CHAPTER 3

CHARACTERIZATION OF EXOPOLYSACCHARIDE OF
RHIZOBIUM RADIOBACTER BE1
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*RHIZOBIUM RADIOBACTER BE1*

PART A
CHEMICAL AND PHYSICAL CHARACTERIZATION
CHAPTER SUMMARY

For the determination of complete covalent or primary structure of a polysaccharide, answers must be given to questions of regarding its composition, constitution and configuration. Upon elemental analysis, the EPS/BE1 was found to be composed of carbon: 33.19%, hydrogen, 5.54%, and oxygen, 61.27%. Sulfur and nitrogen were not detected indicating that EPS/BE1 did not contain sulfate and amino groups, respectively.

The EPS/BE1 was found to be anionic in nature as analysed by ion-exchange chromatography. EPS/BE1 contained 84.0% as total sugar, of which 89.72% was reducing sugars. EPS contained 68.25% glucose and 22.2% as uronic acid(s). When the hydrolysate of EPS/BE1 obtained using 2.0 M trifluoroacetic acid for 2 h, was analysed through paper chromatography, glucose and glucuronic acid were found to be present as monosaccharide components. The presence of glucose in the EPS/BE1 as the major component was further confirmed by analysis of the alditol acetates of hydrolysate using GC-MS. The presence of pyruvate and formate were detected using high performance liquid chromatography (HPLC). The overall composition of the EPS/BE1 was found to be: glucose, 68.25%; glucuronic acid, 22.2%; formate, 3.72%, and pyruvate 2.30%.

The infrared and Raman spectra indicated the functional groups characteristic of hydroxyl, carboxyl and linkages of sugar ring.

The methylation analysis of EPS/BE1 indicated that D-Glucuronic acid and α-D-glucose were linked to either 2 or 4 position of β-D-glucose in a trisaccharide repeat unit.

The $^1$H-NMR, and $^{13}$C-NMR of both native and partially hydrolysed EPS/BE1 indicated that in the repeating unit of the EPS/BE1, α-D-GlcpA was linked equatorially (α-) to the β-D-Glcp in the main chain and α-D-Glcp was present as a substitution or branch point on β-D-Glcp.
Further, to determine the nature of linkages present, when the action of enzymes on EPS/BE1 was studied, of the enzymes used, cellulase and glucoamylase did not act on the EPS/BE1. However, α-amylase showed a moderate activity, indicating the presence of α-linkages in EPS/BE1.

On the basis of information obtained through above mentioned physical, chemical and enzymatic methods, the EPS/BE1 is proposed to be comprised of the following repeat units:

\[
\alpha-D-\text{Glc}p (1\rightarrow2) \xrightarrow{\rightarrow4} \alpha-D-\text{Glc}p\beta-D-\text{Glc}p (1\rightarrow)
\]

When the molecular weight of the EPS/BE1 was analysed through gel permeation chromatography, the number average molecular weight (Mn), weight average molecular weight (Mw) and the peak average molecular weight (Mp) were found to be \(1.6 \times 10^6\), \(3.9 \times 10^6\) and \(4.8 \times 10^6\) respectively. The molecular weigh distribution analysis suggested that 70.0% of the EPS/BE1 molecules had a molecular weight of more than \(1.9 \times 10^6\) Da.

The EPS/BE1 exhibited a three-stage thermal decomposition pattern as analysed by thermogravimetric analysis (TGA). EPS/BE1 even at 400°C exhibited only a weight loss of a meagre ~18% indicating its high thermal stability. The activation energies for the stages I (95 to 225°C), II (380 to 420°C) and III (425-500°C) observed in the TGA thermogram were found to be 23.6, 108.6 and 21.2 kJ/mol respectively.

In the Differential Scanning Calorimetry (DSC) thermogram of EPS/BE1 showed, an endothermic peak at 94.9 °C (\(\Delta H = -184.16\) J/g) due to evaporation of water and an exothermic transition at 272.9 °C (\(\Delta H = -127.58\) J/g) and final decomposition around 350 °C.
3.1 INTRODUCTION
The primary structure of an EPS molecule is defined by its monomer composition (both absolute and anomic configurations), the sequence and ring size of the constituting monosaccharides, the location of the glycosidic linkages, and the type and location of non-carbohydrate substituents. Since the polysaccharides exhibit tremendous diversity in composition and irregularity in structure, their characterization becomes a formidable task. Also, because of the interchain interactions, polysaccharide chains are seldom dispersed in solvent as a single chain. Hence, the understanding of the polysaccharide chains is still in its premature state with respect to structure in solid and in solution (Aspinall, 1982; Kajiwara and Miyamoto, 1998). No single technique capable of assigning all the parameters in terms of unique carbohydrate structure is available. Therefore a combination of several techniques is necessary for characterization of an exopolysaccharide molecule (Ruas-Madiedo and de Los Reyes-Gavilan, 2005).

3.1.1 Methods for structural determination

3.1.1.1 Sequencing methods
There is no universal approach for isolation and sequencing of polysaccharides due to the structural differences existing between various types of polysaccharides and the way they are linked. In polysaccharides, the monosaccharide residues are linked through O-glycosidic bonds. Determination of sugar components present in the repeating unit of a polysaccharide is carried out by hydrolyzing the polysaccharide with either acids or methanolic hydrochloride. Since glycosidic bonds have different susceptibilities to acid and the resulting monosaccharides exhibit variable lability under the conditions used, no single hydrolysis method provides a quantitative account for each monosaccharide residue of a polysaccharide. The conditions generally employed for hydrolysis are 2 to 4 M trifluoroacetic acid (TFA) or 2 to 4 N HCl for 3 to 6 h at 100°C. Monosaccharides formed after acid hydrolysis are reduced to alditols with sodium borohydride or its deuterium analogue, followed by acetylation of the alditols using acetic anhydride in pyridine to their alditol acetates. The alditol acetates are then separated and identified on the basis of their characteristic fragmentation pattern through GC-MS. Alternatively, the monosaccharides liberated through acid hydrolysis can be determined using High
Performance Liquid chromatography (HPLC). When hydrolyzed using methanolic hydrochloride, the resulting methyl glycosides can be converted to trimethylsilyl ethers and analyzed by GC-MS (Welply, 1989; Blakeney et al., 1983; Kennedy and Sutherland, 1987; Anumula and Taylor, 1992).

3.1.1.2 Linkage analysis

Each monosaccharide is linked to one of its neighbour through its anomeric carbon C-1 and to other adjacent monosaccharides through the remaining carbon atoms. In case of polysaccharides containing ketoses, such as in sialic acid, there is no anomeric carbon, instead a glycosidic linkage always occurs through C-2. Since the positions of the glycosidic linkages within an oligosaccharide/polysaccharide and the extent of branching can be determined unambiguously by methylation analysis, its use for elucidation of polysaccharide structure has become indispensable today (Anumula and Taylor, 1992).

In methylation analysis, the free hydroxyls (those that are not involved in formation glycosidic linkages to other monosaccharide residues) are methylated using a strong base such as Corey-Chakovsky base (methylsulfinyl carbon anion) in presence of methyl iodide in dimethyl sulfoxide (DMSO) converting the free hydroxyls to alkoxides (Hakamori, 1964; Ciucanu and Kerek, 1984). The permethylated polysaccharides are then hydrolyzed and the newly freed hydroxyls involved in the linkages are then acetylated. The positions of the glycosidic linkages are thus determined by analyzing which carbon atoms are acetylated. These methylated/partially methylated sugars are separated by Gas Chromatography (GC) and identified by chemical ionization or electron impact mass spectrometry (EI-MS) through their characteristic retention time in GC and fragmentation pattern of ions in MS. In pyranoses, C1 and C-5 are involved in glycosidic linkage and formation of ring structure, respectively, and thus they are not free for reaction with methyl iodide. Methylation analysis although provides unambiguous information about the positions of glycosidic linkages between residues, it does not address the sequence of the residues within the polysaccharides. Use of enzymes such as glycanases and exoglycosidases and chemical techniques such as acetylation and smith degradation could also be used to determine the location of linkages in a polysaccharide (Welply, 1989).
3.1.1.3 Anomericity and sequence

In structural elucidation of bacterial polysaccharides, NMR experiments are an important complement to component analysis. The nuclei of interest for NMR analysis are $^1$H and $^{13}$C and in some cases $^{31}$P and $^{15}$N. Proton NMR ($^1$H) provides complete information about anomericity, linkage positions and composition (Welplpy, 1989). For the above purpose, both one dimensional and two dimensional NMR of various types have been successfully used. The $^1$H NMR spectrum of a polysaccharide can generally be divided into three major regions: the anomeric region ($\delta_H$ 4.5-5.5 ppm), the ring proton region ($\delta_H$ 3.1-4.5 ppm) and the alkyl region ($\delta_H$ 1.2-2.3 ppm). In order to obtain a good $^{13}$C NMR spectrum, concentrated samples are required. The sensitivity of the $^{13}$C-NMR spectroscopy is lower due to the low natural abundance (1.1%) and lower magnetogyric ratio of the $^{13}$C nucleus.

The $^{13}$C NMR spectrum can also be divided into different regions. The anomeric carbons are found at $\delta_C$ 95 – 110 ppm, the ring carbons at $\delta_C$ 50 – 85 ppm, the alkyl carbons at $\delta_C$ 15 – 25 ppm and the carbonyl carbons at $\delta_C$ 165 - 180. The chemical shifts of the carbon resonances for substituted positions are shifted downfield compared to the corresponding unsubstituted monosaccharides by about 6-10 ppm. Molecular recognition (affinity binding) by the use of monoclonal antibodies against the carbohydrate, use of lectins which bind like antibodies and FABMS which provides precise molecular weight of the oligosaccharide have been suggested as other methods for determination of anomericity of linkages present in polysaccharides (Welplpy, 1989; Aspinall, 1982; Yalpani, 1988).

3.1.1.4 Thermogravimetric Analysis (TGA)

Thermogravimetric analysis is a technique in which the mass of a substance is measured as a function of time or temperature while the substance is subjected to a controlled temperature program. Because mass is a fundamental attribute of a material, any change in mass is more likely to be associated with a chemical change, which may in turn reflect a compositional change and ultimately the thermal stability of the polymers. The changes in mass are a result of the rupture and/or formation of
various chemical and physical bonds at elevated temperatures either due to random coil scission or systematic chain scission, or a combination of two that lead to the evolution of volatile products or the formation of heavier reaction products. Thermogravimetric curves are characteristic for a given polymer or compound and depends on its molecular structure.

3.1.1.5 Differential scanning calorimetry (DSC)
Differential scanning calorimetry measures the excess apparent specific heat of a system ($\Delta C_p$) in a continuous manner as a function of temperature at a fixed scan rate. Biologically relevant macromolecular and polymolecular structures in their native state are stabilized by numerous weak forces and usually undergo conformational and/or phase transitional changes on heating and/or cooling in the temperature range of -20 to 130 °C. Through DSC macroscopic thermodynamic parameters can be related to the microscopic structural/conformational changes. Assuming that there is no difference in the heating rates for the sample and the reference and that the DSC curve returns to the original base line after the transition the enthalpy can be described in the following way:

$$\Delta H = \int C_p dT = \int \left( \frac{K \Delta T}{q} \right) dT$$

Where, $\Delta H$ is the enthalpy of transition, $C_p$ is the heat capacity, $T_i$ and $T_f$ refer to the initial and final temperatures of the transition, $K$ is the thermal conductivity, $\Delta T$ is the difference in temperature between the reference material and the sample. Below the glass transition temperature, $T_g$, an amorphous polymer has characteristics of a glass, whereas it becomes more rubbery above the $T_g$. On the molecular level, the $T_g$ is the temperature of the onset of motion of short chain segments. Either an increase in heat capacity at $T_g$ due to the onset of these additional molecular motions or melting of a crystalline solid results in an endothermic peak in the DSC curve. Today, DSC has become an integral part of the studies on thermal characteristics of the polysaccharides (Chowdhry et al., 1989; Sandler et al., 1998).
3.1.1.6 Composition and structure of bacterial polysaccharides

Exopolysaccharides have considerable heterogeneity in their structure from simple α-(1-4) linked unbranched glucose polymers called dextrans to the highly complex branched and substituted heteropolysaccharides made up of oligosaccharide repeating units such as xanthan. Exopolysaccharides have substitutions (normally as an ester or N-linked) such as pyruvate, acetate, formate, sulfate, phosphate and other side groups.

Part of structural diversity of exopolysaccharides is due to the fact that two identical sugars can bond to form 11 different disaccharides. Additionally exopolysaccharides contain a wide variety of sugars such as glucose, galactose, xylose mannose, rhamnose, uronic acids (glucuronic, galacturonic, mannanuronic, guluronic) hexosamines, aminouronic acids, aldoses, diaminohexoses, 2,3 diamino-2,3-dideoxyuronic acid and 5,7-diamino-3,5,7,9-tetraideoxyxynulosonic acid. Furthermore, the non-carbohydrate side groups found in the bacterial EPS add to their heterogeneity. As a consequence, the diversity found in bacterial polysaccharides far exceeds that of proteins and is reflected in the hundreds of O-antigen serotypes of enterobacteria (Weiner, 1995). Compared to 20 different monosaccharide residues observed in plant and animal polysaccharides, more than 100 different monosaccharide components and 50 different non-sugar compounds have been identified in bacterial polysaccharides, providing enormous structural diversity (Lindberg, 1998).

3.1.1.7 Exopolysaccharides of Agrobacterium sp.

As already discussed in Chapter 2, Young et al. (2001) amalgamated all species of Agrobacterium Conn 1942 and Allorhizobium undicola de Lajudie et al. (1998) into a single genus Rhizobium. As a consequence, the new combination of Rhizobium radiobacter (Young et al., 2001) encompassed the strains previously allocated to Agrobacterium radiobacter and Agrobacterium tumefaciens. Hence, in this section, only the exopolysaccharides found hitherto in Agrobacterium sp. has been described without going into the details of exopolysaccharides produced by other Rhizobium sp. Most of the Agrobacterium strains produced succinoglycan and curdlan-type polysaccharides having octasaccharide repeat units (Hisamatsu et al., 1978 a,b; 1982; Zevenhuizen, 1983). Succinoglycan contained an octasaccharide repeat unit composed of glucose and galactose in a molar ratio of 7:1 with succinate, pyruvate...
and acetate as substituents, whereas curdlan is a β-(1,3) glucan (Harada, 1965). Significantly, the capacity of *Alcaligenes* and *Agrobacterium* to produce succinoglycan was reported to be unstable (Hisamatsu et al., 1978a,b).

When a search was made in **Bacterial Carbohydrate Structural Database** (BCSDB, accessible at [http://www.glyco.ac.ru/bcsdb/start.shtml](http://www.glyco.ac.ru/bcsdb/start.shtml)), out of a total of 8331 entries for bacterial polysaccharides, only 12 records were found for *Agrobacterium radiobacter* whereas around 81 records were obtained for *Rhizobium* sp. Among the 12 different polysaccharides, irrespective of whether O-polysaccharides or EPS, sugars such as Glc, rha, Fuc, Man, Gal, and Fru were found to be present along with non-sugar substituents such as acetate, pyruvate and succinate, either individually or in combinations with one another. Half esters of methylmalonic and succinic acid were also present in the cyclic β-1,2 D-glucans from *Agrobacterium* and *Rhizobium* species (Harada, 1979). Surprisingly, the repeat unit of an EPS of *A. radiobacter* ATCC 53271 had 17 glycosyl residue/unit.

Succinoglycan and acidic polysaccharides of *A. radiobacter* ATCC 53271 were branched, the branches bearing single or more residues (Matulova et al., 1994; O'Neill et al., 1992). Because of emulsion stabilizing, suspending or thickening abilities, succinoglycan is being produced industrially by Shell and marketed as Alphaflo. Moreover, cyclic β-1,2 glucans have also been isolated from *Agrobacterium* sp. These glucans are known to be localized within the periplasmic space and play an important role in the osmotic adaptation of the bacteria and in the virulence of *Agrobacterium tumefaciens*. The β-(1,4) glucans present in Rhizobia and *Agrobacterium* may function in anchoring these species to the plant cell and thus to facilitate them during the infection process (Zevenhuizen, 1997; Stredansky, 1999).

