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Structure characterization of a fucose-containing exopolysaccharide produced by *Enterobacter cloacae* Z0206

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ABSTRACT

A novel high molecular weight (1.1×10^6 Da) exopolysaccharide (EPS) produced by *Enterobacter cloacae* Z0206 strain was isolated by column chromatography. Complete hydrolysis of the EPS followed by gas chromatography mass spectrometry (GC–MS) and high performance liquid chromatography (HPLC) analyses showed that the EPS is composed of L-fucose, D-glucose, D-galactose, D-glucuronic acid and pyruvic acid in the approximate molar ratio of 2:1:3:1:1. Partial acid hydrolysis of the purified EPS followed by gel permeation chromatography (GPC) yielded a hexasaccharide. A combination of chemical analysis coupled with mass spectrometry and 1D and 2D NMR spectroscopy applied to the oligosaccharide showed that the EPS comprises a heptasaccharide repeating unit.

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1. Introduction

Microbial polysaccharides have been reported to possess diverse biological activities including antiviral (Arena et al., 2009) and immunostimulatory properties (Liong, 2007). Oligo- and polysaccharides containing fucose found in microbial strain to have potential anti-cancer and anti-inflammatory effects (Cescutti et al., 2005; Freitas et al., 2011). Numerous members of the family *Enterobacteriaceae* commonly produce polysaccharides composed of fucose, glucose, galactose, glucuronic acid and the pyruvic and acetyl groups (Freitas et al., 2011).

In our previous study, *Enterobacter cloacae* Z0206, a bacterial strain, was screened and acclimated, and it was found to produce large amounts of exopolysaccharides in a selenium accumulated medium. A neutral fraction isolated from the exopolysaccharides was composed of glucose, mannose and galactose with a molar ratio of 8.530:0.061:0.706 (Jin et al., 2010; Xu, Wang, Jin, & Yang, 2009). However, in this study, another acidic fucose-containing fraction was isolated. For this reason a thorough investigation in structure was carried out with the same crude exopolysaccharides.

2. Materials and methods

2.1. Extraction and purification of EPS

The crude exopolysaccharides (EPSs, 50 g) secreted by the *E. cloacae* Z0206 strain were dissolved in water and precipitated with alcohol (Xu et al., 2009). After deproteinization using the Sevage method (Tong, Liang, & Wang, 2008), the supernatant containing the EPSs were dialyzed against deionized water (8000–14,000, molecular weight (MW) cut-off), and then lyophilized. The dried crude EPSs (5.5 g) were dissolved in water and applied to a DEAE-52 cellulose (Whatman Ltd., England) column (6 cm × 35 cm) (Li, Zhou, & Han, 2006). Elution was performed sequentially with water and NaCl gradient from 0 to 0.3 M at a flow rate of 1 mL/min (8 min/fraction); the fractions eluted with 0.3 M NaCl were collected. After dialysis and lyophilization, the fractions were further fractionated on a Superose (Healthcare, Sweden) column (2.5 cm × 60 cm) eluted with water to obtain purified EPS (2.2 g). Fractions (0.2 mL) were analyzed using the phenol-sulfuric acid method (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956).

2.2. Homogeneity and molecular weight determination

The homogeneity and MW of the EPS were determined by gel permeation chromatography (GPC) using a Waters Alliance 2695 HPLC system with a 2414 refractive index detector (Waters, Milford, MA. USA). The sample (10 μL, 2 mg/mL buffer consisting of

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0.1 M NaCl) was injected into the Ultrahydrogel columns (250, 500 and 1000 columns, connected in series) and was eluted with 0.1 M NaCl at a flow rate of 1.0 mL/min (Wu, Hu, Pan, Zhou, & Zhou, 2007). The MW was calculated according to a calibration curve generated using a series of dextran standards.

2.3. Sugar composition analysis

EPS (15 mg) was dissolved in a solution of 0.5 mL xylose (8 mg/mL) and 3.5 mL trifluoroacetic acid (TFA, 3 M) in a sealed vessel, heated at 120 °C for 5 h (Zhang, Wang, Zhang, & Wang, 2011) and then dried under nitrogen (N_2).

A modification of the peracetylated procedure was designed for mercaptalated monosaccharides (Eleonora, Natalia, Jianjun, Annie, & Susan, 2003; Yoichiro, Mika, & Toshihisa, 1998). A mixture of 0.5 mL of the stock solutions of each monosaccharide was dried under a flow of N_2 at 55 °C. 3 mL Ethanethiol and TFA (2:1, v/v) were added and maintained at room temperature (25 °C) for 25 min. After removal of the solvents under a flow of N_2, the residue was acetylated with 4 mL Ac_2O–pyridine (1:1, v/v) at 55 °C for 5 h and 0.4 μL of the solution was directly injected into a GC–MS for analysis (Pitthard & Finch, 2001).

