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Characterization of an exopolysaccharide with potential health-benefit properties from a probiotic *Lactobacillus plantarum* RJF_4

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ABSTRACT

Exopolysaccharides (EPS) are macromolecules composed of sugar and sugar derivatives and the function and applications mainly depend on their monosaccharide composition, molecular weight and branching. EPS from the food-grade Lactic acid bacteria (LAB) serves as natural alternatives to commercial additives. One heteropoysaccharide produced by a recently isolated (from rotten jack fruit) *Lactobacillus plantarum* RJF_4 was extracted and purified. HPLC analysis and FTIR spectroscopy revealed that it is composed of glucose and mannose. The EPS was tolerant to temperature up to 225 °C. *In vitro* assays showed that the EPS possesses total antioxidant capacity (32%), DPPH radical scavenging ability (23.63%) and reduction potential (50% compared to the control ascorbic acid). It also displayed cholesterol lowering property (42.24%) and α-amylase inhibition (40%), which is an *in vitro* assay for evaluating anti diabetic property. The cytotoxicity studies (MTT assay and Alamar Blue assay) revealed cancer cell specific anti proliferative effects of EPS. It showed to be toxic to MiaPaCa2-pancreatic cancer cell line in dose dependent manner and remained nontoxic to normal cell line (L6 and L929 fibroblast cells).

1. Introduction

Exopolysaccharides are composed of monosaccharide residues of sugar and sugar derivatives and are produced by plants, algae, fungi and bacteria. EPS produced by food grade lactic acid bacteria with GRAS (Generally Recognized As Safe) status can be used as an alternative for EPS of plant and animal origin. They are mainly used for improving texture, rheology and mouth feel of milk and milk derived products such as cheese, yogurt, curd etc. (Ismail & Nampoothiri, 2010).

They are either capsular or slime polysaccharides and depending upon their composition they are divided into two groups, homopolysaccharides and heteropolysaccharides. In case of bacteria, the outer layer of EPS protects them from dessication, phage attack and helps to form biofilm. Microbial EPS have a wide range of application in food and pharmaceutical industries. They are used as biosurfactants in petrochemical waste management with negatively charged functional groups which can efficiently remove heavy metals (Bhaskar & Bhosle, 2006; Zhang, Wang, & Pan, 2006). EPS are also used as thickeners, gelling agents and emulsifiers in food industry to improve the quality and shelf life of packaged food materials (Mishra & Jha, 2013).

The term “probiotic” was first proposed by Fuller (1989) and its definition was further refined as “live microorganisms which when administered in adequate amounts confer a health benefit on the host” (FAO/WHO, 2006). Probiotic foods belong to the functional food sector and probiotic LAB are a representative of live food ingredients that exert a beneficial effect on the host's health (Tsuda, Hara, & Miyamoto, 2008). EPS from LAB has health benefit properties like antioxidant, anti-ulceretic, anti-tumor and immunomodulating activities. Immunomodulating activity of EPS from LAB is mainly due to induction of IL-6, IL-1β, and TNF-α production and thus phagocytosis (Liu et al., 2011). High levels of free radicals like reactive oxygen species (ROS) in our body are detrimental because they cause damage to the biological macromolecules and result in tissue damage. Recently, much attention has been given to the

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health benefits and therapeutical applications of EPS. Oxidative stress plays major role in several diseased conditions like cancer, liver cirrhosis, fatty liver etc. It is due to increase in concentration of reactive oxygen species (ROS) and free radicals in the biological system. Consumption of antioxidants may be beneficial for human health because they can quench ROS and free radicals (Xing et al., 2015). The present study is about the purification, physico chemical characterization and analysis of the therapeutical properties of an EPS produced by a probiotic *Lactobacillus plantarum* RJF_4.

2. Materials and methods

2.1. Microorganism

A newly isolated culture, *Lb. plantarum* RJF_4 identified by 16s rRNA sequencing and reported earlier by our group for its probiotic characterizations (Varsha, Priya, Devendra, & Nampoothiri, 2014) was used for EPS production. The organism was subcultured in de Man–Rogosa–Sharpe (MRS) medium every two weeks and was stored in MRS plates at 4 °C for immediate use and it was preserved by preparing glycerol (20% v/v) stocks at −20 °C.

2.2. Inoculum, EPS production medium and culture conditions

Pre-inoculum (10^9 CFU/ml) was prepared by incubating cells in MRS broth for 18 h at 37 °C at static condition. EPS production medium contains (g/L) lactose (40); yeast extract (40); ammonium sulphate (5.5), sodium acetate (5), dipotassium hydrogen phosphate (2), magnesium sulphate (0.1), manganese sulphate (0.05), and tween 80, pH 7.3 (Ismail & Nampoothiri, 2010) was used. 1% (v/v) of the pre-inoculum was added to EPS production medium and incubated at 37 °C for 72 h under static condition.

