4.1 Introduction

Lactic acid bacteria (LAB) are known to produce several industrially important biomolecules including exopolysaccharides (EPS) from fermented foods (Patel and Prajapathi, 2013). EPS production is well documented among various species of LAB belonging to *Leuconostoc* spp., *Lactobacillus* spp., *Lactococcus* spp. and *Streptococcus* spp. (Navarini *et al.*, 2001). Homopolysaccharides secreted by *Lactobacillus* spp. contain glucose or fructose as sole monosaccharides and are classified as glucans (dextrans) and fructans, respectively (Kralj *et al.*, 2005). EPS are profusely used as bio-absorbants, bio-flocculants, encapsulating constituents, heavy metal confiscating agents, drug distribution agents and ion-exchange resins (Ismail *et al.*, 2010). Bacteria produce biofilms to defend the microbial community against the environmental stress (Ciszek-lenda *et al.*, 2011). Bacterial exopolysaccharides are the most important constituent of extracellular polymer forming biofilm and mediate most of the cell-to-cell and cell-to-surface connections and stabilization in the intestine (Flemming *et al.*, 2010). The EPS produced by LAB act as an immuno-stimulator, anti-tumour agent and blood cholesterol-lowering agent. EPS are used as drug conjugates, coatings, and matrix agents to develop the specificity of drug release in colon cancer treatment (Vandamme *et al.*, 2002). Current research is mainly focussed on developing polymeric matrices with tuned characteristics such as transparency, barrier and mechanical properties, biocompatibility and bioactivity. The *in vitro* manipulations of polymer have been carried out to form structured
materials (e.g. nanoparticles, scaffolds or hydrogels) that can be used for numerous industrial
and medical applications and also as edible coatings for numerous food products (Lavanya et
al., 2011, Ruas-Madiedo et al., 2002b, Bounaix et al., 2009). Some of the EPS (exopolysaccharide) producing LAB species isolated from idli batter are Leuconostoc
mesenteroides, Weissella confusa, Weissella cibaria, Pediococcus parvulus (Patel et al.,
2014; Sawale and Lele, 2010). Furthermore EPS was used as a bio-floccuants, stabilizing,
emulsifying, bio-absorbants, and act as drug delivery, heavy metal removing agents (Liu et
al., 2010). Dextrans are produced through various LAB especially Leuconostoc and
Streptococcus species (Leathers, 2002). Leuconostoc spp produced α-1-6 and α-1-4 linkages
polysaccharide (Dextran) can be used as an anti-tumor agent (Sawale et al., 2010). Glucan
was produced by Leconostoc dextraniucm exhibited a highly linear structure with about 96%
of α 1-6 and α-1 -4 linkages (Majumder et al., 2009). α-glucans produced from Streptococcus
mutans and Streptococcus sobrinus contains 90% of α-1-3 linkages. Leuconostoc
mesenteroides produced high molecular weight α-glucans predominantly α-1-6 linkages and
also contains α-1-4 and α-1-3 linkages (Cerning, 1990). Glucan produced by Leconostoc
garium PR contains 95% of α-1-6 glucopyranose linkage carrying low content branches of α-
1-2, α-1-3 and α-1-4 linkage (Capek et al., 2011).The polysaccharide from Pantoea
agglomerans KFS-9 showed highly scavenging activity, superoxide radicals and hydroxyl
radical’s activity were isolated from mangrove forest (Wang et al., 2008).Exopolysaccharides
having the antioxidant capacity can securely interact with free radicals and terminate the
chain reaction previous adverse deterioration arises (Ye et al., 2012). Keissleriella sp. YS
4108 and Penicillium sp. F23-2, produced polysaccharides revealed good free-scavenging
activities, particularly scavenging capabilities on superoxide radicals and hydroxyl radicals
(Sun et al., 2004:2009). EPS was intensively used as food additives to improve the texture
which impact on the development of innovative food products with enhanced appearance,
mouth feel, firmness, and rheological properties (De Vuyst et al., 2001). EPS-producing cultures in dairy foods are known to promote alleviating and water-binding functions (Lavanya et al., 2011). EPS-producing probiotic microflora has been suggested as effective functional starter cultures for preparing fermented foods (Ruas-Madiedo et al., 2002). The exopolysaccharide (Dextran) produced by LAB which enhance the texture and sensory properties in many Indian fermented foods (Patel and Prajapati, 2013). This chapter is planned to screen and identify the EPS-producing microorganism and characterize the EPS produced by a selected strain.
4.2 Materials and Methods

4.2.1 Preliminary screening for EPS-producing isolates

The cell suspension was prepared in MRS broth by growing the strains for overnight at 30 °C. Then the cells were grown on MRS agar medium supplemented with 40 g/L sucrose. The 10 μL of suspension was inoculated on MRS sucrose agar medium. After two days of incubation at 30 °C, the strains producing slimy colonies were considered as EPS producers by their visual appearance (compact, creamy or liquid, slime) (Bounaix et al., 2009).

