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Physico-chemical characterization of a new heteropolysaccharide produced by a native isolate of heterofermentative *Lactobacillus* sp. CFR-2182

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Abstract A heterofermentative *Lactobacillus* sp. CFR-2182 was isolated from *dahi* samples and it was found to produce 8.0 and 20.5 g/L heteropolysaccharide (HePS) in EPS medium (a simplified synthetic medium) and modified MRS broth, respectively, after 72 h at 30 °C. The total carbohydrate, reducing sugar and moisture contents of the purified HePS were 74, 10.6 and 2 g, respectively, per 100g on dry weight basis. The HePS produced in EPS medium had glucose and mannose in 17:1 ratio. The HePS was non-gelling and non-film forming type. It was completely soluble in water and 1N sodium hydroxide solution. Gel permeation chromatography and HPLC analysis indicated considerable heterogeneity of the HePS, having three fractions with molecular weights ranging from $3.3 \times 10^4 - 1.32 \times 10^6$ Da. The enzymatic hydrolysis of the HePS with pullulanase and α-amylase (with α(1→4) linkage) indicated the presence of α(1→6) and traces of α(1→4) linkages, respectively. NMR analysis of the EPS revealed unique chemical shifts.

KEYWORDS chromatography · EPS medium · fermentation · heteropolysaccharide · *Lactobacillus*

Introduction

Many lactic acid bacteria (LAB) produce exopolysaccharides (EPS) (Schiraldi et al. 2006). LAB cultures are categorized as generally regarded as safe (GRAS) organisms with no reports on production of harmful compound(s) or causing illness (Tallon et al. 2003). Hence, the EPS produced by LAB can be used as natural, safe additives to enhance the rheology and texture of novel food products (Dueñas et al. 2003). Besides technological applications, EPSs of LAB are also known to lower cholesterol level (Pigeon et al. 2002), minimize detrimental effect of putrefactive bacteria in the gut (Vasiljevic and Shah, 2007), protects gut mucosal immunity (Vinderola et al. 2006), stimulate the growth rate and biomass productivity of common constituent cultures of probiotic dairy starters, such as *Bifiobacterium lactis, Lactobacillus acidophilus, Streptococcus thermophilus* (Semjonovs and Zikmanis, 2008), reduce the colitis in rats (Şengül et al. 2005) and have immunomadulatory activity (Cobb and Kasper, 2005; Makino et al. 2006). Therefore, EPSs from LAB have potential for development and exploitation as food additives or functional ingredients with both health and economic benefits and these novel microbial biopolymers may fill the gap in the market-available polymers or may replace traditional food products in terms of improved rheological and stability characteristics (Ayala-Hernández et al. 2008; De Vuyst et al. 2001; Jaworska et al. 2005; Ketabi et al. 2008; Laws and Marshall, 2001; Purwandari et al. 2007; Tieking et al. 2003; Zisu and Shah, 2005). As a result, all over the world, many researchers are working on biosynthesis, genetic and metabolic engineering, characterization, and application of the EPS from new strains of these bacteria and it became a subject of many reviews (De Vuyst et al. 2001; Welman and Maddox, 2003; Ruas-Madiedo and de los Reyes-Gavilán, 2005). Isolation of EPS producing *Lactobacillus* cultures from traditional fermented foods of Thailand (Smitinont et al. 1999), in an Oat-based non dairy milk substitute (Mårtensson et al. 2000), Burkina Faso fermented milk (Savadogo et al. 2004) and fermented green olives (Sánchez et al. 2006) was reported.

The chemical, structural and functional properties of EPS produced by LAB vary with type of strain, culture conditions and composition of the media in which it is produced (Looijesteijn and Hugenholtz, 1999). Heteropolysaccharides (HePS) of LAB differ in sugar composition and the ratio of different sugars. HePS production from thermophilic LAB strains appears to be growth associated. The molecular mass of HePS varies from 10^4 to 10^6 Da (De Vuyst and Degeest, 1999; Vijayendra et al. 2008). The amount of HePS produced by lactobacilli is very low (<2 g/L) (Dueñas et al. 2003; Tallon et al. 2003; Torino et al. 2005; Sánchez et al. 2006; Lin and Chang Chien, 2007; Mozzi et al. 2007), which is not enough to make the production process economically viable. However, production of large amount of HePS (18.38 g/L) in just 4 h of fermentation by *Leuconostoc* sp. CFR 2181 was reported recently (Vijayendra and Sharath Babu, 2008). Therefore, the present study was focused on isolation of a high HePS yielding native strain of *Lactobacillus* sp. and physico-chemical characterization of the derived new HePS.