GC–MS was performed on a Thermo (Trace 2000 GC/DSQ MS, USA) instrument with a HP-5MS column (0.32 mm × 30 m) using the following temperature profile: 80 °C for 2 min, 80–200 °C at 15 °C/min, 200–210 °C at 1 °C/min, 210–280 °C at 25 °C/min and held for 5 min.

2.4. Pyruvic acid analysis

EPS (15–20 mg) was dissolved in 3 M TFA and hydrolyzed at 120 °C for 5 h. The sample was cooled to ambient temperature, transferred to a volumetric flask and the volume was made up to 100 mL.

Quantitative analysis of pyruvic acid in the EPS was performed on a Waters Alliance 2695 HPLC system with a 2998 PDA detector (Waters). The sample was filtered and then applied to an XBridge^{TM} C_{18} column (250 × 4.6 mm, 5 μm, Waters) and eluted with 98% K_2HPO_4–H_3PO_4 buffer (0.1 M K_2HPO_4–H_3PO_4, pH 2.9)–2% CH_3OH at a flow rate of 0.25 mL/min. The UV wavelength for detection was set at 215 nm (Hallstrom, Carlsson, Hillered, & Ungerstedt, 1989).

2.5. Methylation analysis

EPS (55 mg) was methylated by using NaOH as a catalyst and CH_3I in Me_2SO. The entire process was repeated four times according to a previously described protocol (Ciucanu & Kerek, 1984). The partially permethylated product was then hydrolyzed in 3 M TFA for 5 h at 120 °C, and reduced by $NaBD_4$ for 12 h at 25 °C and finally acetylated with Ac_2O–pyridine (1:1, v/v) for 5 h at 55 °C and analyzed by GC–MS as described in Section 2.3.

2.6. Determination of absolute configuration of sugars

The absolute configuration of neutral sugars was determined using a modification of the methods described by Gerwig, Kamerling, and Vliegenthart (1979) and Leontein, Linaberg, and Lonngren (1978). Butanolysis of the dry neutral sugar (6 mg) both in D and L configuration was performed in a sealed system under a flow of N_2 by addition of 50 μL TFA and 0.5 mL (+)-2-butanol followed by incubation in an oil bath at 130 °C overnight. The solution was dried under a flow of N_2 at room temperature and then treated with 1 mL Ac_2O–pyridine (1:1, v/v) for 5 h at 55 °C. The same procedure was adopted for butanolysis of D-glucuronic acid, although in this case, both (−)-2-butanol and (+)-2-butanol were used. The products were immediately analyzed by GC–MS analysis as described in Section 2.3.

2.7. Periodate oxidation and Smith degradation study

EPS (55 mg) was dissolved in 0.015 M $NaIO_4$ buffer (50 mL) and kept in the dark at 4 °C. The OD value at 233 nm was monitored until this value stabilized. The reaction was completed after 122 h and excess of oxidant was destroyed by addition of ethylene glycol. The reaction mixture was dialyzed against deionized water for 48 h (8000–14,000, MW cut-off) and then lyophilized. Following reduction with $NaBH_4$ (80 mg dissolved in 3 mL deionized water) for 22 h at room temperature (Hu, Kong, Yang, & Pan, 2011) the reduced product was subjected to hydrolysis with 3 M TFA (2 mL) for 5 h at 120 °C. The acid was removed by co-distillation with CH_3OH under N_2. Finally, the product was acetylated using the previously described process and analyzed by GC–MS using the following temperature profile: 120 °C for 2 min, 120–190 °C at 25 °C/min and maintained for 5 min, 190–220 °C at 20 °C/min, 220–280 °C at 30 °C/min.

2.8. Preparation and purification of oligosaccharides

EPS (1.5 g) was hydrolyzed with 0.1 M TFA for 3 h at 100 °C to obtain oligosaccharides (partial acid hydrolysis). After removal of the acid under N_2, oligosaccharides were fractionated using Bio-Gel P-2 (Bio-Rad, 2.5 cm × 60 cm) at a flow rate of 0.2 mL/min (8 min/fraction) with 0.1 M NH_4HCO_3 as the eluent (Richard et al., 2000).

The elution was monitored and pooled based on paper chromatography (PC) and mass spectrometry (MS) detection simultaneously. For PC detection, Whatmann 1 mm filter paper sheet was used, on which the fractions were developed using *n*-BuOH–pyridine–H_2O (5:3:3, v/v/v) as the solvent (Woranovicz, Gorin, Marcellij, Torri, & Iacomini, 1997). The MS detection was performed on a Waters Xevo TQ MS.

