Chapter 3

Isolation, purification and chemical characterization of exopolysaccharide produced by a marine fouling bacterium
Chapter 3

INTRODUCTION

Metallic and non-metallic surfaces immersed in an aquatic environment get readily colonized by a variety of microorganisms including bacteria, diatoms, fungi and protozoa as well as by organic and inorganic detritus. While colonizing the surface microorganism produce a wide range of extracellular polymeric substances (EPS). EPS composed of polysaccharides, proteins (including enzymes), nucleic acids and lipids (Costerton et al, 1981; Platt et al, 1985; Frolund et al, 1996; Nielsen et al, 1997; Dignac et al, 1998; Neu 1996; Takeda et al, 1998; Sand & Gehrke, 1999; Spaeth & wuertz, 2000). They play important roles in biofilms. These compounds protect cells against changes in pH, water and salt content and hydraulic pressure. EPS also play an important role in adsorption of nutrients, detoxification of toxic chemicals and influence the specific properties of biofilms based on their physico-chemical characteristics. Several species of bacteria are able to synthesize more than one chemically distinct EPS, although normally only one type is expressed under any set of specific growth conditions. Growth phase and nutrient status of the surface-associated bacteria may influence the quality and the chemical composition of the EPS produced (Decho, 1990). The functions of the EPS vary with the composition and quality of the exopolymers. Polysaccharides, produced by microorganisms are either homopolysaccharides or heteropolysaccharides. Most polysaccharides produced by the fouling bacteria are of heteropolysaccharides, which are acidic in nature (Sutherland, 1990). The acidic nature of the EPS is due to the presence of uronic acids, pyruvate, and
inorganic residues such as phosphate or sulphate (Sutherland, 1990; Majumdar et al, 1999; Iarpin et al, 2002; Tallon et al, 2003).

In recent years there has been a growing interest in the isolation and characterization of microbial polysaccharides owing to their importance in adhesion, biofouling and biocorrosion. EPS appear to play an important role in adhesion of the bacteria to the surface (Geesey et al, 1977; Allison & Sutherland, 1987). Moreover, the involvement of functional groups of EPS in biofouling and biocorrosion processes has been demonstrated by using surface analytical techniques (Geesey et al, 1986; Jolley et al, 1988). Despite their importance, very few studies have been carried out in chemical characterization of EPS by fouling bacterial (Corpe, 1970; Rodrigues and Bhosle, 1991; Jahn et al, 1999; Majumdar et al, 1999; Muralidharan & Jayachandran, 2003). In this present research work the isolation, partial purification and chemical characterization of the EPS produced by a fouling bacterium, Bacillus sp. (SS-15) are presented and discussed.

MATERIAL AND METHODS

Isolation of Bacteria

The stainless steel panels were deployed in the surface waters of the Dona Paula Bay as detailed in the Chapter 2a. In order to isolate bacterial cultures, the biofilm material was removed using a brush and sterile seawater. The biofilm samples were then serially diluted. A known aliquot was plated onto ZoBell marine agar plates. The plates were incubated at room temperature (28 ± 2 °C) for 24 h, colonies were randomly selected, purified and stored on ZoBell
marine agar slants at 4 °C. Twenty cultures were randomly selected to assess their potential for exopolysaccharide production.

**Culture Conditions**

Bacterial cultures for EPS production were grown in the basal salt solution (BSS) containing 1 ml of traced metal solution (TMS). The composition of the BSS and TMS is given in Table 1. The carbon and nitrogen sources and concentrations and concentrations of phosphate, magnesium, calcium, and sodium chloride were varied as required. The pH of the medium was adjusted to 7.5 with 1N NaOH. The medium was sterilized by autoclaving for 20 min. at 121 °C. Medium was inoculated with 1 % (V / V) of a 24h old culture grown in the same medium at room temperature on a rotary shaker at 150 rpm.

**Assessment of EPS Production**

Bacterial cultures were grown in the BSS medium supplemented with trisodium citrate (6.0 g/l) as carbon source. The culture was grown for 5 days at room temperature on rotary shaker at 150 rpm. The cells were removed by centrifugation at 7,000 rpm at 4 °C for 10 min. One milliliter of the clear supernatant was than used for the estimation of EPS using the phenol-sulphuric acid method (Dubois et al, 1956). Of the sixty cultures screened, the culture SS-15 produced the highest amount of EPS. This culture was identified and was used for further studies on EPS production and characterization as described below.
Characterization of the Culture

The selected culture SS-15 was studied for morphological, physiological and biochemical characteristics and identified upto generic level. A taxonomic scheme for the identification of bacteria given in the Bergey's manual was used for the characterization of this isolate. The culture was tentatively identified as *Bacillus* sp. and hereafter referred as *Bacillus* sp. (SS-15).

Effect of Carbon Source and Concentration on EPS Production by *Bacillus* sp. (SS-15)

The effect of carbon source on the production of EPS was studied using BSS medium supplemented with 1 % of either, galactose, glucose, maltose, sucrose and xylose as the carbon and energy source. Sucrose concentration in the growth medium was varied from 0.25 to 5 % in order to evaluate the influence of carbon concentration on EPS production by *Bacillus* sp. (SS-15). The culture was grown for 5 days and the cells were removed by centrifugation at 7,000 rpm for 10 min at 4 °C. The supernatant was passed through 0.2 μm pore size filter (Neucleopore). Filtrate was dialysed against distilled water at 4 °C to eliminate any low molecular weight sugars and salts using dialysis bags (MW cut-off of 8,000). The dialysed supernatant was again concentrated using rotary vacuum evaporator at 40 °C and adjusted to a known volume. A known aliquot was used to estimate EPS by the phenol sulphuric acid method.

Effect of Nitrogen Source and Concentration on the EPS Production

The BSS containing 1 % sucrose and 0.013 % of nitrogen either as ammonium chloride, ammonium sulphate, sodium nitrate, glycine or urea was used to assess the effect of nitrogen source on EPS production. The nitrogen (as
ammonium chloride) concentration in the growth medium was varied from 0.005 to 0.1 % to assess the effect of nitrogen concentration on the exopolysaccharide production. The growth medium was inoculated with the overnight grown Bacillus sp. (SS-15) culture grown in the same medium. The culture was grown at room temperature for 5 days on rotary shaker at 150 rpm. The exopolysaccharide was estimated following the method described as above.