2.3. Production and extraction of EPS

After the desired interval of time, the culture was centrifuged at 11,000 g for 10 min and the supernatant was collected. Double volume ethanol was added to the supernatant and kept for overnight incubation at 4 °C to precipitate EPS. Precipitated EPS was collected by centrifuging at 2,500 g for 20 min. The pellet was dissolved in distilled water and again precipitated by adding double volume chilled ethanol. It was further centrifuged at 2,500 g for 20 min and pellet was collected and the total sugar present in the pellet was determined by phenol sulphuric acid method (Dubois, Gilles, Hamilton, Reber, & Smith, 1956).

2.4. Purification of EPS

Ethanol precipitated EPS was dialyzed using 12 kDa cellulose membrane (Sigma) against deionized water for 24 h with two times water change and lyophilized (Scanvac coolsafe freez dryer, Denmark). Lyophilized crude EPS was purified by the protocol of Dabour and LaPointe (2005). 1% solution of EPS (w/v) was extracted two times with an equal volume of phenol: chloroform: isoamyl alcohol (25:24:1). It was then precipitated overnight at 4 °C with an equal volume of chilled acetone. Precipitated EPS was collected by centrifuging at 2,500 g for 20 min. Pelleted EPS was dissolved in distilled water and dialyzed using 12 kDa membrane against deionized water for 24 h with two times water change and it was lyophilized and was used for further analysis.

2.5. Physico chemical characterization

2.5.1. FTIR analysis

The Functional groups of EPS were recorded by Fourier Transform Infra Red spectroscopy using Shimadzu FTIR spectrophotometer (IR Prestige-21, USA). The sample pellets were prepared by mixing the fine freeze dried EPS (1 mg) with 100 mg KBr and it was scanned from 500 to 4000 cm^{-1}.

2.5.2. Thermo gravimetric analysis

Thermal behavior of the EPS was studied by thermo gravimetric analysis using TG-DTA 6200 (SII Nano-technology Inc., Japan). The substance was subjected to a temperature range of 30–500 °C under nitrogen atmosphere at a rate of 10 °C/min and the corresponding weight loss was determined.

2.5.3. Monosaccharide composition analysis

20 mg of purified EPS was hydrolyzed by 1M sulphuric acid at 100 °C for 2 h. The hydrolysate was then neutralized with 1N NaOH. The monosaccharide composition was analyzed by HPLC (Shimadzu, Japan) using Aminex HPX-87P carbohydrate analysis column (Biorad, USA) with RI detector. Deionized water was used as the mobile phase at a flow rate of 0.6 mL/min.

2.5.4. One dimensional ^1H and ^{13}C nuclear magnetic resonance (NMR) spectroscopy

One-dimensional (1D)-NMR spectra of the polysaccharide solution was recorded at room temperature using a Bruker Avance II-500 spectrometer, (Germany). Both ^1H and ^{13}C NMR were performed. 25 mg of purified EPS sample was dissolved in 1 mL of 99.96% D_2O and analyzed. For ^1H NMR spectra the sample was submitted to a delay (D1) and acquisition time (AQ) of 1.00 s and 3.17 s, respectively, whereas for ^{13}C NMR, the D1 and AQ were 2.0 and 1.1 s respectively. Chemicals shifts were expressed in parts per million (ppm).

2.6. Biochemical characterization

2.6.1. In vitro determination of antioxidant property

2.6.1.1. DPPH radical scavenging ability. The ability of the *Lb. plantarum* EPS to scavenge the DPPH free radical was evaluated using he protocol mentioned by Shimada, Fujikawa, Yahara, and Nakamura (1992). The EPS samples were taken in concentrations 2, 4, 6, 8 and 10 mg/mL. 0.5 mL of the EPS samples were mixed with 0.5 mL of 0.1 mmol/L DPPH solution (in 95% ethanol) and reacted in dark for 30 min. The absorbance was measured at 517 nm. Blank/control was prepared by replacing the EPS with methanol. Ascorbic acid was used as positive control. The ability of EPS to reduce DPPH was indicated by a color change of the purple color of DPPH to yellow color of DPPHH and is quantified by the using given equation.

$$\text{DPPH radical scavenging ability} = (1 - \text{OD of sample}/\text{OD of control}) \times 100.$$