2.9. Compositional analysis of oligosaccharides

Purified oligosaccharide (5 mg) was completely hydrolyzed and derivatized. The derivatives were analyzed by GC–MS as described in Section 2.3.

2.10. ESI-MS analysis of oligosaccharides

For MS detection, elution introduction was performed at a flow rate of 0.4 mL/min on a Waters Xevo TQ MS detector in the electrospray ionization mass spectrometry (ESI-MS) with negative mode. Nitrogen was used as sheath gas. MS analyses (10 μL) were carried out using a Waters Acquity Ultra Performance LC with H_2O/acetonitrile (80:20, v/v). The voltages of the capillary and cone were set to 3 kV and 50 V, respectively.

2.11. NMR analysis of EPS and oligosaccharide

The hydroxyl groups of sugar residues were exchanged by repeated addition of D_2O (99.99%) prior to NMR analysis. ^1H and ^{13}C NMR spectra were recorded with a Bruker Avance III 400 MHz NMR spectrometer (Germany) at 25 °C. Chemical shifts were reported relative to internal (3-trimethylsily)-propane sulfonic acid sodium salt (DSS). Standard homo- and heteronuclear correlated 2D techniques: 2D ^1H–^1H correlated spectroscopy (COSY), ^1H–^{13}C heteronuclear single quantum coherence (HSQC), ^1H–^1H nuclear overhauser effect spectroscopy (NOESY), ^1H–^{13}C heteronuclear multiple quantum coherence (HMBC) were used to assign

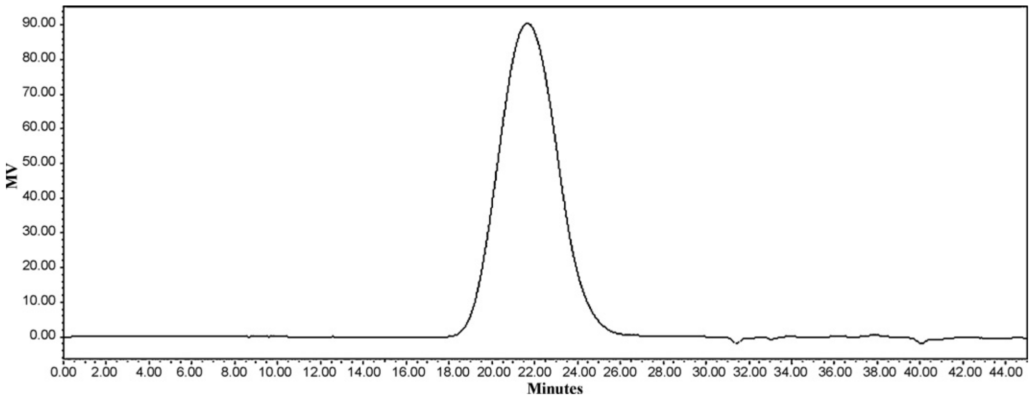

Fig. 1. HPGPC profile of EPS isolated from *Enterobacter cloacae* Z0206.

signals and determine the sequence of sugar residues (Liu et al., 2008).

3. Results and discussion

3.1. Composition analysis

Lack of absorption at 280 nm indicated the absence of protein in the EPS. A single peak was obtained by HPGPC analysis of EPS, indicating a homogeneous EPS (Fig. 1) with a MW of approximately 1.1×10^6 Da.

The reaction route developed for monosaccharides of the EPS was based on a previous report (Pitthard & Finch, 2001) with some modifications. For the derivative of glucuronic acid, the —OH presented in the C-3 position and the —COOH in the C-6 position were dehydrated to form a 5-carbon lactone ring. A scheme of the mercaptalation reaction of aldose and uronic acid with subsequent peracetylation was determined using the retention time and ions of the molecule. Contrary to a prior report (Pitthard & Finch, 2001), uronic acid gave useful derivatives upon acetylation. The EPS and hexasaccharide were composed of fucose, glucose, glucuronic acid and galactose in the molar ratios 2:1:1:3 and 2:1:1:2, respectively, with xylose as an inner standard.

The pyruvic acid was quantitatively analyzed by HPLC using the external standard method. The average content in the EPS was 7.64%, and the molar ratio to glucuronic acid was 1:1.

The absolute configuration of the sugars in the EPS was determined by GC–MS analysis of acetylated (+)-2-butyl glycosides (acetylated (−)-2-butyl glycoside of D-glucuronic acid and acetylated (+)-2-butyl glycoside of L-glucuronic acid had the same retention time). Fucose was found to be in the L-configuration, while glucose, glucuronic acid and galactose were in the D-configuration.