### 3.2 THE PRESENT STUDY

Although, in literature, chemical, physical and rheological characteristics of a large number of exopolysaccharides have been reported, the applications of most of such polysaccharides are still unknown. Also, it is difficult to relate the chemical structure an EPS elucidated to its physical functionality. Further the number of potential polysaccharide structures is almost limitless but in practice many such polymers are
unlikely to possess useful physical properties (Sutherland, 1996). To realize the potential application of EPS of *R. radiobacter* BE1, a detailed physical, chemical and rheological characterization was undertaken.

### 3.3 MATERIALS AND METHODS

#### 3.3.1 Cultivation and extraction of EPS/BE1

*Rhizobium radiobacter* BE1 was cultivated in synthetic medium (pH 7.0) containing (g/l): sucrose, 15.0; KNO₃, 1.0; K₂HPO₄, 0.5; MgSO₄.7H₂O, 0.2; CaCl₂.2H₂O, 0.1, and NaCl, 0.2. The flasks (50 ml medium in 250 ml Erlenmeyer flasks) were inoculated with a suspension of *R. radiobacter* BE1 cells (A₆₀₀= 0.5) at 15.0% (v/v) level and incubated on a rotary shaker at 200 rpm and 30±1°C on an orbital shaker (200 rpm) for 72 h. The culture was diluted 20 times with distilled water and centrifuged at 10 000 g for 30 min at 30±1°C to remove bacterial cells. EPS/BE1 from the cell free supernatant was precipitated by adding 3 volumes of acetone. The precipitate was redissolved in distilled water and reprecipitated. The above step was repeated thrice and then dialysis of the EPS/BE1 solution (in distilled water with three changes at every 12 h) was carried out to remove any low molecular weight ingredient of the culture broth present. The precipitate was further dried at 50±1°C to a constant weight.

#### 3.3.2 Protein estimation

Protein was estimated by the method of Bradford et al. (1976), using bovine serum albumin as standard. To 0.10 ml of sample, 1.0 ml of Coomassie Brilliant Blue G-259 reagent (10 mg/100 ml, made in a mixture of water, absolute ethanol and O-phosphoric acid in the ratio of 8:1:1) was added. After incubation for 2 min, the absorbance was noted at 595 nm.

#### 3.3.3 Elemental analysis

Elemental analysis of EPS/BE1 was carried out on a Perkin Elmer 2400 CHNS analyzer by first decomposing the polysaccharide at high temperature and the estimating the combustion products using a Thermal Conductivity Detector (TCD). Prior to use, the system was calibrated using acetonitrile supplied by Perkin Elmer.
3.3.4 Determination of ionic nature

The ionic nature of EPS/BE1 was determined by measuring its efficiency of binding to anion (Dowex 1) and cation (Dowex 50) exchange resins as described previously by Ashtaputre and Shah (1995). Resins (5.0 g each) were activated by treating with either NaOH (1.0 N) or HCl (1.0 N), washed with water and then equilibrated with appropriate buffers of 10X strength (100 mM) i.e. Tris HCl (pH 8.9) for Dowex 50 and acetate buffer (pH 5.0) for Dowex 1. Columns (1 x 6 cm) were packed with the resins and 0.5 mg/ml EPS/BE1 was loaded on to each of the columns. EPS was then eluted with the respective buffers (10 mM), and the fractions (0.5 ml each) were collected were subjected to total sugar estimation using anthrone reagent. The bound polymer was quantitated using a calibration curve.

3.3.5 Estimation of total sugars

Total sugars were estimated by the phenol-sulfuric acid method of Dubois et al. (1956). To various aliquots of EPS/BE1 sample (1% w/v), 1.0 ml of 5% phenol was added and mixed. Using a fast flowing pipette, 5.0 ml of concentrated H₂SO₄ was added directing the stream of acid onto the surface of the liquid and shaking the tube simultaneously. The tubes were allowed to stand for 10 min, cooled to 30±1°C and the absorbance was measured at 488 nm. Glucose was used as the reference sugar in the range of 20 to 100 µg.

3.3.6 Estimation of total neutral sugars

Neutral sugars in EPS/BE1 were estimated using the method of Morris (1948). Various aliquots (up to 1.0 ml) of polysaccharide samples (1.0% w/v) were mixed with 5.0 ml of anthrone reagent [200 mg in 100 ml of 75% (v/v) H₂SO₄] and heated at 95°C for 10 min. The reaction mixture was cooled in ice and the readings were noted down immediately at 625 nm. Glucose was used as the reference sugar in the range of 10 to 100 µg.

3.3.7 Estimation of reducing sugars

Reducing sugars were estimated by the method of Miller (1959). To 3.0 ml of sample in test tubes, 1.0 ml of DNS reagent was added and the test tubes were then placed in boiling water bath for 5 min, cooled to room temperature and the absorbance at 540
nm was measured. Glucose (up to 250 μg) was used as standard. DNS reagent was prepared by mixing solutions, (A) 300 g sodium potassium tartarate in 500 ml distilled water, and (B) 10.0 g of 3, 5 dinitrosalicylic acid in 200 ml of 2 M sodium hydroxide. The final volume of the solution was made up to 1.0 l with distilled water.

3.3.8 Estimation of Uronic acid
Uronic acid content in EPS/BE1 was estimated using the method of Bitter and Muir (1962). To 1.0 ml of sample, 5.0 ml of borate reagent (0.025 M sodium tetraborate in concentrated sulfuric acid) was added, mixed, heated in a boiling water bath for 10 min and cooled to room temperature. 0.2 ml of carbazole reagent [0.125% (w/v) in ethanol] was then added and the mixture was again heated for 15 min in a boiling water bath, cooled and the absorbance was taken at 530 nm. Glucuronic acid (5 to 100 μg) was used as the standard.

3.3.9 Determination of amino sugars
The amino sugars in EPS/BE1 was estimated using the method of Rondle and Morgan (1955). The pH of the hydrolyzed EPS/BE1 samples (1.0% w/v) and the standard sugar (glucosamine: 5 to 150 μg) was adjusted to 8.0±0.2 with 1.0 N NaOH. Acetylacetone (1.0 ml) was added to 1.0 ml aliquot, heated at 95°C for 20 min and cooled to room temperature. Subsequently, 5.0 ml of ethanol and 1.0 ml of dimethyl aminobenzaldehyde reagent (1.3% w/v in ethanol: HCl, 1:1) were added, incubated at 70°C for 10 min and the absorbance was measured at 530 nm.

3.3.10 Estimation of pyruvate
Pyruvyl content in EPS/BE1 was estimated by the method described by Slonekar and Orentas (1962). EPS/BE1 solutions of various concentrations (2.0 ml each) were hydrolyzed in 1 N HCl for 3 h, and incubated with 1.0 ml of 2,4, dinitrophenyl hydrazine reagent (0.5% w/v in 2 N HCl) for 5 min. The reaction mixture was extracted with 5.0 ml of ethyl acetate and the aqueous layer was discarded. The ethyl acetate fraction was further extracted with 5.0 ml of 10% (w/v) Na₂CO₃ and the concentration of pyruvic acid was determined by measuring the absorbance at 375 nm. Pyruvic acid was used as a standard in concentration range of 0.01 to 0.05 μg/ml.
3.3.11 Estimation of acetyl content

The estimation of O-acetyl groups in the EPS/BE1 was carried out as described by Hestrin (1949), using acetylcholine (0.1 to 1.0 mg) as a standard. To 1.0 ml aliquot of hydrolyzed polysaccharide sample (1.0% w/v), 2.0 ml of freshly prepared alkaline hydroxylamine reagent (hydroxylamine-HCl 2.0 M and NaOH 3.5 N, 1:1) was added. The pH of the reaction mixture was adjusted to 1.2 ± 0.2 using 6.0 N HCl, 1.0 ml of FeCl₃ solution was added and the absorbance was read at 540 nm.

3.3.12 Complete hydrolysis of EPS

Hydrolysis of the EPS/BE1 (1.0% w/v) was routinely carried out using 2.0 M trifluoroacetic acid (TFA) at 100°C for 2 h unless otherwise mentioned. TFA was evaporated in vacuo, the residue was redissolved in distilled water and used for further analysis.

3.3.13 Partial hydrolysis of EPS

Partial hydrolysis of the EPS/BE1 (4.0% w/v) was carried out using 0.2 M TFA at 100 °C for 1 h. TFA was evaporated in vacuo and the residue was lyophilized.

3.3.14 Paper chromatography

Paper chromatography of the EPS/BE1 hydrolysates (2.0 M TFA, 100°C) was performed on a Whatman No. 1 filter paper using the solvent system, n-butanol: pyridine: water (6:4:3). The developed chromatograms were dipped (for 1 min) into silver nitrate reagent. After drying, the chromatograms were sprayed with ethanolic 0.5 N NaOH solution to visualize the spots. For preparing silver nitrate reagent, to 15.0 ml of a saturated aqueous solution of AgNO₃, three liters of acetone was added and the precipitates formed were then dissolved by adding 70.0 ml distilled water.

3.3.15 Glucose estimation

Glucose content in EPS/BE1 hydrolysates was measured by the glucose oxidase-peroxidase method of Trinder (1969) using a Glucose Kit (Qualigens Diagnostics, Mumbai, India). An aliquot (10 µl) of EPS/BE1 hydrolysate was incubated with 1.0 ml of enzyme reagent containing: glucose oxidase, peroxidase, aminoantipyrine and phenol in phosphate buffer for 15 min at 37°C following which, absorbance at 505 nm
was recorded. Glucose was used as a standard in the concentration range of 10 to 50 μg.

3.3.16 Preparation of alditol acetates
Two hundred μl of an aqueous solution of EPS/BE1 containing ~4.0 mg of hydrolysed EPS/BE1 (2.0 M TFA, 100°C for 2 h) with or without added meso-inositol (72 μg) was lyophilized in a dry vial. To the above lyophilized sample, 100 μl of freshly prepared solution of sodium borohydride (20 mg/ml in 3.0 M NH₄OH) was added, mixed vigorously and allowed to stand for 1 h at 30±1°C. The reaction was stopped by adding glacial acetic acid (10 μl) until bubbling ceased. Subsequently, 400 μl of methanol was added and the mixture was evaporated to dryness (repeated 3 times). After drying the contents, 200 μl of pyridine:acetic anhydride mixture was added and incubated at 100°C for 10 min and then evaporated to dryness. Prior to analysis by GC-MS, the contents were dissolved in 25 μl of acetone. Similarly prepared alditol acetate of glucose was used as a standard for GC-MS analysis.

3.3.17 GC-MS analysis
GC-MS analysis of the alditol acetate derivatives of the EPS/BE1 hydrolysate was performed on a Perkin Elmer Autosystem XL equipped with a flame ionization detector and coupled with Perkin-Elmer Turbo Mass spectrometer. The samples were analyzed using a PE-5MS column (Perkin-Elmer, 30 m length and ID 0.25 mm) using the following temperature program: after injection of the sample (4.0 μl) the initial temperature was held at 100°C for 2 min, then increased to 240°C at a rate of 8.0°C per min and finally maintained 240°C for 8.0 min. Throughout the analysis, the injector temperature was maintained at 250°C. The mass spectra obtained were compared against a library of standard mass spectra of sugars.

3.3.18 High performance liquid chromatography
High performance liquid chromatography (HPLC) of the EPS/BE1 hydrolysates for detecting the presence of organic acid(s) in the EPS/BE1 was performed on a Merck HPLC system using RP-18 reverse phase column. Aqueous H₃PO₄ at a flow rate of 1.0 ml/min was used as mobile phase. 20 μl of either authentic standards of respective
organic acids (50 mM) or EPS/BE1 hydrolysate (10 mg/ml) was injected and the peaks were detected using a UV detector at 210 nm.

### 3.3.19 Methylation analysis

50 mg of EPS/BE1 was suspended in 25.0 ml of dry DMSO, stirred at ambient temperature for 15 min and then sonicated at 40°C for 10 min. The above step was repeated until a clear solution of EPS/BE1 was obtained. To the above solution, 250 mg of pulverized NaOH and 25.0 ml of methyl iodide were added and incubated for 30 min at 30±1°C. The methylation reaction was stopped by adding 25 ml of cold water. The excess methyl iodide from the reaction mixture was removed by bubbling a stream of nitrogen through the solution. The permethylated product formed was extracted with dichloromethane and used for linkage analysis. The permethylated products were initially hydrolyzed using 2.0 M TFA for 2 h at 100°C, converted to alditol acetates, and subsequently analyzed using GC-MS.

GC-MS analysis of the methylated alditol acetate derivatives of the EPS/BE1 hydrolysate was performed on a Perkin Elmer Autosystem XL equipped with a flame ionization detector and coupled with Perkin-Elmer Turbo Mass spectrometer. The samples were analyzed on a PE-5MS column (Perkin-Elmer, 30 m, ID 0.25 mm) using the following temperature program: after injection of the sample (4.0 μl), the initial temperature was held at 150°C for 10 min, then increased to 250°C at a rate of 5.0°C per min and finally maintained at 250°C for 20 min. Throughout the analysis the injector temperature was maintained at 250°C. The mass spectra obtained were compared against a library of standard mass spectra of sugars.

### 3.3.20 Infrared spectroscopy

The infrared (IR) spectrum of KBr pellet of EPS/BE1 was obtained using Perkin Elmer FTIR spectrometer.

### 3.3.21 Raman spectroscopy

The FT-Raman spectra were recorded (at 28±1°C) on a Bruker FRA 106 FT-spectrometer using a Nd:YAG laser with an excitation wavelength of 1064 nm.
3.3.22 NMR spectroscopy
The NMR spectrum of the native EPS/BE1 was recorded at 70°C in D_2O (1.0% w/w) using a Bruker 200 NMR spectrometer. The NMR spectrum of partially hydrolyzed EPS/BE1 was recorded in D_2O on a Jeol GSX-400 spectrometer at 23°C and 75°C. For both native and hydrolysed EPS/BE1 samples, ^1H NMR peaks were recorded relative to the water peak at δ 4.8 ppm.

3.3.23 Action of cellulase
The assay systems containing 1.0 ml of EPS/BE1 and other polysaccharide solutions (5.0 and 10.0 g/l), and 15 units of cellulase enzyme from Penicillium funiculosum in 10 mM citrate buffer (pH 5.0) were incubated at 37°C for 30 min. The released reducing sugar was estimated using DNS reagent as described by Miller (1959).

3.3.24 Action of amylases
The effect of glucoamylase and α-amylase on EPS/BE1 and other polysaccharides was checked separately by incubating EPS/BE1 as well as other polysaccharides with either 20 units of glucoamylase or 15 units of α-amylase in 0.01 M citrate buffer (pH 5.0) and 0.10 M phosphate buffer (pH 7.0) respectively, at 30°C for 1 h. The released reducing sugar was estimated using DNS reagent as described by Miller (1959).

3.3.25 Thermogravimetric analysis (TGA)
TGA thermogram was recorded in a temperature range of 25 to 600°C using a Shimadzu thermal analyzer DT 30B at a heating rate of 10°C min^{-1} and under nitrogen atmosphere.

3.3.26 Differential scanning calorimetry (DSC)
DSC analysis of the EPS/BE1 was carried out on a Shimadzu DSC 50 instrument in the presence of nitrogen gas at a heating rate of 10°C min^{-1}.

3.3.27 Determination of molecular weight by gel filtration column chromatography
Gel filtration of EPS/BE1 was carried out on Sepharose 4B column (1.8x50 cm). Samples (250 μg) were loaded onto the column and eluted with 10 mM phosphate
buffer (pH 7.2 ± 0.05). Fractions of 2.0 ml were collected and subjected to total sugar estimation by the anthrone method (Morris, 1948). While blue dextran was used to determine the void volume of the bed, dextrans of different molecular weight ranging from 9000 to 2×10^6 Da were used as standards.