Influence of Potassium Phosphate, MgCl₂, CaCl₂ and NaCl Concentration on EPS Production
The BSS medium containing 1 % sucrose and appropriate concentration of ammonium chloride was supplemented with various concentrations of dipotassium hydrogen phosphate and potassium dihydrogen phosphate (phosphate concentration 0.025 to 0.2 %) to assess the effect of phosphate concentration on the EPS production. The magnesium sulphate, calcium chloride and sodium chloride concentration in the BSS varied individually between 0.1 to 0.9 %, 0.005 to 0.05 % and 0.5 to 4.5 %, respectively to assess the effect of these compounds on ESP production by the Bacillus sp. (SS-15). The culture was grown and the EPS was estimated as described above.

Effect of pH on the EPS Production
The effect of pH on the exopolysaccharide production was assessed by adjusting the pH of the BSS medium from 5 to 10 using 1N NaOH. The BSS medium containing 1 % sucrose and optimized concentration of various nutrients was used. The culture was grown at room temperature and the EPS was estimated as above.
Effect of High Temperature and pH on the EPS Production by *Bacillus* sp. (SS-15).

The influence of high temperature (50 °C) and high pH (10) on EPS production was studied by using the optimized BSS medium containing 1 % sucrose as carbon source. The pH of the medium was adjusted to 10 using 1 N NaOH and the culture was grown at 28 °C and at 50 °C. The EPS was then estimated as above.

Effect of Culture Condition on EPS Production

The BSS medium containing 1 % sucrose and optimized concentration of various nutrients (Table 1) was used to assess the influence of static and shaking condition on polysaccharide production by the *Bacillus* sp. (SS-15). The culture was grown for 5 days under static and shaking condition at room temperature (28 ± 2 °C) and the EPS was estimated as detailed above.

Growth Kinetics and EPS Production by *Bacillus* sp. (SS-15)

To assess the growth and EPS production, the culture *Bacillus* sp. (SS-15) was grown in optimized BSS medium containing 1 % sucrose as carbon source. Culture was grown on rotary shaker (150 rpm) at room temperature. At regular intervals 5 ml aliquots were removed. Growth was monitored by measuring optical density at 540 nm. Samples were centrifuged (7000 rpm for 10 min) and 1 ml supernatant was removed and dialysed as described above. A suitable aliquot was used to estimate EPS concentration using the phenol sulphuric acid method. The experiment was carried out until the culture reached the stationary growth phase.
Table 1. Chemical composition of basal salt solution (BSS), trace metal solution (TMS) and optimized basal salt solution.

<table>
<thead>
<tr>
<th>Chemical Composition</th>
<th>per liter</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Basal Salt Solution (BSS)</strong></td>
<td></td>
</tr>
<tr>
<td>NaCl</td>
<td>25.0 gm</td>
</tr>
<tr>
<td>KCl</td>
<td>0.75 gm</td>
</tr>
<tr>
<td>MgSO₄</td>
<td>7.0 gm</td>
</tr>
<tr>
<td>NH₄Cl</td>
<td>1.0 gm</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>0.2 gm</td>
</tr>
<tr>
<td>Glucose</td>
<td>10.0 gm</td>
</tr>
<tr>
<td>K₂HPO₄ (10 %)</td>
<td>7 ml</td>
</tr>
<tr>
<td>KH₂PO₄ (10 %)</td>
<td>3 ml</td>
</tr>
<tr>
<td>TMS*</td>
<td>1 ml</td>
</tr>
<tr>
<td>Distilled Water</td>
<td>1000 ml</td>
</tr>
<tr>
<td><em><em>Trace Metal Solution (TMS</em>)</em>*</td>
<td></td>
</tr>
<tr>
<td>H₃BO₃</td>
<td>2850 mg</td>
</tr>
<tr>
<td>MnCl₂·4H₂O</td>
<td>1800 mg</td>
</tr>
<tr>
<td>FeSO₄·7H₂O</td>
<td>2490 mg</td>
</tr>
<tr>
<td>Na-tartarate</td>
<td>1770 mg</td>
</tr>
<tr>
<td>CuCl₂</td>
<td>26.9 mg</td>
</tr>
<tr>
<td>ZnCl₂</td>
<td>20.8 mg</td>
</tr>
<tr>
<td>CoCl₂</td>
<td>40.4 mg</td>
</tr>
<tr>
<td>Na₂MoO₄·2H₂O</td>
<td>25.2 mg</td>
</tr>
<tr>
<td><strong>Optimized Basal Salt Solution</strong></td>
<td></td>
</tr>
<tr>
<td>NaCl</td>
<td>5.0 gm</td>
</tr>
<tr>
<td>KCl</td>
<td>0.75 gm</td>
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<tr>
<td>MgSO₄</td>
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<tr>
<td>CaCl₂</td>
<td>0.2 gm</td>
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<tr>
<td>Sucrose</td>
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<tr>
<td>K₂HPO₄ (10 %)</td>
<td>10 ml</td>
</tr>
<tr>
<td>KH₂PO₄ (10 %)</td>
<td>4.3 ml</td>
</tr>
<tr>
<td>TMS*</td>
<td>1 ml</td>
</tr>
<tr>
<td>Distilled Water</td>
<td>1000 ml</td>
</tr>
</tbody>
</table>
Isolation of EPS Produced by *Bacillus* sp. (SS-15)

Large-scale production of EPS by *Bacillus* sp. (SS-15) was studied in batch cultures. *Bacillus* sp. (SS-15) was grown in five liter conical flasks containing 1000 ml of the optimized BSS medium and 1 % sucrose as carbon source. The medium was inoculated (1 %) with overnight grown cells of *Bacillus* sp. (SS-15). Flasks were incubated on a rotary shaker at room temperature on a rotary shaker at 150 rpm. Flasks were removed after 5 days following inoculation. A flow diagram to isolate the EPS from the cell free broth is shown in Figure 5. In brief, the cells were removed by centrifugation at 10,000 rpm for 10 min at 4 °C. Supernatant (2 l) was concentrated (30 ml) using amicon ultrafiltration cell fitted with an ultra-filter of MW cut-off 10,000. The EPS was then precipitated by adding 3 volumes of chilled ethyl alcohol. EPS was collected on a glass rod and left overnight at 4 °C for complete precipitation. The EPS was collected after decanting the alcohol and partially dried in an oven at 40 °C. EPS was dissolved in small volume of distilled water and dialyzed using dialysis bags (MW cut-off of 8,000) against distilled water at 4 °C. After dialysis the solution was concentrated using rotary vacuum evaporator and the EPS was again precipitated. Precipitate was collected and dried in an oven at 40 °C. After this the EPS was further purified following the procedures described below.