Materials and methods

Materials

Pullulanase, α-amylase, T-series dextran standards and dialysis membrane (10,000 cut-off) were obtained from Sigma-Aldrich Corporation (St. Luis, USA). All other chemicals were procured from SD Fine Chemicals, Mumbai, India.

Isolation and identification of EPS producing *Lactobacillus* sp.

Twenty five samples of *Dahi*, a traditional fermented dairy product, fifteen samples of buttermilk and vegetables (cabbage and cucumber) were collected from various parts of India (Bangalore, Coimbatore and Mysore) and processed for the isolation of EPS producing lactic cultures. All the samples were subjected to serial dilution using sterile saline (0.85 g/100ml) and suitable dilutions were spread on to the modified deMan Rogosa Sharpe agar (modified MRS agar- the glucose present in the original MRS agar formulation was replaced with sucrose at 5 g/100g (w/v) level) plates and incubated at 37 °C for 24-48 h. Mucoid colonies were selected, purified and preserved at 4 °C on modified MRS agar slants. The selected isolate was characterized by morphological, cultural and biochemical tests (Sharpe, 1979; Holt et al. 1994) and identified up to the genus level.

Production of the HePS by the isolate

Production of the HePS by the isolate was studied using modified MRS broth and EPS medium. The latter consisted of (g/L) Na_2HPO_4-5.0, KH_2PO_4-6.0, tri ammonium citrate-2.0, sucrose-50.0, $MgSO_4$-1.0 and trace elements solution ((g/L): $FeSO_4.7H_2O$-5.0, $MnSO_4$-2.0, $CoCl_2$-1.0, $ZnCl_2$-1.0 dissolved in 0.1 N HCl solution)-10 ml. Fifty ml of each medium (initial pH - 6.7) was distributed individually in 250 ml Erlenmeyer flasks, sterilized at 121 °C for 15 min and after cooling to 30 °C, inoculated (10 ml/100ml, v/v) with actively growing culture (in modified MRS broth for 5 h) after adjusting the absorbance to 1.0 at OD_{600} nm (~ 10^7- 10^8 cfu/ml) and incubated at 30 °C for 72 h on a rotary shaker (200 rev/min).

Analysis of the fermented broth

The fermented medium was centrifuged (Remi Instruments, India) at 8000 x g, to remove the biomass and the pH of the cell free supernatant was measured using digital pH meter (Henna Instruments, Singapore). The cell pellet was washed twice with sterile normal saline (0.9% w/v, sodium chloride) and collected in a pre-weighed aluminum foil cup. Two volumes of ice-cold isopropyl alcohol were added to one volume of the cell free supernatant and kept overnight at 4 °C for precipitation of the HePS. The alcoholic supernatant was decanted and the HePS was washed with acetone. The biomass and the HePS were dried in a hot air oven at 90 ± 2 °C to a constant weight and the dry weight was expressed in g/L. The reducing sugar content was determined using dinitrosalicylic acid reagent as described earlier (Shivakumar and Vijayendra, 2006).

Analysis of the HePS

The HePS produced by *Lactobacillus* sp. CFR-2182 in EPS medium was purified by dialysis using a membrane, as indicated in section 2.1, at 4 °C for 24 h with three changes of water. It was lyophilized (Edwards Co, England) and subjected to various analyses. To determine the moisture content, known quantity of the HePS was placed in a pre-weighed dish, dried to a constant weight in an oven at 90±2 °C and the moisture content

was calculated. Solubility of the EPS was checked in double distilled water and in 1 N sodium hydroxide solution. The aqueous solution of the HePS (1 g/100 ml, w/v) was boiled for 10 min, cooled and observed for gelling property. The ability of the HePS to form films was tested by pouring the aqueous HePS solution (1 g/100ml, w/v) over a glass plate (10 x 10 cm) up to a thickness of 1 cm and kept for air-drying for 24 h at 37 °C.