3.3.28 High performance gel permeation chromatography (GPC)
The gel permeation chromatography of the EPS/BE1 was carried out using Waters 2690 Alliance System equipped with RI detector 2410 and TSK GEL type PW 3000, 4000 and 5000 columns. 100 μl of either dextran molecular weight standard or EPS/BE1, both at a concentration of 0.1% (w/v), was injected into the column. NaNO₃ solution (0.2 M) was used as mobile phase at a flow rate of 1 ml/min and the peaks were detected using refractive index detector. Throughout the analysis, the oven temperature was maintained at 30°C. The data were analyzed using Millenium³² software.

Chemicals
Enzymes, standard sugars, dextrans, xanthan gum, gel filtration and ion exchange resins and assay reagents were obtained from Sigma Chemical Co., Missouri, USA. All the chemicals used were of analytical grade and the solvents used for spectroscopy were of spectroscopic grade, which were obtained locally.

Reproducibility
All the experiments have been carried out in duplicates and have been performed three times.

3.4 RESULT AND DISCUSSION

3.4.1 Purification of EPS/BE1
For characterization of an EPS, the sample used should be free from extraneous contaminants such as cells of the producer organism, protein and nucleic acids. After cell removal, repeated precipitation of EPS and dialysis, no protein either of soluble or insoluble (cellular) nature was detected indicating that the cells from the EPS were removed completely. In an earlier report, using a similar approach involving protein
estimation, the quality of the purified exopolysaccharides from a *Pseudomonas* sp. was confirmed (Manresa et al., 1987).

### 3.4.2 Elemental Analysis

Upon elemental analysis, the purified EPS/BE1 was found to be composed of carbon: 33.19%, hydrogen: 5.54% and oxygen: 61.27%. Theoretically, an oxygen content of 44.4% was expected in the EPS/BE1, if it was solely composed of glucose. However, the higher content of oxygen present (61.27%) indicated that EPS/BE1 was a heteropolysaccharide composed of sugars having either carboxyl groups or carboxyl side chain substituents or sulfate. However, absence of sulfur indicated that EPS/BE1 was not sulfated. Similarly, nitrogen was also not detected indicating that (i) the EPS/BE1 did not contain amino sugars, and (ii) the EPS sample used for the chemical analysis was free from proteins, nucleic acids or cellular content.

### 3.4.3 Ionic nature

A large number of native polysaccharides carry anionic (carboxyl, phosphate and sulphate) or cationic functions. Anionic polysaccharides are more predominant compared to cationic. Chitosan bearing amine functions is one of the few such cationic polysaccharides. The number, position and degree of dissociation of the ionic polyelectrolyte groups influence the intrinsic viscosity, solubility, degree of aggregation, gel forming capacity, and the susceptibility of the polyelectrolytes to enzymatic degradation (Yalpani, 1988).

The EPS/BE1 could be precipitated using a quaternary ammonium compound, cetyl pyridinium chloride (1.0% w/v), indicating that the EPS was anionic and acidic in nature (Scott et al., 1965; Danishefsky, 1970; Kumar et al., 2004). Also, the ability of the insoluble complex to be redissolved as a major fraction indicated that the EPS/BE1 was a single complex heteroglycan. The anionic nature of EPS/BE1 was further confirmed by ion exchange chromatography. Almost 86% of the EPS loaded on to the column of anion exchange resin remained bound to the resin, whereas only 4.0% got bound to the cation exchange resin. Anionic nature of a polymer conferred onto it properties such as lower retention on the rock surfaces and a low reactivity with the reactive dyes. EPS/BE1 being anionic has potential for use in oil exploration (Parker, 1983) and textile printing (Lapasin and Pricl, 1999).
3.4.4 Gross chemical composition

Although several advanced methods for detection and determination of sugars are available today, often the use of a combination of simple (e.g. paper chromatography and TLC) as well as advanced methods (e.g. GC-MS, HPLC, and HPTLC) is required for the determination of chemical composition of polysaccharides as evidenced by the reports available. For characterization of exopolysaccharides of *Butyrivibrio fibrisolvens* (Stack, 1988), *Pseudomonas putida* and *Pseudomonas fluorescence* (Read and Costerton, 1987) apart from the GLC of alditol acetates of the hydrolysates, monosaccharides were also identified using TLC. Similarly, high performance thin layer chromatography (HPTLC) was used to identify the components of a novel EPS of *Azotobacter vinelandii* (Vermani et al., 1995). Moreover, colorimetric methods belonging to both chemical and enzymatic category have been widely used for quantitative determination of individual sugars either directly in the EPS itself or in its hydrolysate (Manresa et al., 1987; Kennedy and Sutherland, 1987).

Total sugar content in the EPS/BE1 was found to be 84.0 % (w/w) and 81.9 % (w/w) as estimated by phenol-sulfuric acid method (Dubois et al., 1956) and anthrone method (Morris et al., 1948) respectively. The difference in the values obtained for the total sugar content of EPS/BE1 using the above methods was due to the difference in the reactivity of various classes of sugars to the respective reagents. For example, the anthrone reagent does not react with hexuronic acids and hexosamines and provides essentially neutral sugar content (Herbert et al., 1971). Reducing sugar content in the EPS/BE1 was found to be 89.72 %, which correlated well with the values obtained using total sugar methods. Glucose content of the EPS/BE1 as estimated using glucose oxidase-peroxidase method was found to be 68.25%, which accounted for 76.06% of the total reducing sugar content in the EPS/BE1.

Since during hydrolysis using strong acids the uronic acids are subjected to decomposition by decarboxylation, their accurate determination in a polysaccharide is not an easy task. Most of the methods used for uronic acid determination either individually (Dische, 1962; Bitter and Muir, 1962) or in their mixtures (Knutson and Jeans, 1968) depend upon the use of either carbazole reagent or meta-hydroxydiphenyl reagent (Blumenkrantz and Asboe-Hansen, 1973). Due to the
increased sensitivity for the detection of uronic acids such as mannuronic acid and glucuronic acid (Sutherland, 1970), a modified carbazole method described by Bitter and Muir (1962) was used for determination of uronic acid in the EPS/BE1. Uronic acid content in the EPS/BE1 was found to be 22.2%, which was comparatively high for an EPS. With the exception of bacterial alginate and gellan (containing glucuronic acid up to 21%), no other bacterial exopolysaccharide of industrial interest contains such a large amount of uronic acid (Guezenec, et al., 1994). Since uronic acids along with ester-linked substituents such as sulfates and pyruvate ketals were found to confer a net negative charge to polymer, the ion/metal binding characteristics exhibited by EPS/BE1 might be attributed to its high uronic acid (Guezenec et al., 1994). Significantly, no acetyl content was detected in the EPS/BE1.

3.4.5 Hydrolysis of EPS/BE1

In order to determine their monosaccharide composition, glycosidic linkages present in polysaccharides are usually hydrolysed using strong acids such as H2SO4 or HCl (Kennedy and Sutherland, 1987; Shanta Raju et al., 2001; van Casteren, et al., 1998). Use of such strong acids for hydrolysis of EPS has disadvantages such as the degradation of aldopentoses, deoxy sugars, uronic acids (through decarboxylation) and loss of acetyl groups in N-acetylated sugars. Hence, in the present study, 2.0 M trifluoroacetetic acid (TFA) was used for the hydrolysis of uronic acid containing EPS/BE1. The effective hydrogen ion concentration for 2.0 M TFA (derived from the equation \( H_0 = -\log_{10} h_0 \), where \( H_0 \) is the Hammett acidity function) was 6.92 (Tait et al., 1990). The significance of the above parameter is that for a range of polysaccharides the rate of hydrolysis was proportional to \( h_0 \) (Tait and Sutherland, 1990). In an earlier report, for determination of monomeric composition, an EPS of Pseudomonas sp strain ATCC 53923 was hydrolysed using 2.0 M TFA for 4 h at 120 °C (Dasinger, 1994).

In case of EPS/BE1, in order to determine the time required for complete hydrolysis using 2 M TFA at 100°C, initially, hydrolysis was carried out for various time intervals up to six hours. When the extent of hydrolysis was determined using paper chromatography, as the time of hydrolysis was increased from 0.5 h to 5 h, the smear that was observed in the hydrolysate obtained after 0.5 h disappeared progressively
and after 2 h almost complete hydrolysis of the EPS was observed. Compared to native EPS/BE1, since partially hydrolyzed EPS would contain reducing ends, all oligosaccharides obtained as partial hydrolysis products are reducing substances (Aspinall, 1982). Hence, when the reducing sugar content was estimated in the hydrolysates, beyond 2 h of hydrolysis, no further increase in reducing sugar content in the hydrolysate was observed, supporting the results obtained from paper chromatography. Hence, in the present study, routinely hydrolysis was carried out using 2.0 M TFA for 2 h at 100°C.

3.4.6 Paper chromatography
For preliminary check on the likely composition of the EPS hydrolysates, paper chromatography of the hydrolysates can be carried out (Kennedy and Sutherland, 1987; Shanta Raju et al., 2001; Linker and Jones, 1966). When the hydrolysate (2.0 M TFA, 2 h) of EPS/BE1 was analyzed for the monosaccharides through paper chromatography, glucose (spot ‘a’ with Rf 0.388) was found to be present as major component (Figure 1).

Figure 1. Paper chromatogram of EPS/BE1 hydrolysate

EPS/BE1 was hydrolyzed using 2.0 M trifluoroacetic acid (TFA) at 100°C for various time periods. 2.0 μl of authentic standards (10 mg/ml) and lyophilized hydrolysates (50 mg/ml) were applied on Whatman No. 1 filter paper and the chromatograms were developed using solvent system: n-butanol: pyridine:water (6:4:3). Spots were visualized using silver reagent. (A) Spot no. 1=glucose, 2= galactose, 3=fructose, 4= hydrolysate of EPS/BE1 (0.5 h), 5=hydrolysate of EPS/BE1 (1.0 h), hydrolysate of EPS/BE1 (2.0 h), hydrolysate of EPS/BE1 (3.0 h), and hydrolysate of EPS/BE1 (5.0 h). (B) 9=glucuronic acid, 10=galacturonic acid, 11=mannuronic acid, 4= hydrolysate of EPS/BE1 (0.5 h), 5=hydrolysate of EPS/BE1 (1.0 h), hydrolysate of EPS/BE1 (2.0 h), hydrolysate of EPS/BE1 (3.0 h), and hydrolysate of EPS/BE1 (5.0 h). In both the chromatograms (A) and (B), spots a, b and c corresponds to Rf 0.388, 0.254, and 0.162 respectively.
A spot corresponding to glucuronic acid (a spot 'c' with Rf 0.162) was also seen, which showed tailing due to the alkaline nature of the solvent system used. In fact, in case of uronic acids, the applied sample whether authentic standard or hydrolysate, always contains an equilibrium mixture of the acid and its lactone. Compared to neutral and acidic systems where lactones resolve from acids, in alkaline systems, however, a gradual conversion of lactones into acids takes place causing tailing of the spots. In a previous report, using a similar solvent system employed in the present study, the monomer composition of *Klebsiella aerogenes* Type 8 polysaccharide was determined (Sutherland, 1970).

### 3.4.7 Monosaccharide composition by GC-MS analysis

For identification of the neutral sugar components of EPS/BE1, the sugars present in EPS hydrolysate (2.0 M TFA at 100°C for 2 h) were converted to their alditol acetates and subsequently identified using GC-MS. Since from the paper chromatography, the major probable sugar component of the EPS was found to be glucose, for GC-MS analysis, the alditol acetate of glucose was prepared and used as an authentic standard. When the alditol acetate of glucose was analyzed, two unresolved peaks at Rt 19.29 minutes and 19.38 minutes were obtained (figure 2A).

In gas chromatography, since the alditols acetate of sugars cannot anomerize, ideally only a single peak should be obtained. However, the two peaks obtained for glucose might have been due to (i) formation of multiple derivatives of sugars contributed by existence of equilibrium mixtures of pyranose ring form and acyclic form in solution, (ii) incomplete reduction of the aldehyde groups resulting in acetate derivatives of different anomic forms, and (iii) acetylation was carried out in the presence of catalysts such as pyridine and sodium acetate, which were reported to be effective only in the absence of borate (Blakeney et al., 1983). Incomplete removal of borate (which complexes with alditols) from the reaction mixture after the reduction step might have resulted in incomplete acetylation of the alditols. Similar to the authentic standard of glucose, the gas chromatogram of the alditol acetate derivatives of EPS hydrolysate also showed an unresolved peaks at Rt 19.30 and 19.39 min (figures 2A and 2B).
Upon mass spectral analysis of these peaks and subsequent comparison of the fragmentation pattern obtained using a mass spectral library, the above peaks were found to conform to D-glucose 2,3,4,5,6 pentaacetate and its pyranose ring form, α-D-glucopyranose. The above results confirmed that the two peaks obtained at \( R_t \) of 19.30 and 19.39 min in case of alditol acetate of EPS/BE1 hydrolysate were those of glucose only (figures 3 and 4).

Using alditol acetate derivatives, uronic acids, ulosonic acids (e.g. KDO), 4-aminosugars, or charged species like phosphorylated sugars could not be detected in EPS/BE1, since (i) during formation of alditols, the carboxylic acid groups of the uronic acid might have got converted to aldehyde/hydroxyl groups through their reduction thereby forming neutral sugars, and (ii) the sodium salt of the uronic acid that was formed on addition of NaBH₄ was not volatile (Aspinall, 1982).

### 3.4.8 High performance liquid chromatography

Since the EPS/BE1 was anionic in nature, to determine if it contained any acidic sugar(s) in the main chain or organic acid group as side chain substituents, HPLC analysis of the hydrolysate (2.0 M TFA, 2 h) was carried out. On the basis of retention time of each peak obtained, the following acidic components were found to be present in the EPS/BE1: glucuronic acid (\( R_t \) 2.18 min), formate (?) (\( R_t \) 2.73 min) and pyruvate (\( R_t \) 3.13 min) (figure 5).

Previously also, organic acid substituents in xanthan were estimated using HPLC (Tait et al., 1990; Tait and Sutherland, 1990). From the above observations, the overall composition of the EPS/BE1 was determined to be: Glucose, 68.25%; Glucuronic acid, 22.2%; Formate (?), 3.72%; Pyruvate, 2.30%. Nevertheless, the monomeric composition of EPS/BE1 was found to be different from those of the earlier reported bacterial exopolysaccharides indicating it to be a new polymer.
Figure 2. Gas chromatogram of alditol acetates of glucose and hydrolysate of EPS/BE1

(A)

EPS was hydrolysed using 2.0 M TFA for 2 h at 100°C. GC was carried out using Perkin Elmer Autosystem XL (coupled with Turbo Mass spectrometer) equipped with a flame ionization detector and PE-5MS column. (A) The unresolved peaks at 19.29 and 19.38 corresponded to alditol acetate of authentic glucose sample, and (B) alditol acetate of hydrolysate of EPS/BE1. The peaks observed at 3.52, 3.55 and 4.50 were found to be artefacts.
Figure 3. Mass spectrum of peaks obtained for peracetylated glucitol.

(A) 

(B) 

GC-MS of the peracetylated glucitol was carried out using Perkin Elmer Autosystem XL (coupled with Turbo Mass spectrometer) equipped with a flame ionization detector and PE-5MS column. Mass spectra of GC obtained peaks at (A) 19.29 min, and (B) 19.38 min.
EPS was hydrolysed using 2.0 M TFA for 2 h at 100°C. GC-MS of the alditol acetates of hydrolysate was carried out using Perkin Elmer Autosystem XL (coupled with Turbo Mass spectrometer) equipped with a flame ionization detector and PE-5MS column. Mass spectra of GC peaks at (A) 19.30 min and (B) 19.39 min.
EPS was hydrolysed using 2.0 M TFA for 2 h at 100°C. HPLC analysis of the EPS/BE1 hydrolysate was performed using a Merck HPLC system equipped with RP-18 reverse phase column. Aqueous H₃PO₄ at a flow rate of 1 ml/min was used as mobile phase and the peaks were detected using a UV detector at 210 nm. 20 μl of either standards (50 mM) or hydrolysate (10 mg/ml) was injected.