2.6.1.2. Reducing power assay. The reducing power of the EPS was determined as described by Oyaizu (1986). The polysaccharide solution at concentration of 10 mg/mL was at first mixed with 1 mL of 0.2 mol/L phosphate buffer (pH 6.6) and 1 mL of (1%) potassium ferricyanide. The mixture was incubated at 50 °C for 20 min. 1 mL of 10% TCA was then added and the mixture was centrifuged at 3000 × g for 10 min. 2.5 mL of the upper layer was pipetted out. 2.5 mL of deionized water was added followed by 0.5 mL of 0.1% freshly prepared ferric chloride solution. The absorbance was measured at 700 nm using a spectrophotometer (UV-1601 UV Visible Spectrophotometer, Shimadzu, Japan). The ability of the EPS to reduce the Fe^{3+} to Fe^{2+} was indicated by the formation of Perl's

Prussian blue complex. A higher absorbance indicates a higher reducing power. Ascorbic acid was used as positive control.

2.6.1.3. Total antioxidant capacity. The method of Mitsuda, Yuasumoto, and Iwami (1996) was used to evaluate the total antioxidant capacity of EPS to reduce the Mo^{6+} to Mo^{5+} ammonium molybdate, forming phosphomolybdenum which showing maximum absorbance at 695 nm. 7.45 mL of sulfuric acid (0.6 M), 0.9942 g of sodium sulfate (28 mM) and 1.235 g of ammonium molybdate (4 mM) were mixed together in 250 mL distilled water and labeled as total antioxidant capacity reagent. 0.1 mL of the EPS extract (2 mg/mL) was dissolved in 1 mL of total antioxidant capacity and absorbance was read at 695 nm after 15 min. Ascorbic acid was used as standard.

2.6.2. Alpha amylase enzyme inhibition assay

The ability of the EPS samples to inhibit the alpha amylase enzyme was analyzed in order to check its anti-diabetic role. The assay was done by the procedure explained as follows. Different concentrations (100 μg/mL and 800 μg/mL) of *Lb. plantarum* RJF$_4$ EPS and standard acarbose were prepared in dimethyl sulfoxide from 1 mg/mL stock solution and 500 μL of test and standard was added to 500 μL of α-amylase (0.5 mg/mL) and was incubated for 10 min at room temperature. Then 500 μL of 1.0% starch solution was added and incubated for another 10 min. After that 1 mL of the DNS reagent was added to the reaction mixture and heated in a boiling water bath for 5 min. After cooling, it was diluted with10 mL of distilled water. The absorbance was then measured at 540 nm against reagent blank. The α-amylase inhibition was expressed as percentage of inhibition. It was calculated employing the following formula (Sivashanmugam & Chatterjee, 2013).

$$\%\ \text{inhibition} = \frac{\text{OD of control} - \text{OD of test}}{\text{OD of control}} \times 100$$

2.6.3. Cholesterol adsorption by EPS

The ability of EPS to lower cholesterol concentration was studied by the protocol reported by Soh, Kim, & Lee, 2003). According to the protocol, 1 mL of reaction mixture containing 0.1% polysaccharide and 30 μg cholesterol was mixed at 25 °C for 20 min. Then the mixture was added to 50 μL hexadecyl trimethyl ammonium bromide. It was centrifuged at 12,500 × g. Supernatant was analyzed for total cholesterol concentration by Zak's method (Zak & Epstein, 1961). Cholesterol lowering activity of the compound was calculated by the following equation.

$$\%\ \text{cholesterol lowering activity} = \{\text{Conc. of cholesterol in control} - \text{Conc. of cholesterol in test}/\text{Conc. of cholesterol in control}\} \times 100$$

2.7. Measurement of cytotoxicity

2.7.1. MTT assay

The effect of *Lb. plantarum* RJF$_4$ EPS on normal cells was evaluated using the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay. L6 normal fibroblast cells were trypsinized and seeded on 96 well plate. Different concentrations of EPS (1 μg/mL, 10 μg/mL, 100 μg/mL, 250 μg/mL, and 500 μg/mL) were treated and incubated at 37 °C for 24 h. Treated media were removed and 100 μL MTT reagent (50 μg/well) was added and incubated for 4 h in the dark. The reagent was removed and 200 μL DMSO was added to all wells covered with aluminum foil and agitated using a shaker for 45 min. Absorbance at 570 nm was measured using multimode reader. Cell viability was calculated as

$$\text{Cell viability}\ (\%) = (\text{OD570 of sample})/(\text{OD570 of control}) \times 100.$$