Characterization of the EPS

The total carbohydrate (Dubois et al. 1956), protein (Bradford, 1976) and uronic acid (Dische, 1947) contents of the dialyzed HePS were determined using D-glucose, bovine serum albumin and glucuronic acid, respectively, as standards. The monosaccharide composition of the hydrolyzed HePS was determined, as reported earlier (Vijayendra et al. 2008), after hydrolyzing the polymer with concentrated sulphuric acid derivatization into alditol acetates and following gas chromatography (Model: GC–15A, Shimadzu, Japan) equipped with OV-225 column (3%) and flame ionization detector, using N_2 as a carrier gas at a flow rate of 40 ml/min. The column temperature was maintained at 200 °C and the injector and detector were maintained at 250 °C. The HePS (1 g/100 ml, w/v) dissolved in 50 mM sodium acetate buffer (pH 5.0) was subjected for enzyme hydrolysis using α-amylase (E.C.3.2.1.1) and pullulanase (E.C.3.2.1.41), both from Sigma, USA. The glucose and reducing sugar contents were determined by the glucose oxidase (Dahlquist, 1964) and dinitrosalicylic (Bernfeld, 1955) methods, respectively. The infrared analysis of the dialyzed HePS was carried out by micro-KBr pellet technique using Nicolet-57000 Fourier-Transform Infrared (FTIR) spectrophotometer (Thermo Electron Corporation, USA). Purified dextran (Sigma, USA) was used as a standard.

The homogeneity and molecular weight of the HePS were determined by gel permeation chromatography (GPC) using Sepharose CL-2B column (94 cm x 2 cm). The HePS was eluted with triple distilled degassed distilled water (18 ml/h). Elution volumes (V_e) of standard dextrans of different molecular weights (T series dextrans, Sigma, USA) dissolved in triple distilled water were determined. Similarly, the void volume (V_o) was determined by using blue dextran solution (10 mg/ml). From the calibration curve of log Mw Vs V_e/V_o, the approximate molecular weight of the each fraction was computed. The ^{1}H-NMR and ^{13}C-NMR analyses of the dialyzed HePS dissolved in D_2O (10 mg/ml) were carried out using an ultra shield spectrophotometer (AC 500 MHz, Bruker, Germany) equipped with 5-mm broadband probe. ^{1}H-NMR measurements were obtained at 300 K and the chemical shifts (ppm) were referred indirectly to acetone. The spectral width was 10330.578 Hz and the digital resolution was 0.157 Hz, with an acquisition time of 3.17 sec. The spectrum was obtained with 16 scans. ^{13}C-NMR spectrum was also obtained at 300 K and the chemical shifts (ppm) were referred indirectly to tetramethylsilane. The spectral width was 26455.02 Hz with the digital resolution of 1.61 Hz and acquisition time of 0.30 sec. The spectrum was obtained with 1024 scans.

Results

Isolation and identification of the culture

While processing fermented dairy products (*dahi* or butter milk samples) and vegetables (cucumber and cabbage) for the isolation of EPS producing *Leuconostoc* sp. in our previous study (Vijayendra et al. 2008), we

could also observe one mucoid colony in the modified MRS agar plates spreaded with cucumber sample and incubated at 37 °C. The purified culture of this colony was found to be Gram positive, catalase negative and the isolate fermented glucose both in aerobic and anaerobic conditions. Upon microscopic observation, the mucoid culture appeared as rod shaped cells, arranged in single or in pairs. The results of the biochemical and physiological tests are presented in Table 1.

Production of HePS by the isolate

The ability of the isolated culture to produce EPS was determined by growing it in modified MRS broth and EPS medium prepared with sucrose (5 g/100 ml) as a carbon source. Production of EPS by the isolate was found to be growth associated (data not shown). The results indicated that the isolate produced more biomass and HePS in modified MRS medium than in EPS medium (Table 2).

Characterization of the HePS

The dialyzed HePS produced in the EPS medium was tasteless, smooth and puffy in appearance, non-gelling and non-film forming type and was completely soluble in water and 1N sodium hydroxide solution. The total carbohydrate, reducing sugar, uronic acid and moisture contents of the purified HePS were 74, 10.6, 0.68 and 2 g, respectively, per 100 g on dry weight basis. The major monosaccharides of the HePS were found to be glucose and mannose (88 and 5 g/100g, respectively), indicating that the EPS produced is a HePS. Presence of very low levels of rhamnose, arabinose, xylose and inositol (0.9 g/100g) was also noticed.

