*O*-(1-carboxyethylidene)]-α-D-Gal*p*-(1→3)[4,6-*O*-(1-carboxyethylidene)]-α-D-Gal*p*-(1→3)-α-D-Gal*p*-(1→. By enrichment cultures from various ground and compost heap samples a polysaccharide-degrading culture was obtained that produced an endo acting enzyme able to degrade the EPS described. The enzyme hydrolysed the EPS to a large extent, releasing oligomers that mainly consisted out of two repeating units.

Keywords: *Methylobacterium* sp.; EPS; Pulp and paper industry; Pink slime; Enzyme; Galactan; Pyruvate; NMR

1. Introduction

Slime deposits in the paper industry are the cause of 70% of all web breaks, blockages and pump failures and therefore are a significant problem within a paper mill.[1] These slime deposits and the problems related to them are generally referred to as biofouling.[2]

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.[2–4] Under these conditions the bacteria present form microbial biofilms, which are accumulations of microorganisms, exopolysaccharides (EPS), multivalent cations, biogenic and inorganic particles as well as colloidal and dissolved compounds. EPSs are the major component of a biofilm and are responsible for the structural and functional integrity of the biofilm.[5]

The EPSs found in paper mill environments are largely heteropolysaccharides mainly consisting of fucose, rhamnose, glucose, mannose and glucuronic acid.[4,6,7] Apart from the biofilm itself slime deposits also contain material derived from the paper manufacture process, such as fibre and organic and inorganic precipitates from the process waters.[7]

Prevention of the problems related to slime deposits is still largely performed by the use of more or less toxic biocides. However, due to environmental considerations nowadays alternative methods are being investigated for slime control. One of these alternative approaches is the

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use of enzymes to degrade the EPSs responsible for the structural integrity of the slime deposits.

Levanase is an example of an enzyme that degrades the specific EPS called levan.[8] Another example is the use of an enzyme able to degrade colanic acid[9] an EPS that is produced by several bacteria belonging to the Enterobacteriaceae family.[10] Furthermore, Rättö and coworkers screened several bacterial strains that showed enzyme activities towards EPSs produced by bacteria isolated from paper machines.[11] For a more targeted search of novel enzymes able to degrade these EPSs more knowledge about the chemical fine structure of these EPSs is necessary. However, only knowing the sugar composition is not sufficient to define a (mixture of) enzymes required to degrade these EPSs, since the monosaccharides present can be linked in many different ways. Furthermore, these EPSs can be substituted with several different organic and inorganic substituents like acetyl, pyruvate or phosphate esters.[12]

Until now hardly any chemical structures for the EPSs in paper mill biofilms have been published. It has been suggested that a fructose-containing polysaccharide levan is the EPS secreted by several species of *Bacillus* and *Pseudomonas* bacteria in recirculated water of the paper machine.[8] Furthermore, the structure of the EPS produced by *Brevundimonas vesicularis* isolated from a Finnish paper mill has been elucidated. *B. vesicularis* produces a linear polysaccharide containing both glucuronic- and galacturonic acid next to rhamnose and glucose in its repeating unit.[13]

Bacteria belonging to the *Methylobacterium* species are known generally to be pink-pigmented bacteria that produce so called pink slime and they were first isolated by Oppong and coworkers,[14] but the structure of the EPS produced by *Methylobacterium* strains has not been studied. Therefore, this publication deals with the structural elucidation and enzymatic degradation of the EPS produced by a bacterium belonging to the *Methylobacterium* sp. VTT-E-011929 isolated from a Finnish cardboard producing paper mill using chemical pulp as raw material.

2. Experimental

2.1. Isolation of bacterial species and extraction and purification of the EPS produced

The bacterial species *Methylobacterium* sp. VTT-E-11929 was isolated from a slime sample obtained from a Finnish cardboard producing paper machine. After isolation and purification of the strain the EPS 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), $MgSO_4 \cdot 7 H_2O$ (0.3 g/L), $CaCl_2$ (0.05 g/L), $ZnSO_4 \cdot 7 H_2O$ (0.2 mg/L), $CuSO_4 \cdot 5 H_2O$ (0.2 mg/L), $MnSO_4 \cdot H_2O$ (0.2 mg/L), $FeSO_4 \cdot 7 H_2O$ (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. 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 12–14,000 Da) and freeze-dried.

2.2. 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.

2.3. HPSEC of the native EPS

The EPS (2 mg) was dissolved in 1 mL of distilled water and analysed by high performance size exclusion chromatography (HPSEC) using pullulan for calibration. HPSEC was performed on a Thermo Quest HPLC using three TOSOHAAS TSK-Gel columns in series (6000-, 4000-, 3000PWXL) preceded by an TSK guard column (40 × 6 mm). Enzyme digests were analysed using three TOSOHAAS TSK-Gel columns in series (4000-, 3000-, 2500PWXL) preceded by an TSK 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.