3.4.9 Vibrational spectroscopy

3.4.9.1 FT-IR spectroscopy

The bands at 1623.57 cm⁻¹, 1728.44 cm⁻¹ and 1418.43 cm⁻¹ indicated the presence of carboxyl group bearing residues in the EPS/BE1 (Guezzeneec, 1994; Navarini et al., 1997). The peak observed at 1043 cm⁻¹ was due to uronic acid moieties (Coimbra et al., 1998). EPS/BE1 did not contain inorganic substituents such as sulphate or amide bonds as indicated by the absence of bands at 1250 cm⁻¹ and 1550 cm⁻¹ respectively (figure 6 and table 1). A 2 b type bending C1-H vibrational mode observed at 894.28 cm⁻¹ indicated the presence of β-hexopyranose(s) as reported earlier for succinogluccan 10C3, exopolysaccharides of Rhizobium heydsari HCNT-1 and R.meliloti strain L5-30 (Harada, 1965; Mody et al., 1989; Da Costa Castro et al., 1983).
3.4.9.2 FT-Raman spectroscopy

The bands obtained in the FT-Raman spectra of EPS/BE1 (figure 7) were assigned as shown in table 2. The peak at 905.2 cm⁻¹ in FT-Raman was associated with the CH bending at the anomeric carbon in β-glucose residues. The peak at 1094.4 cm⁻¹ in FT Raman spectra indicated contributions from several modes, namely to the C-O and C-C stretchings and to the C-C-O and C-O-H deformations.

Figure 6. FTIR spectrum of the EPS/BE1

Infra red spectrum of KBr pellet of EPS/BE1 was obtained using Perkin Elmer FTIR spectrometer.

The peak at 1094.4 cm⁻¹ indicated glycosidic linkage (Pereira et al., 2003). Overall, both IR and Raman spectroscopic observations indicated the presence of -OH, -CH,
CH$_2$, C=O, C-O-C, -COOH, ketals and hemicyclic ring formation characteristic of sugars. Bands at 1388.2 cm$^{-1}$ in Raman spectra and 1166.39 cm$^{-1}$ in IR spectra indicated the presence of pyruvate ketals in the EPS/BE1.

<table>
<thead>
<tr>
<th>Frequency at which bands were observed (cm$^{-1}$)</th>
<th>Functional Group Assigned</th>
</tr>
</thead>
<tbody>
<tr>
<td>1043.19</td>
<td>Free OH related deformation</td>
</tr>
<tr>
<td>3435.92</td>
<td>H bonded OH</td>
</tr>
<tr>
<td>2922.59</td>
<td>C-H stretching</td>
</tr>
<tr>
<td>1418.43, 1623.57 and 1314</td>
<td>C=O stretching indicating COOH groups, carboxylate anion</td>
</tr>
<tr>
<td>1728.44</td>
<td>CO stretching indicating COOR and aldehydes</td>
</tr>
<tr>
<td>1261.83</td>
<td>C=O stretching from alcohols</td>
</tr>
<tr>
<td>1728.44</td>
<td>C=O stretching from COOH</td>
</tr>
<tr>
<td>1166.39</td>
<td>Ketals</td>
</tr>
<tr>
<td>1374.15</td>
<td>OH bending vibration</td>
</tr>
<tr>
<td>2922.59</td>
<td>C-H stretch, carboxylic acids, methylene</td>
</tr>
<tr>
<td>1314.15</td>
<td>C=O stretching</td>
</tr>
<tr>
<td>1623.57</td>
<td>Carboxylate ion</td>
</tr>
<tr>
<td>1374</td>
<td>C-H</td>
</tr>
</tbody>
</table>

Infra red spectrum of KBr pellet of EPS/BE1 was obtained using Perkin Elmer FTIR spectrometer.

<table>
<thead>
<tr>
<th>Bands (cm$^{-1}$)</th>
<th>Groups assigned</th>
</tr>
</thead>
<tbody>
<tr>
<td>430.1</td>
<td>C-C</td>
</tr>
<tr>
<td>905.2</td>
<td>C-O-C</td>
</tr>
<tr>
<td>1094.4</td>
<td>C-O-C involved in ring formation</td>
</tr>
<tr>
<td>1388.2</td>
<td>CH$_3$</td>
</tr>
<tr>
<td>1461.4</td>
<td>CH$_2$</td>
</tr>
<tr>
<td>2904.7</td>
<td>CH</td>
</tr>
</tbody>
</table>

The FT-Raman spectra were recorded (at 28±2°C, 2500 scans) on a Bruker FRA 106 FT-spectrometer using a Nd:YAG laser with excitation wavelength of 1064 nm.
3.4.10 Methylation analysis

The EPS/BE1 was methylated, hydrolyzed and converted to alditol acetates prior to GC-MS analysis. In the GC-MS of methylated alditol acetates of the hydrolysate of EPS/BE1, three peaks were obtained at 5.219, 10.65 and 19.557 min (figure 8 A, B). Upon mass spectral analysis, the peaks were assigned to D-glucose 2,3,5,6 tetramethyl (figure 9A), α-D-glucofuranose and methyl 2,4 diacetyl-3,6-di-O-methyl-β-D-glucopyranoside (figure 9B), respectively. The presence of α-D-glucofuranose indicated that the EPS/BE1 contained an equatorially (α-) linked D-glucose residue.

Figure 7. FT-Raman spectrum of EPS/BE1

Interestingly, the presence of a methylated product, D-glucose 2,3,5,6 tetramethyl indicated that a D-Glc residue was present at a branch point. Detection of a methyl 2,4 diacetyl-3,6-di-O-methyl-β-D-glucopyranoside indicated that a β-linked glucose was present as a terminal residue. Other two sugar residues i.e. D-GlcP A and α-D-glucose were linked to either 2- or 4- positions of β-D-GlcP as these positions were not methylated. The crystalline sugars exist in a single form, whereas, the sugars in solution are present as either furanose or pyranose forms, between which exists the
intermediate acyclic structure at a rapid equilibrium. In general, since pyranose ring forms are more stable than furanose structures in aqueous solutions, and almost all bacterial polysaccharides are composed of pyranosides (Bush et al., 1999), in the present study also, the reducing sugars are shown in pyranose ring forms.

Figure 8. Mass spectra of methylated EPS/BE1

(A)

(B)

. Mass spectra of peaks of methylated alditol acetates of hydrolysate obtained at (A) 5.219 min (B) 19.557 min

3.4.11 Nuclear Magnetic Resonance Spectroscopy (NMR)

To assign anomeric configurations to individual glycosidic bonds and to determine the sequence of sugar residues in the EPS/BE1, $^1$H and $^{13}$C NMR spectroscopy were carried out. One of the major problems encountered during the recording of NMR spectra was that for getting adequate signals, a concentrated solution of EPS was required. When concentrated solution of EPS was used, due to higher viscosity of the
solution, the peaks obtained were of less intensity and broad (Mody et al., 1989; Leeflang et al., 2000). To alleviate the above problems and to increase the sensitivity, following approaches have been suggested: (i) raising the operating temperature to 60-90°C (Aspinal, 1982) (ii) reduction of molecular weight of the EPS by partial acid hydrolysis (Lemoine et al., 1997; de Pinto et al., 1998), and (iii) the use of high field of NMR along with the above suggested methods.

Figure 9. Products of methylated and acetylation of EPS/BE1

(A) D-glucose 2,3,5,6 tetramethyl glucopyranoside  
(B) 2,4 diacetyl-3,6-Di-O-methyl-β-D- 

Hence, on the basis of evidences obtained from methylation analysis, either of the following structures for repeat units of EPS/BE1 may be suggested:

\[
\begin{align*}
\text{α-D-Glc}(1-4) & & \text{α-D-Glc}(1-2) \\
\text{D-GlcPA (1-2) D- Glc} & & \text{D-GlcPA (1-4) D- Glc} \\
(I) & & (II)
\end{align*}
\]

3.4.11.1 Native EPS/BE1

NMR of the native EPS/BE1 was recorded at 70°C at an operating frequency of 200 MHz. The chemical shifts were recorded with reference to D$_2$O peak at 8 4.8 ppm as suggested earlier (Rodriguez-Carvajal et al., 2001; Flatt et al., 1992). For native EPS/BE1, a moderately sharp $^1$H spectrum was obtained exhibiting broad and scarce number of peaks as shown in figure 10.
In the $^1$H spectra of the native polysaccharide a peak was observed at δ 2.151 ppm which suggested the presence of acetyl groups in the EPS/BE1. However, neither colorimetric analysis nor HPLC analysis provided any evidence for presence of acetyl groups in the EPS/BE1 and hence the signal at δ 2.151 ppm might be attributed to other alkyl groups. Similarly, although CH$_3$CON gives a peak at δ 1.8-2.1 ppm, the resonance at 2.151 ppm could not be attributed to amino sugars, as their absence in the EPS/BE1 had already been established.

Figure 10. $^1$H NMR spectrum of native EPS/BE1

![Figure 10](image)

The spectra was recorded at 70°C in D$_2$O using Bruker 200 MHz instrument. The broad peak at δ 4.8 is that of HOD (water).

Surprisingly, in the $^1$H NMR of native EPS/BE1, no peaks indicative of pyruvate was observed even though its presence in the EPS was confirmed through HPLC. Peaks were also absent at δ 2.49 ppm and δ 2.65 ppm indicating the absence of succinyl group in the EPS/BE1 as a side chain substituent (Leigh and Lee, 1988). The resonances observed between δ 3.2-4.7 ppm were due to ring protons (Louch et al., 2001). As H-2 to H-6 is indicated by resonances at δ 3.5 to 4.5 ppm presence of signals at δ 4.182 ppm and 3.222 ppm were assigned to ring protons.
The number of peaks in the anomeric region indicate the number of sugar residues in the repeat unit of a polysaccharide (Neu et al., 1992). $^1$H-NMR spectra of EPS/BE1 showed three different peaks in the anomeric region suggesting that the repeat unit structure of EPS/BE1 consists of three sugar residues. An equatorial Cl–H1 bond of an α-glucose residue is normally represented by an H1 signal in the region of δ 5-5.5 ppm whereas axial H-1 of the β-anomer resonate up field closer to δ 4.5 ppm. Hence, a peak at δ 4.706 ppm indicated the presence of β- linkage in the main chain and in addition, the two peaks at observed at δ 5.393 ppm and 5.201 ppm indicated the presence of two α-anomeric linkages. The signals at δ 4.7 ppm, 5.201 ppm and 5.393 ppm were ascribed to β-glucopyranose, α-D-glucose and α-D-glucuronic acid residues.

Unlike $^1$H NMR, the $^{13}$C NMR of native EPS/BE1 recorded at 200 MHz and at an operational temperature of 70°C provided sufficiently sharp peaks as shown in figure 11. In $^{13}$C NMR, the anomeric carbons resonate at δ of 95-110 ppm, ring carbons δ 50-85 ppm, alkyl carbons at δ 15-25 ppm, and carbonyls δ 165-180 ppm. In the EPS/BE1, the $^{13}$C resonances at δ 61.72 and 62.28 indicated CH$_2$OH. δ 61.7 ppm to 179 ppm were assigned to C1 to C6 of various sugar residues (C1-C6 α-D-Glc, β-D-Glcp and α-D-GlcpA) as shown in table 3.

Figure 11. $^{13}$C NMR spectrum of unhydrolysed (native) EPS/BE1

The spectrum was recorded at a temperature of 70°C using a 200 MHz Bruker instrument.
There was no non-anomeric carbon involved in glycosidic linkage as no peaks were seen between δ 80-87 ppm. Characteristically, the chemical shift of C-1 of an α-anomer is expected to be around δ ~100 and 3-4 ppm up-field of that observed for β-anomer. Presence of signals at δ 103.70 ppm and 103.97 ppm indicated the presence of two equatorial glycosidic (α-anomeric) linkages and at δ 104.67 ppm suggested presence of an axial (β-anomeric) glycosidic linkage. Absence of a peak at δ 91.67 ppm indicated the absence of reducing ends in case of native EPS. The evidence for presence of pyruvate was obtained from the 13C NMR of partial hydrolysate. The peaks observed at δ 32.458 and δ 179.9 ppm indicated the CH- group and –CO group of pyruvate respectively. The absence of resonance at δ 96.4 ppm indicated the absence of C-2 as anomeric carbon (Bhattacharjee et al., 1975). Absence of a peak at δ 88.0 ppm negated the possibility of β-(1-3) linkage (Van de Valde et al., 2004).

3.4.11.2 Partially hydrolyzed EPS/BE1
In order to get a more sharp 1H and 13C NMR spectra, the EPS/BE1 was partially hydrolysed using 0.2 M TFA for 1 h at 100°C and after completely evaporating TFA from the sample, NMR spectra were recorded using an operating frequency of 400 MHz. A similar approach for recording NMR spectra using hydrolysed EPS has been reported earlier for NMR spectroscopy of an EPS of *Streptococcus thermophilus* SFi39 and SF112 (Lemoine et al., 1997). Sharp spectra (figure 12) obtained at elevated temperatures and hydrolysed EPS/BE1 indicated conformationally mobile polysaccharide coil (Mody et al., 1989). The assignment of resonances obtained for partially hydrolyzed EPS is as given in table 4. The NMR spectrum of partially hydrolysed EPS/BE1 (75°C), showed signals at δ 5.221 ppm and 4.675 ppm indicating reducing ends of α- and β- anomeric linkages. The above observation was further confirmed by the presence of signals at δ 99 ppm (β-anomer) and 95 ppm (α-anomer) in the 13C spectrum (at 23°C) of partially hydrolyzed EPS/BE1 as shown in figure 13. Further, looking to the resonances obtained, it appeared that there were no terminal glucuronic acid residues present (de Pinto et al., 1995).

Also the absence of a signal at δ 3.64-3.56 ppm indicated that methoxyl groups were not present in the EPS (Rodriguez-Carrajal et al., 2001). Overall, on the basis of NMR resonances obtained it was evident that in the repeating unit of the EPS/BE1, D-
GlcpA was linked equatorially (α-) to the β-D-Glc in the main chain and α-D-Glc was present as a substitution or branch point on β-D-Glc.

Figure 12. Proton NMR spectra of hydrolysed EPS/BE1

The spectra was recorded at a temperature of 75°C using a 400 MHz Jeol GSX 400 instrument. Partial hydrolysis of the EPS/BE1 was carried out using 0.2M TFA at 100°C for 1h. TFA was completely evaporated in vacuo before analysis.

Figure 13. 13C NMR of partial hydrolysate of EPS/BE1

The spectra was recorded at a temperature of 23°C using a 400 MHz Jeol GSX 400 instrument. Partial hydrolysis of the EPS/BE1 was carried out using 0.2M TFA at 100°C for 1h. TFA was completely evaporated in vacuo before analysis.
Table 3. $^{13}$C chemical shifts of native EPS/BE1

<table>
<thead>
<tr>
<th>component</th>
<th>chemical shifts (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C1</td>
</tr>
<tr>
<td>$\alpha$-D-GlcpA</td>
<td>103.7, 103.97</td>
</tr>
<tr>
<td>$\alpha$-D-Glcp</td>
<td>104.67</td>
</tr>
<tr>
<td>$\beta$-D-Glcp</td>
<td>110.20, 110.60</td>
</tr>
</tbody>
</table>

Chemical shifts were recorded at a temperature of 70°C using a Bruker spectrometer operating at a frequency of 200 MHz.

