African Journal of Microbiology Research Vol. 6(34), pp. 6379-6385, 6 September, 2012
Available online http://www.academicjournals.org/AJMR
DOI: 10.5897/AJMR12.848
ISSN 1996-0808 ©2012 Academic Journals

Full Length Research Paper

Identification of pyruvyl groups in a novel microbial polysaccharide

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Accepted 27 August, 2012

A novel microbial exopolysaccharide (EPS) was isolated from *Enterobacter cloacae* Z0206. The pyruvyl groups of the polysaccharide were identified by ultraviolet (UV) spectra, nuclear magnetic resonance (^{1}H NMR, ^{13}C NMR) spectra, quadruple time-of-flight electrospray ionization mass spectrometry (Q-TOF-ESI-MS) and reversed phase-high performance liquid chromatography (RP-HPLC). To measure the pyruvyl groups, a microbial polysaccharide was hydrolyzed using 3M trifluoroaceticacid (TFA) at 120°C and subsequently analyzed by isocratic RP-HPLC and UV detection at 215 nm. Results showed that the linear range for the pyruvate was from 0.0025 to 0.5 g/L with a detection limit (signal-to-noise ratio of 3) of 0.05 mg/L, and the recovery rate ranged from 100.2 to 101.6%. This method can be used to determine the pyruvate content in acid hydrolysates of crude polysaccharides without the need for complex pre-treatment.

Key words: Microbial polysaccharide, pyruvate, identification.

INTRODUCTION

Numerous microbial exopolysaccharides (EPSs) have been reported to exhibit diverse biological activities including antiviral (Arena et al., 2009) and immunostimulatory properties (Liong, 2007). EPSs are regarded as having structural and functional diversity (Mishra and Jha, 2009). In order to clarify the structure-function correlations of polysaccharides, the compositional analysis is the most important step. Polysaccharides are mainly composed of neutral monosaccharides or other substitutive groups, such as acetate esters and pyruvate ketals (Sutherland, 2001). Pyruvyl groups may be estimated after acidic hydrolysis in a solution of 2,4-dinitrophenylhydrazine (Barbara et al., 2000) chromatographic methods (Smith et al., 1990; Rättötto et al., 2006), ion-exchange high performance liquid chromatography (HPLC) using ultraviolet (UV) detection (Yun et al., 2001), reversed phase-HPLC (RP-HPLC) equipped with fluorescence detection (Smith et al., 1981), and enzymatic assays (Smith et al., 1981; 1989; Ben et al., 2010). The mentioned processes involve possible interference (colorimetric) or tedious pre-treatments. In contrast, we present a simple pre- treatment method as part of a more selective and universal technique: RP-HPLC equipped with a UV detector.

In our previous study, *Enterobacter cloacae* Z0206, a bacterial strain, was found to produce large amounts of EPSs and only a fraction of the polysaccharides was composed of glucose, mannose and galactose (Xu et al., 2009). These differ from the present results that show the presence of pyruvyl groups, and for this reason a thorough investigation was carried out with the whole extract of the same strain. Therefore, the objective of this study was to identify the pyruvate and establish a time efficient method for its analysis.

MATERIALS AND METHODS

Apparatus and materials

Mass spectrometry (MS) detection was performed on a MicrOTOF-QII (American, Bruker), and nuclear magnetic resonance (^{1}H NMR and ^{13}C NMR) spectra were determined using an Avance 400 MHz nuclear magnetic resonance spectrometer (Germany, Bruker). A Waters Alliance 2695 HPLC system (American, Waters) with a Waters 2414 Refractive Index detector equipped with ultrahydrogel-250, -500 and -1000 multiple columns were used to determine homogeneity. For pyruvate analysis, A Waters Alliance 2695 HPLC system with a Waters 2998 photodiode array (PDA) detector

