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Purification and structural data of a highly substituted exopolysaccharide from *Pseudomonas stutzeri* AS22

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ABSTRACT

Pseudomonas stutzeri AS22, when grown on media containing starch and yeast extract and incubated at 30 °C and 200 rpm for 24 h, was found to produce an acidic and high-molecular mass exopolysaccharide (EPS22). The EPS22 was purified and a yield of 1.3 g/l was achieved.

The average molecular mass of the EPS22 was determined by high-performance size-exclusion chromatography (HPSEC) and showed an average molecular mass of 9.9×10^5 Da and a polydispersity index M_w/M_n (M_w, weight-average and M_n, number-average) of 1.197 ± 0.015. Structural data of this EPS22 were determined using a combination approach including monosaccharide composition (HPAEC–PAD and GLC), methylation analysis (GC–MS) and NMR spectroscopy analysis. EPS22 was found to be a complex heteropolysaccharide with a repeating unit mainly composed of glucose, mannose and lactyl rhamnose in a molar ratio of 1:1.1:0.7. The acidic nature of the polysaccharide is due to the presence of three non-osidic substituents consisting of a lactyl, acetyl, and pyruvyl groups.

1. Introduction

The ability to produce exopolysaccharides (EPSs) with greatly varying composition and properties is a well-known biological phenomenon among bacteria (Singha, 2012). On the basis of monosaccharide composition in their backbone, bacterial EPSs have been classified into homopolysaccharides and heteropolysaccharides (Donot, Fontana, Baccou, & Schorr-Galindo, 2012). While homopolysaccharides are made up of a single type of monosaccharide, heteropolysaccharides, which constitute the majority of bacterial EPSs, are composed of several types of monosaccharides as well as non-osidic components (Donot et al., 2012; Notararigo et al., 2013) leading therefore to a great structural diversity. Although the acidic nature of some of these polymers is most often due to the presence of uronic acids, other acidic substituents such as lactate, pyruvate, succinate and acetate may also be present.

The interest in the exploitation of microorganisms for the production of valuable polysaccharides has greatly increased in recent years, since these biopolymers often show advantages over other natural polysaccharides derived from plants, marine algae and animals (Donot et al., 2012). The genus *Pseudomonas* can be included among the potential sources of promising biopolymers. For example, alginate produced by *P. aeruginosa* and *P. mendocina*, levan from *P. fluorescens*, succinoglycan synthesized by *P. marginalis*, hyaluronan from *P. aeruginosa* and recently GalactoPol synthesized by *P. oleovorans*, have gained considerable commercial interest (Freitas, Alves, & Reis, 2011).

Increasing attention is being paid to such molecules because of their physical properties such as gelling, stabilizing, thickening and emulsifying, making them suitable for numerous commercial applications in food industries (Freitas et al., 2011). Thanks to their bioactive role such as anti-tumor (Okutani, 1984), immunostimulating (Okutani, 1992), anticoagulant (Colliec et al., 2001), hypoglycemic (Dahech et al., 2011) and antioxidant activities, these biopolymers exhibit therefore large applications in biomedical, biopharmaceutical and cosmetic industries.

Because technological applications and biological properties of exopolysaccharides are quite dependent on their structural properties, the determination of the chemical composition and structure of an EPS is relevant for predicting its potential applications. Therefore, in order to investigate the biological nature of the EPS22 secreted by *Pseudomonas stutzeri* AS22 for future applications, we present in this manuscript the purification and structural data of this polysaccharide.

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2. Materials and methods

2.1. Production, isolation and purification of EPS22

The production of EPS22 by *P. stutzeri* AS22 was performed on liquid medium composed of (g/l): starch 10, yeast extract 5 (ultrafiltered to remove molecular species larger than 3×10^4 Da especially mannan), $MgSO_4$ (7 H_2O) 0.1, K_2HPO_4 1.4, KH_2PO_4 0.7 and NaCl 0.5. The medium was adjusted to pH 8.0. The strain was cultured in 25 ml medium in 250 ml conical flasks maintained at 30 °C and incubated with agitation at 200 rpm for 24 h. Bacterial cells were separated from the EPS preparation by dilution and centrifugation (13,000 rpm for 15 min at 4 °C) of the culture broth. The supernatant, containing the EPS fraction, was filtered under vacuum successively through Sartorius cellulose nitrate filters of 8.0, 5.0, 3.0, 1.2, 0.8 and 0.45 μm pore size (in diameter) to eliminate cells and large cellular fragments and to give therefore the crude exopolysaccharide. Then, to remove simple carbohydrates from culture broth prior to the quantification of exopolysaccharides, ultrafiltration (UF) using a 300 kDa cut-off cellulose membrane was used. Finally, the exopolysaccharide fraction, collected from the retentate, was dialyzed against distilled water, evaporated and lyophilized and used for further study as the purified EPS22. The purified EPS22 dry weight measurement was used as an indication of the EPS yield, expressed as grams of polymer dry mass per liter of fermented medium.

2.2. Structural characterization of EPS22

2.2.1. Molecular mass determination

The average molecular mass of the EPS22 was determined by high-performance size-exclusion chromatography (HPSEC) using Shodex OHpak SB-804 HQ and SB-805 HQ columns in series, coupled to a differential refractometer, a multi-angle laser light-scattering detector (MiniDAWN, Wyatt Technology, Dawn) and a viscometer (Waters). EPS was separated with 0.1 M $NaNO_3$ as eluent.

2.2.2. General procedures

Total carbohydrate content of the EPS22 from *P. stutzeri* AS22 was estimated by the phenol-sulfuric acid calorimetric method (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956).

Protein content was calculated according to the Bradford's method (1976).