Table 4. $^1$H chemical shifts of the partially hydrolysed EPS/BE1

<table>
<thead>
<tr>
<th>component</th>
<th>chemical shifts (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>H1</td>
</tr>
<tr>
<td>$\alpha$-D-GlcpA</td>
<td>5.2</td>
</tr>
<tr>
<td>$\alpha$-D-Glcp</td>
<td>5.31</td>
</tr>
<tr>
<td>$\beta$-D-Glcp</td>
<td>4.67</td>
</tr>
</tbody>
</table>

Partial hydrolysis of the EPS was carried out using 0.2 M TFA for 1h at 100°C. NMR was recorded at 75°C in a Jeol GSX 400 spectrometer operating at a frequency of 400 MHz.

3.4.12 Enzymatic hydrolysis

For a polysaccharide, the configurational assignments at anomeric centres may be made from the results of hydrolysis with specific glycosidases (Aspinall, 1982). It is well known that cellulase acts on $\beta$-(1,4), $\alpha$-amylase on $\alpha$-(1,4) and glucoamylase acts on $\alpha$-(1,6) glycosidic linkages present in polysaccharides. In the present study, cellulase did not have any action against EPS/BE1, starch and pullulan, whereas it hydrolysed curdlan, gellan, xanthan and carboxymethyl cellulose (CMC) as shown in table 5.
### Table 5. Action of glycosidases on EPS/BE1 and several exopolysaccharides

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Concentration (% w/v)</th>
<th>Reducing sugar released (μg/ml)</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>celluase</td>
<td>α-amylase</td>
<td>glucoamylase</td>
<td></td>
</tr>
<tr>
<td>EPS/BE1</td>
<td>0.5</td>
<td>0</td>
<td>452</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>0</td>
<td>660</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Carboxymethylcellulose</td>
<td>0.5</td>
<td>1168</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>3856</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Starch</td>
<td>0.5</td>
<td>0</td>
<td>4816</td>
<td>3620</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>0</td>
<td>4836</td>
<td>5040</td>
<td></td>
</tr>
<tr>
<td>xanthan</td>
<td>0.5</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>472</td>
<td>13</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>gellan</td>
<td>0.5</td>
<td>220</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>78</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Curdlan</td>
<td>0.5</td>
<td>2076</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>1108</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Dextran</td>
<td>0.5</td>
<td>0</td>
<td>228</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>0</td>
<td>166</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Pullulan</td>
<td>0.5</td>
<td>0</td>
<td>300</td>
<td>3960</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>0</td>
<td>240</td>
<td>7104</td>
<td></td>
</tr>
</tbody>
</table>

Assay system for cellulase contained: 1.0 ml (0.5 and 1.0 % w/v) of either EPS and or other polysaccharide solutions in 10 mM citrate buffer (pH 5.0) and 15 units of cellulase. After incubation at 37°C for 30 min, reducing sugar was estimated by DNS method (Miller, 1959). The effect of glucoamylase and α-amylase on EPS/BE1 and other polysaccharides was checked separately by incubating either with glucoamylase (20 units) or α-amylase (15 units) in 0.1 M citrate buffer (pH 5.0) or 0.1 M phosphate buffer (pH 7.0) at 30°C for 1 h and the released sugar was estimated by the DNS method (Miller, 1959).

Interestingly, α-amylase showed activity on EPS/BE1 moderately, while it acted more efficiently on starch and pullulan. Glucoamylase only hydrolyzed starch and pullulan. In a previous study also, when a polysaccharide of *Musa Paradisica* was treated with glucoamylase, it efficiently hydrolyzed α-D-(1,4) glycosidic bonds, compared to α-D-(1,3) and α-D-(1,6) bonds (Shanta Raju et al., 2001). Also, gellan lyase, β-D-glucosidase and β-D-glucuronidase produced by a number of *Sphingomonas* strains exhibited negligible activity against the native acetylated gellan polysaccharides. Similarly, the polysaccharide lyases active against alginate were also strongly inhibited by the presence of O-acetyl or other acyl groups in the polymeric substrates.
(Fialho et al., 1999). The low rate of hydrolysis of EPS/BE1 by \( \alpha \)-amylase could be due to the interference caused by the presence of bulkier pyruvate substituents and/or by D-Glucose branch. Further, since \( \alpha \)-amylase is an endo enzyme, the low activity on EPS/BE1 compared to starch suggested that the \( \alpha \)-linkages present in EPS/BE1 was different from those present in starch. The absence of glucoamylase activity against the EPS/BE1 suggested that the EPS was a branched polysaccharide and the branch point (\( \alpha \)-D-Glc) did not have (1,4) linkage. The moderate activity of \( \alpha \)-amylase towards EPS/BE1 suggested the presence of \( \alpha \)-(1,4) bond in the main chain.

### 3.4.13 Chemical structure of EPS/BE1

The overall chemical structure of the repeat unit of the EPS/BE1 was arrived at on the basis of the evidences obtained from the paper chromatography of complete and partial acid hydrolysates of EPS/BE1, GC-MS analysis, IR and Raman spectroscopy, methylation analysis, \(^1\)H and \(^{13}\)C NMR spectroscopy and enzymatic hydrolysis.

The oligosaccharides obtained from the partial hydrolysis of a polysaccharide provide more detailed and unambiguous information on sequence, anomeric configurations and linkages present in it (Aspinal, 1982). In past, partial hydrolysis has been used as an approach for characterization of an EPS of *Aerobacter aerogenes* (Gahan et al., 1967), *Streptococcus thermophilus* SFi39 and SFi12 (Lemoine et al., 1997) and *Streptococcus macedonicus* Scl36 (Vincent et al., 2001).

As seen in the paper chromatogram (figure 1), the hydrolysis of EPS/BE1 up to 1 h resulted in partial hydrolysis products. When the \( R_Y \) values of these products were compared, the spot at \( R_Y \sim 0.254 \) was found to decrease in the intensity with increase in time of hydrolysis, indicating that the spot belonged to a disaccharide or a higher oligomer. If the spot in question belonged to a higher oligomer (with degree of polymerization more than two), then a few more spots should have been obtained on further hydrolysis. However, absence of such spots indicated that the spot corresponding to \( R_Y 0.254 \) was of a disaccharide. Since the EPS was made up of glucose and glucuronic acid, the disaccharide could be either D-Glc\( \beta \)-D-Glc\( \beta \) or D-Glc\( \beta \)-D-Glc\( \beta \)A.
Unfortunately, as the standards for D-Glcp-D-GlcpA were not available, use of several common disaccharides composed of D-Glcp-D-Glcp having different anomeric configurations were resorted to as standards for comparison. Hence, when the \( R_f \) values of disaccharides containing glucose such as cellobiose [\( \beta \)-D-Glcp-(1\( \rightarrow \)4)-\( \beta \)-D-Glcp], trehalose [\( \alpha \)-D-Glcp(1\( \rightarrow \)1)-\( \alpha \)-D-Glcp] and maltose [\( \alpha \)-D-Glcp(1\( \rightarrow \)4)-\( \alpha \)-D-Glcp] was compared, the spot of the hydrolysate corresponding to \( R_f \sim 0.254 \) fell in between the \( R_f \) of cellobiose (0.255), trehalose (0.255), maltose (0.285) and lactose (0.208). In fact, even though the difference in the \( R_f \) value of disaccharides used was marginal, there was considerable difference in the \( R_f \) value between mono- and disaccharides. The two spots observed at \( R_f \) 0.162 and \( R_f \) 0.370 might correspond to D-Glcp-D-GlcpA because D-glucopyranosyluronic acid residues are difficult to hydrolyze as reported in case of acid hydrolysis of xanthan gum (Danishefsky, 1970; Tait et al., 1990).

Both \(^1\)H and \(^1^3\)C NMR indicated the presence of a trisaccharide repeat structure for EPS/BEl. Also, the IR spectra pointed to the presence of a residue bearing \( \beta \)-anomeric configuration. Since methylation analysis revealed the presence of a disubstituted \( \beta \)-D-Glcp residue (one substitution at 4- position and other at 2- position) constituting a trisaccharide repeat unit, it can be concluded that \( \beta \)-D-Glcp residue was present in the main chain, forming a structure of \( \alpha \)-D-GlcpA-\( \beta \)-D-Glcp to the main chain and the \( \alpha \)-D-Glcp was present as a substitution or branch point on \( \beta \)-D-Glcp. In the paper chromatogram (figure 1) of the partial hydrolysate of EPS/BEl, no spot corresponding to maltose was obtained indicating that \( \alpha \)-D-Glcp (1\( \rightarrow \)4) \( \alpha \)-D-Glcp disaccharide structure was not present. Further, enzymatic hydrolysis showed that it was unlikely that \( \alpha \)-D-Glcp at the branch point was linked through 1,4 linkages to \( \beta \)-D-Glcp as glucoamylase did not act on it. Hence, \( \alpha \)-D-Glcp must be linked to preceding \( \beta \)-D-Glcp through 1,2 linkages. Therefore, in the paper chromatogram (figure 1) either the \( \beta \)-D-Glcp-(1\( \rightarrow \)4)-\( \alpha \)-D-Glcp or \( \alpha \)-D-Glcp-(1\( \rightarrow \)4)-\( \beta \)-D-Glcp oligosaccharide residue resembling more closely to cellobiose might have given rise to the spot corresponding to \( R_f \) 0.254.
On the basis of the above information following structure of the oligosaccharide repeat unit of EPS/BE1 is proposed:

\[ \alpha-D-\text{Glcp} (1\rightarrow2) \]

\[ \rightarrow 4) \alpha-D-\text{GlcpA} (1\rightarrow4) \beta-D-\text{Glc} (1\rightarrow) \]

Pyruvate is usually linked to the 4- and 6- positions of a hexopyranosyl residue, forming a 1,3 dioxane ring. The presence of a signal at 8 32.4 ppm in $^{13}$C NMR indicated the presence of an equatorially linked pyruvate group. Since in case of both the $\alpha$-D-GlcpA and $\beta$-D-Glc the O-4 was involved in the glycosidic linkage, pyruvate must be present as the side chain substitution linked to $\alpha$-D-Glcp at 4- and 6-positions forming a 1,3 dioxane ring as shown below:

\[ \text{Pyr (2- +} \]
\[ \quad / \quad \backslash \]
\[ \quad 6 \quad 4 \]
\[ \backslash \quad / \]
\[ \alpha-D-\text{Glc} (1\rightarrow2) \]

\[ \rightarrow 4) \alpha-D-\text{GlcpA} (1\rightarrow4) \beta-D-\text{Glc} (1\rightarrow) \]

3.4.14 Gel Permeation Chromatography (GPC)

The thickening properties of polysaccharides are usually directly proportional to its molecular weight (Yalpani, 1988). Initially, using column chromatography, the molecular weight of EPS/BE1 was found to lie between $5.3 \times 10^5$ and $2.0 \times 10^6$ Da. Subsequently, when the molecular weight of EPS/BE1 was determined using GPC, the number average molecular weight ($M_n$), the weight average molecular weight ($M_w$) and the peak average molecular weight ($M_p$) and of the EPS/BE1 were found to be $1.6 \times 10^6$, $3.9 \times 10^6$, and $4.8 \times 10^6$ respectively.

On the basis of molecular weight distribution, EPS/BE1 was found to be polydisperse with a polydispersity index of 2.36. In case of polysaccharides, the incomplete stringent control over the number of subunits added to a polymer chain results in the
synthesis of long and short chain polymers having different molecular weights (Danishefsky, 1970).

The molecular weight distribution of EPS/BE1 is shown in Figure 14 as a plot of log (M) versus dwt/d (log M). In all, around 70% of EPS/BE1 molecules had molecular weight more than 1.9x10^6 Da. Interestingly, polydisperse products are desirable in certain applications of polysaccharides. For example, use of carrageenans as binders in toothpaste and as dispersants in chocolate milk require polydisperse products to obtain desired functionality (Yalpani, 1988). The molecular weight of the EPS/BE1 obtained was similar to that reported for other exopolysaccharides. The molecular mass of EPS from two *Schizochytrium* sp. (Jain, et al, 2005), *Microdochium nivale Butyrivibrio fibrisolvens*, *Pseudomonas* (Manresa et al., 1987) and lactan gum (Flatt et al., 1992) were found to be 94 and 320 kDa, 6x10^6 Da, 300 to 800 kDa, 3.17x10^7 Da, 7.0x10^6 Da respectively.

Figure 14. Molecular weight distribution of EPS/BE1

The GPC of the EPS/BE1 was carried out using Waters 2690 Alliance System equipped with RI detector 2410 and TSK GEL type PW 3000, 4000 and 5000 columns. 100μl of the respective samples (both Dextran and EPS/BE1, 0.1% w/v) were injected. 0.2 M NaNO₃ was used as mobile phase at a flow rate of 1 ml/min. Throughout the analysis, oven temperature was maintained at 30° C. The data was analyzed using Millenium™ software.

3.4.15 Thermal Analysis

Besides chemical properties, applicability of polysaccharides in industry is largely dependent on its thermal and rheological behaviour. In literature, most of the studies
on thermal characterization of polysaccharides are focused on cellulose, starch and
some of their convenient derivatives and no systematic comparative study has been
conducted on thermal characterization of microbial exopolysaccharides (Zohuriaan,
and Shokrolahi, 2004). Hence, thermal analysis of EPS/BE1 was carried out to
determine its thermal stability.

3.4.15.1 Thermogravimetric analysis (TGA)
TGA offers a suitable and rapid method to investigate the thermal stability of
polymeric systems, as it is impractical to use time-consuming procedures at the initial
stage for valuation of products developed to have high thermal stability (Doyle,
1961). The threshold decomposition temperature gives an indication of the highest
processing temperature that can be used for a product during its use, while the study
of the kinetics of its decomposition processes may help in the identification of the
degradation mechanisms (Mano et al., 2003; Sandler et al., 1998). Individual stages of
decomposition in a multistage thermal decomposition process often exhibited by
complex polymers can be detected by the presence of points of inflection in the
thermal degradation curves of these materials. In case of multi-component systems the
stages of decomposition can be conjugated with each other (Mamleev, 2000).

EPS/BE1 exhibited a three stage decomposition proves when its thermal stability was
studied using TGA as shown in figure 15. Interestingly, even at 400°C, only a weight
loss of ~18% was recorded. Overall, between 95 and 500°C, a weight loss of 63% was
observed, of which 51% weight loss occurred in three well defined stages. In the first
stage of decomposition (95 to 225°C) a 14 % weight loss occurred. Above this
temperature the weight remained constant till a second degradation process started at
380°C, which further continued till 420°C, with a weight loss of 22%. In the third
stage of decomposition, which occurred between 425°C and continued till 500°C, a
final weight loss of 15% was noted. In a recent report on thermal studies of various
plant derived polysaccharides and xanthan gum, a double stage decomposition pattern
was observed (Zohuriaan, and Shokrolahi, 2004).

Unlike for EPS/BE1 where the decomposition started only at around 380°C, the main
decomposition of the above polysaccharides started at 200°C itself, indicating the
high thermal stability of EPS/BE1. Thermal analysis of exopolysaccharides of four cyanobacterial strains, *Cyanothece* sp., *Oscillatoria* sp., *Nostoc* sp. and *Nostoc carneum*, revealed that the above polysaccharides were stable up to 250°C (Parikh and Madamwar, 2006). In case of an EPS of a haloalkalophilic *Bacillus* sp. I-471, the onset of thermal degradation occurred about 250°C (Kumar et al., 2004). It has been suggested that the polysaccharide degradation proceeds in four distinct phases: desorption of physically absorbed water, (ii) removal of structural water (dehydration reactions) (iii) depolymerization accompanied by the rupture of C-O and C-C bonds in the ring units resulting in the evolution of CO, CO$_2$ and H$_2$O, and (iv) formation of polynuclear aromatic and graphitic carbon structures (Zamora et al., 2002).