2.4. Sugar composition

The EPS sugar composition was determined using methanolysis as described by De Ruiter and coworkers.[15] The EPS was treated with 2 M HCl in dry CH_3OH for 16 h at 80 °C, followed by 1 h of 2 M CF_3CO_2H (TFA) at 121 °C. The released sugars were analysed using high performance anion exchange chromatography (HPAEC) with pulsed amperometric detection (PAD) as described by Verhoef and coworkers.[13]

2.5. Absolute configuration

The absolute configurations of the monosaccharides present in the EPS were determined as described by

Gerwig and coworkers.[16] The GC-FID analyses of the tri-methylsilated (−)-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 (approx. 0.5 μL) were injected directly on the column without a stream splitter.

2.6. Sugar linkage analyses

The EPS sample was methylated according to Hakomori[17]and subsequently dialysed against water and evaporated in a stream of dry air. The methylated samples were hydrolysed using 2 M TFA (2 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.[18] Identification of the compound was performed using GC–MS as described by van Casteren and coworkers.[19]

2.7. Determination of the pyruvate content

The amount of pyruvate was determined using a method reported by Troyano and coworkers.[20] The EPSs (1 mg) were dissolved in 1 mL of 1 M TFA and hydrolysed for 6 h at 120 °C. The hydrolysate was centrifuged and analysed at 40 °C using a Termo Quest HPLC system equipped with both a Shodex RI71 refractive index detector and a Spectra Physics UV2000 UV detector at 220 nm. HPLC separation was performed using a Bio-Rad Aminex HPX-87H column using 0.6 mL/min 0.01 M H_2SO_4 as a mobile phase. The amount of pyruvate was calculated using a calibration curve of 0.2–2 mM pyruvic acid.

2.8. Partial hydrolyses of the native EPS

Since enzymes are known to facilitate the recognition of repeating units, enzyme-producing bacterial cultures were enriched from compost heap samples by using the EPS produced by *Methylobacterium* as the only carbon source. Culture filtrate from an enrichment culture showing clear reduction of EPS viscosity was used as a crude enzyme preparation to incubate with the EPS in order to obtain oligomers preferably on a repeating unit level. The EPS (1 mg/mL 50 mM NaOAc buffer) was incubated 50 μL/mL culture filtrate at 30 °C for 15 h in an incubation shaker. The reaction was stopped by keeping the solution at 100 °C for 15 min, the sample was centrifuged before analysis. The released

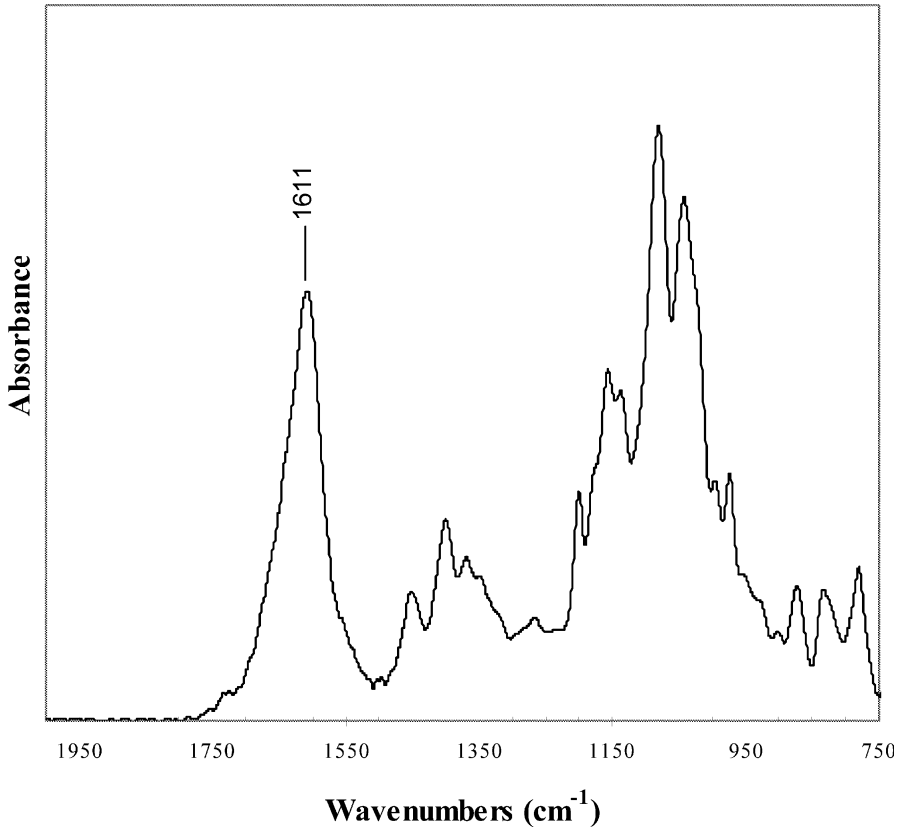


Fig. 1. FT-IR spectrum of the native EPS produced by *Methylobacterium* sp. after drying 2 mg/ml of the sample on an ATR crystal.

Table 1
Glycosidic linkage composition in mol% of the partially methylated alditol acetates of the native and depyruvated EPS of *Methylobacterium* sp.