Purification of EPS

EPS (10mg) obtained, as above was dissolve in distilled water. The EPS was treated with deoxyribonulease and ribonuclease (1-2 µg.ml⁻¹ final concentration; Merck). The solution was then incubated at 37 °C for 4 hrs in order to remove the nucleic acids. After this, the solution was then treated with protease K (0.1 mg.ml⁻¹, Sigma) at 37 °C overnight. The sample was then extensively dialysed.
using dialysis bags (MW cut-off of 8,000) against distilled water at 4 °C to remove low molecular weight compounds. The sample was centrifuged to remove insoluble material. The supernatant was recovered and further purified using size exclusion and anion exchange chromatography as detailed below.

Size Exclusion Chromatography of the EPS

Purity of the EPS was evaluated by size exclusion chromatography. EPS was dissolved in 0.05M phosphate buffer prepared in 0.05 M NaCl and loaded on the Sephadex-G-200 column (70 x 1.5 cm). The same buffer was used as a mobile phase. Sample was eluted at the rate of 20 ml per hour. Fractions of 3 ml were collected and then analyzed for the presence of carbohydrate using the phenol-sulphuric acid method. The fractions containing EPS were pooled together and dialysed as above. The dialysed EPS was concentrated on a rotary vacuum evaporator and lyophylized. The lyophylized EPS sample was then subjected anion-exchange column chromatography.

Anion-Exchange (DEAE) column chromatography

The uniformity of the EPS material was further examined by anion-exchange DEAE-Sepharose column (Sigma). Purified EPS was dissolved in 10 mM K₂HPO₄ (pH 7.5) + 0.1 M NaCl and was applied as 1ml sample to a column (25 x 1.5 cm) of DEAE-Sepharose. Samples were eluted with 20 ml of starting buffer, then with a linear increasing gradient from 0.1 to 1.0 M NaCl (in phosphate buffer) at a flow rate of 20 ml per hour. Fractions of 1 ml were collected and analysed for total carbohydrates.
Viscosity Measurement

The apparent viscosity of the pure EPS was determined with a Brookfield Model DV-III+ Programmable Rheometer equipped with a CPE-40 spindle following the method of Ha et al, 1991. The dried EPS was dissolved in distilled water by stirring and hydration for 24 h at room temperature prior to testing for viscosity. The viscosity of the EPS solutions was measured at various shear rates (0-1100 per s).

Molecular Weight of EPS

The viscosity at different concentrations of the EPS solutions was measured at various shear rates (0 — 1100 per s). The specific viscosity of the EPS solution was calculated by employing the formula:

$$\eta_{sp} = \eta/\eta_0 - 1$$

where $\eta_{sp}$, specific viscosity; $\eta$, viscosity of EPS solution; $\eta_0$ = viscosity of solvent.

The molecular weight of the EPS was calculated using the following formula (Muralidharan and Jayachandran, 2003).

$$M_W = 0.9(\eta_{sp}) \times 10^6$$

Where $M_W$, the molecular weight of EPS, $\eta_{sp}$, = the specific viscosity of EPS solution at 0 % concentration. Employing regression equation, the specific viscosity was calculated at 0 % EPS concentration and the value was used in the above formula to determine the average molecular weight.
Chemical Analysis of EPS

Total carbohydrate of the EPS was estimated by the phenol-sulphuric acid method (Dubois et al, 1956). Protein content was analysed using the method of Smith et al, (1985). Pyruvate was determined by the method of Stonecker and Orentas, (1962). Uronic acids were estimated by the method of Filisetti-Cozzi and Carpita, (1991). Sulphate was determined using turbidatory method of Dogdson & Price, (1962). Hexosamines was estimated following the method of Johnson, (1971). Inorganic content of the EPS was estimated by the gravimetric method. The pre-weighed EPS sample was ashed at 400 °C for 4 h and cooled to room temperature. The resulting ash quantifies the inorganic content of the EPS and the organic material lost as CO₂ was estimated from the difference in weight before and after ashing.

Sugar Composition of the EPS

In order to determine monosaccharide composition of the EPS, 2 mg of the polysaccharide was hydrolyzed with 2N HCl for 2 h at 100 °C in ampoules flushed with nitrogen before sealing. After cooling an internal standard (myo-inositol) was added to the hydrolyzate. The solution was evaporated to dryness under reduced pressure at 40 °C. Distilled water (1 ml) was added and the pH of the resulting solution was raised to 8-9 by adding 10 % V/V triethylamine solution in water for the hydrolysis of lactones. The neutral sugars were converted to their alditol acetates and were analysed by capillary gas chromatography as describe in detailed in Chapter 2a.

A capillary gas chromatograph (Agilent 6890 series) equipped with a fused silica capillary column coated with CP Sil-88 (25 m, i.d. = 0.32mm) and flame ionization detector (FID), was used to separate the alditol acetate mixture.
Sample (0.4 μl) was injected using an on-column injector when the initial temperature was 70 °C. The oven temperature was then rapidly raised to 150 °C and further raised at 3 °C/min to 230 °C and maintained at this temperature for 15 min. The injector and the detector temperature were kept at 300 °C. Quantification of the component was achieved by peak area integration of the GC results using a HP Chemstation Data Handling System installed in the instrument.

**Amino acid Composition of the EPS**

5 mg of the EPS was hydrolyzed with 6N HCl for 24 h at 110 °C in ampoules flushed with nitrogen before sealing. After hydrolyses the samples was neutralised with 6N NaOH and centrifuged. The supernatant was evaporated to dryness under vacuum. 2ml of HPLC grade distilled water was added and a suitable aliquot was then taken for the analysis of amino acids by HPLC method as detailed in Chapter 2b.

**Fourier Transform Infrared Analysis (FTIR)**

Major structural groups of the EPS were detected using Fourier transformed infra-red spectroscopy (FTIR). The FTIR adsorption spectra were obtained on Shimadzu Spectrophotometer (FTIR 820IPC) using KBr techniques. Pellets were obtained by carefully grinding a mixture of 2 mg of EPS with about 200 mg of dry KBr. Infrared spectra were recorded in a range from 4000-400 cm⁻¹.
Nuclear Magnetic Resonance Spectroscopy (NMR)

$^1$H and $^{13}$C nuclear magnetic resonance (NMR) spectra were obtained at ambient temperature with EPS (20 mg) dissolved in D$_2$O (0.75 ml) in NMR tubes. A Bruker Avance-300 spectrometer was used in the Fourier pulsed transform mode with complete proton decoupling for $^{13}$C. For $^1$H, operation was at 300 MHz and $^{13}$C at 75 MHz. TMS was used as internal reference. The values are given in $\delta$ for proton NMR and ppm for $^{13}$C NMR.