4.2.2 Extraction and purification of EPS

Fifty millilitres of cell suspension was grown in MRS medium supplemented with sucrose under shaking condition of 100 rpm at 30 °C for 24 h. The cell suspension was heated to 100 °C for 10 min for inactivating the enzymes, and then, the suspension was cooled to room temperature and centrifuged at 4100 g for 20 min to remove the biomass. The crude solution was further treated with Sevage reagent (chloroform: n-butanol at 5:1 v/v) three times to remove the proteinaceous materials. EPS was precipitated with cold ethanol (three times of the volume) and left overnight. The precipitate was collected through centrifugation at 19200 g for 15 min and dissolved in Milli Q water. Afterwards, it was encased in a dialysis bag (12-14 KDa) and dialysed at 4 °C with Milli Q water for 48 h for partial purification (Liu et al., 2010). The fraction of EPS was carried out for purification in a DEAE-52 anion-exchange chromatography column (1.5 cm - 50 cm) with consecutive elution using distilled water and NaCl_2 solution in a linear gradient of 0.2-1M NaCl_2 at the flow rate of 1 mL/5min. The main fraction was further purified over a Sephadex G-100 column (2.6 cm - 50 cm) and the column was eluted through 0.01M NaCl_2 solution at a flow rate of 1mL/min. The carbohydrate
content was then measured using the phenol-sulfuric acid method (Dubois et al., 1956). The main fraction was dialyzed, concentrated, and used for further analysis (Ye et al., 2012).

4.2.3 Sugar analysis through HPTLC

EPS were hydrolysed with 2M trifluoroacetic acid (TFA) at 121 °C for 4 h. The hydrolysate (2 µL) was uniformly applied using autosampler on the HPTLC plate (MerkSilica GelG, size 20 mm-10 mm) through HPTLC (CAMAG Switzerland) along with the standards (glucose, xylose, mannose, galactose, arabinose and fructose). Sugar analysis was carried out as per the method of Pan and Mei, (2010) with a slight modification. A mixture of 1-propanol–ethyl acetate (3:1 v/v) was used as the evolving solvent. A solution containing 2% aniline, 2% diphenylamine, and 85% phosphoric acid in the ratio of 5:5:1 was used as the developer system. When the solution had migrated to 70 cm, the TLC plate was taken out of the chamber, air-dried, then soaked for 1 minute in the developer and air-dried again. The HPTLC plate was heated at 80 °C for 20 min. The bands were visualized under white light, and image was scanned and analysed using CAMAG densitometer.

4.2.4 Fourier transforms infrared spectroscopy analysis

The purified EPS powder was prepared with dry potassium bromide (KBr) followed by pressing the combination and compressed them into a pellet form. The spectrum was recorded between the wavelength ranges of 400 and 4000 cm⁻¹ in Fourier transform infrared (FT-IR Model: 6700) (Kanmani et al., 2011).
4.2.5 Nuclear magnetic resonance spectroscopy analysis

The purified exopolysaccharide was dissolved in 99.96% D_2O. ^1H, ^13C and DEPT-135 NMR spectra were recorded with a Bruker DRX Avance 400 MHz spectrometer. Structural analysis of exopolysaccharides are usual homo- and heteronuclear correlated two-dimensional (2D) methods were used for overall assignments of EPS: heteronuclear multiple quantum coherence (HMQC), correlation spectroscopy (COSY), and heteronuclear multiple bond coherence (HMBC) (Liu et al., 2007).

4.2.6 MALDI-TOF/TOF mass spectrometry

Matrix-assisted laser desorption ionization (Ultraflex TOF/TOF) mass spectrometry was performed in the positive and negative modes, using a matrix of alpha-cyano-4-hydroxycinnamic acid matrix (10 mg/mL in 50% acetonitrile, 0.1% TFA). 0.5 µl sample were taken up with a nitrogen laser (κ 330 nm) via a detector. Mass spectra were recorded over a range of 0–16000 m/ and minimum 5000 laser shots were taken per spectrum with the laser repetition rate 2000Hz (Ismail and Nampoothiri, 2010).

4.2.7 Thermogravmetric analysis and X-ray diffraction analysis

TG-DTA analysis of EPS was conceded with the thermal system (TG-DTA/DSC Model: Q600 SDT). About 10 mg of dried sample was used for the TG-DTA experiment. TG-DTA thermograms were attained in the range of 0–400 °C under the flow of nitrogen air at the rate of 10 °C min⁻¹. Their individual graphs were plotted with weight (percentage) loss and heat flow against temperature (Wang et al., 2010). To study the physical characterization of EPS, X-ray diffraction scan at diverse ranges of two-theta angles (10-70 °C) was performed. XRD was accomplished on X-ray powder diffractometer (Philips X’pert pro, the Netherlands) with
a Cu tube X-ray produced at 40 kV and 30 mA with PW3011/20 proportional detector. Crystallinity index \( (C_{I_{\text{XRD}}}) \) was measured from the area under crystalline peaks standardized with equivalent to total scattering area (Ricou et al., 2005).