Elemental analysis on the carbon (C), nitrogen (N), phosphorus (P), sodium (Na), chloride (Cl) and sulfur (S) weight percentages of the purified EPS22 was realized using an elemental analyzer (SCA Instrumentation, CNRS Solaize, France) at "Service Central d'Analyses" (SCA) (CNRS, Solaize-France).

2.2.3. Glycosyl composition analysis using HPLC and GLC

The monomer composition of the *P. stutzeri* exopolysaccharide was determined after complete acid hydrolysis in acidic conditions. The purified EPS22 (4 mg/ml) was treated with 2 N trifluoroacetic acid (TFA) containing *myo*-inositol (1.05 mg/ml) at 100 °C for 4 h. The identity of the released monomers was determined using high performance anion exchange chromatography (HPAEC) with pulsed amperometric detection (PAD) on a Dionex CarboPac PA1 column (4 mm × 250 mm). Neutral monosaccharides were eluted isocratically using 18 mM of sodium hydroxide at a flow rate of 0.7 ml/min. The ratio of monomers was determined by comparison of the detector response to calibration standards of the individual monomers.

The results were confirmed by gas–liquid chromatography (GLC) analysis of the per-*O*-trimethylsilylated methyl glycosides (TMS). Briefly, these derivatives were prepared by methanolysis of 400 μg of EPS22 (3 N MeOH/HCl; 4 h at 110 °C), followed by TMS-derivatisation performed with bis(trimethylsilyl)trifluoroacetamide (BSTFA) and trimethylchlorosilane (TMCS) reagents in dry pyridine, at 80 °C for 20 min. After evaporation to dryness under a stream of nitrogen gas, per-*O*-TMS-glycoside samples were recovered from the dichloromethane organic phase before injection. GLC analysis of the TMS methyl glycosides was performed on an Agilent 6850 Series GC System, equipped with a HP-5MS column (30 m × 0.25 mm) (Agilent Technologies, Palo Alto, CA, USA) and using He as carrier gas and a Flam Ionization Detector.

2.2.4. Methylation analysis

A sample of EPS22 (10 mg) was methylated by the method of Hakomori (1964). After methylation, the reaction mixture was diluted with water, dialyzed and freeze-dried. The methylation procedure was repeated twice following the method of Purdie and Irvine (1903). Methylated polysaccharide was hydrolyzed with 90% formic acid at 100 °C for 2 h and then heated with 2 M trifluoroacetic acid for 3 h at 100 °C. Methylated sugars were converted into their alditol acetates (Albersheim, Nevins, English, & Karr, 1967) and analyzed by GC-MS. Peaks identification was based on their typical electron-impact break down profiles and retention times compared to partially methylated alditol acetates standards.

GC-MS analyses were performed using an Agilent 6850 Series GC System coupled with an Agilent 5975 C MSD mass spectrometer (France) connected to a capillary SP2380 column (30 m × 0.25 mm) (Supelco), using He as carrier gas. The GC oven temperature was set to 150 °C and held for 2 min, then increased to 240 °C at 3 °C/min and held for 5 min. For MS detection, the ion source and transfer line temperatures were set at 250 °C, with electron ionization (EI) mode at 70 eV.

2.2.5. Partial acid hydrolysis

Exopolysaccharide (20 mg/ml) was hydrolyzed with TFA 1 N at 100 °C for 0, 5, 15, 30, 60 and 240 min. After analysis of the different hydrolysates, oligosaccharides obtained from 5 min EPS22 hydrolysis were then fractionated by gel-filtration chromatography on a Bio-Gel P2 column (1.5 cm × 200 cm, Biorad, Richmond, California), eluted with water at a rate of 0.5 ml/min. The different collected fractions were freeze dried before analysis by mass spectrometry and NMR spectroscopy.

2.2.6. Electrospray ionization mass spectrometry (ESI-MS)

ESI mass spectra, in the positive-ion mode, were recorded on a ZQ Waters micromass spectrometer (capillary 3.5 kV, cone voltage 80 V).

2.2.7. Nuclear magnetic resonance spectroscopy (NMR)

NMR spectra were recorded for EPS samples that were dissolved (2–30 mg/ml) directly in D_2O in 5 mm tubes. Chemical shifts are given relative to external tetramethylsilane (TMS) (0 ppm) and calibration was performed using the signal of the residual protons of the solvent (HOD) as a secondary reference.

^1H NMR and ^{13}C NMR experiments were recorded on a Bruker Avance 400 spectrometer. Samples were examined as solution in D_2O at 300 K in 5 mm o.d. tube (internal acetone ^1H (CH_3) at 2.1 ppm relative to Me_4Si).

Two-dimensional spectra COSY (Correlation Spectroscopy) and HMQC (HeteroMultiQuantum Coherence) were recorded using the standard Bruker procedures.