3.2. Periodate oxidation

Periodate consumption of EPS was 0.44 mol/mol sugar residue. GC–MS analysis of the periodate-oxidized, reduced product of EPS following hydrolysis with TFA showed the presence of glucose, galactose, fucose and erythritol. The formation of glucose, galactose, fucose suggested substitution at position 3, while the formation of erythritol suggested the presence of 4-substituted sugars. HPLC as described above confirmed the absence of formic acid produced in the periodate oxidation.

3.3. Methylation analysis

GC–MS analysis of the permethylated EPS following hydrolysis, reduction and acetylation revealed minor amounts of terminal fucose and galactose residues and six main peaks corresponding to 2,3-bi-O-methyl fucose, 2-O-methyl fucose, 2,4,6-tri-O-methyl galactose, 2,3,6-tri-O-methyl galactose, 2,4,6-tri-O-methyl glucose and 2,3-bi-O-methyl galactose at an approximate molar ratio of 1:1:1:1:1:1 (Table 1). This was consistent with the results of periodate oxidation and sugar component analysis. According to previous studies of EPSs, pyruvic acid is linked with α-D-galactose (Choy & Dutton, 1973; Yun Yang, Brand, & Montgomery, 2001), β-D-galactose (Baumann, Tzianabos, Brisson, Kasper, & Jennings, 1992) or β-D-glucose (Evans, Linker, & Impallomeni, 2000) as a 4,6-O-ketal substituent. In this study, it was concluded that a pyruvic acid group was linked to a terminal D-galactose as 4, 6-O-ketal substituent.

These results indicated that the repeat unit of the EPS consisted of a unit containing single molecules of 1,4-linked L-fucose, 1,3,4-linked L-fucose, 1,3-linked D-galactose, 1,4-linked D-galactose, 1,4,6-linked D-galactose (1 pyruvate D-galactose) and 1,3-linked D-glucose and D-glucuronic acid. The chain was composed of 1–4 and 1–3 linkages with branching occurred solely from the fucose residue at C-3 or C-4.

3.4. Electrospray mass spectrometry

The partial hydrolysate mixture was subjected to gel filtration chromatography on Bio-Gel P_2 to yield pure oligosaccharide. The oligosaccharides from the partial acid hydrolysate of the EPS and Bio-Gel P-2 fractions were analyzed by ESI-MS and PC. For ESI-MS

Table 1
GC–MS results of partially O-methylalditol acetates of methylation analysis of EPS.

Alditol acetate	Retention time [a]	Molar ratio[a]	Fragments (diagnostic ions, *m*/*z*)	Proposed structure
2,3-Bi-O-methyl-fucose	0.94	0.93	101, 102, 118, 143, 162, 203	1,4-Linked fucose
2-O-methyl-fucose	1.00	1.00	118, 129, 202, 215, 231, 275	1,3,4-Linked fucose
2,4,6-Tri-O-methyl-galactose	1.07	1.12	101, 118, 161, 217, 234, 277	1,3-Linked galactose
2,3,6-Tri-O-methyl-galactose	1.08	0.83	113, 118, 162, 173, 233, 234	1,4-Linked galactose
2,4,6-Tri-O-methyl-glucose	1.09	1.05	101, 118, 129, 161, 234, 277	1,3-Linked glucose
2,3-Bi-O-methyl-galactose	1.24	0.82	102, 118, 162, 201, 234, 261	1,4,6-Linked galactose

[a] Relative to 2-O-methyl-fucose.