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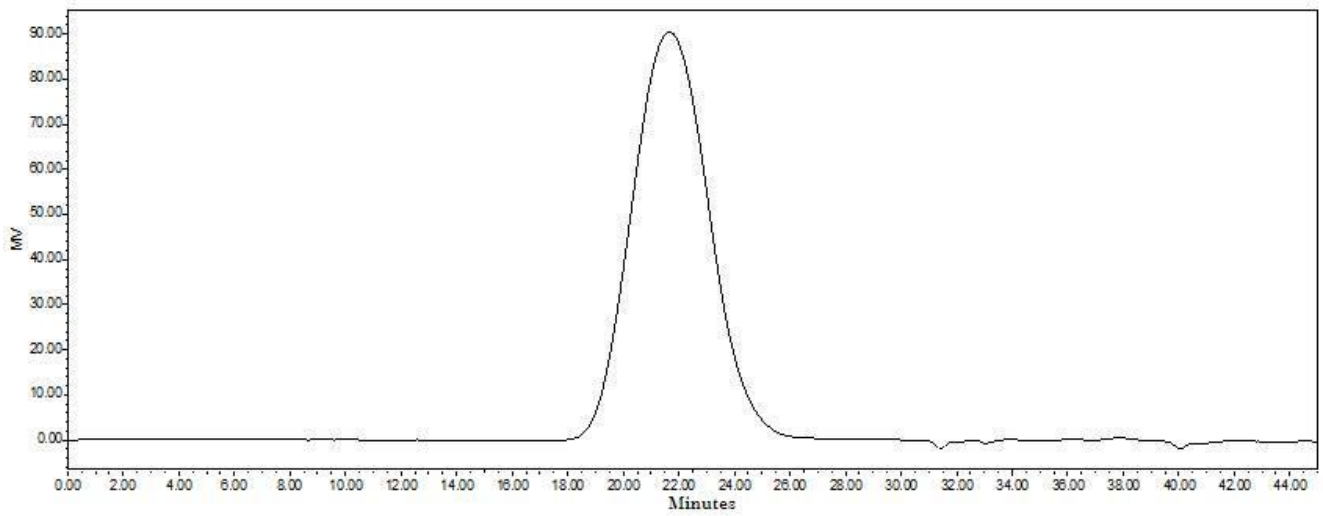

Figure 1. HPGPC profile of EPS isolated from *E. cloacae* Z0206.

equipped with an XBridge^{TM} C_{18} column was used.

The identity of the bacterial strain, *E. cloacae* Z0206, was confirmed and grown in our laboratory. The crude EPSs were obtained as previously described (Jin et al., 2010). DEAE-52 cellulose and Superose were purchased from Whatman Ltd. (England) and GE Healthcare (Sweden), respectively. HPLC-grade methanol was from Burdick & Jackson and pyruvate utilized as an external standard was from Aladdin. All other chemicals were of analytical reagent grade and made in China.

Separation and purification of the polysaccharide

The crude EPSs were extracted with hot water (80°C) for 5 h. The associated proteins were removed using the Sevag method (Tong et al., 2008). After removing the Sevag reagent, the water phase was dialyzed against water and lyophilized. An aliquot (3 g) of lyophilized EPSs was resuspended in 10 ml distilled water, the insoluble material was removed by centrifugation and the supernatant was fractionated using a DEAE-52 cellulose column (6 × 35 cm). Elution was performed sequentially with a water and sodium chloride (NaCl) gradient from 0 to 0.3 M at a flow rate of 1 ml/ min (8 min/fraction); the fractions eluted with NaCl solution were collected. After dialysis and lyophilization, the fractions were further fractionated on a Superose column (2.5 × 60 cm) and eluted with water at a flow rate of 0.2 ml/min (8 min/fraction). This resulted in one primary fraction that was determined with the phenol-sulfuric acid method. This fraction was dialyzed and lyophilized to give purified EPS, and the homogeneity of the EPS was identified by high-performance gel permeation chromatography (HPGPC) (Figure 1).

EPS (15 to 20 mg) was dissolved in 3M trifluoroaceticacid (TFA; 4 ml) in a closed vial with a butyl-lined screw cap and heated at 120°C for 5 h. The samples were allowed to cool to ambient temperature and transferred to a volumetric flask and the final volume was made up to 100 ml.

Chromatography conditions

XBridge^{TM} C_{18} column (250 × 4.6 mm, 5 µm, Waters); column temperature: 30°C; flow: 0.25 ml/min; mobile phase: solvent A: 98% K_2HPO_4-H_3PO_4 buffer (0.1 M K_2HPO_4-H_3PO_4, pH 2.9), solvent B: 2% methanol; UV wavelength 215 nm (Hallstrom et al., 1989).