In case of EPS/BE1, the initial loss of weight observed up to 180°C (stage I) was due to the loss of water content of the polymer as suggested earlier in case of an EPS of haloalkalophilic *Bacillus* sp. I-471 (Kumar et al., 2004). In case of EPS/BE1, the weight loss observed in stage II and III was due to depolymerization reactions resulting from thermal break up of glycosidic linkages present in the polysaccharide. The acceleration of thermal degradation can be quantitatively represented by determining the apparent activation energy (E$_a$) for different stages of degradation of the polymer using the well known Broido method (Mathakiya et al., 2004). The activation energy (E$_a$) for decomposition of EPS/BE1 was calculated using the equation,

$$\ln\ln(1/Y) = (-E_a/R)(1/T) + \text{constant}$$

Where $Y = (W_t-W_\infty)(W_0-W_\infty)$ is the fraction of the number of initial molecules not yet decomposed; $W_t$ is the weight at any time $t$; $W_0$ and $W_\infty$ stand for initial and final weights of EPS/BE1 sample, respectively. $T$ is the absolute temperature recorded in the thermogram.

On plotting ln/ln (1/Y) versus 1/T a straight line was observed, the slope of which yielded activation energy (E$_a$). The activation energy for the stages I, II and III for thermal decomposition for EPS/BE1 were found to be 23.6, 108.6, 21.22 kJmol$^{-1}$ respectively. In a previous report, on the basis of activation energy for thermal decomposition, the various gums studied were ranked in the order tragacanth
TGA was recorded at 25 to 500°C using a Shimadzu thermal analyzer DT 30B under nitrogen atmosphere at a heating rate of 10°C min⁻¹.

3.4.15.2 Differential Scanning Calorimetry (DSC) analysis
In the DSC thermogram of EPS/BE1, the endothermic peak observed at 94.9°C (onset at 68.7°C and endset 121.8°C) resulted from the evaporation of water (figure 16). The enthalpy change for this transition i.e. the energy required to vaporize water present in the polymer was found to be −184.16 Jg⁻¹. The above endothermic activity corresponded to the initial loss in weight observed in the TGA plots of EPS/BE1 up to 180°C. In a previous study, for vaporization of water, xanthan gum and sodium alginate showed ΔH of +181.5 and +49.5 Jg⁻¹ respectively, which suggested that xanthan gum was more hydrophilic than sodium alginate (Zohuriaan and Shokrolahi, 2004). The high enthalpy change observed in case of vapourization of water for EPS/BE1 (ΔH −184.16 kJ⁻¹), indicated that it was highly hydrophilic similar to xanthan gum. In the previous reports, the early endotherms (between 63 to 134°C)
observed for gum arabic, gum tragacanth, xanthan, sodium alginate, chitosan, CMC, HEC, methylcellulose and locust bean gum were also associated with water loss (2-10%) (Zohuriaan and Shokrolahi, 2004; Aydinli et al., 2004; Cervera et al., 2004). It may also be argued that the early endothermic transition observed at 94.9 °C (ΔH = 184.16 kJ/mol) at could be due to the melting of the polymer and that the T_g transition peak was obscured by the moisture endothermic peak (Zohuriaan and Shokrolahi, 2004).

Figure 16. DSC thermogram of EPS/BE1

DSC was carried out on a Shimadzu DSC 50 instrument in the presence of nitrogen gas at a heating rate of 10°C min⁻¹.

However, if that were the case than there would have been an increase in the energy of the system and not a loss in weight due to the phase transition in the TGA curve. However, loss in weight of the EPS/BE1 sample observed at 94.9°C range proved that the endothermic transition was due to the reduction in the water content of the polymer only and not due to the melting of EPS/BE1 or glass transition. The absence of endotherms in the DSC thermograms, besides the one at 94 to 95°C, implied that the EPS/BE1 was an amorphous polysaccharide. In case of polysaccharides exhibiting certain degree of crystallinity (chitin, amylopectin and cellulose), the temperature of
onset for evaporation of water was always found to be higher compared to totally amorphous polysaccharides such as amylose and mannan. Although the onset temperature of cellulose, chitin and amylpectin was higher than that of EPS/BE1, the enthalpy change was found to be low indicating that the bound water in these polysaccharides evaporated more easily than that present in EPS/BE1. As a practical consequence, although more energy may be required for drying of the recovered EPS/BE1, the above nature is common to most of the hydrocolloids, which bind water strongly and give highly viscous solutions (Ramos-Sanchez et al., 1991).

The exothermic transition in case of EPS/BE1 observed at 272.9°C (onset at 259.44 °C and end set at 294.15°C) with a ΔH of -127.58 Jg⁻¹ indicated restructuring/phase transition of the polysaccharide chains. The decomposition of the polysaccharide started around 350°C, which supported the TGA observation that at 350 °C only 18% in the weight loss was observed, including the weight loss due to evaporation of water. In past, most of the DSC thermograms of polysaccharides indicated a major intense exothermic transition around 300°C followed by weaker exotherms (Zohuriaan and Shokrolahi, 2004). Also, the locust bean gum based edible films and chitosan powder exhibited decomposition around 280 to 300°C (Aydinli et al., 2004; Cervera et al., 2004). In case of EPS/BE1, the exothermic effect at or more than 300°C appeared to involve further elimination of the polyhydroxyl groups, accompanied by depolymerization and early decomposition of EPS/BE1 molecules with evolution of CO₂ and CO. At further higher temperatures, the decomposition of EPS/BE1 was probably accomplished, with the formation of methane and unsaturated hydrocarbons as suggested previously (Ramos-Sanchez et al., 1991; Zohuriaan, 2004).

### 3.5 CONCLUSION

On the basis of the chemical composition and structure, the EPS produced by *Rhizobium radiobacter* BE1 was found to be a novel one.
BIBLIOGRAPHY


CHAPTER 3

CHARACTERIZATION OF EXOPOLYSACCHARIDE OF RHIZOBIUM RADIOBACTER BE1

PART B
RHEOLOGICAL CHARACTERIZATION
CHAPTER SUMMARY

The industrial value of polysaccharide lies in its capacity to alter the rheological properties of their aqueous solutions either through gelling or alteration of the flow characteristics. The intrinsic viscosity (at 30±0.05 °C) of EPS/BE1 in water and 0.1 M NaCl was found to be 2700 ml/g and 2100 ml/g, respectively. For EPS/BE1 solution in water, the Huggin’s constant k’ and Kraemer’s constant k” were determined as 0.36 and 0.30, respectively. While, EPS/BE1 solution in 0.1 M NaCl, showed Huggin’s constant (k’), Kraemer’s constant (k”) as 0.29 and 0.22, respectively.

Solutions of alginate above a concentration of 75 g/l showed pseudoplastic rheology. At and below 50.0 g/l, sodium alginate solutions showed Newtonian behaviour. Sodium alginate solution at a concentration of 125.0 g/l exhibited a viscosity of 6276 cP. EPS/BE1 at 10 times less concentration (1.0% w/v) exhibited equal viscosity to that observed for sodium alginate at 10.0% (w/v). At identical concentration (10.0 g/l), EPS/BE1 exhibited 1.6, 8.6 and 6 times higher viscosity compared to that obtained for guar gum, CMC, and carrageenan, respectively.

The consistency index of EPS/BE1 solution (10.0 g/l) was 5.8 times higher than that observed for sodium alginate solution (125.0 g/l). The flow index of EPS/BE1 solution (1.0 % w/v) was 2 times higher than that observed for xanthan solution (1.0% w/v) and 4.3 times higher than that observed for sodium alginate solution (12.5% w/v). Overall, among the gums tested, guar gum, CMC and carrageenan fared poorly in terms of viscosity, consistency index and degree of pseudoplasticity. EPS/BE1 just at a concentration of 1% (w/v) exhibited 1.74 times higher yield stress (151.7 dynes/cm²) compared to sodium alginate at 12.5% concentration.

In the temperature range tested (30 to 100°C), no significant change in the viscosity of the EPS/BE1 solutions (1.0% w/v) was observed indicating a high thermal stability of the viscosity of EPS/BE1 solutions. When the EPS/BE1 solution (1% w/v) was subjected to various temperatures, concomitant to increase in the prevailing temperature, a sharp decrease in viscosity of the solution was observed around 60°C,
which was characteristic of other polysaccharides also. The activation energy of flow for EPS/BE1 solution (1.0% w/v) obtained (114.2 kJ/ml) indicated a high thermal stability of the solution.

No significant change in the viscosity of the EPS/BE1 solution was observed in the pH range of 6.0 to 10.0, however, below a pH of 6.0, a marginal increase in the viscosity was observed. When the effect of NaCl concentration on the viscosity of EPS/BE1 solution was tested, up to 150 g/l NaCl concentration tested, the viscosity was found to be stable. However, a drastic drop in viscosity of solutions of CMC and xanthan was observed by NaCl concentration of 20 g/l.

When the effect of presence of various salts (at a concentration of 5.0% w/v) on the viscosity of EPS/BE1 solution (0.5% w/v) was studied, addition of NaCl, CaCl\(_2\).2H\(_2\)O, Na\(_2\)CO\(_3\), ZnSO\(_4\).7H\(_2\)O, and CoCl\(_2\).6H\(_2\)O caused an increase in the viscosity of the EPS solution. Upon aging at 30°C and pH 7.0 (for 90 days), except in case of Na\(_2\)CO\(_3\), MgCl\(_2\).6H\(_2\)O and CuSO\(_4\).5H\(_2\)O where an increase in the viscosity was noted, in other cases a marginal decrease in the viscosity of the EPS/BE1 solution was observed.

At pH 9.0 and 30°C, addition of various salts resulted in an increase in the viscosity of EPS solution. Upon aging at pH 9.0 and 75°C, in majority of the cases, a decrease in the viscosity of the solution was observed. When the EPS/BE1 solutions, which had registered a decrease in viscosity upon aging at pH 9.0 and 75°C, were further incubated at 30°C for 90 days, an increase in viscosity of the solutions containing respective salts was observed suggesting that the decrease in viscosity observed after aging was not due to the depolymerization of the EPS chains. When the effect of various acids on the viscosity of EPS/BE1 solution was tested, in presence of phosphoric acid, a 50% decrease and in presence of citric acid and tartaric acid, a moderate decrease in viscosity of the solution was observed.
Rheology is the science of the deformation and flow of matter which is concerned with the response of polymeric materials to mechanical stress. The industrial value of polysaccharides lies in their capacity to alter the rheological properties of aqueous solutions either through gelling or through the alteration of their flow characteristics (Lapasin and pricil, 1999). The capacity of the carbohydrate polymers to generate high solution viscosities at low concentration is due to the restricted rotation around the glycosidic bonds between adjacent residues, which in turn restricts chain flexibility leading to large overall coil dimensions for a disordered polysaccharide (Aspinall, 1982).

### 3.6.1 Rheology of polysaccharide systems

Polysaccharides and their derivatives exhibit a broad variety of rheological behaviours ranging from Newtonian to various types of non Newtonian behaviours (figure 1). At low shear rates, solutions containing low concentration of polysaccharides may display Newtonian behaviour ($\eta_0$), whereas at high concentrations, non Newtonian behaviour is commonly observed due to interchain interactions. Gum Arabic, a high molecular weight polysaccharide display extremely low viscosities and Newtonian flow behaviour at concentrations below 40% (w/v). However, it exhibited pseudoplastic behaviour at concentrations above 50% (w/v). The solution behaviour of random coil polysaccharides was found to depend upon their concentration. In case of polymer solutions, three different concentration regimes could be recognized: dilute concentration ($c<c^*$) where the polymer chains exist as isolated hydrodynamically non-interacting species, (ii) semi-dilute concentration ($c=c^*$) where coil overlap or entanglement occurred, and (iii) high concentration ($c>c^*$) (Oertel and Kulicke, 1991).

### 3.6.2 Intrinsic viscosity

Intrinsic viscosity is the intrinsic ability of a polymer to increase the viscosity of a particular solvent at a given temperature. If the viscosity of a solvent is $\eta_0$ and the viscosity of polymer solution is $\eta$, then the relative viscosity is the ratio of the two,
\[ \eta_{rel} = \eta_\eta / \eta_\phi. \] Intrinsic viscosity can be obtained by the use of Huggin's Equation, 
\[ \eta_{sp}/c = [\eta] + k'[\eta]^2 c; \] and Kraemer's equation \[ \ln \eta_{rel}/c = [\eta] - k'' [\eta]^2 c. \]

3.6.3 Newtonian and Non Newtonian flow behaviour

The generalized relationship between shear stress and shear rate for a fluid is given by the equation:

\[ \tau = \tau_0 + K(\gamma)^n \]

where, \( \tau \) is the shear stress applied on the fluid, \( \tau_0 \) is the yield stress, \( K \) is the consistency index, \( n \) is the dimensionless flow index and \( \gamma \) is the fluid velocity gradient referred to as the shear rate.

For Newtonian fluids \( \tau_0 = 0 \) and \( n = 1 \) and hence, \( \tau = K\gamma \). Newtonian fluids have a linear relationship between shear stress \( \tau \) and shear rate \( \gamma \) and the proportionality constant (slope of the straight curve) is referred to as viscosity, which is uniquely defined and is independent of shear rate.

For non Newtonian fluids \( n \neq 1 \). Non Newtonian fluids may be further classified into pseudoplastic, dilatant, Bingham plastic and Casson body. For pseudoplastic fluids \( \tau_0 = 0 \) and \( n < 1 \) and the equation \( \tau = K(\gamma)^n \) applies. For dilatant fluids \( n > 1 \). For both pseudoplastic and dilatant fluids, which are called as power law fluids, the apparent viscosity is not constant but depends on the fluid shear rate, the consistency index \( K \) and flow index \( n \). From a plot of \( \log \tau \) versus \( \log \gamma \) which is a straight line, \( n \) can be obtained from the slope and \( K \), from the intercept on Y axis (at a shear rate of \( 1 \text{s}^{-1} \)).

Bingham plastic fluids show a finite yield stress which is the force needed to initiate flow and \( n = 1 \), which means that they behave like Newtonian fluids after the yield stress has been applied. \( K \) is a direct measure of viscosity at a given rate of shear; the larger the \( K \), the greater is the viscosity of the solution at a given rate of shear. For pseudoplastic fluids, the lower the value of \( n \), the more pronounced is the effect of pseudoplasticity (Charles, 1978).
The power law fluids exhibit three different flow regions depending upon the shear rate applied. At low shear rate \( \gamma \), \( \eta \) approaches a constant value \( \eta_0 \), which is the zero shear rate viscosity (upper Newtonian plateau). As the shear rate \( \gamma \) is increased, \( \eta \) decreases and the flow curve exhibits a linear profile known as power-law region. Finally, at a very high shear rate, \( \eta \) may again become independent of shear rate and approaches another constant value, the so called infinite shear rate viscosity \( \eta_\infty \) (lower Newtonian plateau). In fact, \( \eta_\infty \) cannot be generally evaluated since no experimental data can be easily obtained at very high shear rates (Lapasin et al., 1991).

Figure 1. The flow behaviour of fluids

\[ \text{Apparent viscosity (cP)} \]

\[ \text{Rate of shear (sec}^{-1}\text{)} \]

3.7 PRESENT STUDY

In general, rheological research in industry can play a major role in quality control of raw materials and final products, optimization of processes, in product formulations and their eventual modifications, to evaluate the time evolution of the macroscopic properties of a given system and its structural and/or molecular characteristics (Lapasin and Pricl, 1999). The industrial researcher has to choose appropriate polymers and determine optimum concentrations of that polymer in presence of many others for specific applications. The examination of the rheological properties exhibited by the final product is a fundamental step to ensure quality of the product. Since EPS/BE1 was intended to be evaluated as a substitute for sodium alginate, a detailed study of the rheological characteristics of EPS/BE1 was undertaken in comparison to sodium alginate and other gums.
3.8 MATERIALS AND METHODS

3.8.1 Intrinsic viscosity measurements
The intrinsic viscosities of the EPS/BE1 (0.2 and 0.6 g/dl) were determined using Ubbelohde viscometer placed vertically in a thermostat at 30 ± 0.05°C.