RESULTS

Screening of Cultures for EPS Production and Identification of the Bacterial Culture

Twenty fouling cultures were randomly selected and screened for EPS production (Table 2). All the cultures produced EPS, however the production of EPS was highest with the culture SS-15. This culture was, therefore selected, identified and used for further studies on EPS production. The morphological, biochemical and physiological characterization of the culture SS-15 was; Short fat rods, motile, Gram positive, oxidase and catalase positive, facultatively anaerobic, hydrolysed starch, reduce nitrate to nitrite and showed the presence of spores (Table 3). These morphological, biochemical and physiological characteristics of culture was similar to those described for *Bacillus* sp. in the Bergey's manual and thus the culture was tentatively identified as *Bacillus* sp. (Krieg & Holt, 1984).
Table 2. Screening of fouling bacteria for EPS production grown in BSS medium using citrate as carbon source.

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>OD (540 nm)</th>
<th>EPS (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SS-1</td>
<td>0.113</td>
<td>11.44</td>
</tr>
<tr>
<td>SS-2</td>
<td>0.101</td>
<td>9.54</td>
</tr>
<tr>
<td>SS-3</td>
<td>0.091</td>
<td>7.95</td>
</tr>
<tr>
<td>SS-4</td>
<td>0.093</td>
<td>8.27</td>
</tr>
<tr>
<td>SS-5</td>
<td>0.084</td>
<td>6.83</td>
</tr>
<tr>
<td>SS-6</td>
<td>0.100</td>
<td>9.54</td>
</tr>
<tr>
<td>SS-7</td>
<td>0.132</td>
<td>14.62</td>
</tr>
<tr>
<td>SS-8</td>
<td>0.146</td>
<td>16.85</td>
</tr>
<tr>
<td>SS-9</td>
<td>0.126</td>
<td>13.67</td>
</tr>
<tr>
<td>SS-10</td>
<td>0.140</td>
<td>15.93</td>
</tr>
<tr>
<td>SS-11</td>
<td>0.110</td>
<td>11.13</td>
</tr>
<tr>
<td>SS-12</td>
<td>0.137</td>
<td>15.41</td>
</tr>
<tr>
<td>SS-13</td>
<td>0.141</td>
<td>16.05</td>
</tr>
<tr>
<td>SS-14</td>
<td>0.111</td>
<td>11.28</td>
</tr>
<tr>
<td>SS-15</td>
<td>0.206</td>
<td>26.42</td>
</tr>
<tr>
<td>SS-16</td>
<td>0.116</td>
<td>12.08</td>
</tr>
<tr>
<td>SS-17</td>
<td>0.192</td>
<td>24.17</td>
</tr>
<tr>
<td>SS-18</td>
<td>0.142</td>
<td>16.22</td>
</tr>
<tr>
<td>SS-19</td>
<td>0.180</td>
<td>17.55</td>
</tr>
<tr>
<td>SS-20</td>
<td>0.197</td>
<td>24.96</td>
</tr>
</tbody>
</table>

Optimization of EPS Production

To elucidate the optimal growth medium composition for maximal EPS production, various factors influencing EPS production by the culture *Bacillus* sp. (SS-15) were assessed. Amongst different carbon and nitrogen sources used, sucrose produced the highest amount of EPS, while ammonium chloride was preferred nitrogen source for EPS production (Figure 1a & c). The EPS production increased with increase in sucrose concentration (Figure 1b). Of the various ammonium chloride concentrations used, 0.06 % produced the highest
Figure 1. Effect of carbon and nitrogen sources and concentration on EPS production of *Bacillus* sp. (SS-15).
amount of the EPS. EPS production increased with the increase in phosphate concentration up to 0.15 %. However, further increase in phosphate concentration (> 0.15 %) did not enhance EPS production (Figure 2a). When the growth medium was supplemented with 0.3 % magnesium sulphate, the EPS production was highest (Figure 2b). The EPS production was not effected by the different concentrations of calcium (0.005 to 0.03 %), and further increase (0.03 %) in calcium concentration reduced the EPS production (Figure 2c). EPS production by *Bacillus* sp. (SS-15) decreased with increasing concentration of NaCl (Figure 2d).

**Effect of Culture Condition on EPS Production**

The *Bacillus* sp. (SS-15) was grown at different pH and the EPS production was monitored. At lower pH the quantity of EPS produced was low and increased with the increase in the pH of the medium. At pH 10, maximal EPS was produced (Figure 4a). In another experiment the pH of the medium was adjusted to 10 and the EPS synthesis was measured at room temperature (28 °C) and 50 °C. The *Bacillus* sp. (SS-15) produced EPS at high pH and at high temperature, however the concentration was relatively low as compared to that observed at room temperature (Figure 4b). EPS production by the *Bacillus* sp. (SS-15) was not effected when the culture was grown under static or shaking condition (Figure 4c).

**Growth Kinetics and EPS Production of Bacillus sp. (SS-15)**

*Figure 3* represents a characteristic growth curve of an exopolysaccharide producing fouling bacterium *Bacillus* sp. (SS-15) when grown in BSS medium. The EPS production was measured during the growth of *Bacillus* sp. (SS-15) in
Figure 2. Effect of phosphate (a), magnesium sulphate (b), calcium chloride (c) and sodium chloride (d) on EPS production by Bacillus sp. (SS-15).
batch cultures. The lag phase of the culture was for about 7 h. Thereafter bacterial cells started growing and highest cell biomass was reached at ~ 30 h. This was followed by a fairly long stationary growth phase. EPS production was observed at all stages of culture growth. However, the EPS production was higher during the stationary phase of growth, and its concentration did not shown any decline during 96 h growth period.

![Graph showing growth curve and EPS production](attachment:figure3.png)

**Figure 3.** Growth curve and EPS production by *Bacillus* sp. (SS-15) when grown at room temperature in optimized BSS medium supplemented with 1 % sucrose.

**Purification of EPS**

The elution profile of the EPS by gel filtration showed a single broad peak. The EPS got eluted in the void volume indicating that it is a high molecular weight compound. The EPS was also eluted as a single peak in anion-exchange chromatography (Figure 6).
Table 3. Biochemical characteristics of the culture SS-15.