\[
C_{I_{\text{XRD}}} = \frac{\sum A_{\text{crystal}}}{\sum A_{\text{crystal}} + \sum A_{\text{amorphous}}}
\]

### 4.2.8 Atomic force microscopy and scanning electron microscopy analysis

The EPS (2–3 mg) was dissolved in Milli Q water and stirred constantly to get a uniform dispersion in a water bath at 40 °C and cooled to room temperature. Then the solution was diluted to 0.1 mg/mL. About 5–10 µl was dispersed on a mica disc (Pelco mica disc 10 mm), and absolute ethanol was dropped over the sample to fix it on the mica disc. Then the mica sheet was air-dried to remove the residual ethanol. Subsequently, the AFM images were captured by scanning probe microscope (Brukers MM8) in tapping mode. The cantilever oscillated at its appropriate frequency (158 kHz), and the ambitious amplitude was 0.430 V. Then the EPS was fixed above the aluminium stub and then examined through SEM (Hitachi, Model: S-3400N) (Ahmed et al., 2013).

### 4.2.9 Water solubility index

The water solubility index for EPS was determined as per the method of Anderson et al., (1969). Two hundred milligrams of sample was dissolved in 5mL of Milli Q water and stirred for 30 min in a water bath at 40 °C to get a uniform suspension. Then the suspension was centrifuged at 5000 x g for 10 minutes. The supernatant was positioned in a petri dish and dehydrated at 105 °C for 4 h to attain the dry solid weight. Afterwards, centrifuged wet residue was also weighed (Reddy et al., 2013).

\[
\text{WSI} = \frac{\text{Weight of dry solids in supernatant}}{\text{Weight of dry sample}} \times 100
\]
4.2.10 Water-holding capacity

The EPS sample was characterized for its water-holding capacity (WHC) by dissolving 0.2 g sample in 10 mL of Milli Q water and kept at 40 °C for 10 min for uniform dispersion. Dispersed sample was centrifuged at 16,400 x g for 30 min and the supernatant was discarded. The pellet was dropped on pre-weighed filter paper for comprehensive drainage of water. Then the filter paper was weighed and the value recorded (Ahmed et al., 2013). The percentage of water-holding capacity was expressed through this equation

\[
\text{WHC\%} = \frac{\text{Total sample weight after water absorption}}{\text{Total dry sample weight}} \times 100
\]

4.2.11 2, 2-diphenyl-1-picrylhydrazyl radicals scavenging activity

The assay was executed according to the method of Yang et al., (2006) with slight modification. The 100 µl DPPH solution (0.2 Mm dissolved in 95% ethanol v/v) and 100 µL of EPS with various concentrations (100-500 µg) sample solution. After incubation in a dark room at room temperature for 30 mins and the absorbance of the mixture solution was observed at 517 nm and compared with the value observed for control solution of DPPH in the absence of EPS samples. Ascorbic acid was used as a positive control. The percentage of RSA was evaluated by the following equation.

\[
\% \text{ of RSA} = (\frac{A_{\text{Control}} - A_{\text{Test}}}{A_{\text{Control}}}) \times 100
\]

Whereas A control is the absorbance with only DPPH solution, A test is the absorbance with DPPH solution and test samples. The IC 50 value (mg/mL) is the concentration at which DPPH scavenging activity was 50% compared with Ascorbic acid. Values are done in triplicates with standard deviation.
4.2.12 Hydroxyl radical (OH) scavenging activity

Hydroxyl radical scavenging activity of Exopolysaccharides was evaluated by Ye, et al (2012) method with slight modification. The reaction mixture consist of 2.0 mL PBS (pH 7.4, 0.15 mM), 0.2 mL safranine T (0.52 mg/mL), 1.0 mL EDTA-Fe(II) (6 mM) 0.8 mL H2O2 (6%, v/v) and 1.0 mL EPS sample isolation with various concentration. After incubation at 40°C for 30min, the absorbance was evaluated at 520 nm. Ascorbic acid (Vc) was used as a positive control. The scavenging activity was calculated by the following equation:

\[
\text{% of Scavenging activity} = \left( \frac{A_{\text{Control}} - A_{\text{Test}}}{A_{\text{Control}}} \right) \times 100
\]

Whereas, A control is the absorbance in the absence of test samples, A test is the absorbance with samples. The IC 50 value (mg/mL) is the concentration at which hydroxyl scavenging activity was 50% compared with ascorbic acid. Values are done in triplicates with standard deviation.

