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Physico-chemical characterization of an exopolysaccharide produced by a non-ropy strain of *Leuconostoc* sp. CFR 2181 isolated from *dahi*, an Indian traditional lactic fermented milk product

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Abstract

Exopolysaccharide (EPS) producing non-ropy strain of lactic acid bacteria (LAB) was isolated from *dahi*, an Indian traditional fermented milk product, and on the basis of morphology, physiological and biochemical tests it was identified as *Leuconostoc* sp. CFR 2181. In shake flask fermentation for 72 h at 22 °C, the quantity of EPS produced by the isolate was 13.8 g/L in modified MRS broth and 25.4 g/L in EPS medium (a newly formulated simplified synthetic medium). The EPS was non-gelling and non-film forming. It was completely soluble in water and 1N sodium hydroxide solution. The purified EPS contained 84% of total carbohydrates, 11.2% of reducing sugars, 2% of moisture, 0.8% of proteins and 0.6% uronic acid. The EPS consisted mainly of glucose (91%) with minor quantities of rhamnose and arabinose (1.8% each). Gel permeation chromatography indicated considerable heterogeneity of EPS, having three fractions with molecular weights ranging from 1.0×10^4 to 1.5×10^6 Da. Enzymatic hydrolysis with pullulanase and α-amylase and NMR analysis of the EPS indicated the presence of α $(1 \rightarrow 6)$ linkages and the absence of α $(1 \rightarrow 4)$ linkages.

Keywords: *Dahi*; EPS medium; Exopolysaccharide; Fermentation; *Leuconostoc*

1. Introduction

Exopolysaccharides (EPS) of microbial origin are gaining in importance because of their applications in food and other industries. Several species of lactic acid bacteria (LAB) produce different types of EPS, which vary in composition, structure and size. The isolated EPS can be used as natural, safe additives to enhance the rheology and texture of novel food products (Dueñas-Chasco et al., 1998). LAB are classified as generally regarded as safe (GRAS) organisms and there are no reports of any illness involving these bacteria or production of any harmful compound(s) by them.

The ability to produce EPS is widespread among LAB. As a result, all over the world, many researchers are working on the production, characterization and applications of the EPS from new strains of these bacteria. Many reviews have been published covering the screening, isolation and characterization, biosynthesis, genetic and metabolic engineering and functional properties of the EPS produced by LAB (De Vuyst, De Vin, Vaningelgem, & Degeest, 2001; De Vuyst & Degeest, 1999; Jolly, Vincent, Duboc, & Neeser, 2002; Ruas-Madiedo & de los Reyes-Gavilán, 2005; Welman & Maddox, 2003).

Although having no taste of their own, the EPS from LAB increase the residence time the milk products spend in the mouth and hence impart an enhanced perception of taste (Duboc & Mollet, 2001). An additional hypothesized, physiological benefit is that EPS will remain longer in the gastro-intestinal tract, thus enhancing the colonization by probiotic bacteria (German et al., 1999). Unlike the EPS produced by other microorganisms, many health benefits such as antitumor effects (Kitazawa et al., 1998),

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cholesterol lowering ability (Pigeon, Cuesta, & Gilliland, 2002) and immunostimulatory activity (Chabot et al., 2001) have been attributed to EPS produced by LAB.

The chemical, structural and functional properties of EPS produced by LAB are dependent on the type of strain, culture conditions and media composition (Looijesteijn & Hugenholtz, 1999). Isolation of EPS producing LAB cultures from cheese (Kojic et al., 1992), wine (Manca de Nadra & Strasser de Saad, 1995), traditional Thai (Smitinont et al., 1999) and Nigerian (Sanni, Onilude, Ogunbanwo, Fadahunsi, & Afolabi, 2002) fermented foods has been reported. Many of them are produced at very low quantity (<500 mg/L) of EPS (Dueñas, Munduate, Perea, & Irastorza, 2003; Kimmel & Roberts, 1998; Lin & Chang Chien, 2006; Torino, Mozzi, & Font de Valdez, 2005). The aims of the present study were isolation and identification of a lactic culture that produces higher amounts of EPS rapidly in a newly formulated low-cost synthetic medium. In addition some physico-chemical properties of the EPS were determined.