Glycosyl residue	Yield (mol%)		Relative retention time [a]
	Native EPS	Depyruvylated EPS	
3-Linked-galactose	39	93	0.643
3-4-6-Linked-galactose	61	7	0.889

[a] Relative to inositol.

oligomers were analysed using MALDI-TOF MS and HPSEC as described by Verhoef and coworkers.[13]

2.9. Auto hydrolyses

Auto hydrolysis was used to remove pyruvate from the polysaccharide backbone. The native EPS (40 mg) was dissolved in 40 mL water and brought to its H^+ form using Amberlite IR 50 ion exchange resin. A suspension of the polysaccharide solution and Amberlite IR 50 was stirred for 0.5 h at 25 °C. After filtering of the Amberlite the EPS solution was stirred at 100 °C under reflux for 18 h. The depyruvated EPS was dialysed against water for 24 h and freeze-dried.

2.10. FT-IR

The EPS (1 mg/mL) was dried on a crystal and the absorption spectrum between 750 and 4000 cm^{-1} was taken using attenuated total reflectance on a BIO-RAD FTS 6000 FT-IR spectrometer.

2.11. ^{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: 2.225 ppm for ^{1}H and 31.55 ppm for ^{13}C. The 1D ^{1}H proton spectra were recorded at 500.13 MHz using 64 scans of 8192 data points and a sweep width of 3000 Hz. The 2D COSY spectrum was acquired using the double quantum filtered (DQF) method with a standard pulse sequence delivered by Bruker. 2D TOCSY spectra were acquired using standard Bruker pulse sequences with 110 ms mixing time. For all homonuclear 2D spectra 512 experiments of 2048 data points were recorded using 16–64 scans per increment. For the 2D HMBC spectrum[21] 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 1024 experiments of 2048 data points were performed with 128 scans per increment.

3. Results and discussion

3.1. Isolation and purification

Bacterial species *Methylobacterium* sp. was isolated from a Finnish cardboard producing paper mill using chemical pulp as raw material. After isolation the bacterial species was grown on laboratory scale and the EPS produced. Prior to the experiments needed for structure elucidation the isolated and purified EPS was analysed for protein content and molecular size distribution. The protein content was found to be 4% (w/w), while the HPSEC elution profile showed the EPS has a molecular weight of 2000 kDa. Furthermore, the elution profile showed that no other carbohydrate populations were present making it possible to further elucidate the EPS fine structure without further purification.

3.2. Sugar composition and absolute configuration

The sugar composition of the EPS was determined by subjecting the EPS to methanolysis followed by TFA hydrolysis. The released sugar residues were analysed by HPAEC and it was found that the EPS only consists of galactose. However, the FT-IR spectrum (Fig. 1) of the native EPS showed a strong peak at 1611 cm^{-1} typical for a carboxylate anion indicating the presence of an organic acid substituent. Since pyruvate is very common as an organic acid substituent[12] and is not ester linked to the sugar residues the pyruvate content was measured by HPLC. This revealed that the EPS consisted of both galactose and pyruvate in a molar ratio of 3:2, respectively. The galactose present was found to be in the D absolute configuration. The results above indicate that the repeating of the EPS would consist of a unit containing 1 D-galactose and 2 pyruvated D-galactoses.