2.7.2. Alamar Blue assay

Effect of EPS on cell viability was also studied by Alamar Blue Assay. Five cell lines were used for the study namely, mouse fibroblast cell line (L929 cells) was used as the normal (control) cell line, Mouse mesenchymal stem cell line (KUSA) ά stem cell line, Glial cell line (G1 cells), Pancreatic cancer cell line (MiaPaCa2) and Colon carcinoma cell line (DLD2). The cell lines were obtained from the Riken Culture Collection Center, Japan. All the mentioned cell lines were routinely grown in Dulbecco's Modified Eagle's Medium (DMEM) with 5% heat-inactivated fetal bovine serum and penicillin/streptomycin (100units/mL) in T25 flasks. All of the cell lines were cultured at 37 °C in a humidified 5% CO_2 atmosphere, and the cells were sub-cultured every 2 days. The cells were maintained in 96 well plates for cytotoxicity studies. Four different concentrations (1, 10, 100and 1000 μg/mL) were tested in cell lines. The fluorescence or absorbance can be measured at 560/590 nm (Excitation/Emission) or 570 nm respectively (Power scan HT Microplate Reader, Dainippon Sumitomo Pharma, Japan). Untreated cells were taken as controls with 100% viability. The relative cell viability was calculated using the formula:

$$\text{Relative cell viability} = (\text{Absorbance of sample}/\text{Absorbance of control}) \times 100$$

3. Results and discussion

Majority of the LAB strains which are studied extensively and widely used in industries have been isolated from dairy products and from the human gastro intestinal tract (GIT) (Almeida Junior et al., 2015). The present culture *Lb. plantarum* RJF$_4$ was isolated from rotten Jack fruit sample collected from Silent Valley National Park, Kerala, India, which is one of the world heritage sites recognized by UNESCO.

3.1. Production and purification of EPS

Dry weight of the crude EPS obtained after ethanol precipitation was 3.5 g/L. After phenol: chloroform: isoamyl alcohol (25:24:1) extraction and acetone precipitation it was 1.5 g and appeared as light brown powder. The yield was much higher than that reported by Tsuda and Miyamoto (2010) for *Lb. plantarum* 301102S.

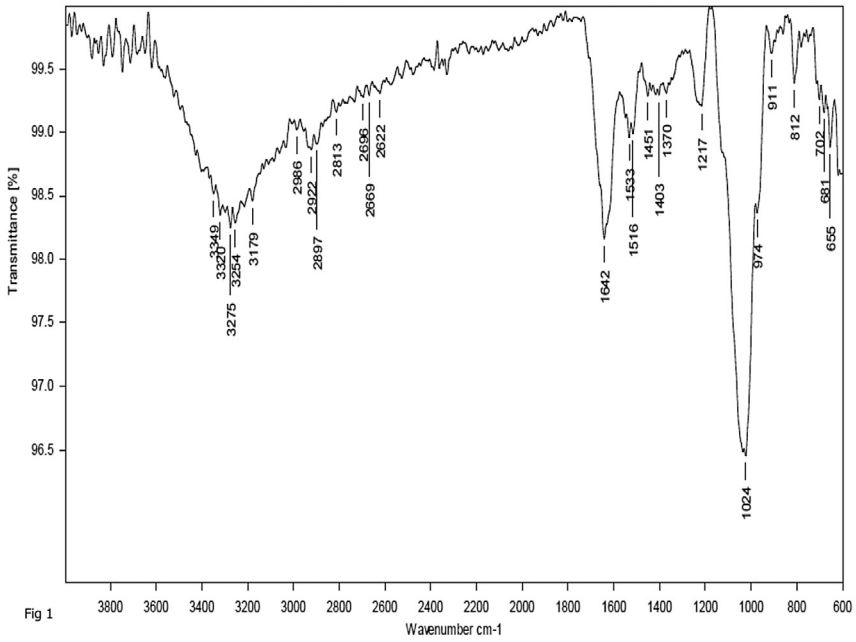

Fig. 1. FT IR spectra of *Lb. plantarum* RJF_4 EPS.