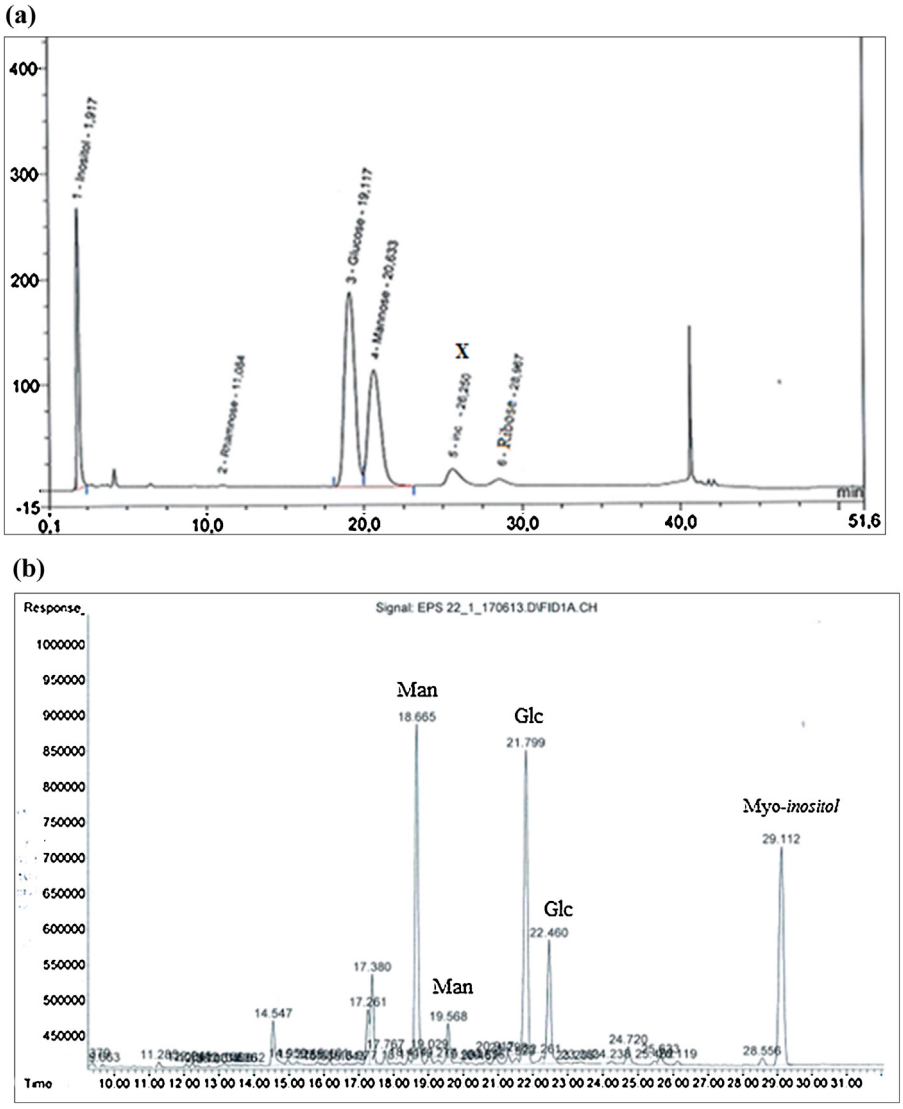


Fig. 1. Composition of EPS22 by (a) HPAEC–PAD and (b) GLC analysis after hydrolysis with TFA 2 N at 100 °C for 4 h.

3. Results and discussion

3.1. Molecular mass estimation

The exopolysaccharide 22 (EPS22) was isolated by high-speed centrifugation and ultrafiltration of the culture supernatant of *P. stutzeri* AS22. Under these conditions, a 1.3 g/l yield of purified EPS, estimated gravimetrically, was achieved.

The HPSEC elution profile (data not shown) showed that EPS22 from *P. stutzeri* AS22 was a homogeneous and high molecular mass polymer with an average mass of 9.9×10^5 Da and a polydispersity index M_w/M_n (M_w, weight-average and M_n, number-average) of 1.197 ± 0.015.

The EPS22 M_w value was found to be in the same range as that of alginate ($0.3–1.3 \times 10^6$), gellan (5.0×10^5), galactoPol (1×10^6), cellulose (1.0×10^6), levan ($1.0–3.0 \times 10^6$) and hyaluronan ($1.0–2.0 \times 10^6$) (Freitas et al., 2011; Freitas, Alves, Reis, Crespo, & Coelhoso, 2014). Besides the molecular mass, the polydispersity index (PI), which reflects the degree of heterogeneity of the polymer's chain lengths, is an important parameter influencing the functional properties of polysaccharides and thereafter will determine their suitability for specific applications.

In the present study, the PI of EPS22 was close to 1, confirming the homogeneity of the polymer. EPS from *P. oleovorans*, with a weight-average molar mass of $1.0–5.0 \times 10^6$, have shown PI that ranged between 1.43 and 2.15 (Freitas et al., 2009). Moreover, Fishman et al. (1997), when studying the physical properties of various *Pseudomonas* EPSs, reported a weight average molar mass ranging from about 0.71 to 2.85×10^6 with a PI range of 1.0l–1.37.

3.2. Composition analysis

Colorimetric assays of the purified EPS22 showed a total sugars and protein amounts about 70% and 1% (w/w), respectively. Elemental analysis indicated that the C and N content were 40.14% and 1.22%, respectively. However, the S, P, and Na content were less than 1%. In addition, the EPS22 was shown to be acidic since it forms a precipitate in presence of cethyl trimethyl ammonium bromide (CTAB).

The glycosyl residue composition was determined by high performance anion exchange chromatography coupled with a pulsed amperometric detector (HPAEC-PAD) after the total acid hydrolysis of EPS22 and by gaz-liquid chromatography (GLC) analysis of trimethylsilyl derivatives after methanolysis of the

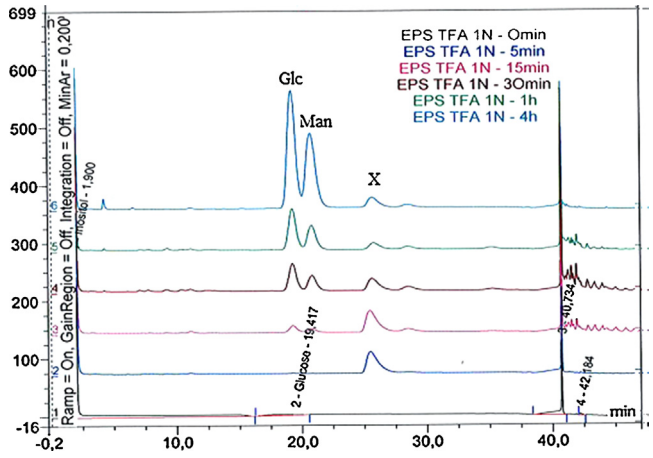


Fig. 2. HPAEC–PAD analysis of TFA hydrolysates obtained after different hydrolysis conditions of EPS22.Glc: glucose; Man: mannose; X: unknown sugar.