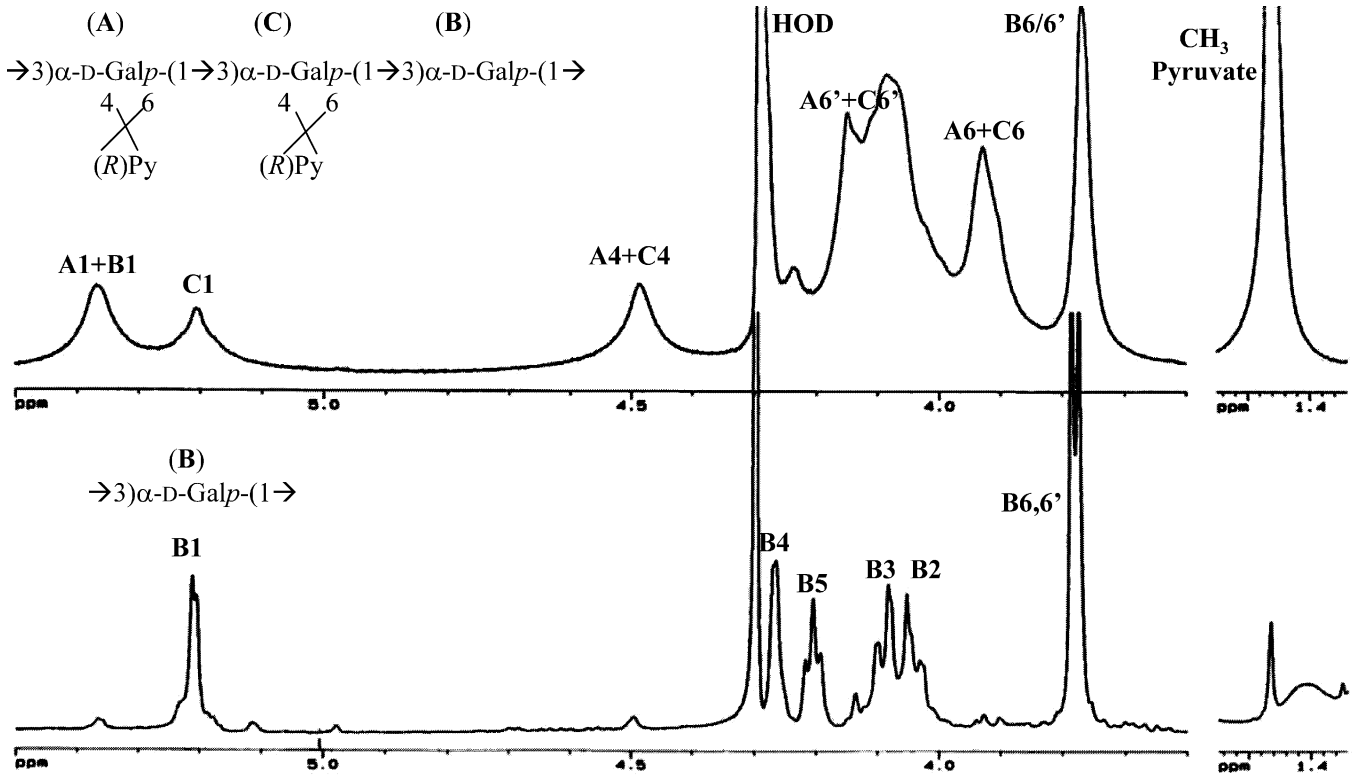


Fig. 2. ^{1}H NMR spectra of both the native (upper trace) and depyruvated EPS (bottom trace) produced by *Methylobacterium* sp.

3.3. Depyruvylation using autohydrolysis

Pyruvate is a acid labile group and thus can be removed under mild acid conditions[22] to obtain a depyruvated sample for both NMR and linkage analysis without degrading the galactan backbone of the EPS. Therefore, the EPS was subjected to autohydrolysis, which is known to remove pyruvate ketals.[23]

By autohydrolysis the removal of 90% of the pyruvate ketals from the galactan backbone was achieved, resulting in a depyruvated EPS sample necessary for linkage analysis. The depyruvated sample was analysed by HPSEC revealing a significant decrease in molecular weight, which resulted in a less viscous sample for NMR measurement.

3.4. Linkage analysis

Both the native and depyruvated EPS were subjected to permethylation according to Hakomori.[17] Table 1 shows the amounts of the different partly methylated alditol acetates released after per methylation followed by hydrolysis of the EPS. Analysis of the GC–MS spectra of the different partly methylated alditol acetates from the native EPS after hydrolysis resulted in the recognition of 39-mol% of (1→3)-linked galactose and 61-mol% of 1,3,4,6-linked galactose. This agrees with the conclusions made from the EPS composition that two out of three galactoses found is substituted with a pyruvate ketal. However, from the results found for the native EPS it is not possible to distinguish between a 4–6 and a 3–4 bound pyruvate ketal.

The GC–MS spectrum of the different partly methylated alditol acetates of the depyruvated EPS revealed the presence of 93-mol% 3-linked-α-galactose and 7-mol% 3,4,6-linked-α-galactose in the EPS. From these results we could conclude that the EPS consists of only 3-linked-α-galactose residues. Furthermore, this proved that the repeating unit of the EPS consists of 1 D-galactopyranose and 2 [4,6-*O*-(1-carboxyethylidene)]-D-galactopyranoses, confirmed by the NMR analysis below.

3.5. ^{1}H NMR analysis of the native EPS

Fig. 2 shows the 1D proton NMR spectrum of both the native and depyruvated EPS. The 1D proton NMR spectrum of the native EPS showed 3 signals (A, B, C) in the α anomeric region at 5.36, 5.38 and 5.21 ppm, respectively. Revealing that the 3 D-galactopyranose are in the α anomeric conformation. Furthermore, the 1D proton NMR spectrum showed a signal at 1.48 ppm typical for the pyruvate ketals found to be substituted to the galactan backbone.[22,24] By recording both a 2D TOCSY (result not shown) and 2D COSY (Fig. 3) NMR spectrum of the native EPS it became possible to assign the proton chemical shifts of the sugar residues present (Table 2).The anomeric signal C has a chemical