<table>
<thead>
<tr>
<th>Test</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gram Stain</td>
<td>Gram +ve</td>
</tr>
<tr>
<td>Motility</td>
<td>Motile</td>
</tr>
<tr>
<td>Oxidase</td>
<td>+</td>
</tr>
<tr>
<td>Catalase</td>
<td>+</td>
</tr>
<tr>
<td>Hugh Leifson</td>
<td>Facultative</td>
</tr>
<tr>
<td>Spores</td>
<td>+</td>
</tr>
<tr>
<td>ONPG</td>
<td>-</td>
</tr>
<tr>
<td>Lysine decarboxylase</td>
<td>-</td>
</tr>
<tr>
<td>Ornithine decarboxylase</td>
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</tr>
<tr>
<td>Urease</td>
<td>-</td>
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<tr>
<td>Nitrate Reduction</td>
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<td>H$_2$S</td>
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<tr>
<td>Citrate</td>
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<tr>
<td>Voges Proskauer</td>
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<td>Methy Red</td>
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<td>Indole test</td>
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<td>Melonate</td>
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<td>Geletin Liquification</td>
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<td>Starch hydrolysis</td>
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<td>Esculin</td>
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<td>lactose</td>
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<td>sucrose</td>
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</table>

Viscosity Measurement

Figure 7 shows the flow curves made for different concentration of EPS solution for the viscosity as a function of the shear rate. The viscosity of the EPS solution increased with increasing EPS concentration and showed a small decrease
throughout a range of increasing shear rate from 0 to 1100 per s. Using viscosity data the molecular weight of the EPS was $1.63 \times 10^7$ Da (Figure 8).

Figure 4. Effect of culture conditions (a) pH, (b) temperature (using pH 10) and (c) static and shaking condition on EPS production by *Bacillus* sp. (SS-15).
Culture

Cells (discarded)

Supernatant (cell free broth)

Filtered through 0.7 & 0.2 μm

Ultrafiltration

Precipitate with ethanol

Collect EPS

Dissolved in Dist. H₂O

Dialysed

Precipitate with ethanol

Lyophilized

Chemical Characterization
(IR, NMR, GC, HPLC & chemical analysis)

Figure 5. Flow chart for the isolation of EPS from the bacterial culture
Chemical Characterization of EPS

The EPS composed of carbohydrate, protein, hexosamines, uronic acids, pyruvate, sulphate and inorganic material (Table 4). Capillary gas chromatography analysis showed the presence of glucose, galactose and mannose as monosaccharide in the EPS (Figure 9). Glucose and Mannose were the major components while galactose was the minor component of the EPS (Table 4). The amino acid composition of the EPS of *Bacillus* sp. (SS-15) is shown in Figure 9. Glutamic acid was the most abundant (74 %) whereas other amino acids such as alanine, serine and glycine were minor components of the protein associated with the EPS (Table 5).

The chemical and the monosaccharide composition of the EPS produced by *Bacillus* sp. (SS-15) at high pH and temperature (50 °C) was slightly different than the EPS obtained at ambient temperature (28 °C) and pH 7.5 (Table 4). For example, pyruvate and uronic acid concentrations were slightly low and sulphate concentration was high in the EPS isolated from the culture grown at high pH and temperature (50 °C).

Fourier Transform Infrared Analysis (FTIR)

Figure 10 showed the infrared spectra of the purified EPS. A well knows bands were recorded with in the range of 4000 to 400 cm⁻¹. The FTIR spectra of the EPS exhibit bands at 3309 m⁻¹, 2885 cm⁻¹, 1652 cm⁻¹, 1469 cm⁻¹, 1267 cm⁻¹, 1080 cm⁻¹ and 810 cm⁻¹.
Table 4. Chemical and monosaccharide composition of EPS produced by *Bacillus* sp. (SS-15) grown at 28 °C, pH 7.5 and 50 °C, pH 10

<table>
<thead>
<tr>
<th>Chemical composition (%)</th>
<th>EPS (28 °C, pH 7.5)</th>
<th>EPS (50 °C, pH 10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carbohydrate</td>
<td>66.54</td>
<td>63.36</td>
</tr>
<tr>
<td>Protein</td>
<td>3.86</td>
<td>3.08</td>
</tr>
<tr>
<td>Pyruvate</td>
<td>11.82</td>
<td>8.14</td>
</tr>
<tr>
<td>Sulphate</td>
<td>1.26</td>
<td>2.22</td>
</tr>
<tr>
<td>Hexoamines</td>
<td>0.74</td>
<td>--</td>
</tr>
<tr>
<td>Uronic acid</td>
<td>2.65</td>
<td>0.22</td>
</tr>
<tr>
<td>Inorganic content</td>
<td>2.23</td>
<td>5.67</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Monosaccharides (%)</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Rhamnose</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Fucose</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Ribose</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Arabinose</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Xylose</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Mannose</td>
<td>33.6</td>
<td>34.3</td>
</tr>
<tr>
<td>Galactose</td>
<td>6.4</td>
<td>7.0</td>
</tr>
<tr>
<td>Glucose</td>
<td>60.0</td>
<td>58.7</td>
</tr>
</tbody>
</table>

**DISCUSSION**

**EPS Production by Bacterial Cultures**

Bacteria are known to produce EPS when grown as biofilms and/or as free cells (planktonic biofilms). Twenty bacterial isolates were screened for EPS production. All these cultures produced EPS although at various concentrations. Of these, the culture SS-15 produced the highest amount of EPS, and therefore this culture was identified and further used for detailed study on optimization, isolation and chemical characterization of EPS. Based on the morphological,
biochemical and physiological characterization, the culture SS-15 was tentatively identified up to generic level as *Bacillus* sp. (SS-15) (Table 3).

**Growth and EPS Production by *Bacillus* sp. (SS-15)**

The growth pattern and the EPS production by *Bacillus* sp. (SS-15) resemble in many aspects with the most common exopolymer producing bacteria (Rodrigues and Bhosle, 1991; Majumdar et al, 1999). The kinetic of exopolysaccharide production by *Bacillus* sp. (SS-15) showed that EPS was produced during exponential growth phase and the production continued in the stationary phase of growth. Similar observations were reported for other EPS-producing bacterial strain (Read & Costerton, 1987; Majumdar et al, 1999; Torino et al, 2000; Duenas et al, 2003). The EPS production during the growth of the fouling bacterium appear to be advantageous as it may help in attachment of bacteria to the surface and also to provide protection to the cells in the biofilm (Read & Costerton, 1987). Many studies showed a decrease in the total EPS amount upon prolong incubation (De Vuyst et al, 1998; Pham et al, 2000). It was interesting to note that in my study no polymer degradation was evident during the stationary phase. This implies that the organisms did not utilize the EPS as a carbon source.