Gel permeation chromatography of the HePS produced by the isolate indicated considerable heterogeneity (Fig. 1). The elution profile indicated 3 peaks with absorption maxima ($OD_{480\ nm}$) of 0.90, 0.80 and 0.43 for fraction number 19, 30 and 42 with molecular weight values of $3.3x10^4$, $2.3x10^5$ and $1.32x10^6$ Da, respectively. This was also confirmed by HPLC analysis of the dialyzed EPS (data not shown), which gave 3 peaks at 10.78, 16.08 and 17.87 min, respectively. ^{13}C-NMR spectrum (Fig. 2) of HePS produced by the isolate indicated six signals at 97.46, 73.14, 71.15, 69.93, 69.30 and 65.33 ppm, corresponding to the six ring carbons C-1, C-4, C-5, C-3, C-6 and C-2, respectively. The signal in the region of 97.46 ppm corresponds to C-1 of α type configuration. On the other hand the ^{1}H NMR spectrum (Fig.3) showed the anomeric proton (C-1) at 4.86 ppm, attributed to the α-anomer, whereas the other protons appeared as a complex series of overlapping signals in the range of 3.3 to 4.1 ppm. The FTIR spectrum of the EPS (Fig.4) showed absorption around wave numbers 3388, 2926, 1651 and 1455 cm^{-1}, indicating stretching of -OH, C-H, carboxylate groups and symmetric bending of CH_3, respectively.

Discussion

Isolation and identification of the lactic culture

Keeping in view of the various potential applications of EPS of LAB cultures, the present study was focused on the isolation of the indigenous lactic cultures that are producing high amounts of EPS. Based on the observations that the isolated culture of the present study is catalase negative and able to ferment glucose both

in aerobic and anaerobic conditions, it was considered to be lactic culture (Sharpe, 1979). The morphology of the isolate and the physiological and biochemical performance of the isolate, especially production of the acid and gas with glucose fermentation indicated that the isolate is a heterofermentative *Lactobacillus* sp. (Holt et al. 1994) and assigned with the identification number CFR-2182.

Production of HePS by the isolate

Production of the EPS was studied in modified MRS and EPS medium. The idea of the using EPS medium from the production of polysaccharide was to simplify the down stream processing of the EPS. It was observed that glucomannan present in yeast extract and peptone interferes in the EPS quantification (Vaningelgem et al. 2004). Hence, a new EPS medium, a simplified synthetic medium, devoid of beef extract, yeast extract and protease peptone was formulated for easy recovery of EPS from the fermented broth, thereby reducing the impurities in the EPS, besides minimizing the cost of EPS production. In the preliminary studies, we found production of higher amount of EPS when fermentation was carried out at 30 °C, which is lower than the optimum growth temperature of 37 °C (data not shown). This is in tune with the observation of our earlier study (Vijayendra et al. 2008) wherein 3 fold higher specific EPS production was noticed with *Leuconostoc* sp. CFR 2181. This is in agreement with the hypothesis that, if cells are growing slowly, then wall polymers synthesis will also be slow, thereby making more isoprenoid phosphate available for EPS synthesis (Sutherland, 1972). Although the amount of HePS produced by *Lactobacillus* sp. CFR-2182 in EPS medium was lower than that of modified MRS broth, it was much higher than the amount reported earlier for different *Lactobacillus* sp. (Dueñas et al. 2003; Torino et al. 2005; Lin and Chang Chien, 2007). The reason for low yield of biomass and HePS in EPS medium might be due to lack of rich nutrients like peptone, extracts of beef and yeasts. Similarly, depending on the medium composition, variation in the quantity of HePS produced by *Strep. thermophilus* was also noticed. It was 152 mg/L in whey medium (Ricciardi et al. 2002) and 600 mg/L in skim milk medium (Cerning et al. 1988). It is very much low when compared to the yield obtained in the present study. This observation is in tune with the report of Degeest et al (2001), which indicated that the yield of EPS produced by LAB is influenced by the composition of medium and growth conditions.

Characterization of the HePS

Although EPS was produced using modified MRS and EPS medium, for characterization the EPS produced from EPS medium was used as the inherent impurities from the production medium are less than compared to the other medium (modified MRS). The EPS produced did not have any taste. However, the EPSs can increase the residence time of the milk products in the mouth, which impart an enhanced perception of the taste (Duboc and Mollet, 2001). The amount of uronic acid present in the EPS is insignificant to be called as an acidic polysaccharide. Presence of glucose and mannose indicated that the EPS produced by the isolate is a heteropolysaccharide. Although the amount of mannose is very small, we strongly feel that the mannose might be a constituent sugar of the HePS, as the analysis was carried out using the dialyzed HePS and it was produced in EPS medium, which is free of peptone or yeast extract. Production of HePS, although in very low quantities, has bee reported earlier. Very recently, Sánchez et al (2006) reported production of a low molecular weight EPS