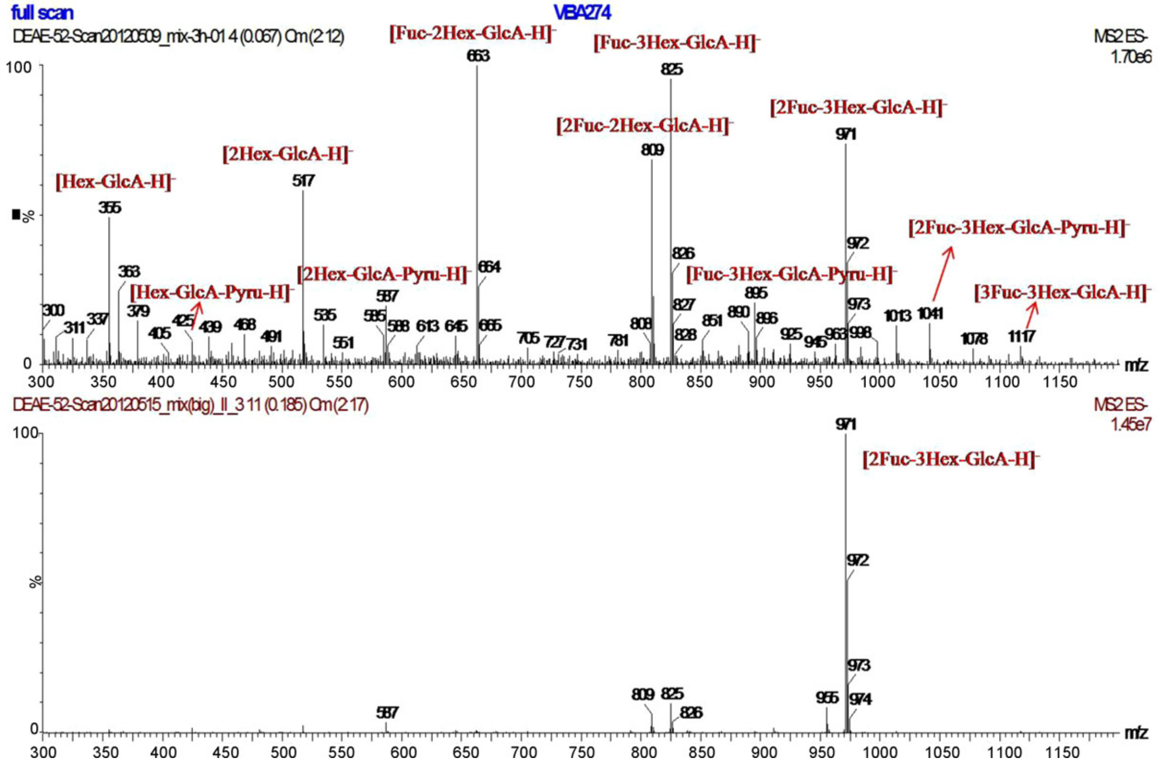

Fig. 2. ESI mass spectrum of partial acid hydrolyzate and hexasaccharide. (Fuc, fucose; Hex, glucose or galactose; GlcA, glucuronic acid; Pyru, pyruvate.) In the negative ion mode; the voltages of the capillary and cone were set to 3 kV and 50 V, respectively.

detection, considering of a mixture of those oligosaccharides would result in an overlapping isotopic cluster at the same value. The eluants were developed on PC to examine purity simultaneously, the hexasaccharide gave a single spot showed in the negative ion mode (Fig. 2).

Mass spectrometry revealed that almost all ions contained glucuronic acid and some ions also contained a pyruvic acid group. The terminal D-galactose with the 4, 6-O-ketal substituted pyruvic acid linked to GlcA was inferred by the presence of a peak at *m*/*z* 425. Considering the only branching took place from the fucose residue at C-3 or C-4. Therefore, the peak at *m*/*z* 587 was observed due to two hex, one glcA and one pyruvate, indicating the linear sequence of an oligosaccharide. The remaining series of deprotonated ions and corresponding compositions are summarized in Fig. 2.

3.5. 1D and 2D NMR spectroscopy

Attempts to assign the EPS proton signals in the low field region were unsuccessful (Fig. 3a). However, it was possible to observe signals typical of methyl protons of fucose and pyruvic acid group at δ 1.203 and δ 1.370 in the high field region. In accordance with previous report (Evans et al., 2000), resonance at δ 25.05, approximately 100 and approximately 176 (Kohno et al., 2009) in the EPS ^{13}C NMR spectrum (Fig. 3b) were attributed to the carbonyl, tertiary carbon and methyl resonance respectively of the ketal-substituted pyruvate residue.

The hexasaccharide was also subjected to NMR analysis and complete structural characterization was achieved following HSQC, COSY, HMBC, and NOESY analysis. The six sugar residues present in the repeating unit are shown in Table 2. The anomeric region (δ 4.5–5.5) of the ^{1}H NMR spectrum contained a total of six peaks that were ascribed to anomeric protons (H-1) of sugar residues labeled A–F. In the ^{1}H NMR, the signals exceeding δ 4.9 ppm (doublets with coupling constants ($J_{1,2}$) in the range 3.2–3.8 Hz) were those of α-anomeric protons, whereas the signals between δ 4.5–4.6 ppm (doublets with coupling constants ($J_{1,2}$) in the range 7.8–8 Hz) were those of β-anomeric protons. Based on their chemical shifts and the value of the coupling constant for anomeric signals, residues A–C were determined to have α-configurations and residues D–F were determined to have β-configurations. The ^{13}C NMR spectrum (Fig. 4) of the hexasaccharide contained six anomeric carbons (δ 98.33, 99.41, 100.44, 103.86, 96.54, 102.76), two CH_3—C groups (C6 of fucose) at δ 15.35 and δ 15.51, three free hydroxyl C6 of glucose and galactose at δ 61.63, 61.38 and 60.62 and one C═O group at δ 175.42. Similar data were reported by Agrawal (1992). The ^{1}H–^{13}C HSQC spectrum of the hexasaccharide contained signals for six pairs of anomeric protons and carbons.