4.2.13 Fe-chelating activity

Fe\(^{2+}\) chelating activity of exopolysaccharides was measured through Decker and welch method 1990 with slight modification. 10 µL of diluted sample at various concentration was mixed with 0.5 mL of 2 mM FeCl\(_2\) and 1mL of 5 mM ferrozine solutions were added and kept at dark room for 20 minutes. Afterwards absorbance of the reactant solution was recorded at 562 nm. The control was set in the identical manner instead of samples add distilled water. Fe\(^{2+}\) chelating activities were then deliberate as follows.

\[
\text{Fe}^{2+}\text{Chelating activities} = \left( \frac{A_{\text{Control}} - A_{\text{Test}}}{A_{\text{Control}}} \right) \times 100
\]
4.3 Results and Discussion

4.3.1 Preliminary screening of EPS-producing bacteria

In total, 200 isolates were screened for the production of EPS from MRS and nutrient agar with supplemented of sucrose. Figure 4.1 shows the EPS-producing strains, in which 80, 24, 25 and 33 strains showed compact, creamy, liquid, and smooth colony morphology, respectively. One isolate having a compact morphology was identified genotypically, and databases were submitted in NCBI (*Leuconostoc lactis* KC117496) and the same was selected for further characterization of EPS which can be used as a functional starter culture for preparation of *idli*.

Figure 4.1 Colony morphology of EPS producing strains.

Figure 4.2 Purification of Exopolysaccaride through Chromatography
Figure 4.2 Purification of EPS was carried out at different concentration of NaCl in DEAE 52-cellulose column and Sephadex G-100 column. At 0.2 M, 0.4 M, 0.6 M NaCl showed impurities compare to 0.8 and 1 M NaCl indicating that 0.8 and 1M eluted purified EPS.

4.3.2 HPTLC analysis

The practical application of TLC method to detect the monosaccharides present in acid hydrolysate biological material (polysaccharides). Figure 4.3 The separations of monosaccharides in TLC are slow due to their polar nature and solvent system employed is mostly polar solvent. The acid-hydrolysed EPS resulted in single sugar spot with a retention force (Rf) of 0.54. Based on the Rf value, the monomer was identified as glucose, indicating that the EPS is a homopolysaccharide (Glucan).
FT-IR spectroscopy analysis

FT-IR spectroscopy was carried out to analyze the vibration of polar bond molecules between dissimilar atoms. Structure of monosaccharides such as functional groups, glucosidic bonds, and type of monosaccharides can be predicted through FT-IR. UV spectra of the EPS only showed single peak at 210 nm, and no other peak was detected in 260–290 nm. It clearly indicates that the purified EPS is free from the presence of any protein and nucleic acid (Kanmani et al., 2011). Total carbohydrate was found to be 36 mg/100 mL based on an analysis using phenol sulphuric acid method. Figure 4.4. FT-IR has been a powerful and valuable tool for perceiving structural and functional groups in bio-molecules. The FT-IR spectrum of purified EPS showed numerous peaks from 3441 cm⁻¹ to 516 cm⁻¹. Presence of high levels of hydroxyl groups (O–H) was indicated by broad absorption peak around 3250–3500 cm⁻¹, confirming the polysaccharide nature of the material (Kanmani et al., 2011). The signal at 2926 cm⁻¹ was assigned to the stretching vibration of C–H (Sun et al., 2009).
stretch three vibrations present between 1100-1010 indicating the pyranoside form (Yang and Zhang, 2009). The intense absorption peaks at 1790–1680 cm\(^{-1}\) resemble the amide C=O and carboxyl group (Wang et al., 2008). The broad stretch of C–O and C–O–C at 1040–1200 cm\(^{-1}\) corresponds to the presence of carbohydrates. The intense peak at 1090 cm\(^{-1}\) is recognized to the main properties of polysaccharide (Nataraj et al., 2008). The weak stretch band at 840.9 was characterized for α-D-glucose (Yang et al., 2009). The weak adsorption band at 763.8 and 516.92 cm\(^{-1}\) was considered as the glycosidic linkage peak for polysaccharide (Singh et al., 2011).

**Figure 4.4 FT-IR Spectrum of EPS produced by Leuconostoc lactis at the region 4000 cm\(^{-1}\)-400 cm\(^{-1}\)**
4.3.4 Nuclear magnetic resonance spectroscopy analysis

$^1$H NMR spectra of EPS produced by *Leuconostoc lactis* KC117496

The NMR spectroscopy is a main technique for determining the structure of organic molecules. The study of carbohydrates through NMR spectroscopy provides guidelines for interpretation of their 1- and 2-D spectra against a background of their tautomeric, configurational and conformational equilibria in solution and consideration of their biosynthetic diversity. The influence of structural character on chemical shifts and coupling constants is showed from correlation spectroscopy (COSY), total correlation spectroscopy (TOCSY), hetero-nuclear single quantum coherence (HSQC), hetero-nuclear multiple bond correlation (HMBC) and nuclear over-hauser enhancement spectroscopy (NOESY) (Bubb, 2003). Signals at 4.8-5.5 ppm in anomeric region was observed in $^1$H spectrum confirm the presence of residues or repeating units are in the anomeric configuration. Figure 4.5. The $^1$H NMR spectra of *Leuconostoc lactis* KC117496 indicated the resonance of hydrogen proton corresponding to the glucosyl residue as the repetitive unit of the biopolymer. One single peak in the anomeric proton signal at δ 5.020 ppm indicated the presence of α-(1→6) linked Glucan, and low intensity peak at δ 5.365 ppm showed the presence of α-(1→3) linkage. Similar results were reported earlier in other EPS from *Leuconostoc strains* (Bounaix et al., 2009) and α-(1→3→6) linked sugar was observed in *Leuconostoc garlicum PR* (Capek et al., 2011). Exopolysaccharides produced by *Leuconostoc mesenteroides, Leuconostoc citreum, Weissella confusa* shown a low intensity anomeric signal at δ 5.32 ppm contained 4.1%, 3.5%, and 2.7% (α-1→3) branched linkage (Maina et al., 2008). This study explains the exopolysaccharide formed by the bacteria resembles a replication unit's of 95% α-(1→6) and 5% branching α-(1→3) D-glucosyl residual.
Figure 4.5 400-MHz $^1$H NMR spectra in D$_2$O of EPS produced by *Leuconostoc lactis* KC117496