**Effect of Nutrients on EPS Production**

A number of physiological conditions (medium composition, physico-chemical and kinetic parameters) are known to influence EPS production by fouling bacteria. The amount of EPS produced by *Bacillus* sp. (SS-15) was influenced by the nutrient concentrations in the medium. For example, the amount of EPS produced by *Bacillus* sp. (SS-15) was influenced by the carbon and nitrogen
sources and concentration (Figure 1). Bacillus sp. (SS-15) grew and produced EPS from a variety of carbon sources. Sucrose was the most efficient carbon source for EPS production, whereas glucose was not an efficient carbon source. Xylose and maltose supported a high growth (OD 540 nm) but produced less EPS. The apparent variations in EPS concentration were probably due to differences in the uptake and metabolism of the substrates by the organisms. Furthermore, some of the metabolic steps involved in EPS synthesis from
different carbon sources may also influence the production of EPS (Linton, 1990; Grobben et al., 1996). In contrast, EPS production by some bacteria was unaffected by the source of carbon (Williams & Wimpenny, 1977). The sugar concentration had a marked effect on EPS yield. Increasing the sucrose concentration resulted in increased EPS production; the maximum EPS production occurred when 50 g of sucrose per liter was used in the medium. The increase in EPS production was not correlated with growth, which remained unchanged. Therefore, it seems likely that sucrose stimulates EPS production by *Bacillus* sp. (SS-15). It has been reported that EPS is produced even when little or no growth occurs (Cerning et al., 1994; Gamar et al., 1997).

*Bacillus* sp. (SS-15) produced relatively high amount of EPS when the growth medium was supplemented with ammonium chloride (1 g/liter) and low yield when glycine was used as nitrogen source. Ammonium chloride and glycine resulted in the different amounts of growth and EPS production indicating the influence of complex and simple nitrogen sources. When grown on organic nitrogen source (glycine) *Bacillus* sp. (SS-15) produced less growth and less EPS yield. In contrast, when grown on simple inorganic N sources (ammonium chloride, ammonium sulphate and sodium nitrate) *Bacillus* sp. (SS-15) produced relatively higher amounts of the EPS. This contrast with the observation that some bacteria produced high EPS yields when grown on complex nitrogen sources (Souw & Demain, 1979; De Souza & Sutherland, 1994).

Bacteria, such as *Xanthomonas*, *Pseudomonas* and *Rhizobium* sp. when grown under nitrogen limiting condition produced high EPS (Sutherland, 1990; Shu & Yang, 1990; Wachenheim & Patterson, 1992). These results compare well with the EPS production by *Bacillus* sp. (SS-15). EPS production by *Bacillus* sp. (SS-15) increased with the decrease in ammonium chloride concentration from 1 %
to 0.06%. However, further, decrease in ammonium chloride concentration resulted in decrease EPS production as well as growth of *Bacillus* sp. (SS-15). This suggests enough nitrogen concentration is necessary for the synthesis of essential cell components, as the cell actively produce exoply saccharides in the presence of an appropriate carbon source (De Vuyst et al, 1998). Therefore, limiting the amount of nitrogen (0.005 %) below saturation level resulted in reduced EPS production as well as growth of *Bacillus* sp. (SS-15).

At low phosphate concentration the EPS production and the growth of *Bacillus* sp. (SS-15) was low. As the concentration of phosphate increased the growth and EPS production also increased. It has been reported that the amount of EPS produced is high in fermentation with pH control as in those without pH control (Mozzi et al, 1996; Kimmel et al, 1998). Lowering the amount of phosphate in the medium reduces the buffering capacity and hence did not enhance polysaccharide production. Thus, a certain surplus of phosphate was apparently required to maintain the pH for *Bacillus* sp. (SS-15) for exopolysaccharide production.

The effect of magnesium on EPS production is not surprising. Magnesium play a vital role in many of the reactions leading to production of cells and of energy (De Souza & Sutherland, 1994), and in several of the reactions yielding biosynthetic precursors of polysaccharides (Sutherland & Norval, 1969). Maazouzi (1991) reported that EPS production, by a strain of *Enterobacter aerogenes* was stimulated in the presence of Mg$^{2+}$. Mg$^{2+}$ ion activates the phosphogulcomutase enzyme, which catalyse the transfer of a phosphate group between carbon $C_1$ and $C_6$ and thus participates in EPS biosynthesis.

The addition of Ca$^{2+}$ did not alter the EPS production, however at high concentration of Ca$^{2+}$ the EPS production was decreased. Very low
concentrations of metal ions are known to stimulate growth and even high concentrations inhibit growth. Metal elements such as calcium are known to serve as enzyme cofactors and an important building block of various cell constituents. It has been reported that calcium ions have a threshold ion concentration beyond which the solution is toxic to bacteria (Kim et al, 2000). Thus the decrease in the EPS may be because of the toxic effect of calcium.

Maximum polysaccharide production was found when the salt concentration was low (0.5%). Increasing salt concentration decreased growth as well as EPS production. This could be because of the composite stress, having both an ionic as well as osmotic component (Lloret et al, 1998).

Effect of Growth Condition on EPS Production by *Bacillus* sp. (SS-15)
The pH of the growth medium influences the production of EPS by *Bacillus* sp. (SS-15). As the pH of the growth medium increased the EPS production and the growth of *Bacillus* sp. (SS-15) also increased. One of the reasons for increased EPS production at higher pH is the result of increasing the time the culture is in the exponential growth phase (Gassem et al, 1997; Gamar-Nourani et al, 1998). Higher pH also results in a longer stationary phase i.e. slowly growing cells which exhibit much slower cell wall polymer biosyntheses of peptidoglycan and teichoic acid, making more isoprenoid lipid carrier precursor molecules available for the EPS biosynthesis (Sutherland, 1972; Degeest et al, 2001). Both growth and EPS production increased with increased in pH of growth medium (Figure 4a). This suggests that EPS production was growth related. Similar results of growth associated EPS-production was observed by Grobben et al, (1995). This suggests that optimizing the growth conditions of bacteria would result in maximal EPS production. Growth associated EPS production
does not occur with some other EPS-producing bacteria. For example, in *Xanthomonas* and *Alcaligenes* sp. no correspondence was recorded in growth and EPS production (Sutherland, 1990).