Mass spectrometry conditions

Accurate MS experiments were performed on a micrOTOF-QII electrospray time-of-flight mass spectrometer (ESI-MS). ESI-MS was performed in the negative ion mode. The capillary voltage was 3500 V. An ESI flow rate of 180 µl/h was used. The experiment was conducted at 180°C with a mass scanning range (m/z) from 50 to 600 Da. The instrument was calibrated with solution of sodium formate to achieve maximal sensitivity.

RESULTS AND DISCUSSION

Pyruvate identification

Attempts to assign the EPS proton signals in the low field region were unsuccessful (Figure 2). However, it was possible to observe signals typical of methyl protons of pyruvate group at δ1.241 in the high field region. In accordance with previous reports (Evans et al., 2000; Perry and Maclean 1994), resonance at δ25.030, approximately 100 and approximately 176 (Kohno et al., 2009) in the EPS ^{13}C NMR spectrum (Figure 3) were attributed to the carbonyl, tertiary carbon and methyl resonance, respectively of the ketal-substituted pyruvate residue.

EPS was hydrolyzed as previously described and the residual TFA was removed by adding water three times under nitrogen flow. The sample was diluted with water and methanol to give a final concentration of approximately 10 pM and was then subjected to micrOTOF MS. The quasi-molecular ion $[M-H]^-$ peak at m/z 87.0075 Da suggested the chemical formula of pyruvate $[C_3H_3O_3]^-$ by accurate calculation with an absolute error of 1.3 mDa (Figure 4).

The final confirmation of the presence of pyruvate was based on the UV absorption curve (Hallstrom et al., 1989)

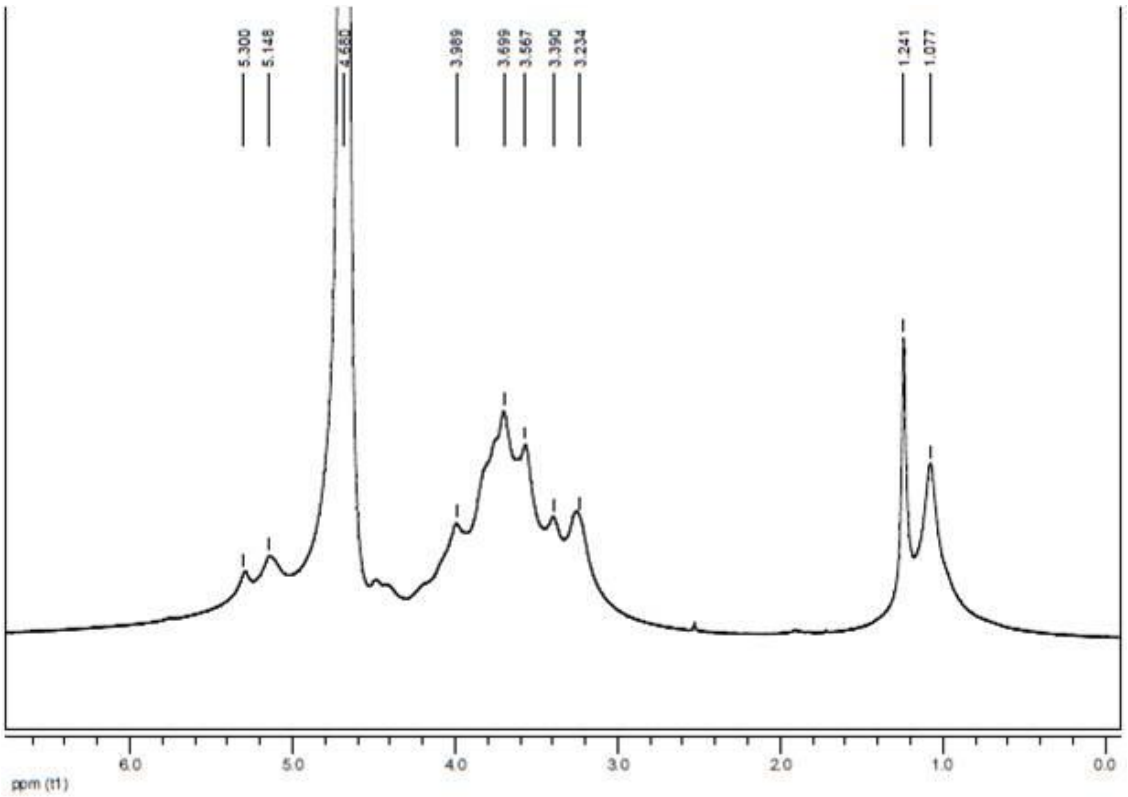

Figure 2. [1] HNMR of the EPS.