2. Experimental

2.1. Isolation and identification of EPS producing LAB culture

Several samples of *dahi*, buttermilk, and vegetables such as cabbage and cucumber collected from various parts of India were used for the isolation of EPS producing lactic cultures. Ten grams of each sample was weighed individually into Erlenmeyer flaks containing 90 ml of sterile saline (0.9% sodium chloride) and shaken thoroughly to dislodge the microorganisms and subjected to serial dilution and appropriate dilutions were plated onto modified deMan Rogosa Sharpe agar (M-MRS agar, the glucose present in the original formulation was replaced by sucrose) containing sucrose (5%, w/v) by following the spread plate method and incubated at 30 and 37 °C for 24–48 h. Plates that had mucoid colonies were picked up and they were purified by following the streaking method and subsequently preserved at 4 °C on M-MRS agar slants. The isolate was characterized for phenotypic and chemotaxonomic characteristics by morphological, cultural and various biochemical tests. It was identified up to genus level according to Sharpe (1979) and Holt, Krieg, Sneath, Staley, Williams (1994).

2.2. Screening of the isolate for EPS production

The isolated mucoid culture was used for EPS production using M-MRS broth and EPS medium, a synthetic 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 – 10 ml (consisting of (g/L): $FeSO_4{\cdot}7H_2O$ – 5.0, $MnSO_4$ – 2.0, $CoCl_2$ – 1.0, $ZnCl_2$ – 1.0 dissolved in 0.1 N HCl solution). Each medium (50 ml) with an initial pH 6.7 was taken individually in 250 ml Erlenmeyer flaks, sterilized at 121 °C for 15 min and later inoculated (10%, v/v), after adjusting the absorbance of actively growing culture to 1.0 at OD_{600} nm and incubated at 30 °C for 72 h on a rotary shaker (200 rpm). The fermented medium was analyzed for EPS and biomass content.

2.3. Analysis of the fermented broth

2.3.1. Measurement of pH

The fermented medium was centrifuged (Remi Instruments, India) at 8000 rpm for 20 min and the cells were separated. The pH of the cell-free supernatant was measured using digital pH meter (CD Instruments Pvt. Ltd., India).

2.3.2. Biomass content

The cell pellet obtained after centrifugation was washed once with distilled water and collected in pre-weighed aluminum foil cups and dried in a hot air oven at 90 ± 2 °C to constant weight. The dry cell weight (biomass) was expressed as g/L.

2.3.3. EPS content

Ice-cold isopropyl alcohol (2 vol) was added to the cell-free supernatant (1 vol) under continuous stirring, and kept overnight for precipitation in the refrigerator. The alcoholic supernatant was decanted and the EPS precipitate was washed with acetone. It was dissolved in water (10 ml) and lyophilized. The EPS content was expressed as g/L.

2.3.4. Reducing sugar content

The reducing sugar content was analyzed (Miller, 1959) after inversion with concentrated HCl at 70 °C for 10 min and reacting with dinitrosalicylic acid (DNS) reagent as described earlier (Shivakumar & Vijayendra, 2006).

2.3.5. Determination of viscosity

Viscosity of the cell-free fermented broth was determined using a viscometer (Model: R1: 3: M, Rheology International Shannon Ltd., Ireland) with spindle no. 2 (50 rpm) at 25 °C and it was expressed as milliPascal second (mPa s).

2.4. Analysis of EPS

The EPS was dissolved in distilled water and dialyzed using a membrane of molecular weight cut-off 10,000 (Sigma, USA) at 4 °C for 24 h with three changes of water. The partially purified EPS was lyophilized (Edwards Co., England) and used to determine the moisture content, physical properties and chemical composition.