3.2. Physico chemical characterization

3.2.1. FTIR analysis

Fourier transform-infrared spectroscopy has been a useful tool in monitoring structural changes in biopolymers (Belton, Goodfellow, & Wilson, 1991). FT IR reveals the fact that molecules possess specific frequencies at which they can rotate or vibrate corresponding to vibrational modes (Mishra & Jha, 2009). FT IR spectra of EPS from *Lb. plantarum* RJF_4 showed the characteristic peaks for EPS (Fig. 1). A broad stretching around the region 3320 cm^{-1} corresponds to the stretching vibration of the hydroxyl groups of carbohydrates. The absorption peak at 2922 cm^{-1} is attributed to the asymmetrical C–H stretching vibration of methyl group. The absorption peak at around 1642 cm^{-1} indicates the characteristic IR absorption of polysaccharides (Bremer & Geesey, 1991) and it corresponds to the stretching vibration of C=O (Singh et al., 2011). The carbohydrate shows high absorbance between the wave numbers 1200–950 cm^{-1} which is considered as the finger print region of exopolysaccharides (Guillermo et al., 2002). The intense peak at 1024 cm^{-1} indicated the existence of α-(1 → 6) glycosidic bond (Das & Goyal, 2014). The presence of characteristic absorption at 812 cm^{-1} in the anomeric region (950–700 cm^{-1}) corresponds to the existence of mannose (Mathlouthi & Koenig, 1986). There were three peaks near 1000–1200 cm^{-1}, indicating that the polysaccharide contained α-pyranose (Chi, Su, & Lu, 2007).

3.2.2. Thermo gravimetric analysis

TGA involves the measurement of change in sample mass with change in temperature. It measures the variation of heat flux in a sample with variation in temperature. There was an initial loss of weight in the thermo gram of EPS from *Lb. plantarum* RJF_4 and may be due to the loss of surface bound water from EPS. Thereafter, the weight was remained stable up to 225 °C and thus the EPS can withstand higher temperatures up to 225 °C (Fig. 2) and this property can be exploited for industrial applications. Another strain of *Lb. plantarum* has been reported to produce an EPS at a degradation temperature of 279.59 °C which is higher than that of locust gum and lower than that of xanthan gum (Wang et al., 2010). EPS from *Lb. plantarum* YW32 has the ability to tolerate temperature up to 283.5 °C (Wang, Zhao, Yang, Zhao, & Yang, 2015). The property to tolerate higher temperatures makes EPS strong enough to withstand the processing procedures in food industry.

3.2.3. Monosaccharide composition analysis

Monosaccharide composition analysis was done by HPLC. The results revealed that EPS from *Lb. plantarum* RJF_4 is an heteropolysaccharide, since it is composed of glucose and mannose sugar residues (Supplementary Fig. 1, 2, & 3). Similar heteropolysaccharides have been reported by Ismail and Nampoothiri (2010). However, it can vary for a different strain of the same species as in the case of *Lb. plantarum* 70810 where it was a homopolysaccharide of galactose as capsular exopolysaccharide (Wang et al., 2014). Tallon, Bressollier, and Urdaci (2003) reported that glucose and galactose are the major components of c-EPS produced by *Lb. plantarum* EP56. It can be much more complex, as *Lb. plantarum* KF5 produces an exopolysaccharide composed of mannose, glucose and galactose moieties in an approximate ratio of 1: 4.99: 6.90 (Wang et al., 2010).

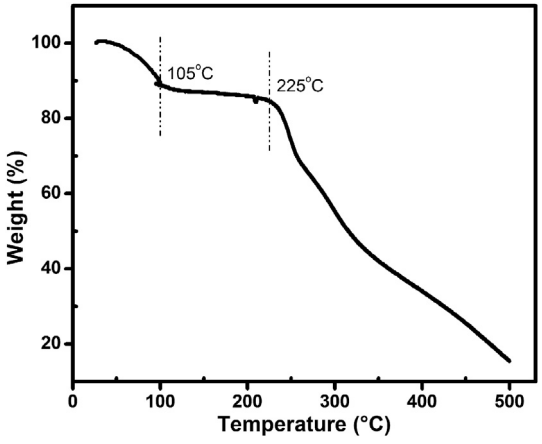

Fig. 2. TGA spectrum of EPS produced by *Lb. plantarum*.

3.2.4. One dimensional ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectroscopy

The ^{1}H NMR spectrum of a polysaccharide can be generally divided into three major regions: the anomeric region (δ H 4.5–5.5), the ring proton region (δ H 3.1–4.5) which is assigned to protons attached to C2–C6. The third group is alkyl region (δ H 1.2–2.3) (Wang et al., 2014). In the spectrum, signals between 5.3 and 4.5 ppm correspond to the anomeric protons of the EPS, and these signals often serve as signatures for differentiating complex carbohydrate structures (Ismail & Nampoothiri, 2010). The chemical shift at 5.308 corresponds to the anomeric proton of α-(1 → 3) of glucosidic linkage (Das & Goyal, 2014). The signals obtained in the spectrum between 4.5 and 3.1 ppm were due to protons attached to C2–C6. They cannot be resolved properly due to the overlapping chemical shifts (Fig. 3). Chemical shifts at δ5.13corresponds to a α-hexapyranosyl residue and those at δ 4.91, δ 4.54, δ 4.53, δ 4.23 were corresponds to pyranose ring forms in β-anomeric configuration (Maeda, Zhu, Suzuki, Suzuki, & Kitamura, 2004). All peaks were not fully resolved in the NMR spectrum obtained by ^{13}C NMR spectroscopy (Supplementary Fig. 4). The presence of signals with a chemical shift above 102 ppm indicates β-configuration of glucose (Usui et al., 1973). The strong signal in the spectrum at 69.46 and 73.22 ppm were corresponds to C-4 and C-5 of α-D-mannose. The chemical shift at 75.60 indicates the presence of β-D-glucose (Ismail & Nampoothiri, 2010).