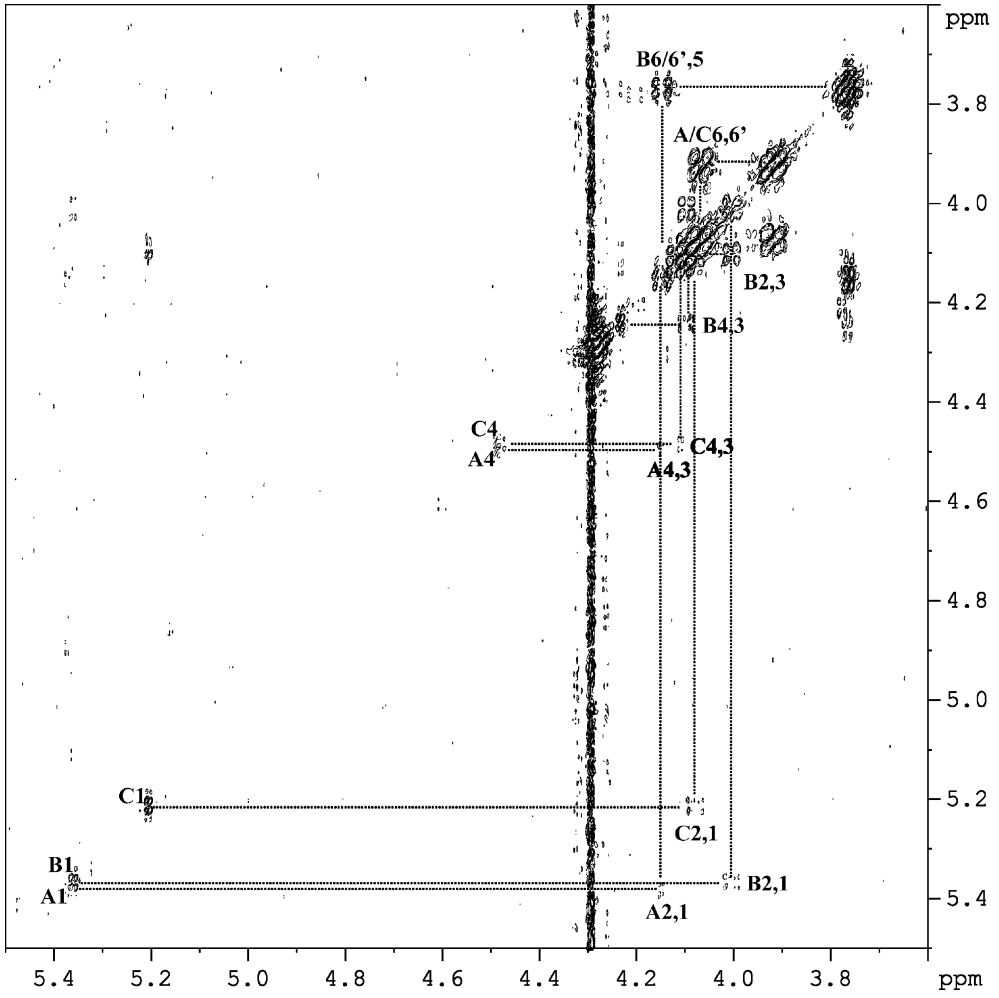


Fig. 3. Homonuclear 2D COSY NMR spectrum of the native EPS produced by *Methylobacterium* sp. recorded in D_2O at 70 °C. The code A1 stands for the diagonal peak belonging to A H-1; A 2,1 indicates the cross peak between A H-2 and H-1, etc.

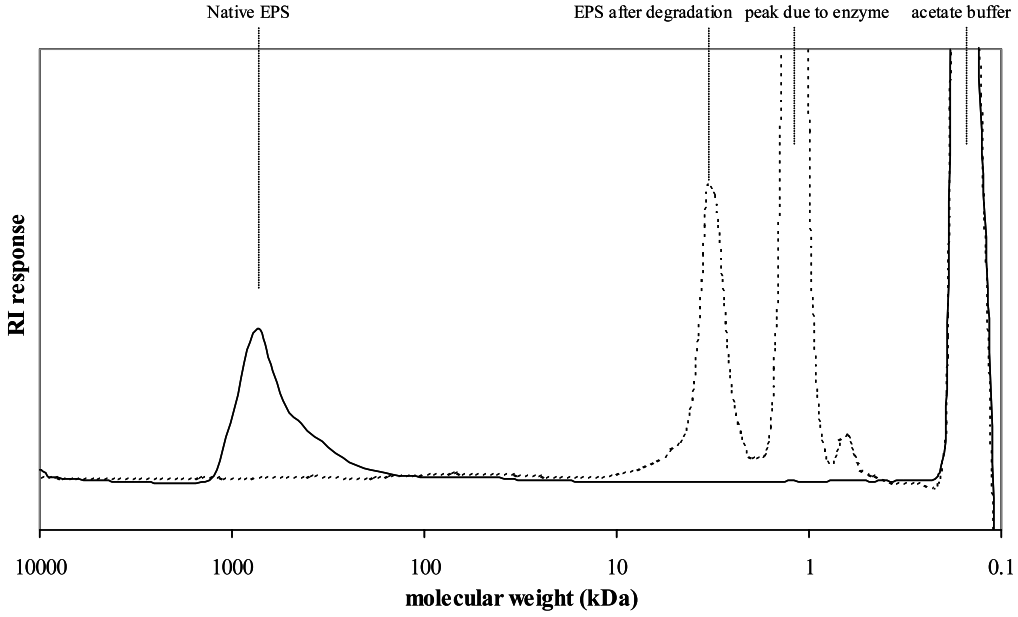


Fig. 4. HPSEC elution profile of both the native (—) and enzyme treated EPS (----) produced by *Methylobacterium* sp.