Optimal condition of temperature results in improved EPS production. Several investigators have found higher EPS production at higher cultivation temperature under optimal growth condition, for instance with respect to pH (Mozzi et al, 1994; 1996a; De Vuyst et al, 1998; Grobben et al, 1998; Gamar-Norani et al, 1998; Petry et al, 2000). It is observed that the growth and EPS production by *Bacillus* sp. (SS-15) was increased with increase in pH to 10, however at high temperature (50 °C) and high pH (10) both the growth and EPS production decreased (Figure 4b). This may be due to limited effects of availability of lipid carriers essential for both cell wall synthesis and EPS production (Sutherland, 1972).

Static and shaking growth did not influence the EPS production by *Bacillus* sp. (SS-15) Since higher EPS yields were obtained even with a lower oxygen tension (De Vuyst et al, 1998; Grobben et al, 1998; Looijesteijn & Hungenholtz, 1999). This implies that aeration was not required for EPS production by *Bacillus* sp. (SS-15).

**Purification of EPS**

The alcohol-precipitated exopolymer was purified using dialysis and treatment with protease and nucleases (DNAase and RNAase). The EPS purity was assessed further using column chromatography. The elution pattern of the exopolymer on sephadex G-200 (Figure 6a) showed one broad peak, which may indicate molecular homogeneity of exopolymer. Further, the EPS eluted in the void volume of the column, as a single peak, indicates that it is a high
molecular weight polysaccharide. Purified polymer was reconstituted in 1ml of 10 mM phosphate buffer (+ 0.1 M NaCl) and further checked for purity by ion exchange chromatography on DEAE-sepharose (Figure 6b). The elution profile also showed a single peak. The presence of single peak after passing through the both the column indicates that EPS contain only one type of polymer. Ethanol precipitated exopolymer obtained by Gorret et al, (2003) showed the presence of two peaks when eluted through a gel permeation and ion exchange chromatography.

Viscosity of the EPS of *Bacillus* sp. (SS-15)

One of the most important prerequisites of a polysaccharide, which determined many of properties useful for industrial utilization are high viscosity of its aqueous solution, capability of forming gel and together with it adequate composition and structure and a high molecular weight (Margaritis & Pace, 1985; Shepherd et al, 1995; De Philippis & Vincenzini, 1998). In this respect, the EPS isolated from *Bacillus* sp. (SS-15) have been tested for viscosity of its aqueous solution. The viscosity of 0.5 % EPS showed a gradual decrease as the shear rate is increased indicating a good pseudoplastic nature of the EPS solution. This property is useful if the EPS is to be used as emulsions, dispersions, and thickening agent (Yalpani & Sandford, 1987; Sutherland, 1990).

Molecular Weight of EPS produced by *Bacillus* sp. (SS-15)

The EPS was retained by dialysis bags with MW cut-off 8,000 as well as by the 10,000 Da ultrafilters indicating that it's molecular weight was in excess of this value. The molecular weight was estimated using viscosity measurement.
Figure 7. Relationship between specific viscosity and the EPS concentrations. Y-intercept value (0.1808) is the specific viscosity of EPS at 0 % concentration. This value was used for the determination of average molecular weight of EPS.

Figure 8. Variation in the viscosity (cP) of 0.5 %, 1 % and 1.5 % aqueous solution of EPS of Bacillus sp. (SS-15) as a function of shear rates (per s)

Molecular mass distributions of all polymers are highly dependent on the viscosity of the polymers (Van den Berg, 1995; Muralidharan & Jayachandran, 2003). The average molecular weight of the EPS produced by Bacillus sp. (SS-15) was $1.63 \times 10^7$ Da.
Chemical Characterisation of EPS

Chemical characterization of the polymer showed the presence of carbohydrates, protein, uronic acid, pyruvate, sulphate, hexosamines and inorganic content (Table 4). The chemical composition of the EPS obtained at 28 °C and pH 7.5 and 50 °C and pH 10 did not show much variation, except for slight increase in the sulphate concentration at high temperature (50 °C) and pH (10). The presence of ~ 65 % of carbohydrates in exopolymer of *Bacillus* sp. (SS-15) indicates the polysaccharide nature of the EPS as reported by others (Fletcher, 1980; Sutherland, 1980; Costerton et al, 1981; Christensen & Characklis, 1990). Capillary gas chromatography analysis of the EPS showed the presence of glucose, galactose and mannose in the ratio 9:1:5. The presence of these sugars suggests that the EPS is a heteropolysaccharide. The EPS produce by each bacterial species is chemically and structurally unique. Further, the growth phase and/or conditions and nutrient status may influence the quality and composition of EPS produced by the same species (Decho, 1990). For example, Rodrigues and Bhosle (1991) reported that EPS produced by *V. fischeri* consisted of glucose, galactose, mannose, arabinose and rhamnose in the ratio of 3.4:3.7:1.5:0.6:0.7. However, Read and Costerton (1987) have reported the presence of glucose and galactose in the ratio 1:1 in the EPS produced by *P. fluorescens*. Two different EPS's i.e. EPS 1 and EPS2 were produced by the same bacterial species *Bacillus thermoantarcticus*. EPS1 was composed of α-D-mannose and β-D-glucose whereas α-D-mannose was present in EPS2 (Manca et al, 1996). Further, medium composition can influence the quality of the EPS produced by bacteria. EPS produced by
Table 5. Amino acid composition of the EPS from *Bacillus* sp. (SS-15)