3.3. Biochemical characterization

3.3.1. In vitro determination of antioxidant property

DPPH radical scavenging ability, reducing power efficiency and the total antioxidant capacity were measured as per the standard protocols.

The DPPH radical is a widely accepted tool for estimating the free radical scavenging activities of antioxidants as it is a stable free radical. When DPPH radicals are scavenged, the color of the reaction mixture changes from purple to yellow and absorbance at 517 nm decreases. At 2 mg mL^{-1} concentration *Lb. plantarum* RJF_4 EPS exhibited 23.63% DPPH radical-scavenging activity (Fig. 4a). This activity might be due to the presence of hydroxyl group and other functional groups in the *Lb. plantarum* EPS, which can donate electrons to reduce the radicals to a more stable form, or react with the free radicals to terminate the radical chain reaction (Shen, Shi, & Xu, 2013).

In the reducing power assay, the yellow color of the test solution changes to various shades of green and blue, depending on the reducing power of each compound. The presence of reducers causes the reduction of the Fe^{3+}/ferricyanide complex to the ferrous form. Therefore, measuring the formation of Perl's Prussian blue at 700 nm can monitor the Fe^{2+}concentration. The reducing power of *Lb. plantarum* RJF_4 EPS was a mean value of 1.38 at 2 mg/mL concentration. It was comparable with the potential antioxidant ascorbic acid which used as the positive control showed an absorbance of 2.906 (Fig. 4b). This EPS has good reductive potential (50% of control) thereby exhibiting its potential antioxidant activity.

Total antioxidant capacity was determined by the ability of EPS to reduce Mo^{6+} to Mo^{5+} forming phosphomolybdenum which shows maximum absorbance at 695 nm as explained by Mitsuda et al. (1996). Ascorbic acid was used as the positive control. Purified *Lb. plantarum* RJF_4 EPS showed 31.32% total antioxidant capacity compared to the potent antioxidant ascorbic acid (Fig. 4c).

3.3.2. Alpha amylase inhibition assay

Lb. plantarum RJF_4 EPS exhibited a dose dependent inhibition of α-amylase enzyme activity. The α-amylase inhibitory effect of EPS