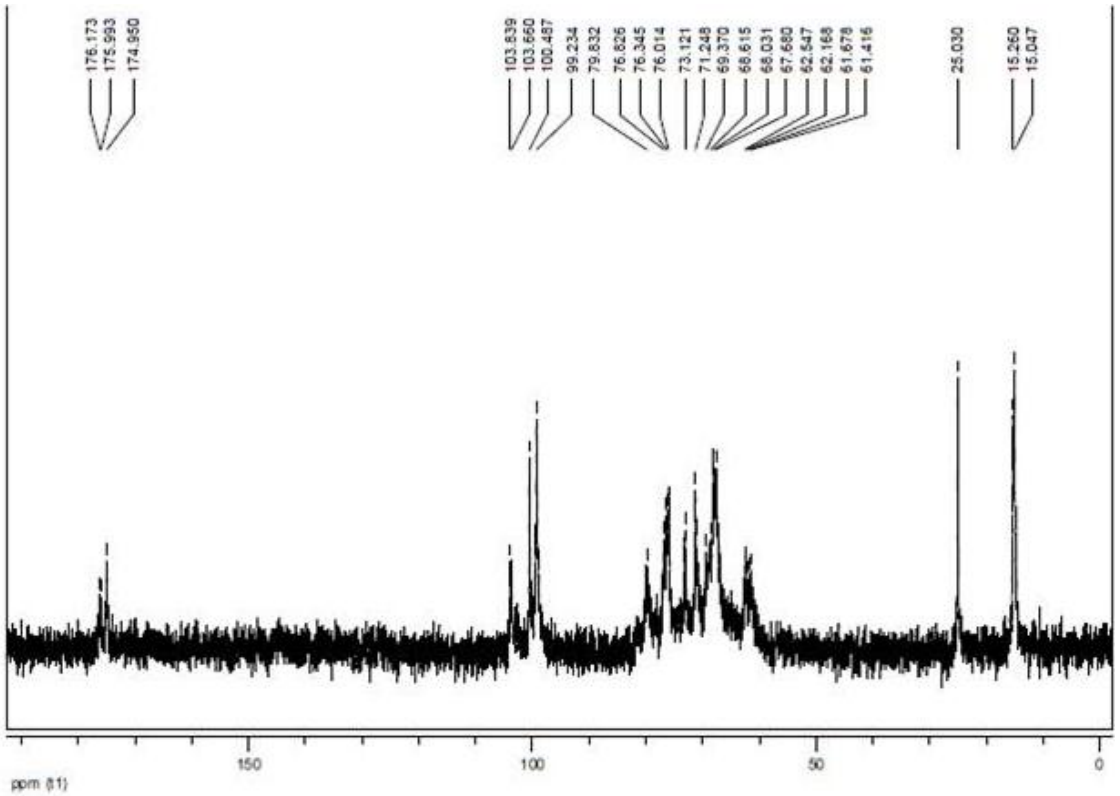

Figure 3. [13]C NMR of the EPS.