same EPS (Fig. 1). EPS22 was found to be composed of mannose/glucose/rhamnose/ribose at a molar ratio of 1.1:1:0.062:0.02, respectively. The later one, detected as trace, could probably be originating from residual biomass. Another unknown sugar labeled X has been detected but not identified. The absolute configuration of the monosaccharides in the polysaccharide was determined from the formation of trimethylsilylated (S)-2-butyl glycosides and from GLC analysis. All the monosaccharides were in the D configuration.

In the same context, it should be pointed out that degradation of some sugars that are known to be sensitive to acidic conditions, may occurs during the acidic treatment. To prove this, many hydrolysis conditions such as 1 N TFA at 100 °C for 5–240 min have been tested. As shown in Fig. 2, the unidentified sugar (X) is released after 5 min of mild hydrolysis. It was also remarkable that while the glucose and mannose concentrations increased gradually from 15 min to 4 h of hydrolysis reaction, the amount of the unknown sugar X was decreasing. Because sensitivity to acid hydrolysis is shown for deoxy-sugars, this result led us to suggest that the uncommon sugar X, the first monosaccharide being released within the first 5 min, is probably a branched deoxy sugar.

Then, the exopolysaccharide EPS22 was subjected to partial hydrolysis with 1 N TFA for 5 min to avoid the contamination of the unknown sugar X by glucose and mannose residues. Then, the hydrolysate was separate by Size Exclusion Chromatography (SEC) to isolate the monosaccharide fraction (P3) and oligosaccharides (P1 and P2) from polysaccharide (Fig. 3a). After that, the purity of the released monosaccharide (P3) was verified by HPAEC–PAD analysis (Fig. 3b) and finally it was structurally characterized using NMR and mass spectrometry experiments.

The ^{1}H NMR spectrum of the purified compound X (Fig. 4a) showed a signal (δ 5.26 ppm) in the anomeric region suggesting the saccharidic nature of this compound. Moreover, the high-field region of the ^{1}H NMR spectrum contained a doublet signal at 1.36 and 1.37 ppm, typical for CH_3-groups of 6-deoxy sugars. We also observe the presence of a signal at δ 1.25 which may be attributed to the CH_3-group of a non-osidic substituent.

According to previous studies on *Pseudomonas* species and especially *P. stutzeri* exopolysaccharides, the acidic nature of these EPSs is most often due to a carboxyethyl (lactic acid) substituent, a common feature in *Pseudomonas* exopolysaccharides (Fett, Osman, Wijey, & Singh, 1993). Consequently this finding together with the presence of a signal at 4.09 ppm which is known to be characteristic of a carboxyethyl (lactyl) substituent (Pillon et al., 2010; Severn & Richards, 1993), let us suggest the presence of such substituent linked to a deoxy sugar.

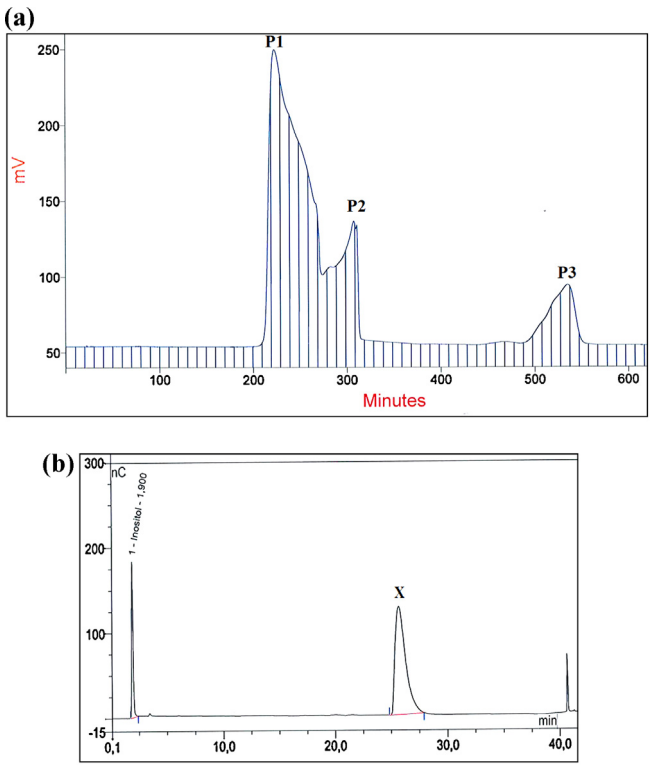


Fig. 3. (a) Elution profile on a Bio-Gel P2 column of the TFA hydrolysate obtained after 5 min of hydrolysis of EPS22 (column 1.5 cm × 200 cm; eluent: H_2O; flow rate: 30 ml/h; temperature: 55 °C). The P1, P2 and P3 peaks correspond to the void volume V_0, to oligosaccharides and to monosaccharides, respectively. Fractions corresponding to the peak P3 are collected and pooled before being subjected to a HPAEC–PAD analysis. (b) HPAEC–PAD analysis of the pool P3. X: the unknown sugar.