In the HMBC spectrum, one methyl proton of fucose at δ 1.247 showed a two-bond coupling to fucose C5 (δ 70.97), which was indicated to be further coupled to the anomeric proton of sugar residue B (δ 5.268). The methyl proton of fucose at δ 1.247 also showed a three-bond coupling to fucose C4 (δ 79.10), which was indicated to be further coupled to H2 (δ 3.880) and H3 (δ 3.880) of sugar residue B and coupled to the anomeric proton of sugar residue C (δ 4.918). The ^{1}H–^{13}C HSQC showed coupling between δ 3.880 and C2 (δ 67.78) as well as C3 (δ 79.52), and the ^{1}H–^{13}C HMBC showed the carbon at δ 79.52 coupled to anomeric proton of sugar residue B (δ 5.268) and another anomeric proton of sugar residue D (δ 4.632). These results indicated that residue B was a 3,4 linked α-L-fucose, which was similar to the fucose residue of the EPS from *Enterobacter amnigenus* (Cescutti et al., 2005), Similarly, the methyl proton of another fucose at δ 1.205 also showed a two-bond coupling to fucose C5 (δ 67.59) and a three-bond coupling to fucose C4 (δ 78.49). In the HMBC spectrum, the C5 also showed coupling with the anomeric proton (δ 4.918) of sugar residue C, and the latter was further coupled to the anomeric proton of sugar residue F (δ 4.505). ^{1}H–^{1}H COSY analysis showed coupling between anomeric proton H1 (δ 4.918) and sugar residue C H2 (δ 3.960). In the ^{1}H–^{13}C HSQC spectra, the H2 (δ 3.960) of sugar residue C showed correlation with C2 (δ 69.18) and the H2 showed a three-bond coupling to fucose C4