2.4.1. Moisture content

The EPS taken in a dish, which was previously dried and weighed, was placed along with its lid in an oven main-

tained at 105 °C for 5 h and cooled in a desiccator. Drying was repeated till a constant weight was obtained and the percentage of moisture content was calculated.

2.4.2. Physical properties

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

2.4.3. Chemical characterization of EPS

The total carbohydrate, protein and uronic acid contents were determined as reported by Dubois, Gilles, Hamilton, Rebers, and Smith (1956, using D-glucose as standard), Bradford (1976, using bovine serum albumin as standard) and Dische (1947, glucuronic acid as standard) methods, respectively. The content of monosaccharides of the dialyzed EPS was determined after derivatization to alditol acetates (Sawardekar, Slonekar, & Jeanes, 1965) and following gas chromatography (GC) (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 homogeneity and molecular weight of the EPS were determined by gel permeation chromatography (GPC) using Sepharose CL-2B column (94×2 cm) eluted with triple distilled degassed water (18 ml/h). Calibration curve was prepared using (V_e, elution volume) standard dextrans of different molecular weights (T series dextrans, Sigma, USA) dissolved in triple distilled water. The void volume (V_o) was determined by using a predialyzed blue dextran solution (10 mg/ml). From the plot of $\log M_w$ vs. V_e/V_o, the approximate molecular weight of the fraction was computed.

The EPS (1%, 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 sugars released were determined by the glucose oxidase (Dahlquist, 1964) and dinitrosalicylic (Bernfeld, 1955) methods, respectively.

The ^{1}H NMR and ^{13}C NMR analyses were carried out on a ultrashield spectrophotometer (AC 500 MHz, Bruker, Germany) equipped with 5-mm broad band probe, using the dialyzed EPS dissolved in D_2O (10 mg/ml). ^{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 s. The spectrum was obtained with 16 scans. ^{13}C NMR spectra were 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 s. The spectrum was obtained with 1024 scans.

The infrared analysis of the dialyzed EPS was carried out by micro-KBr pellet technique using Nicolet – 57,000 Fourier-Transform Infrared (FTIR) spectrophotometer (Thermo Electron Corporation, USA). Purified dextran (Sigma, USA) was used as a standard.

3. Results and discussion

3.1. Isolation and identification of the culture

A total of 32 samples (two each of cucumber and cabbage and rest are *dahi*/butter milk samples) were processed for the isolation of EPS producing LAB. As it is easy to observe the EPS producing colonies by their appearance as mucoid colonies on agar surface, Modified MRS agar prepared using 5% (w/v) sucrose was used as a medium to isolate the EPS producing LAB cultures. MRS agar being a selective agar does not encourage the growth of other bacterial cultures. The samples were serially diluted and plated onto the agar plates by spreading method and one set each of the plates were incubated at 30 and 37 °C for 48 h. The colonies being gram-positive and catalase negative were considered to be LAB. The culture producing mucoid colonies at 30 °C was picked up from M-MRS agar plate spreaded with *dahi* sample. When observed under phase contrast microscope the culture appeared as spherically shaped cells arranged in chains. Using cultural, biochemical and physiological tests as shown in Table 1, the isolate was identified as *Leuconostoc* sp. and given the identification number CFR 2181.

3.2. Production of EPS

The isolated culture, *Leuconostoc* sp. CFR 2181, was grown in M-MRS medium and EPS medium having 5% sucrose. Production of EPS by the isolate was found to be growth associated (data not shown). Recently Vaningelgem et al. (2004) observed that glucomannan present in yeast extract and peptone interferes in the EPS quantification. Hence, a new EPS medium, a simplified semi-synthetic medium, devoid of beef extract and protease peptone was formulated to simplify the recovery of EPS from the fermented broth and to reduce the cost of the production medium. Fermentation was carried out at 30 °C and also at 22 °C for 72 h at 200 rpm. The fermented broth samples were analyzed for final pH, cell biomass, EPS and residual sugar contents. The results, presented in Table 2, show that *Leuconostoc*, at 30 °C produced slightly more biomass in MRS medium than in EPS medium. However, the quantity of EPS produced in EPS medium (22.5 g/L) was much higher than in MRS medium (14 g/L). This indicates the necessity of rich nutrients like peptone, beef