In order to confirm this hypothesis and to establish the identity of the deoxy sugar, this compound (X) was subjected to ESI-MS analysis. The mass spectrum, reported in Fig. 4b, together with literature data, suggest the unknown sugar X to be a rhamnose residue substituted with both acetyl and carboxyethyl substituents, in that ions at m/z = 290 (corresponding to the sodiated parent ion) and 186 (corresponding to the sodiated rhamnose residue) were observed (loss of 104 which was attributed to the carboxyethyl and acetyl substituents).

These data are in good agreement with previous articles on the structural studies of the *Pseudomonas* species exopolysaccharides, where it has been reported the presence of a 1-carboxyethyl (lactic acid) substituent. Osman and Fett (1993) reported that the carboxyethyl substituent was found to be linked to a glucose residue in the case of *P. marginalis* strain ATCC 10844 exopolysaccharide. In *P. stutzeri* strain 17588 (Osman, Fett, & Dudley, 1994) and *P. stutzeri* ATCC-31258 (Hisatsuka, Ishiyama, Inoue, Tsumura, & Sato, 1984) exopolysaccharides, the carboxyethyl substituent was found to be linked to rhamnose residues.

3.3. Glycosyl linkage analysis

The EPS22 was permethylated, then hydrolyzed with acid and converted into alditol acetates, and analyzed by GC–MS (Table 1). Consistent with the glycosyl composition analysis, glycosyl linkage analysis showed that the EPS22 is mainly composed of 4-linked glucopyranose, 3,4-linked mannopyranose and 3-linked rhamnopyranose in a molar ratio of 1:0.92:0.79, with minor peaks for other sugars also being found including 3-linked mannopyranose and terminally-linked rhamnopyranose in a molar ratio of 0.08:0.085.

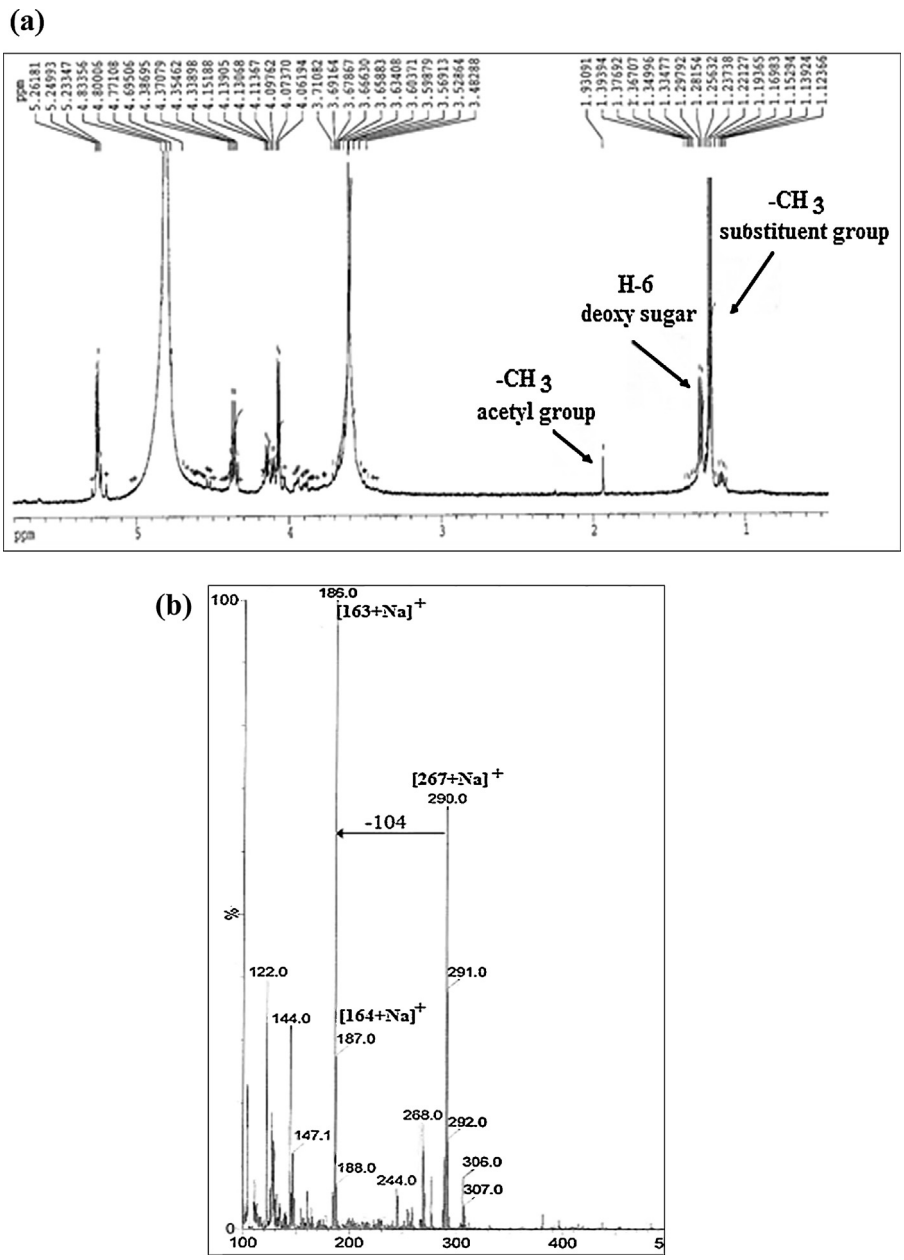


Fig. 4. 1-D ^{1}H NMR spectrum (400 MHz, 300 K) (a) and mass spectrum (b) of the purified unknown sugar X. CH3: protons from methyl group in X residue.