<table>
<thead>
<tr>
<th>Sample</th>
<th>H1</th>
<th>H2</th>
<th>H3</th>
<th>H4</th>
<th>H5</th>
<th>H6</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>EPS</td>
<td>5</td>
<td>3.58</td>
<td>3.7</td>
<td>3.5</td>
<td>3.92</td>
<td>3.99</td>
<td>Paulo <em>et al.</em>, 2012</td>
</tr>
<tr>
<td><em>L. lactis</em> KC117496</td>
<td>5</td>
<td>3.62</td>
<td>3.73</td>
<td>3.55</td>
<td>3.94</td>
<td>3.8</td>
<td></td>
</tr>
</tbody>
</table>
$^{13}$C and DEPT 135 NMR spectra of exopolysaccharides

Carbon spectra can be used to find out the numeral of non-equivalent carbons and to classify the types of carbon atoms present in organic molecules. $^{13}$C NMR provides the structural information of molecules through frame the skeleton of carbon atoms. By using both $^1$H and $^{13}$C NMR together used to determine the structure of unknown molecules. Figure 4.6. The $^{13}$C NMR spectrum of the EPS fraction was observed at C1 δ 97.72 ppm indicated the presence of α-linkage (97–101) which can be explained from Bubb, (2003), and the same pattern was found as α-(1→6) linkage in the anomic region of many lactic acid bacteria isolated from sourdough (Bounaix et al., 2009). There was signal at 90 ppm suggesting that there was only pyranose ring configuration for all sugars. The signal at δ100.78 ppm corresponded to the anomic carbon involved in α-(1→3) linkage (Ismail and Nampoothiri, 2012). CH-1 δ 97.72 ppm, CH-2 δ 71.45 ppm, CH-3 δ 70.23 ppm, CH-4 δ 73.45 ppm, CH-5 δ 69.59 ppm, CH₂-6 δ 65.62 ppm confirmed the presence of sugar through $^{13}$C and DEPT 135.

<table>
<thead>
<tr>
<th>Sample</th>
<th>C1</th>
<th>C2</th>
<th>C3</th>
<th>C4</th>
<th>C5</th>
<th>C6</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>EPS</td>
<td>97.76</td>
<td>71.4</td>
<td>70.2</td>
<td>73.4</td>
<td>69.6</td>
<td>66.6</td>
<td>Kim et al., 2008</td>
</tr>
<tr>
<td><em>L. lactis</em> 95A</td>
<td>99.306</td>
<td>72.996</td>
<td>74.993</td>
<td>71.124</td>
<td>71.775</td>
<td>67.142</td>
<td>Palomba et al., 2012</td>
</tr>
<tr>
<td><em>L. lactis</em> KC 117496</td>
<td>97.72</td>
<td>71.45</td>
<td>73.45</td>
<td>69.59</td>
<td>70.23</td>
<td>65.62</td>
<td></td>
</tr>
</tbody>
</table>
Figure 4.6 $^{13}$C and DEPT 135 NMR spectra of exopolysaccharides
Hetero-Nuclear Single Quantum Coherence (HSQC) Spectrum of EPS

Figure 4.7. Though heteronuclear HSQC spectra showed that α 1-6 linked Glucan in the backbone were dispersed \(((H1/C1) \delta 5/97.75),(H2/C2) \delta 3.6/71.4),(H3/C3) \delta 3.7/73.4),(H4/C4) \delta 3.5/70.),(H5/C5) \delta 3.9/69.6)\) and \((H6,H6 /C6 \delta 4, 3.8/66.6)\). They were in regular agreement with data published for *Leuconostoc garlicum PR* (Capek *et al.*, 2011). Through COSY spectrum represent the chemical shifts of each proton and proton-proton interactions of monosaccharide units present in exopolysaccharides.

Figure 4.7 HSQC spectrum of Exopolysaccharide at room temperature in D$_2$O
Figure 4.8. COSY NMR cross signal between the protons indicating are adjacent to each other. This revealed the position of hydrogen atom in pyranose ring of monosaccharide units.