Table 1
Morphological and biochemical characterization of *Leuconostoc* sp. CFR 2181 isolated from *dahi*

Tests	Observations
Morphological tests	
Morphology	Gram-positive cocci in short chains (4–6 cells)
Size of the cell	0.5 μm diameter
Growth at 45 °C	Negative
Growth at 15 °C	Positive
Growth at pH 9.6	Positive
Growth with 4.0% NaCl	Positive
Growth with 6.5% NaCl	Negative
Biochemical tests	
Arginine utilization	Negative
Gelatin liquefaction	Negative
Utilization of glucose	
Aerobic condition	Positive
Anaerobic condition	Positive
Production of CO_2 with glucose	Positive
Catalase test	Negative
Oxidase test	Negative
Aesculin hydrolysis	Positive
Curdling of milk	Delayed positive
Fermentation of sugars	
Maltose	Positive
Fructose	Positive
Trehalose	Positive
Raffinose	Delayed positive
Lactose	Delayed positive
Arabinose	Positive

extract and yeast extract for the biomass development rather than EPS production. Nevertheless, *Leuconostoc* sp. CFR 2181 could still produce more quantity of EPS (25.4 g/L), when incubated at 22 °C, which is a characteristic feature of this genus. This is in tune with the report of Cerning, Bouillanne, Landon, and Desmazeaud (1992), wherein 50–60% higher EPS production was noticed when grown at 25 °C rather than at 30 °C by slime forming mesophilic lactic acid bacteria.

The specific EPS production (g of EPS per g of dry biomass produced) was 2.5-fold higher in EPS medium (28.12) than in M-MRS broth (11.47) when fermented at 30 °C, whereas nearly 3-fold difference in specific EPS production (10.53 in M-MRS broth and 29.19 in EPS medium) was noticed when grown at 22 °C. 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). The amount of EPS produced by the isolate is significantly much higher than reported (Dueñas et al., 2003; Kimmel & Roberts, 1998; Lin & Chang Chien, 2006; Torino et al., 2005). Although production of dextran like polysaccharide by *Leuconostoc* sp. V41 was reported by Vimala and Lalithakumari (2003), the amount of EPS produced was not mentioned. Earlier studies revealed that though EPS synthesis by thermophilic LAB occurs at an optimum pH of 4.0 (Mozzi, Oliver, Savoy de Giori, & Font de Valdez, 1995) a pH of 5.5 and temperature of 40 °C were found to be optimal for EPS production by *Streptococcus thermophilus* 1275 with a very low yield of 458 mg/L (Zisu & Shah, 2003). Similarly, depending on the medium composition, the quantity of EPS produced by *S. thermophilus* was 152 and 600 mg/L in whey medium (Ricciardi et al., 2002) and skim milk medium (Cerning et al., 1992), respectively. This is very low compared to the yield obtained in the present study. The report of Kimmel and Roberts (1998) indicated that beef extract and peptone account for majority of the impurities in EPS. Therefore these components were excluded while developing the EPS medium to avoid the interference of these components while recovering the EPS from the fermented broth.