Table 2
Proton chemical shift (ppm) of both the native and depyruvated EPS produced by *Methylobacterium* sp. [a]

Residue	H-1	H-2	H-3	H-4	H-5	H-6	H-6′
Native EPS							
(A) →3,4,6)-α-Gal*p*	5.36	4.15	4.15	4.49	nd	3.97	4.08
(B) →3)-α-Gal*p*	5.37	4.01	4.11	4.24	4.08	3.76	3.76
(C) →3,4,6)-α-Gal*p*	5.21	4.08	4.11	4.47	4.13	3.97	4.08
Pyruvate (CH_3)	1.48						
Depyruvated EPS							
→3)-α-Gal*p*	5.21	4.05	4.08	4.27	4.21	3.77	3.77

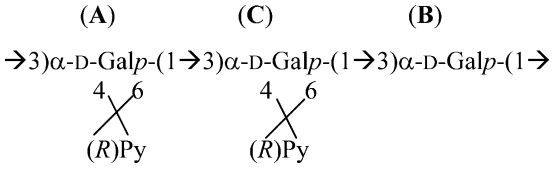
nd = Non-determined.
[a] Chemical shifts relative to internal acetone 2.225 ppm.

shift comparable to the chemical shift found for α-galactopyranoses within literature[25,26] however the anomeric proton of residue A and B show a significant downfield shift compared to that residue C. This indicates that the neighbouring residue at position 1 of A and B is substituted with a pyruvate ketal. The chemical shifts of residue B indicated that this residue is the unsubstituted α-galactose, because apart from the downfield shifted anomeric signal the protons show chemical shifts comparable to the chemical shifts reported[25,26] for 3)-α-galactopyranose. Compared to residue B, A and C show a significant downfield shift with respect to proton 4 and 6 indicating that residue A and C are both substituted with a pyruvate ketal.[25–28]

Fig. 2 also shows the proton NMR spectrum of the depyruvated EPS, by recording a 2D COSY spectrum (results not shown) it became possible to assign all the chemical shifts (Table 2). Fig. 2 shows that after removing the pyruvate ketals only one anomeric signal left at 5.21 ppm and the disappearance of the pyruvate CH_3 singlet at 1.48 ppm. Furthermore, the depyruvated EPS shows similar chemical shifts compared to residue B of the native EPS belonging to the non-substituted galactose, apart from the anomeric signal, which is comparable to the one of residue C in the native EPS. Residue C does not have a substituted galactose at position 1 as neighbouring sugar residue.

A 2D heteronuclear HMBC spectrum was recorded of the oligomers released during enzyme treatment (see below), but it was not possible to solve the spectrum due to low resolution. However, the ^{13}C chemical shifts of the pyruvate ketals could be determined. The carbon of the carboxylic acid group showed a chemical shift of 176.7 ppm and the carbon of the methyl group showed a chemical shift of 26.4 ppm. Together with the proton chemical shift of the methyl group according to Ref. 24, this proves the absolute configuration of the pyruvate ketal is in the R form. Furthermore, the heteronuclear crosspeak of the scalar coupling between the quaternary carbon of the pyruvate ketal at 102.1 ppm with residue A and C H-6′ at 3.97 ppm could be seen, which is in agreement with the 2 [4,6-*O*-(1-carboxyethylidene)]-α-D-galactopyranoses found.

Combining the results found by linkage analysis with the results found for NMR analysis it becomes clear that the EPS is a highly pyruvated α-(1→3)-galactan with the following repeating unit.



3.6. Selective hydrolysis by enzymes from a culture filtrate

To obtain oligomeric fragments, preferably on a repeating unit level, the native EPS was incubated with a crude enzyme preparation obtained using an enrichment culture isolated from a compost heap. HPSEC (Fig. 4) was used to determine the decrease in molecular weight after incubating the EPS for 15 h with the culture filtrate. The HPSEC elution profile showed a significant decrease in molecular weight of the enzyme-degraded EPS compared to the native EPS indicating the presence of oligomeric fragments. Fig. 5 shows the MALDI-TOF MS spectrum of the enzyme-degraded EPS, one intense peak $m/z = 1294$ representing two repeating units consisting of 2 D-galactoses (Gal) residues and 4 [4,6-*O*-(1-carboxyethylidene)]-D-galactoses (Gal-Py) residues is visible. In addition to the peak at $m/z = 1294$ Fig. 5 shows minor peaks at $m/z = 830$, 1061, 1131 and 1455 due to 4Gal2Gal-Py, 5Gal3Gal-Py, 5Gal4Gal-Py and 7Gal4Py, respectively.