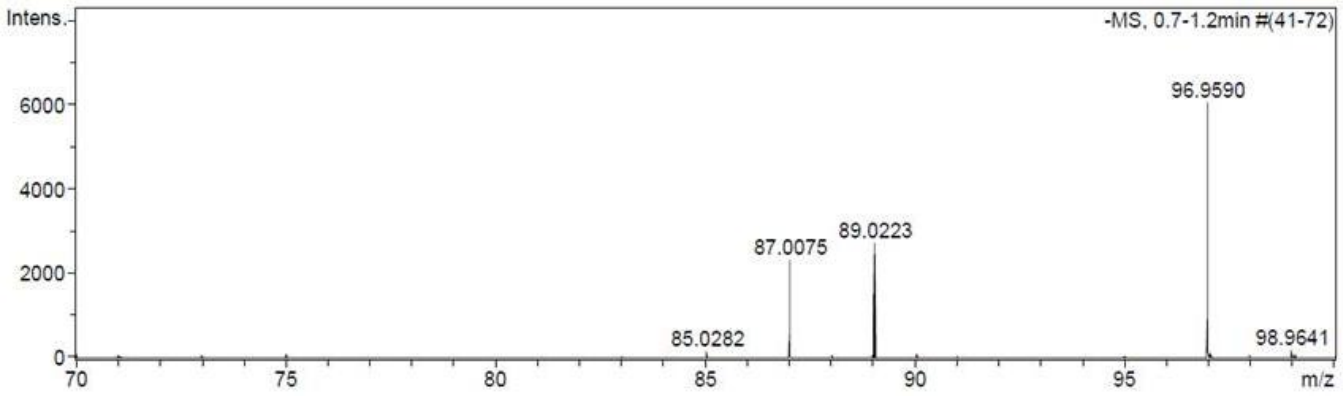

Figure 4. High resolution electrospray mass spectrum of the acidic hydrolysate of the EPS. The instrument was calibrated with a solution of sodium formate to achieve maximal sensitivity in the negative ion mode.

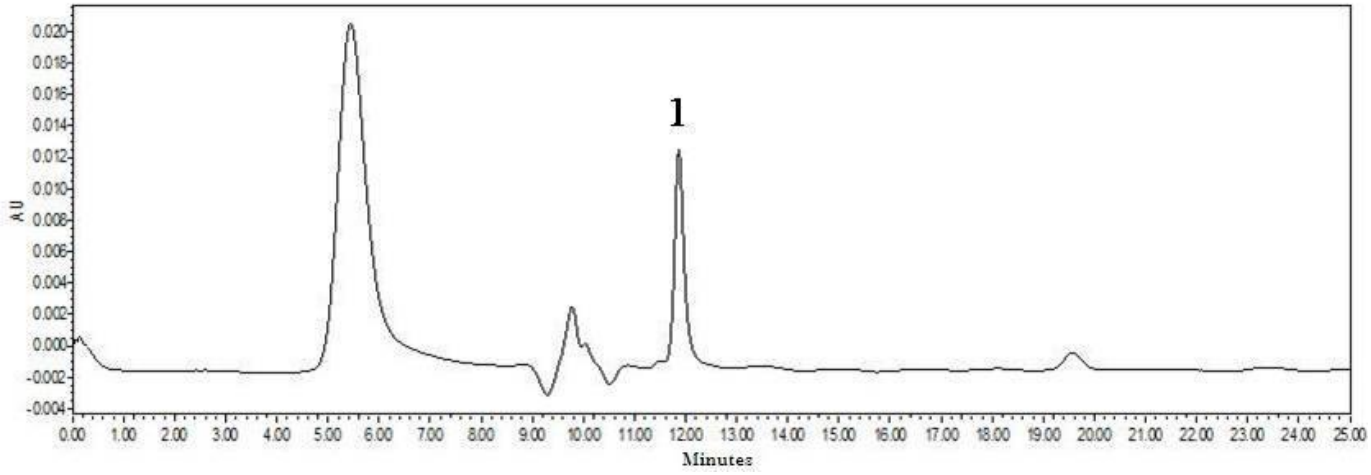

Figure 5. HPLC profile of the EPS hydrolyzed by 3 M HCl. Pyruvic acid (1) released from the EPS by treatment with 3 M HCl. The final volume of the hydrolysate solution was made up to 100 ml.

and retention time of a pyruvate standard.

Acid hydrolysis

To optimize the separation, 3 M HCl (Arnous et al., 2008) and 3 M TFA (Rau et al., 2009) were selected to hydrolyze the purified EPS samples. Figure 5 showed that an impurity was detected before the pyruvate peak following 3 M HCl hydrolysis (Figure 5), However, hydrolysis with 3 M TFA was found to provide baseline resolution (Figure 6).

Diluent volume

TFA peaks were not completely separated from hydrolysates in a total volume of 10 ml. It was observed that the volume of hydrolysates should be adjusted to 100 ml for complete separation of TFA peaks (Figure 7).