The viscosity of the cell-free fermented EPS medium inoculated by *Leuconostoc* sp. CFR 2181 was 16.16 mPa s, whereas, the viscosity of the Mill Milk medium after fermentation with *Lactobacillus delbrueckii* subsp. *bulgaricus* NCFB 2772 for 24 h at 30 °C was found to be 36 mPa s (Mårtensson, Öste, & Holst, 2000). However, they did not indicate the molecular weight and type of linkage of this EPS. Similarly, the viscosity of the 1% (w/v) solution of pullulan (an EPS, wherein maltotriose units are repeatedly polymerized via α (1 → 6) linkages), produced by *Aureobasidium pullulans* CFR-77 was 32 and 160 mPa s, when sucrose and *jaggery* were used as a carbon source, respectively (Vijayendra, Bansal, Prasad, & Nand, 2001). However, the viscosity of a dextran (M_w 2×10^5 Da), a microbial EPS having predominantly α (1 → 6) linkages, solution (50 g/dm^3) as determined by using a modified Zim-Crothers rotary viscometer was indicated to be

Table 2
Production of EPS by *Leuconostoc* sp. CFR 2181 in modified MRS and EPS media at different temperatures

Parameter	Fermentation temperature (°C)			
	30		22	
	M-MRS*	EPS medium	M-MRS	EPS medium
Final pH	4.05 ± 0.04	3.89 ± 0.05	4.05 ± 0.04	3.84 ± 0.11
Dry weight of cell biomass (g/L)	1.22 ± 0.09	0.80 ± 0.06	1.31 ± 0.04	0.87 ± 0.07
EPS content (g/L)	14.00 ± 0.45	22.50 ± 0.47	13.80 ± 0.63	25.40 ± 0.88
Total residual sugar content (g/L)	26.60 ± 0.36	10.00 ± 0.20	30.00 ± 0.36	26.00 ± 0.64

Inoculum rate: 50 ml/L, sucrose content: 50 g/L, fermentation period: 24 h.
Results are means ± standard deviation.
* M-MRS: modified MRS medium prepared with 50 g/L sucrose, instead of 20 g/L glucose.

4.0 mPa s and the viscosity of the dextran solution of same concentration increased from 1.5 to 4.0 mPa s with the increase in the molecular weight of the dextran from 7.1×10^4 to 2.8×10^5 (Mazurkiewicz, Rębilas, & Tomask, 2006). The variation in viscosity may be due to the differences in the molecular mass of the EPSs as also suggested by Laws and Marshall (2001).

3.3. Characterization of EPS

The dialyzed EPS was smooth and puffy in appearance, non-gelling and non-film forming type and was completely soluble both in water and 1 N sodium hydroxide solution. The solubility properties of the EPS could give it a range of applications in the food industry.

The dialyzed EPS produced by *Leuconostoc* sp. CFR 2181 was found to contain moisture – 2.0%, total carbohydrate – 84.0%, protein – 0.8%, uronic acid – 0.6% and reducing sugars – 11.2%. The major monosaccharide identified was glucose (91%), besides rhamnose and arabinose in minor quantities (1.8% each), may be attributed as impurities. The enzymatic hydrolysis of the EPS with pullulanase (E.C.3.2.1.41) and α-amylase (E.C. 3.2.1.1) indicated the presence of α $(1 \rightarrow 6)$ linkages and absence of α $(1 \rightarrow 4)$ linkages. The TLC analysis of the hydrolyzed products of the EPS treated with pullulanase indicated the presence of glucose (data not shown) confirming the α $(1 \rightarrow 6)$ linkages between the glucose molecules. Marshall, Cowie, and Moreton (1995) reported the production of a phosphopolysaccharide by *Lactococcus lactis* subsp. *cremoris* LC330, which consisted of glucose, rhamnose, galactose and glucosamine in an approximate ratio of 6:5:4:1, respectively. Evidence confirming that a single LAB culture produces two homopolymers that have different repeat unit structures is only available for *Lactobacillus* spp. G-77 (Dueñas-Chasco et al., 1998).