**Figure 4.8 COSY spectrum was recorded at room temperature for exopolysaccharide**

<table>
<thead>
<tr>
<th>Proton</th>
<th>Adjacent</th>
<th>Signal</th>
</tr>
</thead>
<tbody>
<tr>
<td>H1</td>
<td>H2</td>
<td>5/3.6</td>
</tr>
<tr>
<td>H2</td>
<td>H1,H3</td>
<td>3.6/5,3.7</td>
</tr>
<tr>
<td>H3</td>
<td>H2,H4</td>
<td>3.7/3.6,3.5</td>
</tr>
<tr>
<td>H4</td>
<td>H3,H5</td>
<td>3.5/3.7,3.9</td>
</tr>
<tr>
<td>H5</td>
<td>H4</td>
<td>3.9/3.5</td>
</tr>
<tr>
<td>H6</td>
<td>H6</td>
<td>4/3.8</td>
</tr>
</tbody>
</table>
Figure 4.9 HMBC Spectrum of EPS recorded at room temperature in D$_2$O
Hetero-nuclear multiple bond correlation spectrum showed the cross signal between C1/H6 (97/3.8) and H1/C6 (5/66) indicating clearly the presence of $\alpha$-1$\rightarrow$6 linkage in Fig 4.9. Based on the 1D and 2D NMR shows the presence of $\alpha$-1$\rightarrow$6, $\alpha$-1$\rightarrow$3 linked glucan (Fig 4.10).

4.3.5 MALDI-TOF-MS analysis

Matrix-assisted laser desorption/ionization (MALDI) mass spectrometry (MS) has been developed successfully as a soft ionization method in molecular mass detection for macromolecules and biopolymers (Hung et al., 2012). Figure 4.11. A main fraction of exopolysaccharide molecular weight was $\sim$4.428 x $10^4$ Da attained from the MALDI-TOF due to the presence repeating unit of hexose sugars in polysaccharides. In earlier report *Leuconostoc mesenteroides* isolated from *idli* batter produced high molecular weight (9.7x10$^5$ Da) with $\alpha$-1-6 and $\alpha$-1-4 linkage polysaccharide (Sawala and Lee, 2010). The major fraction of polysaccharides produced by *Lactobacillus plantarum* MTCC 9510 molecular weight ($\sim$1.31 x 10$^5$Da) was confirmed by MALDI-TOF (Ismail and Nampoothiri,
A novel exopolysaccharide molecular weight $2.8 \times 10^5$ Da produced by *Steptococcus phocae* PI80 (Kanmani et al., 2011). *Pseudomonas PF-6* produced acidic exopolysaccharide with molecular weight of $8.83 \times 10^5$ Da (Ye et al., 2012).

**Figure 4.11 MALDI-TOF-MS analysis of exopolysaccharide**

4.3.6 Scanning electron microscopy (SEM)

Figure 4.12. SEM images of the EPS at 200 X and 500 X revealed the compact and porous structure, essential for the water holding capacity similar to dextran produced by *Leuconostoc mesenteroides* NRRL B-1149 (Shukla et al., 2011). At 5000 X magnifications EPS showed smooth, consistent polymeric matrix indicating the structural reliability important for bio-based films formation. Much of the SEM properties of EPS are comparable to the
characteristics of polymers. Similarly in an earlier report, EPS produced by *Leuconostoc kimchii* strains showed hollow porous structure with polymer matrix (Torres *et al.*, 2014). SEM images of the EPS magnifications showed smooth, consistent polymeric matrix indicating the structural reliability important for bio-based films formation. Much of the SEM properties of EPS are comparable to the characteristics of polymers (Piermaria *et al.*, 2008). Wang *et al.*, (2010) explained the KF5 EPS as smooth surfaces under 5000 X, which is a favourable characteristic of the material used to make the plasticized bio-films. EPS structure forms a hydrated polymer-consistent matrix having mechanical stability of bio-films. The weak physico-chemical interactions between molecules are due to various binding forces such as Van der Waals forces, repulsive forces, electrostatic forces, ionic attractive forces and hydrogen forces. Colonization of microflora on abiotic and biotic surfaces through biofilm in the intestine creates various health benefits (Flemming *et al.*, 2010).

**Figure 4.12 Scanning electron micrograph showing the surface morphology of EPS at various magnifications at 200 x (A), 500 x (B), 5000 x (C)**
4.3.7 Atomic force Microscopy (AFM)

EPS has been studied widely through atomic force microscopy to describe the morphological topographies of polymers. Figure 4.13. AFM images of EPS with rounded and irregular lumps where maximum height of the lump was 42.4 nm and the roughness was 9.82 nm. Recently, Ahmed et al., (2013) and Singh et al., (2011) described the EPS topography with rounded lumps showing maximum height of 31.1 nm and roughness average of 191 nm. However, in some cases, shapes such as spherical lumps and worms can also be observed (Ren et al., 2003). Strongly packed and obligate irregular shaped molecules are also observed in our EPS samples. AFM is an important technique capable of imaging the surface morphology under aqueous solutions, which is used to examine the formation of bio-film (Pradhan et al., 2008).