3.8.2 Rheological characterization
Viscosity was measured using Brookfield LVDV II+ viscometer equipped with a small sample adapter (SSA-8R) and spindle no. 16 at 30±1°C unless otherwise mentioned. Effect of prevailing temperature on viscosity of EPS/BE1 solutions was studied by maintaining the temperature of the sample in the cuvette by circulating water of appropriate temperature through the jacket surrounding the cuvette of the small sample adaptor.

Temperature stability of the viscosity of EPS/BE1 solutions was determined by incubating the solutions at the respective temperature for 1 h. The solutions were cooled to 30±1°C before determination of the viscosity. The effect of pH on rheological properties was studied by adjusting the pH of EPS/BE1 solution using 1 N HCl or 1 N NaOH. The effect of NaCl and other salts on viscosity of polysaccharide solutions was determined by mixing in equal proportions, 2X concentrations of various polysaccharide solutions and 2X concentration of the various salt solutions.

The effect of aging on viscosity of EPS solutions in presence of various salts was studied at both 30±1°C and 75°C at pH 7.0 and 9.0± 0.2 respectively. For aging, the pH of the EPS solution containing respective salts was adjusted to 7.0 and incubation was carried out at 30±1°C for 90 days. Also, the pH of the EPS solution was adjusted to 9.0±0.2 with NaOH (1.0 N) and aging was carried out at 75°C for 20 h. The solutions after aging at 75°C were cooled and further aged at 30°C for 90 days. The effect of acids was studied by mixing the respective acids or their solutions (2X) with the 2X concentration of EPS/BE1 solutions.
Chemicals
Xanthan was obtained from Sigma Chemical Co. Missouri, USA. Welan and gellan were obtained as gift from CP Kelco, Atlanta GA, USA. Other polysaccharides were obtained locally. All chemicals and solvents used were of analytical grade.

Reproducibility
All the experiments have been carried out in triplicates and have been performed three times.

3.9 RESULTS AND DISCUSSION

3.9.1 Dilute solution viscosity of EPS/BE1 solutions

The intrinsic viscosity $[\eta]$ is one of the most important source of information on the size and shape of polymer molecules (Mathakiya et al., 2004). In dilute solutions, chains of dissolved polymer do not interact among themselves and only intramolecular hydrodynamic interactions may occur. This condition is encountered only at very low values of polymer concentration $C$, i.e. for $C$ tending to zero. The individual coils of polymer are well separated from one another and are free to move independently. When a shear field is applied to such systems, the flow behaviour of each single molecule is not affected by the presence of any other molecule at all, and the total rheological response of the solution can be considered as the sum of the individual contributions of the molecules (Lapasin and Pricl, 1999).

The intrinsic viscosity of EPS/BE1 in water and in 0.1M NaCl (to screen the intramolecular electrostatic repulsions) was found to be 2700 ml/g and 2100 ml/g respectively. The decrease in intrinsic viscosity in the presence of NaCl indicated the effective screening of intermolecular repulsion between EPS chains. In either case, both the Huggin’s plot ($\eta_{in}/c$ versus concentration) and the Kraemer’s plot ($\ln \eta_{in}/c$ versus concentration) gave very good linear behaviour in the range of polymer concentrations used as shown in figure 2 (a and b).
The intrinsic viscosities of the EPS/BE1 solutions (initial concentration = 0.05% w/v) in water as well as 0.1 M NaCl were determined using Ubbelohde viscometer placed vertically in a thermostat at 30±0.05°C. Solution of EPS/BE1 was prepared in: (A) water, and (B) 0.1 M NaCl solution. (•) $\eta_{sp/c}$, and (o) $\eta_{re1/c}$.

In an earlier study also, the intrinsic viscosity of polysaccharides from Oscillatoria sp., Nostoc sp. and xanthan decreased in the presence of 0.1 M NaCl aqueous solutions as compared to intrinsic viscosity obtained in water (Parikh and Madamwar,
2006). The intrinsic viscosity of the scleroglucan solution was found to be 113 ml/g (Boeykens et al., 2004). The intrinsic viscosity of alginates from *L. hyperborea* (MW of 209.5 kD) and *M. pyrifera* (MW of 198.1 kD) in 0.1M NaCl at 25°C, was found to be only 7.11±0.009 and 5.48±0.030 respectively, indicating their less viscous nature (Martinsen et al., 1991). The alginates exhibited \( k' + k'' \) value of 0.518±0.008 and 0.544 ±0.002 respectively. The \( k' + k'' \) value observed for EPS/BE1 in 0.1 M NaCl (i.e. 0.51) was in fact more similar to that observed for *L. hyperborea* alginate.

### Table 1. Dilute solution viscosity parameters of EPS/BE1

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Huggin's constant ( k' )</th>
<th>Kraemer's constant ( k'' )</th>
<th>( k' + k'' )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water</td>
<td>0.36</td>
<td>0.30</td>
<td>0.66</td>
</tr>
<tr>
<td>0.1 M NaCl in water</td>
<td>0.29</td>
<td>0.22</td>
<td>0.51</td>
</tr>
</tbody>
</table>

#### 3.9.2 Rheological behaviour of sodium alginate solutions

Since the present study was aimed at finding a suitable substitute for sodium alginate for various potential applications, initially, rheological of sodium alginate solution was investigated. Sodium alginate solution at a concentration of 50.0 g/l exhibited a viscosity of only 102 cP (at a shear rate of 2.9 sec\(^{-1}\)). As the concentration of sodium alginate was increased, up to a concentration of 75 g/l, only a marginal increase in viscosity was observed. However, beyond the concentration of 100 g/l, a significant increase in the viscosity of the solution was observed and at 125.0 g/l, a viscosity of 6276 cP was attained (figure 3). When the shear rate was increased, the viscosity of sodium alginate solutions (50 g/l to 150 g/l) was found to decrease indicating their shear thinning or pseudoplastic behaviour as shown in figure 4 (A and B). The values of rheological constants for sodium alginate solutions were determined according to the power law model (Charles, 1978). For a pseudoplastic solution, the lower the value of \( n \), the more pronounced is the effect of pseudoplasticity (Prabhanjan and Ali, 1990).
Viscosity (at a shear rate of 2.9 sec\(^{-1}\)) was measured using Brookfield LV DV II+ viscometer equipped with a small sample adapter (SSA-8R) and spindle no. 16 at 30 ±1°C.

Although at 50 g/l, the solution of sodium alginate exhibited Newtonian behaviour, at and above a concentration of 75.0 g/l the solutions behaved as pseudoplastic fluids. Commensurate with an increase in the concentration of sodium alginate, the consistency index \(K\) was found to increase, whereas, the flow index, \(n\) was decreased suggesting an increase in the viscous and pseudoplastic nature of the solutions respectively (figure 5). In a previous study, similar to sodium alginate, the shear thinning behaviour of the solutions of an algal polysaccharide from *Monostroma nitidium* was more pronounced at higher concentrations than at lower-concentrations (Chen and Chen, 2001). Similar to a previous report, in the present study, the consistency index \(K\) increased polynomially whereas the flow index \(n\) decreased almost linearly (Cancela et al., 2003).

### 3.9.3 Rheology of EPS/BE1 solutions

Compared to sodium alginate (100.0 g/l w/v), EPS/BE1 at 10 times less concentration gave equal viscosity. As the concentration of EPS/BE1 was increased, the viscosity of its solution was also found to increase. The above behaviour, which was a characteristic feature of a hydrocolloid, is attributed to the intermolecular interaction or entanglement of the polymer chains thereby increasing their effective macromolecular dimensions (Garcia-Ochoa et al., 2000).
Viscosity and shear stress were measured using Brookfield LV DV II+ viscometer equipped with a small sample adapter (SSA-8R) and spindle no. 16 at 30±1° C. sodium alginate (g/l): (●) 50.0, (○) 75.0, (▼) 100.0, (▲) 125.0, and (■) 150.0.
Shear stress (at various shear rates) was measured using Brookfield LV DV H+ viscometer equipped with a small sample adapter (SSA-8R) and spindle no. 16 at 30±1°C. Using power-law model, a plot of log shear rate (X-axis) versus log shear stress (Y-axis) yielded a straight line, whose slope and intercept on Y-axis provided the values of flow index $n$ and consistency index $K$.

The concentration-viscosity relationship observed for EPS/BE1 was similar to that observed for an EPS from the Zoogloea sp. where apparent viscosity and pseudoplasticity increased with an increase in gum concentration (Jang et al., 2002). At all the concentrations of EPS/BE1 used, similar to sodium alginate, as the shear rate was increased the viscosity of the solution was found to decrease, which was more pronounced at higher shear rates (figure 6). For two different solutions of EPS/BE1 having concentrations 0.5% and 1.5% (w/v), the difference in viscosity observed was higher at a shear rate 1.45 sec$^{-1}$ compared to that observed at a shear rate of 29 sec$^{-1}$. At low shear rates, the shear applied was probably insufficient to break the entanglements present in the EPS solution thereby resulting in higher viscosity. On the contrary, at higher shear rates, irrespective of the concentration used, the applied shear was sufficient to overcome the viscosity effect imposed by the EPS/BE1 solution and hence to make it flow. The above property has practical significance as many of the industrial processes that use rheology modifiers such as paper coating and textile printing involve high shear conditions.
Viscosity of the EPS solutions was measured using Brookfield LV DV II+ viscometer equipped with a small sample adapter (SSA-8R) and spindle no. 16 at 30±1°C. Shear rates: (●) 1.45 (○) 2.9 and (▼) 29 sec⁻¹. (A) dilute concentration c<c*, (B) semi-dilute concentration c=c*, and (C) high concentration c>c*

Irrespective of the concentration of EPS/BE1 used, the shear dependence of viscosity suggested a power law behaviour for EPS/BE1 solutions (figure 7 A and B). The consistency index $K$ and flow index $n$ of EPS/BE1 solution (10.0 g/l) was found to be 110 s²/cm² and 0.156 respectively, which indicated the highly viscous and pseudoplastic nature of the EPS solution. Moreover, with an increase in concentration of EPS/BE1, the consistency index of the solution also increased, while the flow index $n$ decreased, indicating that the solution progressively became highly viscous and more pseudoplastic as shown in figure 8. However, beyond 7.5 g/l of EPS/BE1, the decrease in $n$ was only moderate, indicating that beyond this concentration the increase in EPS concentration may not significantly contribute to the pseudoplasticity of the solutions.
3.9.4 Rheology of selected polysaccharide systems: comparison with the rheology of EPS/BE1 solutions

Since, the EPS/BE1 was a novel polysaccharide, a comparison of its rheological properties with the rheological properties of other industrial gums was desirable. In the present study, all the industrial gums tested viz. xanthan, gellan, welan, guar gum, carboxymethylcellulose (CMC) and carrageenan exhibited pseudoplastic behaviour (figure 10 A and B, 11A and B, 12A and B and 13A and B). A comparison of various rheological parameters is as shown in table 2.

Among all the polysaccharides tested, gellan was more superior in terms of the viscosity obtained. At identical concentration (10.0 g/l), EPS/BE1 exhibited 1.6, 8.6, and 6 times higher viscosity than guar gum, carboxymethylcellulose (CMC) and carrageenan respectively. Moreover, the consistency index of the EPS/BE1 solution (10.0 g/l) was 5.8 times higher than that observed for sodium alginate solution (125.0 g/l), indicating highly viscous nature of EPS/BE1 solution. In the case of xanthan, gellan and welan, as the concentration was increased, the pseudoplasticity of the solution was also found to increase. Significantly, pseudoplasticity of EPS/BE1 solution (10.0 g/l) was 2 times higher than that of xanthan (10.0 g/l) and 4.3 times than that of sodium alginate (125.0 g/l). Welan and guar gum solutions showed the highest and the lowest degree of pseudoplasticity respectively.

At identical concentration (10.0 g/l), EPS/BE1 solution showed a 39% less viscosity and 1.6 times less value of consistency index compared to welan. In an earlier study also, at a concentration of 10.0 g/l, the flow index obtained for lactan gum was higher than that obtained for xanthan (Flatt et al., 1992). Overall, in the present study, guar gum, CMC and carrageenan fared poorly in terms of viscosity, consistency index and pseudoplasticity than other polysaccharides tested. In a previous study, a polysaccharide solution (10.0 g/l) from Monostroma nitidium showed $K$ and $n$ values of 0.271 and 0.622 respectively, indicating that the solution was less viscous and less pseudoplastic (Chen and Chen, 2001).
Figure 7. Effect of shear on the viscosity of EPS/BE1 solutions

Viscosity was measured using Brookfield LV DV II+ viscometer equipped with a small sample adapter (SSA-8R) and spindle no. 16 at 30±1°C. EPS/BE1 (g/l) (●) 2.5, (○) 5.0 g/l, (▼) 7.5, (▼) 10.0, and (■) 15.0.
Shear stress (at various shear rates) was measured using Brookfield LV DV II+ viscometer equipped with a small sample adapter (SSA-8R) and spindle no. 16 at 30±1°C. Using power-law model, a plot of log shear rate (X-axis) versus log shear stress (Y-axis) yielded a straight line, whose slope and intercept on Y-axis provided the values of flow index $n$ and consistency index $K$.

Table 2. Comparison of rheological parameters of various polysaccharides

<table>
<thead>
<tr>
<th>Polysaccharide</th>
<th>concentration (g/l)</th>
<th>viscosity (cP) at a shear rate of 2.9 sec$^{-1}$</th>
<th>consistency index $K$</th>
<th>flow index $n$</th>
<th>Yield stress Dynes/cm$^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>EPS/BE 1</td>
<td>10.0</td>
<td>6024</td>
<td>110</td>
<td>0.16</td>
<td>151.7</td>
</tr>
<tr>
<td>Sodium alginate</td>
<td>125.0</td>
<td>6276</td>
<td>19</td>
<td>0.67</td>
<td>87.0</td>
</tr>
<tr>
<td>Xanthan</td>
<td>10.0</td>
<td>8436</td>
<td>84</td>
<td>0.32</td>
<td>178.2</td>
</tr>
<tr>
<td>Gellan</td>
<td>3.0</td>
<td>7734</td>
<td>120</td>
<td>0.19</td>
<td>170.3</td>
</tr>
<tr>
<td>Welan</td>
<td>10.0</td>
<td>9888</td>
<td>180</td>
<td>0.13</td>
<td>247.7</td>
</tr>
<tr>
<td>Guar gum</td>
<td>10.0</td>
<td>3720</td>
<td>7.6</td>
<td>0.84</td>
<td>45.3</td>
</tr>
<tr>
<td>Carboxymethyl cellulose</td>
<td>10.0</td>
<td>702</td>
<td>3.7</td>
<td>0.51</td>
<td>12.3</td>
</tr>
<tr>
<td>carrageenan</td>
<td>10.0</td>
<td>1008</td>
<td>7.7</td>
<td>0.37</td>
<td>19.9</td>
</tr>
</tbody>
</table>

Shear stress (at various shear rates) was measured using Brookfield LV DV II+ viscometer equipped with a small sample adapter (SSA-8R) and spindle no. 16 at 30±1°C. Using power-law model, a plot of log shear rate (X-axis) versus log shear stress (Y-axis) yielded a straight line, whose slope and intercept on Y-axis provided the values of flow index $n$ and consistency index $K$. 

Figure 8. Effect of concentration of EPS/BE1 on its consistency index and flow index
The high pseudoplastic behaviour of polysaccharide solutions is required for many industrial applications such as textile printing, oil exploration and pharmaceutical preparations (Kang and Kotrell, 1979). In a previous study, even at high concentrations of 50 g/l, both starch and gum Arabic yielded very low viscosities of 600 and 190 cPs respectively (Ashtaputre and Shah, 1995).