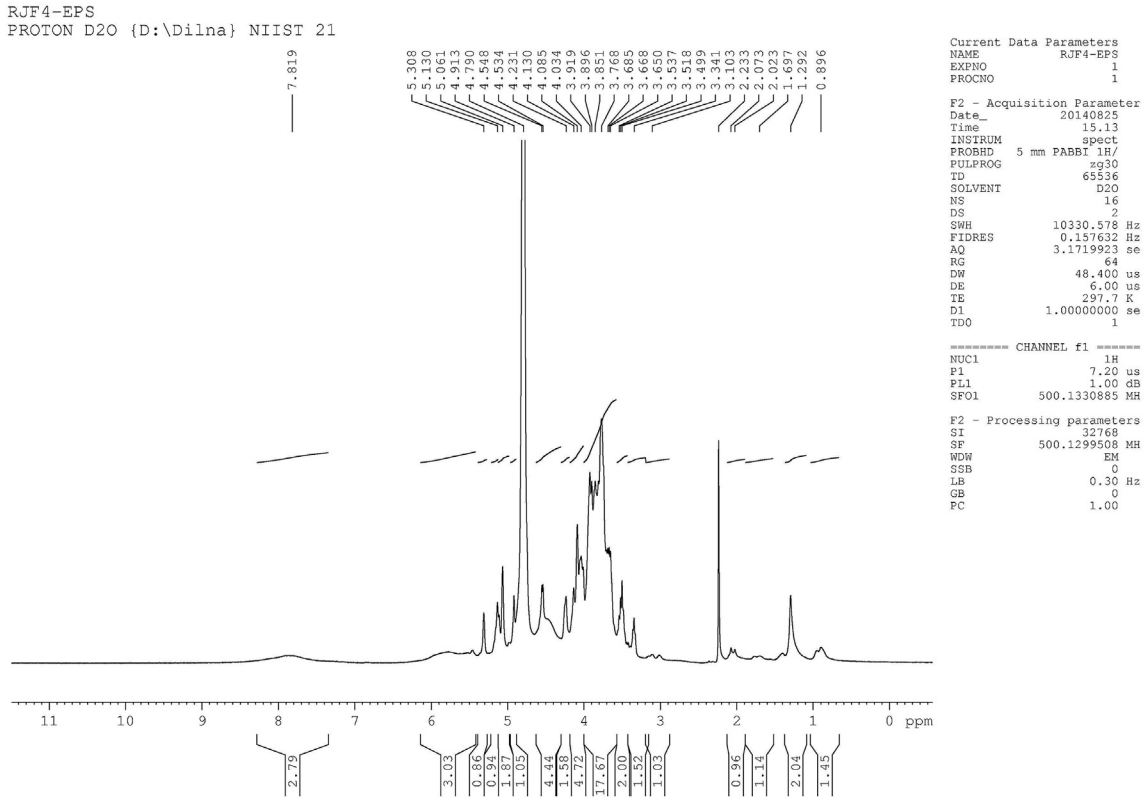

Fig. 3. Proton NMR spectra of EPS produced by *Lb. plantarum*.

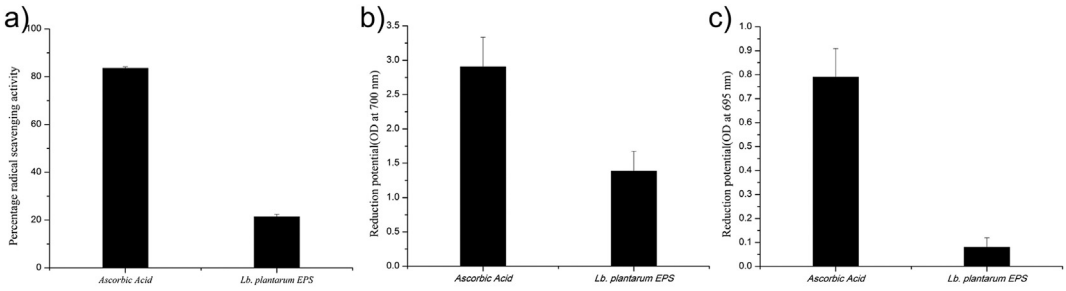

Fig. 4. a. DPPH radical scavenging activity of EPS. b. Reducing power efficiency of the EPS. c. Total antioxidant activity of the EPS.

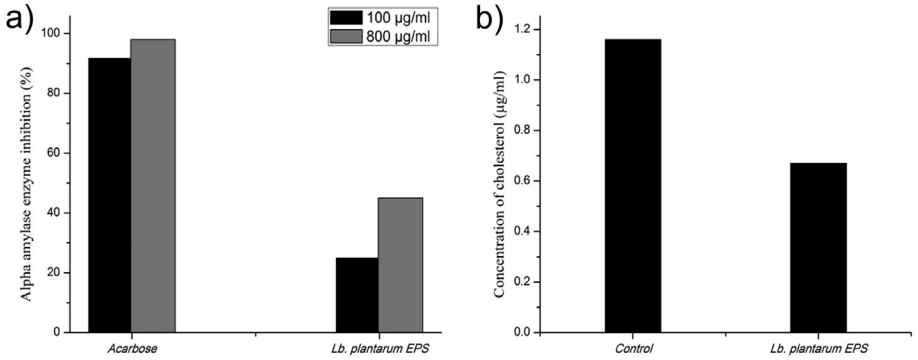

Fig. 5. a. α-amylase inhibition by the EPS. b. Cholesterol lowering activity of the EPS.

was found to be 25% and 40% respectively when studied at concentrations of 100 and 800 µg/mL (Fig. 5a). At the same concentrations the inhibitory effect of standard drug acarbose was 91.75% and 98%. Alpha amylase converts starch to maltose which reduces 3, 5-dinitrosalicylic acid to 3-amino-5-nitrosalicylic acid (Thalapaneni, Chidambaram, Ellappan, Sabapathi, & Mandal, 2008). This reaction produces a color change from orange to red. Acarbose is a drug currently used in anti diabetic therapy for reducing the increase in blood glucose levels after a meal, which is a known α-amylase and α-glucosidase inhibitor. So it is used as a standard to check the anti diabetic property of purified EPS from *Lb. plantarum* RJF_4.