The elution pattern in gel permeation chromatography of the EPS produced by *Leuconostoc* sp. CFR 2181 indicated (Fig. 1) considerable heterogeneity. The elution profile showed three peaks F1, F2 and F3 (Fig. 1), with absorption maximum at OD_{480} nm as 0.98, 0.63 and 0.80 in fraction numbers 16, 26 and 47, respectively. Accordingly, the molecular weight values were 1.5×10^6, 4.7×10^5 and 1.0×10^4 Da for F1, F2 and F3 fractions,

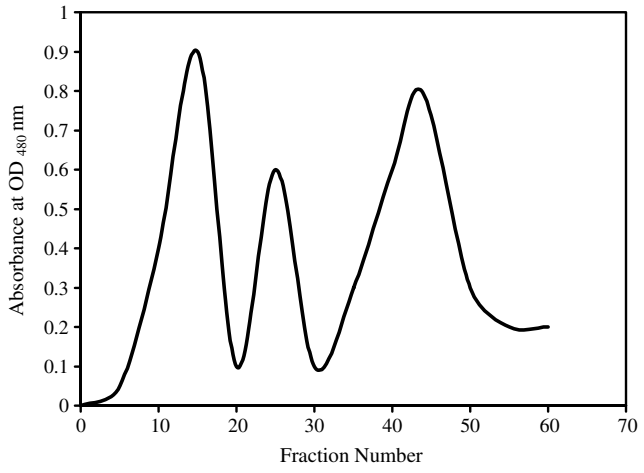

Fig. 1. Elution profile of exopolysaccharide produced by *Leuconostoc* sp. CFR 2181 in Sepharose CL-2B column.

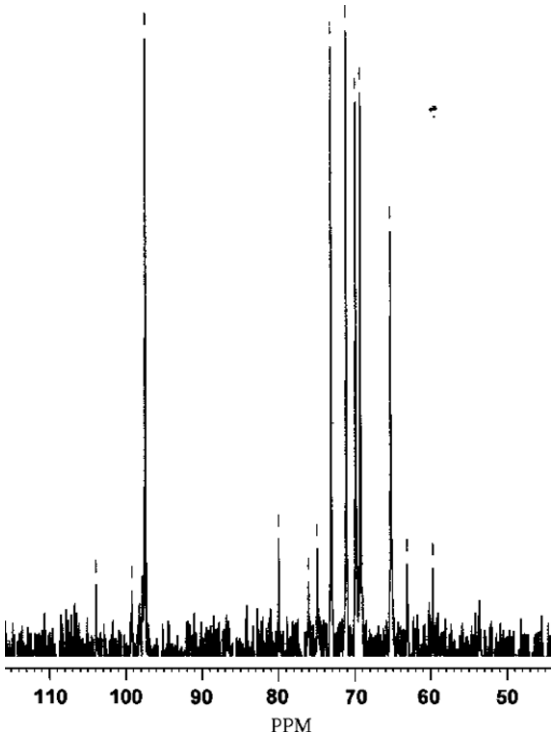

Fig. 2. ^{13}C NMR spectrum of dialyzed exopolysaccharide of *Leuconostoc* sp. CFR 2181.

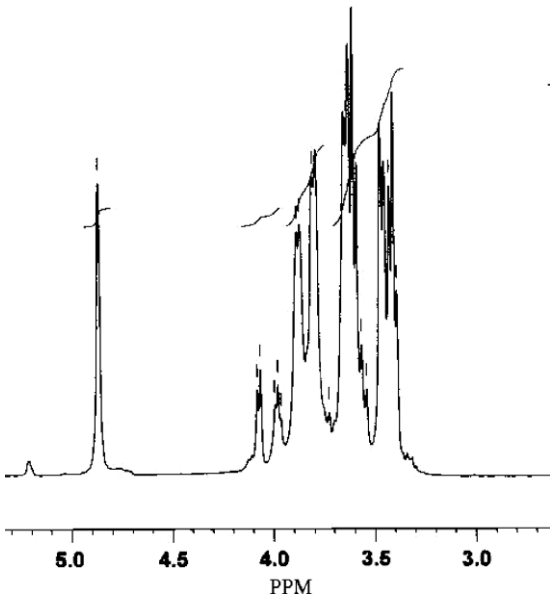

Fig. 3. ^{1}H NMR spectrum of dialyzed exopolysaccharide of *Leuconostoc* sp. CFR 2181.