The presence of a 3,4-disubstituted mannopyranose and both 3-monosubstitued and terminal rhamnopyranosyl residues revealed branch points and substitutions. Moreover, we can deduce that every 10 and 8 repeating units, one mannose residue was not linked (in C-4) to rhamnose and one rhamnose residue was not substituted (in C-3) with lactate residue, respectively.

3.4. NMR spectroscopy analysis

The ^{1}H NMR spectrum of the native EPS22 (Fig. 5a) exhibited in the low-field region (δ 5.5–4.4) the presence of six signals at δ 5.32, 5.2 (A and A′), 5.0 (B), 4.8 (C), 4.65 and 4.47 (D) ppm. However, the HMQC spectrum (Fig. 6a) shows that the low-intensity

Table 1
GC–MS analysis of the methylated EPS22.

Type of sugar	PMAA[a]	Mode of linkage	Molar ratio[b]
Rhamnose	1,3,5-Tri-*O*-acetyl-1-deuterio-6 deoxy 2,4-di-*O*-methyl D-rhamnitol	→3)-Rha-(1→	0.79
Mannose	1,3,5-Tri-*O*-acetyl-1-deuterio-2,4,6-tri-*O*-methyl D-mannitol	→3)-Man-(1→	0.08
Rhamnose	1,5-Di-*O*-acetyl-1-deuterio-6 deoxy 2,3,4-di-*O*-methyl D-rhamnitol	Rha-(1→	0.085
Glucose	1,4,5-Tri-*O*-acetyl-1-deuterio-2,3,6-tri-*O*-methyl D-glucitol	→4)-Glc-(1→	1
Mannose	1,3,5-Tri-*O*-acetyl-1-deuterio-2,4,6-tri-*O*-methyl D-mannitol	→3,4)-Man-(1→	0.93

[a] Partially methylated alditol acetate.
[b] Results are expressed in molar ratios relative to the 1,4-linked-glucose residue.

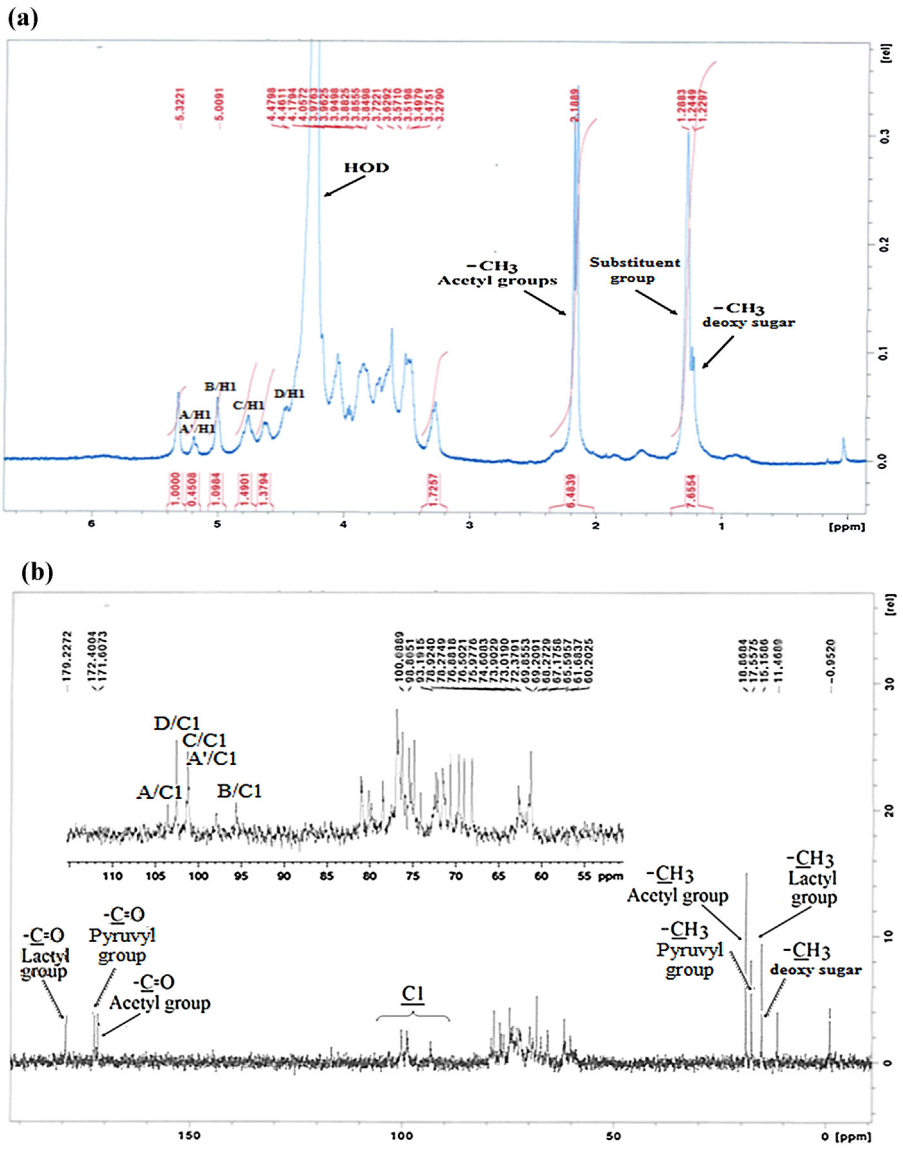


Fig. 5. 1-D ^{1}H NMR (400 MHz, 300 K) (a) and ^{13}C NMR (100 MHz, 300 K) (b) spectra of EPS22 (5 and 30 g/l in D2O). A/H1, A′/H1, B/H1, C/H1 and D/H1: H1 in A, A′, B, C and D residues; A/C1, A′/C1, B/C1, C/C1 and D/C1: C1 in A, A′, B, C and D residues.