Linear relationship and precision validation

A stock solution of a pyruvate standard (1000 mg/L) was prepared and used to generate a series (500, 400, 250, 200, 100, 50, 25, 5, 2.5 mg/L) of standard pyruvate concentrations. Each standard sample analyzed five times (Figure 8) and the peak area versus concentration was measured to obtain the linear regression equation: $A = 30554C - 87020$ ($r^2 = 0.999$). The linear range extended from 0.0025 to 0.5 g/L.

Recovery and reproducibility

Reproducibility of the assay was determined with five samples (15 mg) that were acid hydrolyzed according to

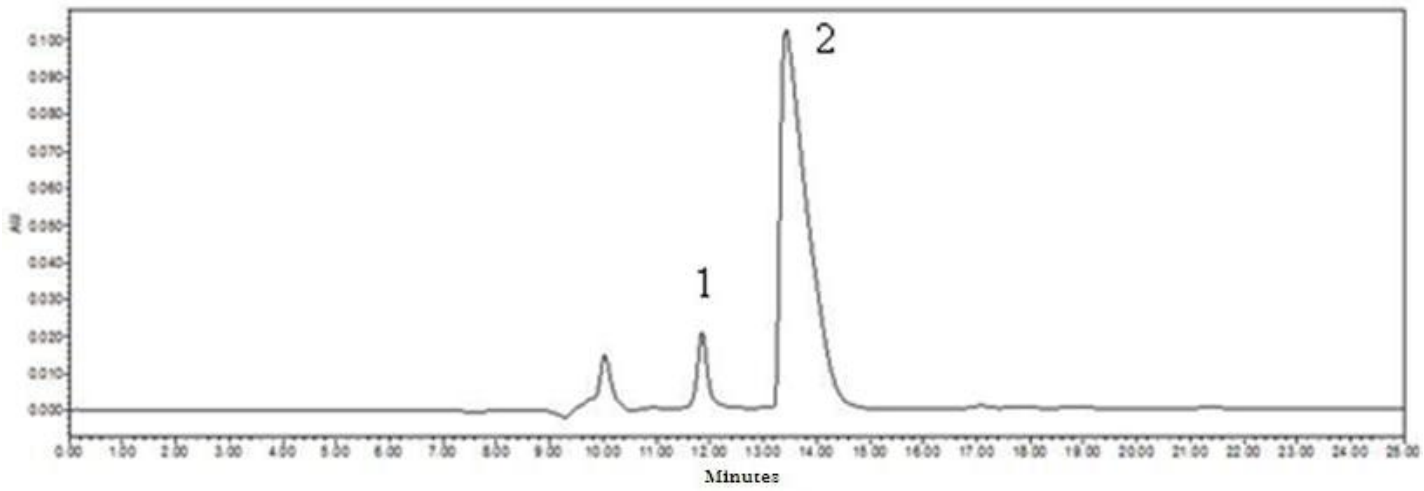

Figure 6. HPLC profile of the EPS hydrolyzed by 3 M TFA. Pyruvic acid (1) released from the EPS by treatment with 3 M TFA (2). The final volume of the hydrolysate solution was made up to 100 ml.