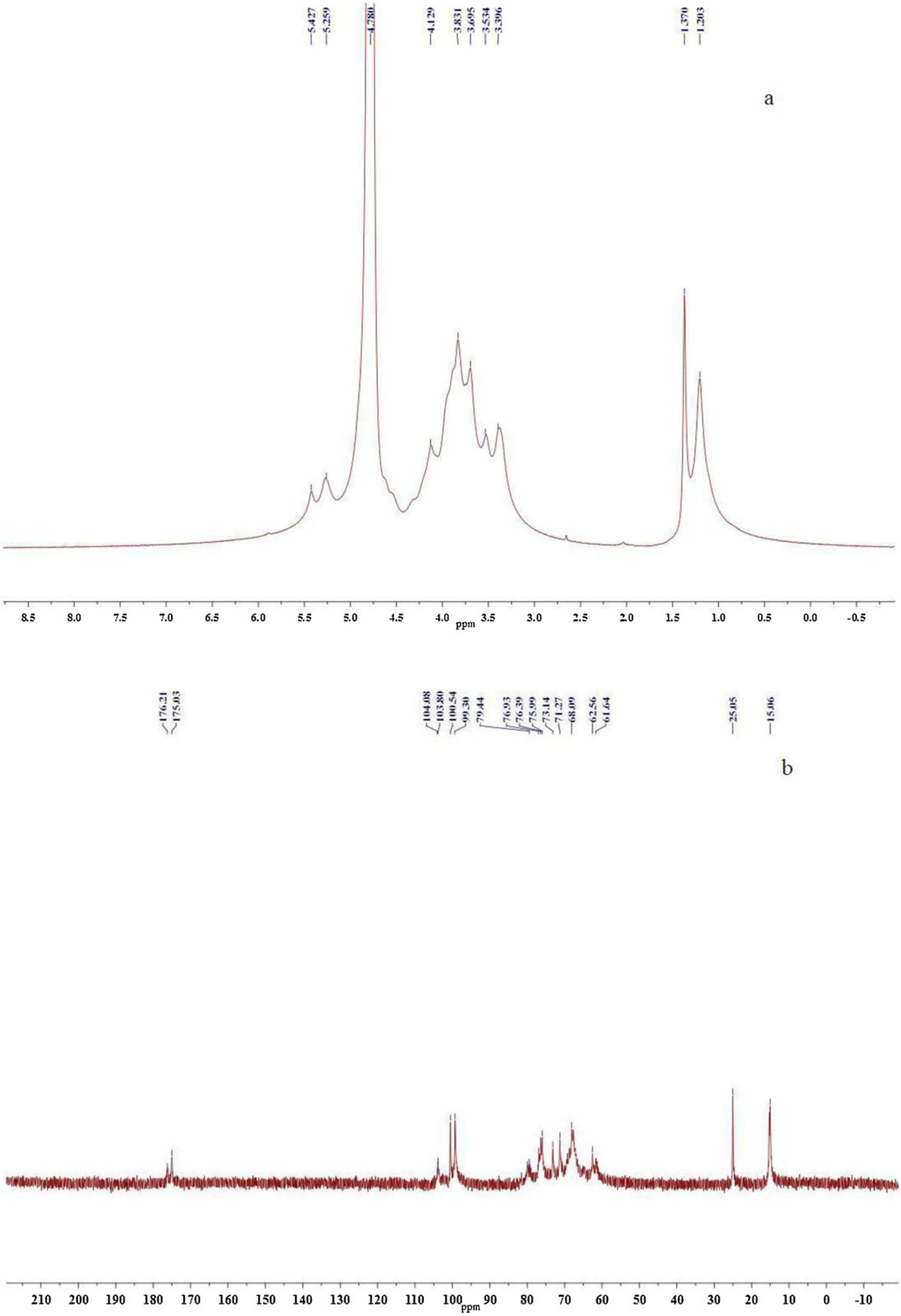

Fig. 3. 400 MHz ^{1}H NMR (a) and ^{13}C NMR (b) spectrum of the EPS recorded in D_2O at 25 °C.

Table 2
^{1}H and ^{13}C NMR chemical shifts of the hexasaccharide recorded in D_2O at 25 °C (δ, ppm).

Sugar residue	Nucleus	Chemical shifts (ppm)					
		1	2	3	4	5	6
A α–D–Gal (1–	^{1}H	5.420	3.730	3.892	3.893	4.195	3.620
	^{13}C	98.33	68.44	70.54	68.91	71.04	61.33
B α–L–Fuc (4–	^{1}H	5.268	3.880	3.880	3.737	3.761	1.247
	^{13}C	99.41	67.78	79.52	79.10	70.97	15.51
C–1)–α–L–Fuc (3,4–	^{1}H	4.918	3.961	4.075	4.156	4.525	1.205
	^{13}C	100.44	69.18	72.51	78.49	67.59	15.35
D–1)–β–D–Glc (3–	^{1}H	4.632	3.374	3.714	3.434	3.714	3.458/3.814
	^{13}C	103.86	73.31	76.36	75.40	76.07	61.38
E–1)–β–D–GlcA (4–	^{1}H	4.532	3.452	3.640	3.708	3.345	–
	^{13}C	96.54	71.32	72.24	76.16	76.65	175.42
Fβ–D–Gal (1–	^{1}H	4.505	3.302	3.371	3.728	4.126	3.603
	^{13}C	102.76	73.83	69.81	68.91	69.47	60.62