signal at δ 5. 2 ppm correspond to two different protons A and A′, with ^{1}H/^{13}C signals at δ 5.2/98.8 and 5.2/104 ppm, respectively, corresponding therefore to two anomeric protons with α configuration. In addition, we note the presence of three other major and well defined anomeric protons, two with α configuration at δ 5.0/93.13 ppm (B) and 4.8/98.8 ppm (C) and another one with β configuration at 4.47/100.08 ppm (D). In contrast, the lowest field signal at δ 5.32 ppm and the signal at δ 4.65 ppm correlated with non-anomeric carbons resonating at δ 69 and 61.8 ppm, respectively and thus couldn't be attributed to anomeric protons. Integration data showed that the ratios between the peak areas of A/A′, B and C were equal to 0.3:0.79:1.08, respectively, which was consistent with glycosyl composition and linkage analysis. Based on these results, major components of the EPS22 repeating unit, designated B, C and D were assigned to α-rhamnose, α-mannose and β-glucose residues, suggesting that the exopolysaccharide under study consists mainly of a trisaccharide repeating unit, in which minor amount of two other residues (A and A′) could be present.

In the high-field region of the ^{1}H NMR spectrum, the signal at δ 2.18 was attributed to the CH_3 of an acetyl group, whereas the methyl signals in the 1.1–1.4 ppm region were assigned to a deoxy sugar (1.22–1.24 ppm) and non-osidic substituents (1.28 ppm), which were identified, based on the results described above and 1D/2D NMR analysis, as a rhamnose residue and both carboxyethyl (lactyl) and pyruvyl substituents, respectively.

The ^{13}C NMR spectrum (Fig. 5b) showed a signal at 15.15 ppm which was correlated with methyl proton signal at 1.28 ppm in HMQC spectrum (Fig. 6a), and another one at 179.22 ppm which was assigned to a carboxyl group. The COSY spectrum (Fig. 6b) showed correlation between the methyl protons CH_3 at 1.28 ppm and CH at 4.17 ppm. Taken together, these results confirmed the presence of a lactyl substituent (Parolis, Parolis, Niemann, & Stirm, 1988). However, the integral of the methyl signal at 1.28 ppm was estimated to be relatively high, suggesting that such signal resulted of the overlapping signals of the lactate and another substituent. The carbon resonances at 17.55 ppm which correlated with the methyl protons CH_3 at 1.28 ppm, and at 172.4 ppm

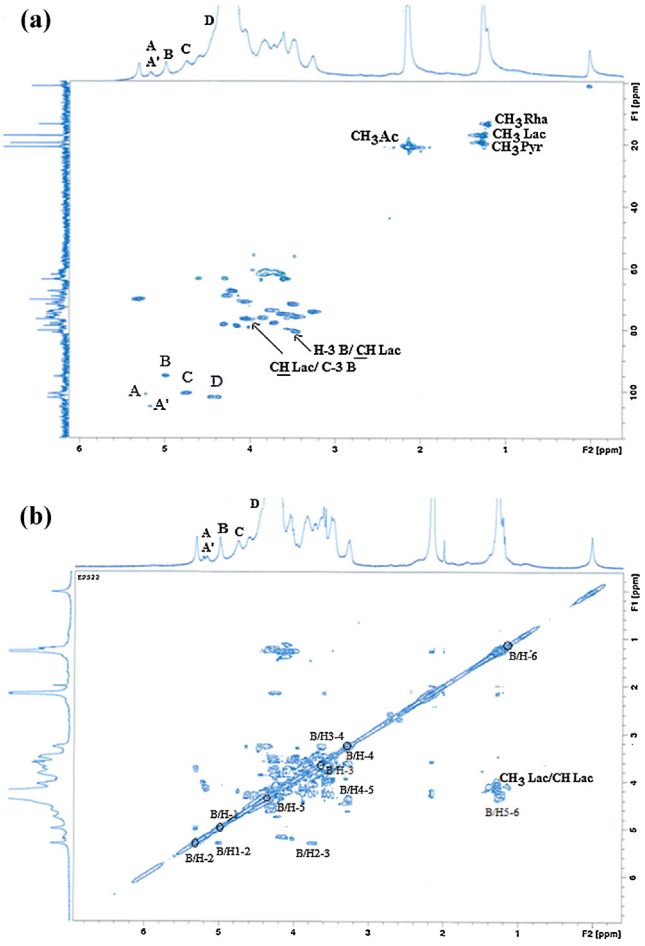


Fig. 6. 2D $^{1}H/^{13}C$ HMQC (a) and $^{1}H/^{1}H$ COSY (b) spectra of EPS22.

are consistent with the presence of a pyruvate methyl and carboxyl groups, respectively. The overlapping signals at 1.28 ppm prevented the estimation of the lactyl and pyruvyl substituent proportions.

Besides, the ^{13}C NMR spectrum of the native EPS showed a signal at 18.86 ppm, which correlated in the HMQC spectrum with the methyl protons CH_3 at 2.18 ppm, and thus may be attributed to an *O*-acetyl substituent. This latter assignment is confirmed by the signal at 171.60 ppm, characteristic of the CO group of such substituent. These results are consistent with the presence of an *O*-acetyl group in the approximate amount of 1.5 *O*-acetyl groups per sugar, estimated, thanks to the integration of protons of acetates reported (1.9–2.2 ppm) to the integration of all H-1 signal integrations (4.6–5.2 ppm) (Tavernier et al., 2008), suggesting therefore a highly acetylated EPS22. Consequently, the signals at 5.32 and 4.65 ppm, which were not observed in the deacetylated EPS (data not shown), were assigned to ring protons attached to *O*-acetyl groups. As a result of the O-acetylation chemical shift effect, these signals shifted into the anomeric region. Since the signal at 5.32 ppm was attributed to H-2 of rhamnose as shown is the COSY spectrum, this indicated the presence of an *O*-acetyl substituent linked to the C-2 of rhamnose, as previously suggested after NMR and MS analysis of the purified sugar X. Concerning the signal at 4.65 ppm, it correlated according to the HMQC spectrum with a carbon signal at 61.6 ppm, suggesting the glucose and/or mannose residues to be acetylated at position 6.