Figure 4.13 Atomic force microscopy 3D images showing the rounded and roughness (A), and irregular lumps (B)
4.3.8 Thermogravimetric analysis (TGA)

Figure 4.14. The weight loss of EPS was up to 15% from 0 °C to 100 °C, due to moisture. Then the first stage of degradation was started slowly (22.82 %) from 100–240 °C. The maximum loss occurs at 240–400 °C and weight loss is 58.56 %. High level of the carboxyl group in the EPS increased the degradation of the first phase as the carboxyl group is bound to more water molecules (Singh et al., 2011). Total weight loss of EPS occurs at the temperature of (Tc) 241.14 °C. Two different peaks of pyrolysis temperature were found at 218.92 °C and 296.51 °C with the average of 272.01 °C and required 25.02 J/g energies. This range of the exothermic process exhibited a substantial thermal transition of EPS amorphous state into a crystalline state. ZW3 EPS showed higher degradation temperature (299.65 °C) similar to our EPS (272.01 °C). It is safe to be used in dairy industry when process temperature seldom overpasses 150 °C (Ahmed et al., 2013). EPS thermostable characteristics play an important role in the food industry especially in rheological properties as the manufacturing and processing of numerous food preparations are carried out at higher temperatures (Sajna et al., 2013).

Figure 4.14 TGA pattern of EPS obtained from Leuconostoc lactis
4.3.9 X-ray diffraction analysis (XRD)

Figure 4.15. Powder XRD spectra revealed the distinguishing diffraction peaks at 43.51 °C and 50.8 °C with interplanar spacing (d-spacing) 2.07806 Å and 1.79433 Å, respectively. The ratio among sharp thin diffraction peaks and wide-ranging peak was used to determine the quantity of crystallinity. From XRD pattern it was found that crystalline peaks were superimposed in the amorphous phase of the EPS imfering a partial crystalline (33.4%) $C_l_{\text{rd}} = 0.334$. Similar patterns were reported earlier for both micro-algae and Bacillus licheniformis EPS (Mishra et al., 2011).

Figure 4.15 XRD of EPS isolated from Leuconostoc lactis at heating rate of 10 °C
4.3.10 Water solubility index and water-holding capacity

The water solubility index and water-holding capacity of the EPS were 14.2 ± 0.208 % and 117 ± 7.5 %, respectively. Ahmed et al., (2013) reported that the solubility and water-holding capacity of EPS from *Lactobacillus kefiranofaciens* ZW3 was 14.2 % and 496 %, respectively. EPS is water soluble with good water-holding capacity due to absorptive structure of the polymer which can hold huge quantities of water through hydrogen bonds (Zhu et al., 2010). Carbohydrates can also improve the textural and rheological properties of food products due to physico-chemical properties such as viscosity, water-holding capacity and solubility (Mudgil et al., 2013).

4.3.11 DPPH radical scavenging activity

The DPPH free radical has been extensively accepted as a tool for assessing the free radical scavenging activities of antioxidants. DPPH free radical scavenging activity was observed using spectrophotometer through the transfer of an electron or a hydrogen atom by antioxidants to DPPH and neutralizing its free radical characteristic forming colour solution (Liu et al., 2011). Figure 4.16. In this present work, the scavenging activity of the EPS is seen increasing with an increase in its concentration. The scavenging effect of control (Vc) at the various concentration (100-500 µg/mL) showed in increasing trend of 2.8-75% with similar trend observed in EPS from 6.8-74%, which specifies that the EPS is having similar scavenging activity as the control (ascorbic acid). The previous reported about DPPH scavenging activity of different concentrations of EPS indicates that 0.125 mg/meal of the three purified EPS fractions showed scavenging abilities of 18.42-22.95% on DPPH free radical. At 4 mg/mL, the scavenging effects increased to 33.53-41.92% (Li et al., 2014). The DPPH radical scavenging activity increased with an increase in the concentration up to 1.0
mg/mL. But increase in -1 concentration did not affect the activity significantly. At a concentration of 4.0 mg/mL, LPC-1 and ascorbic acid showed 52.23% and 88.60% of DPPH radical scavenging activity (Zhang et al., 2013).

**Figure 4.16 DPPH radical scavenging activity**

Results are expressed as means ± standard deviations (n =3).

**Vc** = Ascorbic acid
4.3.12 Hydroxyl radical (OH) scavenging activity

Hydroxyl radicals are extremely strong oxidants, which can easily react with biomolecules present in living cells and create damage to the contiguous macromolecules in biological system. Figure 4.17. The hydroxyl radical scavenging effects of both Vc and EPS were commonly increased with increasing concentrations from 100-500 µg/mL was observed. The hydroxyl radical scavenging activity of the Vc was 41.16-98.63%, the activity of the EPS attained to 27.5-97.8%. This result is comparable to an earlier report where the Pseudomonas PF-6 EPS exhibited higher hydroxyl radical scavenging activity compared to control (Ye et al., 2012). However, the scavenging activities of three crude EPS from Bacillus licheniformis 8 were much lower, 31.67%, 38.61, and 43.17% at the concentration 4.0mg/mL (Liu et al., 2010).