It is evident that the enzyme requires preferably more than two repeating units to efficiently split the polymeric backbone. This is supported by the products of enzymatic reaction: the main peak found representing an hexamer of two repeating units accompanied by several

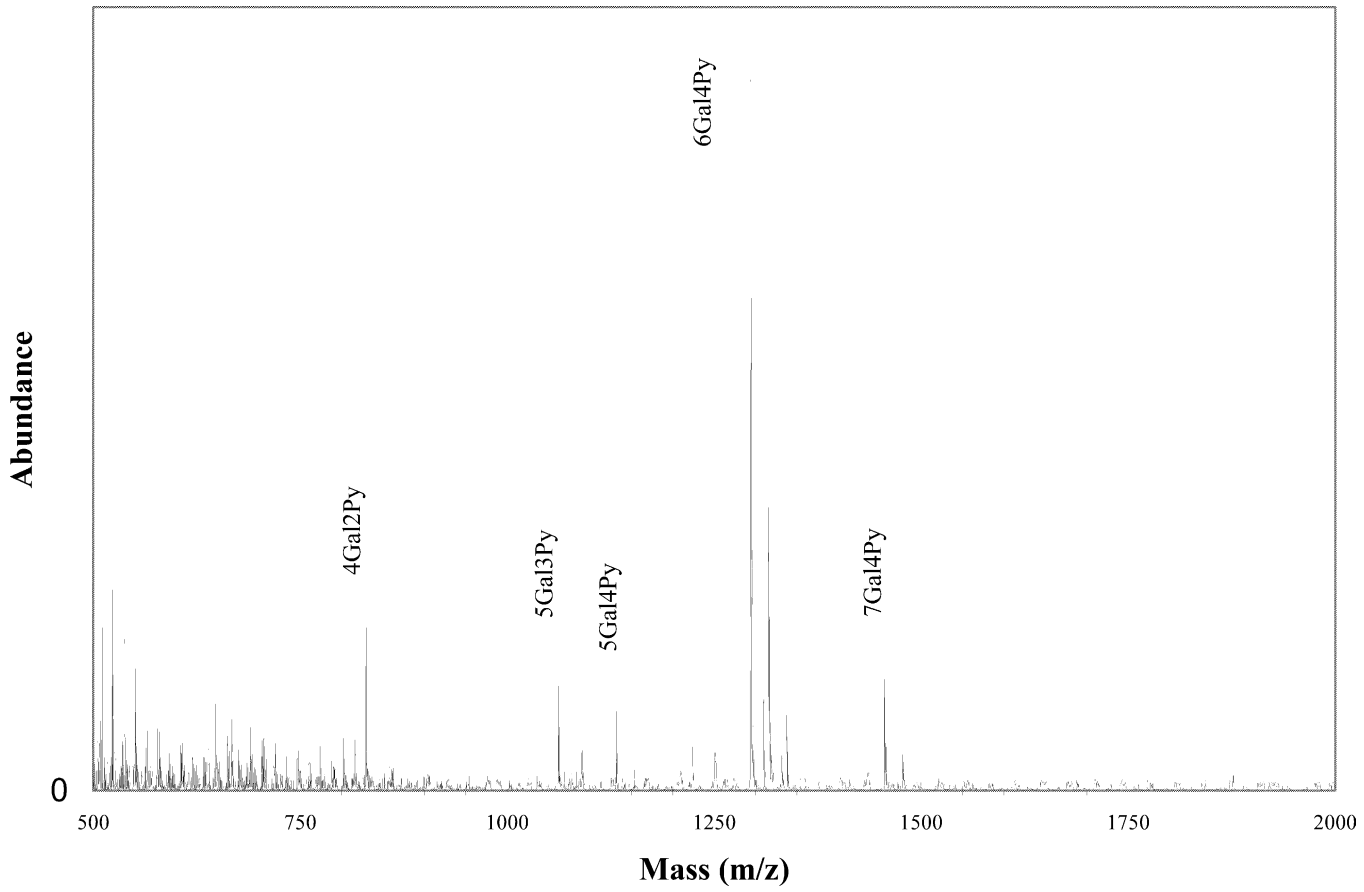


Fig. 5. MALDI-TOF mass spectrum of the enzyme treated EPS produced by *Methylobacterium* sp. (Py = pyruvate).

minor peaks,which indicates that the enzyme shows a relatively low affinity for this hexamer.

According to these results the culture filtrate contained an endo acting enzyme that degrades the α-(1 → 3)-galactan backbone and is tolerant for the two neighbouring [4,6-*O*-(1-carboxyethylidene)]-α-D-galactoses per repeating unit, and mainly releases oligomers consisting out of two repeating units.

4. Conclusions

The overall data showed that this *Methylobacterium* sp. strain, representing a species which is known to be responsible for so called pink slime, produces a α-(1 → 3)-galactan polysaccharide. However, the galactan backbone was found to be highly substituted with pyruvate ketals. This lead to the recognition of a repeating unit consisting of 1 α-D-galactopyranose and 2 [4,6-*O*-(1-carboxyethylidene)]-α-D-galactopyranoses.

Furthermore, an endo acting enzyme that degrades the α-(1 → 3)-galactan backbone, obtained by an enrichment culture, was recognised being tolerant to the two neighbouring [4,6-*O*-(1-carboxyethylidene)]-α-D-galactoses per repeating unit, and mainly releasing oligomers consisting of two repeating units.

Further research will be directed towards the purrification an characterisation of the enzyme found within the culture filtrate.

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References

1. Safade, T. *Paper Technol. Ind.* **1988**, 280–285.
2. Klahre, J.; Flemming, H. C. *Water Res.* **2000**, *34* (14), 3657–3665.
3. Edwards, J. C. *Tappi J. Norcross Ga.: TAPPI* **1996**, *79* (7), 71–77.