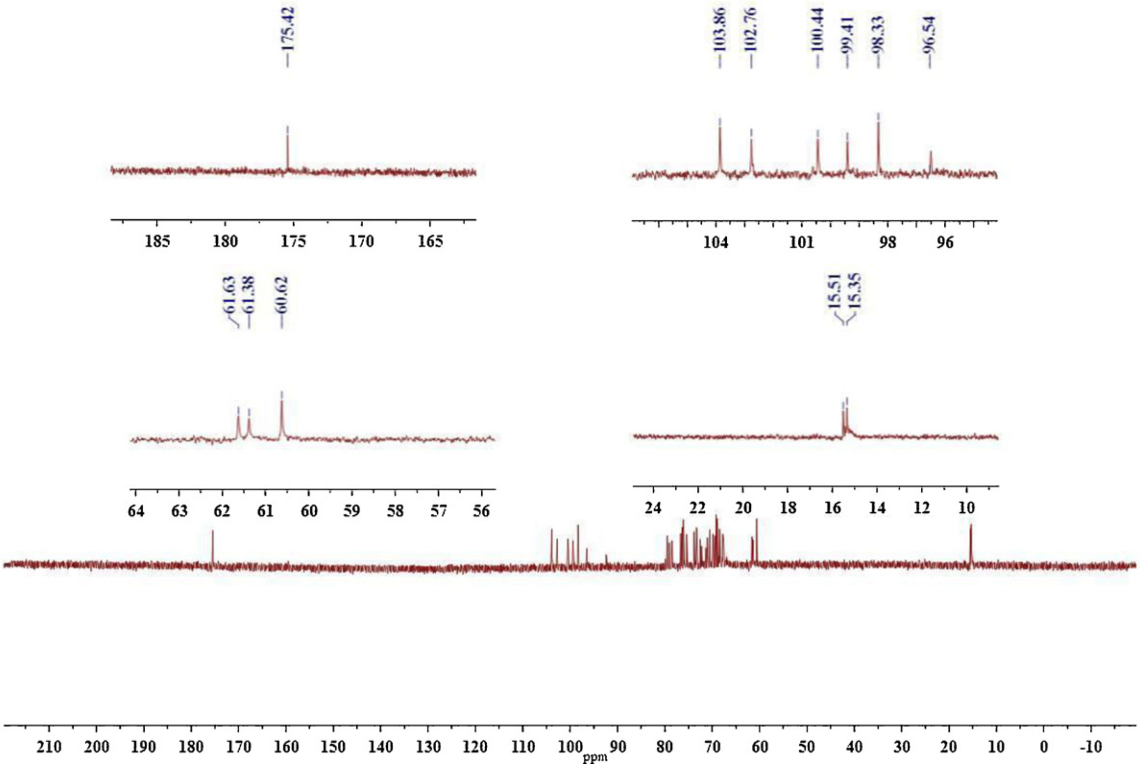

Fig. 4. 400 MHz ^{13}C NMR spectrum of the hexasaccharide recorded in D_2O at 25 °C.

(δ 78.49), thus indicating an α-L-fucose (B)-(4–1)-α-L-fucose (C) linkage and the fucose B branched at position C3 (δ 79.52).

The anomeric proton of residue D at δ 4.632 showed HMBC connectivity with fucose B C3. Proton chemical shifts from H2 to H6 of the residue D were assigned based on ^{1}H–^{1}H COSY, ^{1}H–^{1}H NOESY, ^{1}H–^{13}C HSQC and ^{1}H–^{13}C HMBC data. The chemical shifts for residue D (Table 2) were typical for a β-D-glucose residue (Kohno et al., 2009; Verhoef et al., 2002). Although no HMBC correlation was detected for anomeric proton (δ 4.532) and the anomeric carbon (δ 96.54) of the residue E with β-D-glucose residue, the proposed structure of the hexasaccharide indicated that the residue D was a β-D-glucuronic acid residue. This information was also confirmed by identification of NOESY contact between H4 (3.434) of the β-D-glucose residue and the residue E anomeric proton (δ 4.532). These data are in accordance with previous reports (Verhoef et al., 2002) and suggest that β-D-glucose is linked to β-D-glucuronic acid by a 3-O glycosidic bond.

In the ^{1}H–^{13}C HMBC spectrum, the anomeric proton (δ 5.420) and anomeric carbon (δ 98.33) of residue A correlated with C4 (δ 76.16) and H4 (δ 3.714) of β-D-glucuronic acid residue respectively. Furthermore, the C=O carbon (δ 175.42) correlated with H4 (δ 3.714) of the β-D-glucuronic acid residue. Therefore, the residue A was shown to be an α-D-galactose residue linked to β-D-glucuronic acid at position 4. The assignments of the β-D-glucuronic acid were consistent with the δ values reported elsewhere (Jia et al., 2012).

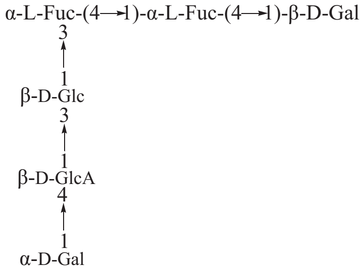

Fig. 5. Structure of the hexasaccharide.

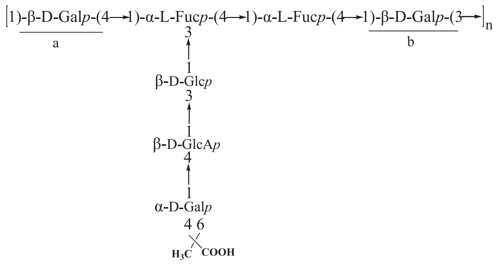

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Fig. 6. Structure of the EPS.

The anomeric proton of the sugar residue F (δ 4.505) was connected with C4 (δ 78.49) of fucose C, thus indicating the residue F was a β-D-galactose.

4. Conclusion

A fucose-containing EPS produced by the bacterium, *E. cloacae* Z0206 was isolated. The high MW (1.1×10^6) EPS was composed of L-fucose, D-glucose, D-galactose and D-glucuronic acid and pyruvic acid in the approximate molar ratio of 2:1:3:1:1. A combination of chemical and ESI-MS as well as NMR analyses was established to elucidate the structure of the hexasaccharide (Fig. 5) and the EPS (Fig. 6).

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