Figure 4.17 Hydroxyl radical scavenging activity

Vc = Acsorbic acid, Results are expressed as means ± standard deviations (n= 3).
4.3.13 Fe-chelating activity

The metal chelating activity plays a critical function as an antioxidant in the biological system. The transition Fe$^{2+}$ can influence lipid peroxidation through producing hydroxyl radicals by Fenton reaction and speed up lipid peroxidation through disintegrating lipid hydroperoxides into alkoxy and peroxy radicals (Benedet and Shibamoto, 2008). The Fe$^{2+}$ chelating activity prevents lipid oxidation through stabilizing transition metals and the activity was observed by preventing the formation of red colour Fe$^{2+}$ ferrozine compound (Jun et al., 2010). Figure 4.18. In this study Fe$^{2+}$ chelating activity was observed from 100-500 µg/mL for both control and EPS. Both increase the chelating ability by increasing in concentration but comparatively EPS was weaker (5.8-72.5%) than control (EDTA-2Na). Similar result was observed in three EPS samples in an earlier report, where, at a concentration of 1.0 mg/mL were 92.4%, 81.1% and 86.5%, compound, 101EP and 102EP were showed 54.18% and 29.34% at 10 mg/mL (Liu et al., 2010, Liu et al., 2011).

**Figure 4.18 Metal chelating activity**

![Metal chelating activity graph](image)

C = Na$_2$EDTA

Results are expressed as means ± standard deviations (n= 3).

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Glucans are synthesized by the action of extracellular enzyme from several bacteria such as *Leuconostoc* spp, *Streptococcus* spp, and *Lactobacillus* spp through sucrose supplied as a supplement in the medium (Naessens *et al.*, 2005). Several *Leuconostoc* species are frequently utilized for the production of dextrans and slime forming α-glucans, which are widely used in food industry as stabilizing, viscosifying, emulsifying, water-binding agents and increase moisture retention (Sarwat *et al.*, 2008). Homopolysaccharides are produced by lactic acid bacteria under normal conditions was lower than 1g/L (Werning *et al.*, 2012). Whereas *Leuconostoc lactis* KC117496 produced glucan yield 360 mg/L in normal condition was comparably similar to EPS produced from different lactic acid bacteria ranged between 100-196 mg/L in normal condition isolated from Nigerian Fermented Foods (Adebayo and Onilude, 2008). *Leuconostoc lactis*-1.8 and *Leuconostoc mesenteroides*-21.2 produced EPS at the range of 800-900 mg/L isolated from raw milk (Van der Meulen *et al.*, 2007). *Leuconostoc dextranicum* NRRL B-1146 produced maximum 1063 mg/L glucan in optimized condition. EPS showed porous structure and pseudoplastic behaviour in SEM (Majumder and Goyal, 2009). Similar results was also observed in our EPS can be used as a food additive to improve the texture.

### 4.4 Conclusions

In the present study, *Leuconostoc lactis* KC117496 was recognized as a potential source for the production of EPS in MRS–sucrose medium. The EPS was found to be homopolymeric polysaccharide and further shown to be a glucan through HPTLC, FT-IR and NMR analysis. Its molecular weight was calculated to be \( \sim 4.428 \times 10^4 \) Da. Structural investigation of exopolysaccharide showed linear 94% α 1-6 linked Glucose and 6% .α 1-3 linked sugar through H\(^1\) and \(^{13}\)C NMR. The dispersion of EPS showed that α 1-6 linked Glucan in the backbone through HSQC spectra. HMBC NMR shown the cross signal C1/H6 (66/5.0) are
adjacent to each other clearly indicating the presence of α 1-6 linkage. The topological image of EPS from the AFM and SEM showed the potential to have physical stability, smooth surface for film formation and cohort of nanostructure for encapsulation of food materials. The EPS has good water solubility and water-binding capacity, with a good potential for use in food industry as a food additive. Antioxidant properties of EPS shown similar trend in DPPH and hydroxyl radical activity as compared the Vc (Control) and less in metal chelating activity. However, the metal chelating activity is considerably high at higher concentrations. These EPS can further be optimised for commercial production and utilisation food and pharmaceutical industry.

**Future perespectives**

Outcome of this work has multiple partical impaction. The cultures isolated and identified from the idli batter and stored in the culture collection can be of much future research invesgation for the potential technological properties some of the selected cultures are already candidates in the development of starter cultures. Some of the culture for examples *Leuconostoc lactis* KC117496, *Enterobacter cloacae* KC607815 with their to produce valuable exopolysaccharide have been optimized for the pilot level production and the EPS is been evaluated for its using food product development as encapsating material. Some of the cultures with potential probiotics properties are been further screened for their probitoic suitability. Cultures with protential phyase activity need to be further optimized for pyhase production and extraction. Five of the culture are been screened for the pontential to bind degrade aflotoxin separately.