having glucose and mannose in 3:1 ratio and a high molecular weight EPS with glucose and rhamonose, also in 3:1 ratio, by *Lact. pentosus* LPS26. Similarly, the HePS produced by *Lact. delbrueckii* was found to have glucose, galactose and rhamnose in the ratio of 1:6.8:0.7 (Grobben et al. 1995) and the EPS of *Lact. rhamnosus* consisted of galactose, glucose and rhamnose in the ratio of 1:1:4 (van Calsteren et al. 2002). However, Harding et al. (2005) noticed that the EPS produced by *Lact. delbrueckii* subsp. *bulgaricus* had a heptasaccharide repeating unit with galactose and glucose in a ratio of 4:3. This clearly indicates that the there is a wide variation in the composition of EPS produced by different species of lactobacilli. The enzymatic hydrolysis of the HePS of our isolate with pullulanase and α-amylase indicated the presence of α(1→6) and traces of α(1→4) linkages.

Purification of the EPS produced by *Lactobacillus* isolate by gel permeation chromatography indicated presence of three peaks, indicating considerable heterogeneity. The molecular weight of these three fractions was found to be different from each other. Similarly, Petry et al (2003) reported the presence of two fractions with different molecular weights in the EPS produced by four different strains of *Lact. delbrueckii* subsp. *bulgaricus* and Tallon et al (2003) observed production of two HePS of different molecular weight by *Lact. plantarum*. Of these the cell bound HePS had 8.5×10^5 Da and the unbound EPS had 4×10^4 Da. There is a substantial evidence in the literature for the synchronous production of EPSs of different molecular masses; a high molecular mass EPS (1.9×10^6 Da) and a low molecular mass EPS (3.3×10^4 Da) by *Lact. pentosus* (Sánchez et al., 2006). They have concluded that culture conditions have a clear impact on the type and concentration of EPS produced by this culture. Evidence confirming that a single lactic culture produces two homopolymers that have different repeat unit structures is also available for *Lactobacillus* spp. G-77 (Dueñas-Chasco et al. 1998). Results of NMR analysis indicated presence of six ring carbons with α-type configuration. In the free glucose, the chemical shift of C6 is generally seen in the range of δ 60-61 (JaganMohan Rao et al. 1982). However, in the present spectrum, no signal at δ 60 could be seen, indicating the possible linkage at C6 position. The chemical shifts of the HePS of the present study were unique, when compared with the chemical shifts of the EPS produced by other *Lactobacillus* species reported earlier (Gruter et al. 1993; Dueñas-Chasco et al. 1998; Harding et al. 2005). The FTIR spectrum of the HePS had close resemblance to the FTIR spectrum of the heteropolysaccharide produced by *Leuconostoc* CFR 2181 (Vijayendra et al. 2008) and not comparable to standard dextran (data not shown).

In conclusion, the newly isolated lactic culture, *Lactobacillus* sp. CFR-2182, was found to produce comparatively good yield of a new heteropolysaccharide in a low-cost synthetic medium. Further work is required to optimize the production so as to make it economically viable to compete with the existing microbial polymers and to find the technological applications for this EPS in food and allied industries.

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Table 1. Morphological and biochemical characterization of *Lactobacillus* sp. CFR-2182 isolated from cucumber

Test	Observation
Morphology	Gram positive short rods (single or pairs)
Size of the cell	2.2 x 0.5 µm
Growth at 45 °C	Weakly positive
Growth at 15 °C	Positive
Growth at pH 9.6	Positive
Growth with 4.0% & 6.5 % NaCl	Positive
Biochemical tests:	
Arginine utilization	Positive
Gelatin liquefaction	Negative
Utilization of glucose under aerobic and anaerobic conditions	Positive at both conditions
Production of acid and gas with glucose	Positive (hetero fermentative)
Catalase & oxidase tests	Negative for both
Fermentation of sugars	
maltose, fructose, trehalose	Positive
cellobiose, xylose, lactose	Positive
arabinose	Positive
rhamnose, mannitol, mellibiose	Negative

ND: Not determined

Table 2. Production of heteropolysaccharide by *Lactobacillus* sp. CFR-2182 in modified MRS broth and EPS medium at 30 °C

Parameter	Fermentation medium	
	Modified MRS*	EPS medium
Final pH	4.13 ± 0.04	6.20 ± 0.05
Dry weight of cell biomass (g/L)	1.36 ± 0.09	0.50 ± 0.06
HePS content (g/L)	20.50 ± 0.45	8.00 ± 0.47
Total residual sugar content (g/L)	37.30 ± 0.36	ND

Inoculum rate: 50 ml/L, sucrose content: 50 g/L, fermentation period: 24 h. ND: Not determined

Results are mean of two experiments in duplicate ± Standard deviation,

* modified MRS broth was prepared with 50 g/L sucrose, instead of 20 g/L glucose

Figure 1. Elution profile of heteropolysaccharide produced by *Lactobacillus* sp. CFR-2182 in Sepharose CL-2B column.