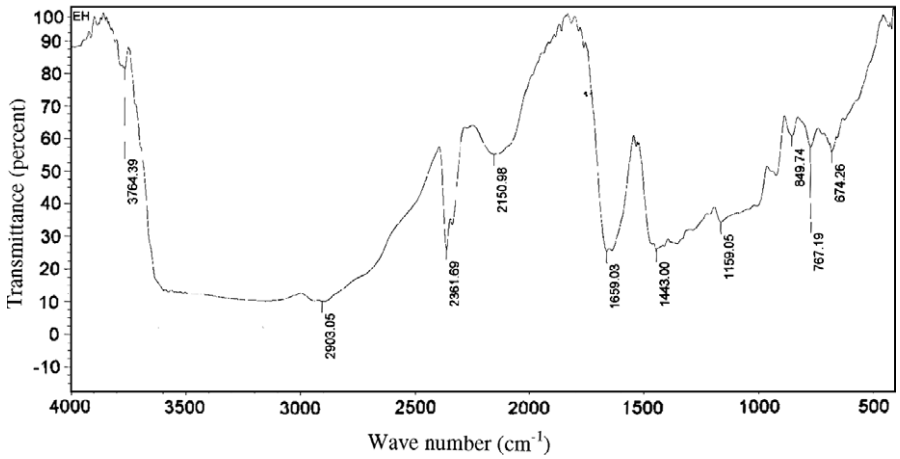

Fig. 4. FTIR spectrum of EPS produced by *Leuconostoc* sp. CFR 2181.

respectively. Similarly, the presence of one low molecular weight fraction and one high molecular weight fraction in the EPS produced by four different strains of *Lb. delbrueckii* subsp. *bulgaricus* was reported by Petry et al. (2003). As reported earlier, there is a substantial evidence for the synchronous production of EPSs having the same structure but different molecular masses. The study conducted by Degeest and de Vuyst (1999) indicated the production of a high molecular mass (1.8×10^6 Da) and low molecular mass (4.1×10^5 Da) EPS by *S. thermophilus* LY 03 and the production of two polysaccharides by *Lactobacillus rhamnosus* has recently been reported (Pham, Dupont, Roy, Lapointe, & Cerning, 2000). However, the low molecular mass material was believed to be generated by the glycosylhydrolase catalyzed hydrolysis of high molecular mass products. Recently, high performance size exclusion chromatography (HPSEC) has been used for the separation of EPSs (Faber, van den Haak, Kamerling, & Vliegenthart, 2001; Faber, Zon, Kamerling, & Vliegenthart, 1998).

^{13}C NMR spectrum (Fig. 2) of EPS produced by *Leuconostoc* sp. CFR 2181 showed 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 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 3.3–4.1 ppm range. In the free glucose, the chemical shift of C6 is generally seen in the range of δ 60–61 (JaganMohan Rao, Krishna Kumari, & Prakasa Rao, 1982). However, in the present spectrum no signal at δ 60 is seen, indicating the possible linkage at C6 position. The FTIR spectrum of the EPS (Fig. 4) indicated absorption around wave numbers 3400, 2900, 1650 and 1440 cm^{-1}, which can be attributed to stretching of –OH, C–H, carboxylate groups and symmetric bending of CH_3, respectively. The FT-IR spectrum was not comparable with that of the standard dextran (data not shown) indicating that the polymer produced might be a new EPS, whose functional properties are yet to be established.

In conclusion, studies on microbial exopolysaccharides are gaining increased attention and production of EPS from lactic acid bacteria, which are considered as safe by its GRAS status, is more encouraging. The newly isolated EPS producing lactic culture, *Leuconostoc* sp. CFR 2181, was found to produce good yields of EPS in a low-cost synthetic medium. Further work is needed to find the technological applications for this EPS.

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