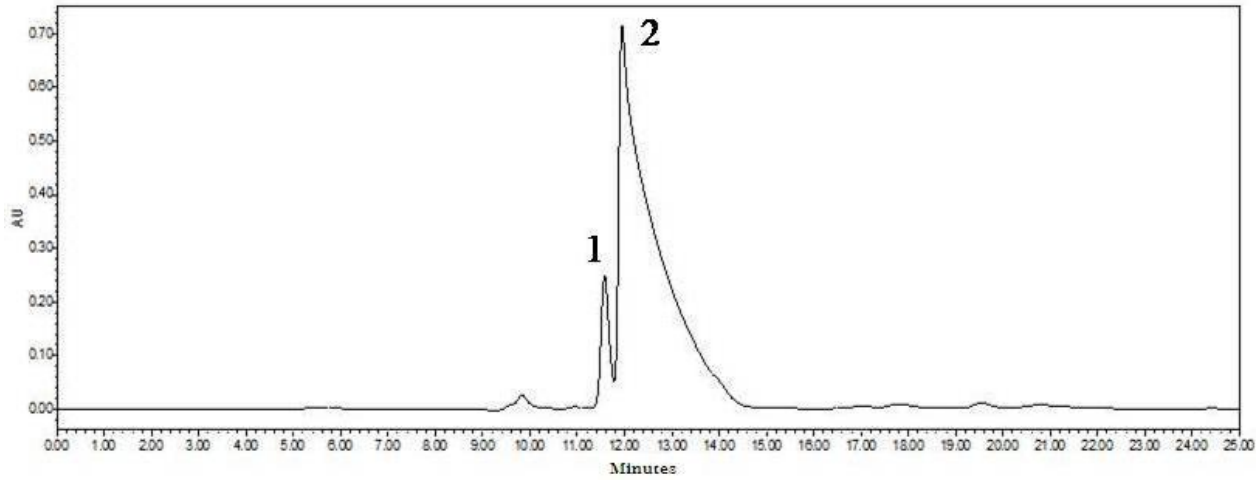

Figure 7. HPLC profile of the EPS hydrolyzed by 3 M TFA. Pyruvic acid (1) released from the EPS by treatment with 3 M TFA (2). The final volume of the hydrolysate solution was made up to 10 ml.

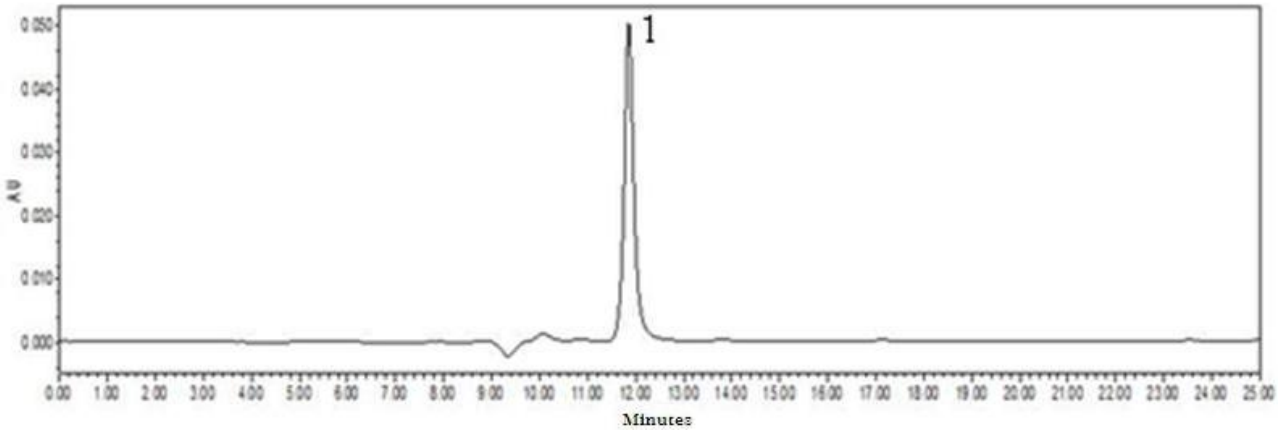

Figure 8. HPLC profile of a standard (pyruvate, 2.5 mg/L).