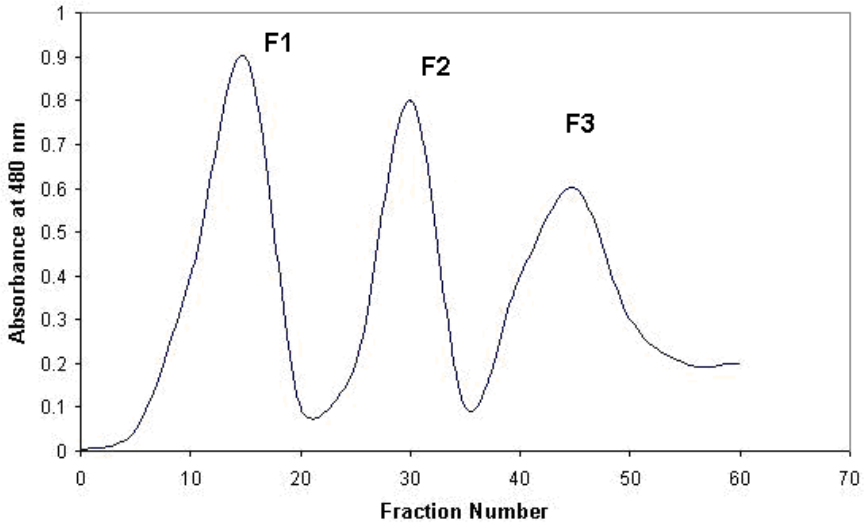

Figure 2. ^{13}C - NMR spectrum of dialyzed heteropolysaccharide of *Lactobacillus* sp. CFR-2182

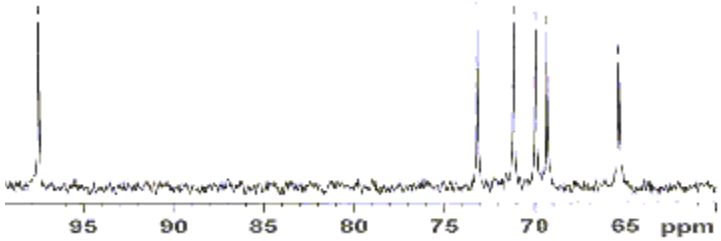

Figure 3. ^{1}H - NMR spectrum of dialyzed heteropolysaccharide of *Lactobacillus* sp. CFR-2182

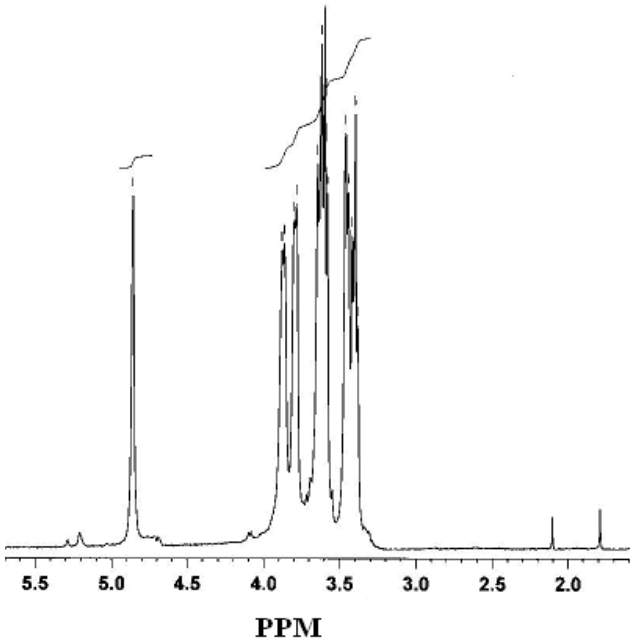

Figure 4. FTIR spectrum of heteropolysaccharide produced by *Lactobacillus* sp. CFR-2182