Signals characteristic of 6-deoxy sugar methyl groups were observed at 1.23 and 1.24 ppm and were assigned to the H-6 of a rhamnose residue. This finding is in agreement with the methylation analysis results that indicated the existence of rhamnose residues as a branch and terminal points and with previously suggested structure of the unknown sugar X purified from the EPS22 acid hydrolysate. Although the complexity of the EPS22 structure due to its high substitution degree, COSY experiments of EPS22 afforded the assignment of most of the rhamnose proton resonances (Fig. 6b) and indicated a correlation between the proton of the lactate CH group and the C-3 of the rhamnose residue B, but also H-3 B and the carbon of the lactate CH group, thus demonstrating the location of the lactyl group with an ether linkage at position 3 of rhamnose. Methylation analysis of the EPS22 revealed a 1,3 linked rhamnopyranose, thus supporting this mode of attachment. In addition, the presence in few amount of a terminal rhamnopyranose also confirms this statement, since a loss of the lactyl substituent may occur during methylation and EPS hydrolysis. Moreover, the detection of few amount of 1,3-linked mannose may be explained by the dissociation of the terminal rhamnose from C-4 of the mannose residue and thus suggesting a Man*p*-(4→1)-Rha*p* linkage.

Unfortunately, the complexity of the EPS22 structure, due to its high molecular mass and to the presence of three different non-osidic substituents (lactyl, acetyl and pyruvyl) prevented the complete assignment of the chemical shifts. The partial chemical shift assignment of the NMR is shown in Table 2.

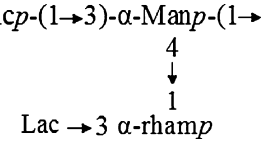
Table 2
^{1}H and ^{13}C NMR data on the residues that occur in EPS22 polysaccharide.

Compound	Residue	Isotope	Chemical shift (ppm) at indicated position					
			1	2	3	4	5	6
B	→3)-α-Rha-(1→	^{1}H	5.14	5.32	3.6	3.4	4.4	1.2
		^{13}C	93.19	69.85	73	75	80	11.4
C	→3,4)-α-Man-(1→	^{1}H	4.8	4.3	4	3.7	3.6	3.8
		^{13}C	98.80	77	75	NI	NI	61.6
D	→4)-β-Glc-(1→	^{1}H	4.52	3.3	3.7	3.7	3.6	3.9
		^{13}C	100.08	75	NI	NI	NI	61.6

Compound	—CH	—CH_3	—CO
Lac	4.17	1.28	–
	78.92	15.15	179.22
O-Ace	–	2.18	–
	–	18.86	171.6
Pyr		1.28	–
		17.55	172.4

Lac, lactate; Ace, acetate; Pyr, pyruvate; Rha, rhamnose; Man, mannose; Glc, glucose; NI, non-identified.

On the basis of all these results, a major structural motif of the heteropolysaccharide was assigned as follows:



It is composed of a backbone of alternating →4)-β-Glc*p*-(1→ and →3,4)-α-Man*p*-(1→ residues, in which a considerable part of the →3,4)-α-Man*p*-(1→ residues is branched at the C-4 position with terminal 1-α-rham*p*, substituted for the greater part (90%) with a carboxyethyl group on O-3 and with an acetyl group on O-2.

Although several *Pseudomonas* exopolysaccharides have been subject of structural studies and revealed the presence of similar type of sugars (glucose, mannose and carboxyethyl rhamnose), it is interesting to note an unusual structural feature of the polysaccharide under study which is the presence of three non-carbohydrate substituents, lactate (carboxyethyl), acetate and pyruvate, responsible for the anionic character of the EPS22 and which may influence its rheological behavior especially its viscosity, as reported by Gianni, Cescutti, Bosco, Fett, and Rizzo (1999).

Another main feature of the EPS investigated in this work, is the high degree of acetylation approximately equal to 1.5 *O*-acetyl groups per sugar. Although these substituents are not fully located due to problems of solubility related to the high molecular mass of this polysaccharide and taking into account the biological importance of acetylation, including the preparation of conjugated vaccines, since *O*-acetyl groups are important immunogenic determinants, this EPS could be seen as a product with potential to be used in the medical field.

4. Conclusion

The research on microbial exopolysaccharides is attracting increased attention. In this study, the EPS22 produced by *P. stutzeri* AS22, was purified and its composition and structure were partially elucidated. The EPS was an anionic, high molecular mass and highly substituted heteropolysaccharide, mainly composed of mannose, glucose and a carboxyethyl-substituted rhamnose in an approximate ratio of 1.1:1:0.7. Although the main sugar components of the *P. stutzeri* AS22 exopolysaccharide were similar to those reported by other researchers, the primary structure of this polymer is rather unusual in three ways; the O-2 acetylation of the carboxyethyl rhamnose residue, the presence of pyruvic groups and the high acetylation degree. All these structural features of the *P. stutzeri* AS22 EPS may provide crucial data for understanding its rheological behavior, investigating its biological properties and thus predicting its potential technological applications.

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