4. Väisänen, O.; Nurmiaho-Lassila, E.; Marmo, S.; Salkinoja-Salonen, M. *Appl. Environ. Microbiol.* **1994**, *60* (2), 641–653.
5. Wingender, J.; Neu, R. T.; Flemming, H.-C. In *Microbial Extracellular Polymeric Substances; Characterisation, Structure and Function*; Wingender, J.; Neu, R. T.; Flemming, H.-C., Eds. What are bacterial extracellular polymeric substances?; Springer: Heidelberg, 1999; pp 1–19.
6. Lindberg, L. E.; Holmbom, B. R.; Vaisanen, O. M.; Weber, A. M. L.; Salkinoja Salonen, M. S. *Appl. Microbiol. Biotechnol.* **2001**, *55* (5), 638–643.
7. Rättö, M.; Perttula, M.; Siika-aho, M. *Mededelingen Faculteit Landbouwkundige en Toegepaste Biologische Wetenschappen Universiteit Gent* **1998**, *63* (4a), 1183–1186.
8. Chaudhary, A.; Gupta, L. K.; Gupta, J. K.; Banerjee, U. C. *Biotechnol. Adv.* **1998**, *16* (5-6), 899–912.
9. van Speybroeck, M. M.; Bruggeman, G.; van Poele, J.; van Pee, K.L.I.; Vandamme Erick, J. *Exopolysaccharide degrading enzyme and the use of the same*, 1996, PCT, WO96/31610.
10. Grant, W. D.; Sutherland, I. W.; Wilkinson, J. F. *J. Bacteriol.* **1969**, *100* (3), 1187–1193.
11. Rättö, M.; Mustranta, A.; Siika-aho, M. *Appl. Microbiol. Biotechnol.* **2001**, *57* (1-2), 182–185.
12. Sutherland Ian, W. In *Cambridge Studies in Biotechnology*; Sir James, Baddiley; Carey, N. H.; Higgins, I. J.; Potter, W. G., Eds. Biotechnology of Microbial Exopolysaccharides; Vol. 9; Cambridge University Press: Cambridge, 1990.
13. Verhoef, R.; de Waard, P.; Schols, H. A.; Rättö, M.; Siika-aho, M.; Voragen, A. G. J. *Carbohydr. Res.* **2002**, *337* (20), 1821–1831.
14. Oppong, D.; King, V. M.; Zhou, X.; Bowen, J. A. *J. Ind. Microbiol. Biotechnol.* **2000**, *25* (2), 74–80.
15. De Ruiter, G. A.; Schols, H. A.; Voragen, A. G. J.; Rombouts, F. M. *Anal. Biochem.* **1992**, *207*, 176–185.
16. Gerwig, G. J.; Kamerling, J. P.; Vliegenthart, J. F. G. *Carbohydr. Res.* **1978**, *62* (2), 349–357.
17. Hakomori, S. *J. Biochem.* **1964**, *55*, 205–208.
18. Englyst, H.; Wiggins, H. S.; Cummings, J. H. *The Analyst* **1982**, *107* (1272), 307–318.
19. van Casteren, W. H. M.; Dijkema, C.; Schols, H. A.; Beldman, G.; Voragen, A. G. J. *Carbohydr. Polym.* **1998**, *37* (2), 123–130.
20. Troyano, E.; Lee, S. P.; Rha, C. K.; Sinskey, A. J. *Carbohydr. Polym.* **1996**, *31* (1-2), 35–40.
21. Bax, A.; Sparks, S. W.; Torchia, D. A. *J. Am. Chem. Soc.* **1988**, *110*, 7926–7927.
22. Duckworth, M.; Madden, J. K. (Eds.) *Determination of pyruvic acid in complex carbohydrates*. Methods in carbohydrate chemistry, J. N. BeMiller, R. L. Whistler, and D. H. Shaw., Eds. John Wiley & Sons Inc., 1993,Vol. 9, pp. 123–127.
23. Thurow, H.; Choy, Y.-M.; Frank, N.; Niemann, H.; Stirm, S. *Carbohydr. Res.* **1975**, *41* (1), 241–255.
24. Garegg, P. J.; Jansson, P.-E.; Lindberg, B.; Lindh, F.; Lonngren, J.; Kvarnstrom, I.; Nimmich, W. *Carbohydr. Res.* **1980**, *78* (1), 127–132.
25. Yang, B. Y.; Brand, J.; Montgomery, R. *Carbohydr. Res.* **2001**, *331* (1), 59–67.
26. Evans, L. R.; Linker, A.; Impallomeni, G. *Int. J. Biol. Macromol.* **2000**, *27* (5), 319–326.
27. Cescutti, P.; Toffanin, R.; Pollesello, P.; Sutherland, I. W. *Carbohydr. Res.* **1999**, *315* (1-2), 159–168.
28. Cerantola, S.; Marty, N.; Montrozier, H. *Carbohydr. Res.* **1996**, *285*, 59–67.