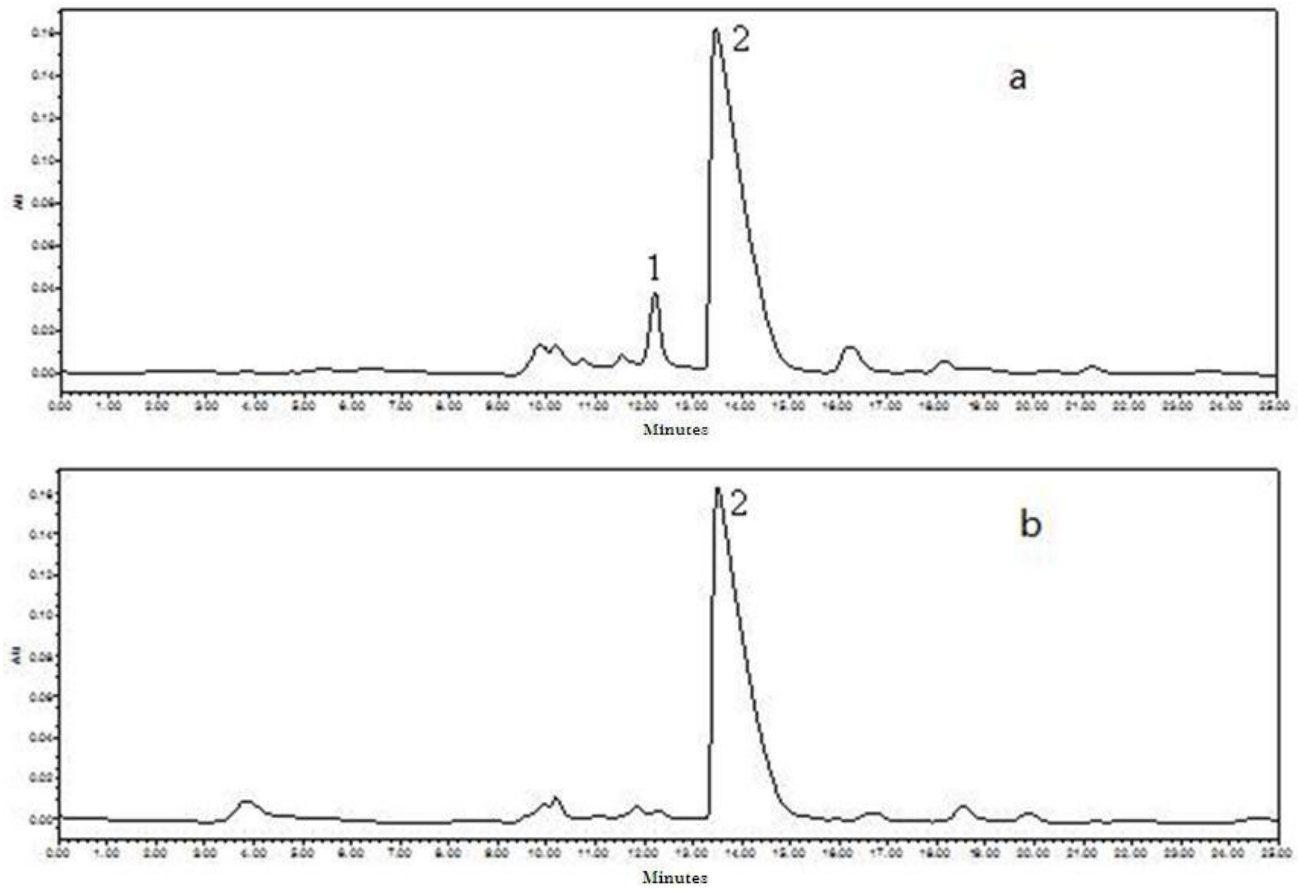

Figure 9. HPLC profiles of the bacterial polysaccharide extract (a) and *Astragalus* polysaccharides extract (b).

the procedure introduced previously. Samples were analyzed five times to obtain the average peak area. Linear regression analysis was applied to these data to determine pyruvate contents of 7.82, 7.54, 7.66, 7.72 and 7.48% (average content ± relative standard deviation (RSD), 7.64 ± 1.80%).

The recovery capacity of the assay was validated using samples containing a known concentration of standard pyruvate. Pyruvate (20 ml, 1000 mg/L) was added to TFA (20 ml, 6 M) to give a final concentration of 3 M TFA containing 500 µg/L pyruvate. Varying volumes (2 ml, 1 ml and 0.5 ml) of this solution were added to 15 mg purified EPS samples and the volumes were made up to 4 ml with 3 M TFA. Recovery rates of 100.8, 100.2 and 101.6%, respectively were obtained (average recovery ± RSD, 100.9 ± 1.54%; N = 15).

Assay specificity

Assay specificity was investigated using crude common bacteria polysaccharides and *Astragalus* polysaccharides (Figure 9). The absence of matrix interference in quantification of the complex polysaccharide extract was confirmed (Figure 9a). In contrast, pyruvate was almost undetectable in the *Astragalus* polysaccharide extract (Figure 9b).

In conclusion, pyruvyl groups in polysaccharides were identified using UV spectra, ^{1}H NMR, ^{13}C NMR spectra, Q-TOF-ESI-MS and RP-HPLC. Moreover, a method was developed for the quantitation of pyruvyl groups in microbial polysaccharides with high recovery and precision. This technique is suitable for quality control of crude polysaccharides.

ACKNOWLEDGMENTS

This work was supported by a grant from the Modern Agro-industry Technology Research System (No. CARS-36).

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