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Mole %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acidic (sum)</td>
<td>75.42</td>
</tr>
<tr>
<td>aspartic acid</td>
<td>1.29</td>
</tr>
<tr>
<td>glutamic acid</td>
<td>74.13</td>
</tr>
<tr>
<td>Basic (Sum)</td>
<td>1.32</td>
</tr>
<tr>
<td>lysine</td>
<td>0.74</td>
</tr>
<tr>
<td>arginine</td>
<td>0.59</td>
</tr>
<tr>
<td>Neutral (Sum)</td>
<td>21.26</td>
</tr>
<tr>
<td>Hydroxy</td>
<td></td>
</tr>
<tr>
<td>threonine</td>
<td>0.46</td>
</tr>
<tr>
<td>serine</td>
<td>3.05</td>
</tr>
<tr>
<td>Straight</td>
<td></td>
</tr>
<tr>
<td>glycine</td>
<td>3.69</td>
</tr>
<tr>
<td>alanine</td>
<td>11.83</td>
</tr>
<tr>
<td>Branched</td>
<td></td>
</tr>
<tr>
<td>valine</td>
<td>1.08</td>
</tr>
<tr>
<td>isoleusine</td>
<td>0.50</td>
</tr>
<tr>
<td>leusine</td>
<td>0.66</td>
</tr>
<tr>
<td>Aromatic (sum)</td>
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<tr>
<td>tyrosine</td>
<td>0.46</td>
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<tr>
<td>phenylalanine</td>
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</tr>
<tr>
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</tr>
<tr>
<td>methionine</td>
<td>--</td>
</tr>
<tr>
<td>Non-Protein (Sum)</td>
<td>0.71</td>
</tr>
<tr>
<td>ornithine</td>
<td>0.50</td>
</tr>
<tr>
<td>β-ABA</td>
<td>--</td>
</tr>
<tr>
<td>γ-ABA</td>
<td>0.21</td>
</tr>
<tr>
<td>β -Alanine</td>
<td>--</td>
</tr>
<tr>
<td>Amino sugars (sum)</td>
<td>0.26</td>
</tr>
<tr>
<td>Galactosamine</td>
<td>--</td>
</tr>
<tr>
<td>Glucosamine</td>
<td>0.26</td>
</tr>
</tbody>
</table>

-- = traces

*Volcaniella eurihalina* contained different monosaccharide when grown on different medium (Bejar et al, 1996)

A proteinaceous moiety has been found in almost all the polymers investigated. Reported protein content of bacterial exopolymers range between 1 and 18 % (Abu et al, 1991; Robijn et al, 1995; Guezennec et al, 1994; Vincent et al, 1994). The protein content of the EPS produced by *Bacillus* sp. (SS-15) was about 3.5
Figure 9. Monosaccharide (a) and amino acid (b) composition of the EPS from *Bacillus* sp. (SS-15).

The amino acid composition of the EPS showed high abundance of acidic amino acids (75%). Whereas other major amino acids alanine, serine, glycine and valine accounted for 20% of the total amino acids. The presence of these amino acids in the EPS could contribute significantly to the hydrophobic interaction, electrostatic interaction and hydrogen bonding of the macromolecule (Flaibani et al., 1989). It has been observed that removal of protein moiety from
the polymer significantly reduced the adhesive capacity of the polysaccharide (Gantar et al, 1995). Thus the presence of high content of acidic amino acids in the EPS of \textit{Bacillus} sp. (SS-15) may facilitate adhesion through hydrogen bonding and electrostatic or hydrophobic interactions.

![Figure 10. FTIR spectra of the EPS from the Bacillus sp. (SS-15).](image)

The chemically characterized EPS of \textit{Bacillus} sp. (SS-15) showed high content of pyruvate (11 \%) along with uronic acid (2.7 \%) and sulphate (1.3 \%). High amount of pyruvated exopolysaccharide are also found in other bacterial species for e.g. \textit{Methylobacterium} sp. (Verhoef et al, 2003). Polysaccharide characterized by the presence of charged components like uronic acids, sulphate or phosphate and pyruvate are usually most promising for the removal of toxic metals from polluted waters (Mittelman and Geesey, 1985; Kaplan et al, 1987; Bender et al 1994; Loaec et al, 1997; De Philippis et al, 2003). Thus, the EPS of \textit{Bacillus} sp. (SS-15) may play an important role in metal-complexing.
Moreover, selective binding of metals ions by microbial EPS may also play an important role in influencing the microbial corrosion of metals. The involvement of functional groups of EPS in biofouling and biocorrosion processes has been demonstrated by using surface analytical techniques (Geesey et al, 1986; Jolley et al, 1988). Further, EPS containing sulphate groups possess inhibitory properties against various types of viruses (Hasui et al, 1995; Witvrouw & Clercq, 1997) and tumors (Itoh et al, 1993; Riou et al, 1996). If this is so, then the presence of sulphate group in the EPS produced by *Bacillus* sp. (SS-15) may offer similar advantage.

The occurrence of pyruvate and other non-sugar components uronic acid, sulphate and proteins confers an overall negative surface charge and acidic properties to EPS. Further, the amino acid composition of the EPS showed high amount of glutamic acids (Table 5) that will assign acidic property to EPS. Such acidic polysaccharides have been observed in biofilm as well as in the bacteria isolated from the biofilm (Fletcher & Floodgate, 1973; Read and Costerton, 1987; Rodrigues & Bhosle, 1991; Majumdar et al, 1999). From these data it appears that acidic heteropolysacharide are of common occurrence in biofilm developed in various environments.

**FTIR and NMR of EPS**

The FTIR spectrum of the exoploymer (Figure 10) revealed prominent characteristic functional groups. FTIR exhibit a broad O–H stretching band at 3309 cm⁻¹ and sharp C–H stretching band at 2895 cm⁻¹. A shoulder at the high frequency side at 1652 cm⁻¹ and 1469 cm⁻¹ bands could be assigned to the presence of carboxylic groups. In addition, a carbohydrate bands were present between 1000 to 1200 cm⁻¹. The bands at 1080 cm⁻¹ and 1128 cm⁻¹ indicate the
Figure 11. $^1$H (a) and $^{13}$C (b) NMR spectra of the EPS isolated from the *Bacillus* sp. (SS-15)

presence of C–O stretching of the carbohydrates. The overall structural identity of the exopolymers shown by FTIR spectrum was that of a polyhydroxy compounds such as polysaccharides (Abu et al, 1991; Titus et al, 1995; Raguenes et al, 1997; Guezennec et al, 1998; Beech et al, 1999). Beside these,
ester sulphate band were found at 1267 cm\(^{-1}\) and 810 cm\(^{-1}\) (Raguènes et al., 1997; Guezennec et al., 1998).

The whole profile of \(^1\)H-NMR spectrum (Figure 11a) is very similar to that of the polysaccharides as reported by others (Masoud et al., 1995; Kawahara et al., 1998). The \(^{13}\)C NMR spectrum showed multiple signals in the region from 60 to 80 ppm, which is characteristic of sugars resonances (Figure 11b). The signal at 104 ppm corresponds to that of anomeric carbon (C1 of sugar). A small peak present in the 176 ppm region, where carbonyl resonances are observed, that may be due to pyruvate group present in the EPS. Hence result obtained with \(^1\)H-NMR and \(^{13}\)C NMR analyses indicate the polysaccharide nature of exopolymers produced by *Bacillus* sp. The FTIR and NMR analyses were in support of the chemical analyses of the EPS done using several analytical techniques.
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