3.3.3. Cholesterol adsorption by EPS

From the assay it was found that *Lb. plantarum* RJF_4 EPS is capable of reducing the cholesterol concentration by adsorption. The concentration of cholesterol in the supernatant was decreased by the addition of EPS. When expressed in percentage the cholesterol lowering ability is 42.24% (Fig. 5b). There was earlier reports that acidic polysaccharides are more efficient to reduce cholesterol than neutral polysaccharides and it was also reported that dextran like EPS failed to remove cholesterol (Soh et al., 2003). The exact mechanism of cholesterol lowering is not known. From studies it was found that certain EPS producing probiotics like *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus* strains could bind free bile acids thus increasing their excretion after digestion. This could result in the synthesis of new bile acids from cholesterol by the liver and thereby decreasing the level of circulating cholesterol (Akalin, Gonc, & Duzel, 1997). So EPS producing *Lb. plantraum* RJF_4 incorporated functional food preparations may help to reduce the increased blood cholesterol level.

3.4. Cytotoxicity analysis

Cytotoxicity was checked by standard MTT assay and by Alamar Blue assay. The viability of normal L6 cells treated with the *Lb. plantarum* EPS showed about 70% up to a concentration of 100 µg/ml and at the concentration of 250 µg and 500 µg it decreased to below 70% when incubated for a period of 24 h (Fig. 6a). Since the polysaccharide does not produce any inhibitory effect on normal cells, it can be regarded as safe while considering the food safety

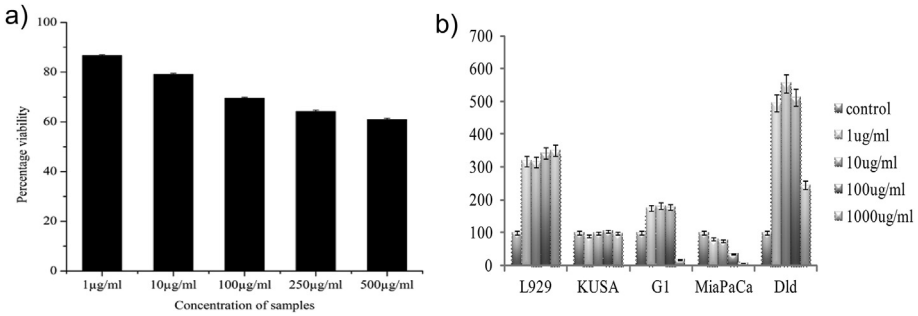

Fig. 6. a. Cytotoxicity analysis of the EPS – MTT assay against L6 cell line. b. Alamar Blue assay.

measures to be used as a food additive and also in other therapeutical applications. Fermentation using probiotic lactic acid bacteria is gaining importance as the products obtained are characterized by hygienic safety, storage stability, and attractive sensory properties. The presence of EPS prolongs the survival of probiotic microorganisms in gastrointestinal tract of the host.

In Alamar Blue assay, only the metabolically active cells convert the non-fluorescent resazurin to fluorescent resorufin. The amount of fluorescence produced is proportional to the percentage of viable cells. From the assay it was found that *Lb. plantarum* EPS exerts antiproliferative effect on MiaPaCa2-pancreatic cancer cell line in dose dependent manner and the highest concentration (1 mg/ml) is toxic to G1 cell line (neural glial cell line), whereas not toxic to colon carcinoma cell line, DLD-2 (Fig. 6b). Cytotoxicity studies showed that *Lb. plantarum* RJF_4 EPS is non toxic to normal cell lines and specifically inhibits some of the cancer cell lines. In contrast to the results obtained an earlier study reports that EPSs from *Lb. plantarum* NRRL B-4496 effectively inhibited the growth of intestinal carcinoma cell line (CACO) *in vitro* in dose dependent manner and were active *in vivo* against Ehrlich Ascites Carcinoma (Haroun, Refaat, El-Menoufy, Amin, & El-Waseif, 2013). The apoptotic and anticancer property of EPS may be due to the its effects on the c-Myc, c-Fos, and vascular endothelial growth factor (VEGF) expression or the B cell dependent mitogenic activity (Madhuri & Prabhakar, 2014; Yang et al., 2005).

4. Conclusions

LAB strain culture would be a useful alternative to produce EPS for food applications if the LAB could be grown in edible and safe culture media and if fermentation conditions were optimized to obtain a high yield. The exopolysaccharides (EPS) produced by food grade lactic acid bacteria (LAB) have gained much importance as biothickeners and texturizers. *Lb. plantarum* RJF_4 produces an heteropolysaccharide composed of glucose and mannose. The EPS showed comparable antioxidant properties to the potent antioxidant ascorbic acid and also has the cholesterol lowering nature and the ability to inhibit α-amylase enzyme. Cell viability tests showed that this EPS specifically inhibits cancer cell lines and non toxic to normal cell lines and thus it has a potential for health benefit applications.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.lwt.2015.07.040.

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