Figure 10. Effect of shear rate on viscosity of xanthan solution

(A)

(B)

Viscosity and shear stress were measured using Brookfield LVDV II+ viscometer equipped with a small sample adapter (SSA-8R) and spindle no. 16 at 30±1° C. Xanthan (g/l): (●) 2.5, (○) 5.0, (▼) 7.5, and (▲) 10.0.
Figure 11. Effect of shear rate on viscosity of gellan solution

Viscosity and shear stress were measured using Brookfield LVDV II+ viscometer equipped with a small sample adapter (SSA-8R) and spindle no. 16 at 30±1 °C. Gellan (g/l): (V) 0.75, (●) 1.0, (○) 2.5, (■) 3.0, and (▲) 4.0.
Figure 12. Effect of shear rate on viscosity of welan solution

Viscosity and shear stress were measured using Brookfield LVDV II+ viscometer equipped with a small sample adapter (SSA-8R) and spindle no.16 at 30±1°C. Welan (g/l): (●) 2.51, (○) 5.0, (▼) 6.0, (▼) 7.5, and (■) 10.0.

A high viscosity is required for stabilizing suspensions such as pigment coating compositions and paint. The pseudoplasticity or reduction of viscosity due to shear helps to make EPS formulations easy to pump, spray or spread. Pseudoplasticity of
EPS also appears to be important in contributing good sensory qualities and flavour release of food industry. Highly branched structure and anionic character of xanthan has been proposed for its higher $K$ value. Similarly, EPS/BE1 being anionic polysaccharide with certain degree of branching showed higher viscosity. In fact, branching in a polysaccharide has been suggested either to interfere with or participate in intra- and intermolecular associations and increase its solubility (Yalpani, 1988).

3.9.5 Mechanism of shear thinning nature of EPS/BE1

The shear thinning behaviour of EPS/BE1 and other polysaccharides can be explained in terms of entanglement formation as reported previously for guar gum derivative solutions. Small angle x-ray scattering (SAXS) data for EPS/BE1 (section 3.14.5) suggested the (i) existence of random coil conformation for individual polysaccharide chains, and (ii) formation of denser domains due to interpenetration of individual EPS chains. Hence, in concentrated solutions of EPS/BE1, in the absence of applied shear or at lower shear rates, the rotational Brownian movement of the molecules and interpenetration of polymeric chains probably gave rise to a dynamic entangled network. At low shear rates, the disruption of existing entanglements by the imposed shear was dynamically balanced by the formation of new entanglements between different chain segments and hence no reduction in viscosity took place.

When the rate of externally imposed deformation exceeded that of the rate of formation of new entanglements, viscosity got reduced and shear thinning behaviour commenced (Castelain, 1987; Lapasin et al, 1991). The shear thinning effect was more pronounced in case of high concentrations of the polysaccharides. As concentration was increased the freedom of movement of individual chains was progressively restricted, with a consequent increase in time required to form new entanglements to replace those disrupted by the externally imposed deformation (Lapasin and Pricl, 1991).

3.9.6 Yield stress

Some fluids behave much like a solid at zero shear rate and they will not flow until a certain amount of force called yield value or yield stress is applied. Yield values are
important for determining (i) power required for a pump to start in a flooded system, (ii) ability of an EPS to prevent separation of phases in suspensions and emulsions, (iii) pourability of the EPS solution and (iv) the efficacy of EPS as a processing aid.

Figure 13. Effect of shear rate on viscosity guar gum, carboxymethyl cellulose and carrageenan

(A) 10000
(B) 1000

Viscosity and shear stress were measured using Brookfield LVDV II+ viscometer equipped with a small sample adapter (SSA-8R) and spindle no.16 at 30±1°C. (●) guar gum, (○) carboxymethyl cellulose, and (▼) carrageenan
When the yield values for various polysaccharide solutions were determined at a concentration of 10.0 g/l, welan exhibited highest amount of yield stress (247.7 dynes/cm²) followed by xanthan (178.2 dynes/cm²) as shown in table 2. At a concentration of 2.5 g/l both EPS/BE1 and xanthan exhibited almost identical yield stress, however, with an increase in the concentration beyond 50 g/l, xanthan showed higher yield stress compared to EPS/BE1 as shown in figure 14. EPS/BE1 just at 10.0 g/l concentration exhibited 1.74 times higher yield stress (151.7 dynes/cm²) compared to sodium alginate (12.5 %, w/v), although the above solutions exhibited an almost identical value of viscosity (~ 6000 cP) (table 2). The values of rheological constants determined in the present study for xanthan were in accordance with those reported by Margaritis and Pace (1985). In fact, it has been reported that aqueous solutions of xanthan having concentration above 0.75% (w/v) showed a rheological yield point (Kang and Cottrel, 1989). Also, in a pervious report, a novel EPS from Sphingomonas paucimobilis GS1 showed yield value of 115 dynes/cm² (Ashtaputre and Shah, 1995). The high yield stress values obtained in case of EPS/BE1 compared to sodium alginate indicated that under low shear the solution of EPS/BE1 would not flow easily, whereas sodium alginate will flow readily.

Figure 14. Effect of concentration of polysaccharide on yield stress

Shear stress were measured using Brookfield LV DV II+ viscometer equipped with a small sample adapter (SSA-8R) and spindle no.16 at 30±1°C. Yield stress was calculated from the plot of log shear rate versus log shear stress. (●) EPS/BE1, and (○) xanthan.
It has been suggested that the yield stress imparts stability to emulsions in low stress situations such as during storage or transportation when the prevailing stress is lower than the yield stress (Ma and Barbosa-Canovas, 1995). In a previous report, a Gum 910 from *Pseudomonas* showed which showed a high yield point (50 mN/m), was suggested to be suitable applications in textile printing, pharmaceutical or paint industries (Manresa et al., 1987).

3.9.7 Effect of temperature on the viscosity of polysaccharides

Thermal stability is an inherent property of the polysaccharide molecules. Unlike, lactan gum, a capsular polysaccharide of *Rhizobium* or agar-agar, EPS/BE1 did not form temperature dependent gels even in presence of monovalent or divalent cations or as a function of pH (Van Casteren et al., 2002; Yalpani, 1988). In the temperature range tested (30 to 100°C), no significant change in the viscosity of EPS/BE1 solution (1.0% w/v) was observed, indicating its excellent thermal stability (figure 15).

Figure 15. Effect of temperature on stability of viscosity of various polysaccharides

EPS solutions were exposed to different temperatures for 1 h and then cooled to 30 °C. Viscosity was measured (at a shear rate of 2.9 sec⁻¹) using Brookfield LVDV II+ viscometer equipped with a small sample adapter (SSA-8R) and spindle no. 16 at 30±1°C. (○) gellan (1.0 g/l); (■) sodium alginate (120.0 g/l); (●) EPS/BE1 (10.0 g/l); (♦) welan (5.0 g/l); (V) guar gum (10.0 g/l); (○) xanthan (10.0 g/l), and (▼) CMC (10.0 g/l).
Of the other polysaccharide solutions tested, in case of xanthan, a steep decrease in viscosity was observed beyond 50°C, indicating a low thermal stability. In fact, in a previous study, the decrease in viscosity of xanthan gum solution started at 45°C (Boyle and Reade, 1983; Manresa et al., 1987). A small maximum observed in case of xanthan gum could be attributed to the unwinding of an ordered conformation, such as a double helix, changing into a random coil conformation with a resulting increase in effective hydrodynamic volume of the molecule and subsequent increase in viscosity as suggested in a previous report (Kang and Cottrel, 1989).

Interestingly, a moderate increase in viscosity was observed in case of welan and gellan gum solutions (figure 15). Welan is known to exhibit high thermostability and has potential in high temperature (deep) oil drilling as an additive of drilling fluids and as a component of workover fluids (Sanderson, 1990; Sutherland, 1996). Similarly, in case of guar gum solution also, a marginal increase in viscosity was observed beyond 60°C, which dropped to its initial value after 90°C. Compared to xanthan gum solution, sodium alginate solution exhibited moderately higher temperature stability. However beyond 60°C, a gradual decrease in the viscosity of sodium alginate solution was observed which might be due to the partial depolymerization of the alginate molecule as reported earlier (Whistler and BeMiller, 1959). In a previous report also, the viscosities of both bacterial alginate from *Pseudomonas syringae* subsp. *phaseolicola* ATCC 19304 and algal alginate decreased above 52°C (Lee et al., 1996).

Several applications of sodium alginate such as a rheology modifier in paper coating and a thickener in textile printing involves operating temperatures in the range of 50 to 100°C. Since in the present study, EPS/BE1 was being evaluated as a substitute for sodium alginate for the above applications, the viscosity of the EPS/BE1 solution as a function of temperature was studied to understand its flow behaviour at various prevailing temperatures. Generally, the viscosity of solutions of most polysaccharides decrease when they are heated (Kang and Cottrell, 1989). When the EPS/BE solution was subjected to various temperatures 30 to 100°C, concomitant to the increase in
temperature, the viscosity of the solution was found to decrease and ultimately reached to minimum (~50 cP) at 70°C (figure 16).

However, upon decrease in temperature, the viscosity of the EPS/BE1 solution was regained indicating that the decrease in viscosity observed was a reversible phenomenon. The pattern of decrease of viscosity of EPS/BE1 solution with temperature was similar to that reported for xanthan gum solution (0.5% w/v) by Kwon et al. (1996). The decrease in viscosity of EPS/BE1 could be due to a helix-coil transition of the backbone followed by progressive decrease of the rigidity of the glucan chains as previously suggested for xanthan gum (Lapasin and Pricl, 1995). Similar to xanthan gum, the gradual transition of EPS/BE1 from the ordered (at lower temperatures) to the disordered state (higher temperature) suggested a lack of significant main-chain breakage to small fragments even at relatively high temperatures (Kierulf and Sutherland, 1988).

The effect of temperature on fluid behaviour of EPS/BE1 was interpreted using Arrhenius equation, \( \eta_a = A \exp \left( \frac{E_a}{RT} \right) \) as described by Kwon et al. (1996). In the above equation \( \eta_a \) = apparent viscosity (cP), \( A \) = frequency factor (cP.s), \( E_a \) = Activation energy of flow (Kcal/mol °K), and \( R \) = Gas constant (8.13 kJ/mol °K). Activation energy \( (E_a) \) of a flow process can be obtained from the plot of \( \ln (\eta_a) \) versus \( 1/T \). The activation energy for the flow process of EPS/BE1 solution (1.0 % w/v) was found to be 114.2 kJ/mol, which indicated a high thermal stability EPS/BE1 solution.

In literature, the activation energies of flow for a polysaccharide solution (5.0 g/l) of *Monostroma nitidium*, hydroxypropyl and sodium carboxymethyl-substituted guar gum, chitosan-butyric acid, chitosan-propionic acid solution and pestan were found to be 9.75 to 15.53 kJ/g mol, 16 to 39 kJ/mol, 22.25 kJ/g, 21.08 kJ/g mol and 1.0263 kJ/g mol, respectively, indicating that the activation energy depends upon the chemical nature of the polysaccharide (Chen and Chen, 2001; Kwon et al., 1996).
Figure 16. The effect of prevailing temperature on the viscosity of EPS/BE1 solution

Viscosity of EPS/BE1 solution (10.0 g/l) was measured (at a shear rate of 2.9 sec⁻¹) using Brookfield LV DV II+ viscometer equipped with a small sample adapter (SSA-8R) and spindle number 16 at various temperatures. Temperature of the sample was varied by circulating water at appropriate temperature through the jacket of the small sample adaptor.

3.9.8 Effect of pH on viscosity of EPS/BE1 solution

The viscosity of polyelectrolyte solutions exhibited pH dependence depending upon the net electric charge on the molecule (Jang et al, 2002). The viscosity of xanthan, gellan and CMC solution was highly susceptible to change in pH, whereas, the viscosity of guar gum and welan was more stable at pH 2.0 to 10.0 (figure 17). The behaviour exhibited by of xanthan and gellan solutions towards pH is a well known characteristic of most of the polysaccharide solutions. At low pH, since the acidic components exist in the free acid form, the polymer chains are presumed to be in the coiled state. As the pH is raised, the acidic component(s) begin to ionize and due to charge repulsion the polymer coils expand causing an increase in the viscosity of the solution. The nonionic nature of the guar gum was responsible for the constant viscosity of its solution over the pH range tested as reported earlier by Kwon et al. (1996). Similar to guar gum and welan, no significant change in viscosity of EPS/BE1 was observed within the pH range of 6.0 to 10.0. In the acidic pH range a marginal increase in viscosity of EPS/BE1 solution was observed. Such an increase in viscosity was also observed in case of solutions of alginic acid salts, where below a pH of 4.5, the viscosity increased because of the lesser solubility of the free acid. In fact, alginate formed gels at pH 3.0 to 3.5 and pH 11.5 to 12.0 (Whistler, 1959).
In general, most microbial exopolysaccharides are unstable under acidic conditions (Jhang, 2002). The gum 910 from a *Pseudomonas* sp. formed stiff gels at pH 3 and 5, whereas it formed weak gels at pH 11 to 12 (Manresa et al., 1987). Also, in case of an EPS designated as PS 3a24, above pH 9.0 and at low pH viscosity of its solution was found to decrease (Boyle and Reade, 1983). Unlike alginate or gum 910, EPS/BE1 did not form gel either at acid or alkaline pH. The print paste used for reactive textile printing, pigment coating composition used for coating of paper/paperboards and water-based paints are formulated to have an alkaline pH. On the contrary, many of the food preparations have acidic pH. The excellent stability of viscosity of EPS/BE1 under both acidic and alkaline pH suggested that it could be used for the industrial applications involving a wide range of pH environments.

3.9.9 Effect of NaCl concentration on viscosity of EPS/BE1

When the various polysaccharide solutions were tested for stability of their viscosity in presence of NaCl, in case of CMC and xanthan solutions, a considerable drop in viscosity was observed at 25.0 g/l of NaCl itself (figure 18).

![Figure 17. Effect of pH on viscosity of various polysaccharide solutions](image)
In case of xanthan, after an initial substantial drop in the viscosity no further decrease was observed whereas a near complete loss of viscosity was observed in case of CMC. The drop in viscosity was attributed to interactions of \( \text{Na}^+/\text{Cl}^- \) ions with the polysaccharide chains and probably it did not involve any hydrolysis. In case of welan also, compared to control, drop in viscosity of the solution was obtained at 25.0 g/l of NaCl beyond which the viscosity attained almost a constant value. However, in contrast, in case of EPS/BE1 a marginal increase in viscosity was noted at 25.0 g/l, which remained almost constant till the maximum range of NaCl tested (150 g/l). The moderate increase in viscosity observed that was observed in case EPS/BE1 at 25.0 g/l indicated an expanded chain conformation of EPS/BE1 chains. As the NaCl concentration was increased, due to charge-charge repulsions (Coulombic repulsion forces), excess energy was dissipated and the viscosity increased. This phenomenon is known as the second electroviscous effect.

Figure 18. Effect of NaCl on viscosity of polysaccharide solutions

Viscosity was measured (at a shear rate of 2.9 sec\(^{-1}\)) using a Brookfield LV DV II + viscometer equipped with a small sample adapter (SSA-8R) and spindle no. 16 at 30±1°C (●) EPS/BE1 (10.0 g/l); (V) welan (10.0 g/l); (○) xanthan (10.0 g/l), and (▼) CMC (10.0 g/l). Polysaccharide solutions (2X) were mixed with NaCl solution (2X) to get a desired concentration of NaCl.
In case of xanthan, such repulsions are probably screened at concentration around 20.0 g/l leading either to a more compact conformation (decrease hydrodynamic domain) and/or decreased in the value of Huggin's coefficient resulting in lower viscosity (Smith and Pace, 1982; Milas, 1985; Yalpani, 1988; Lapasin and Pricl, 1999; Garcia-Ochoa, 2000; Chen and Chen, 2001). In case of water soluble polymer of a Zooglea species also, the addition of NaCl resulted in an increase in the viscosity of the solution (Jang et al, 2002).

3.9.10 The effect of various salts on the viscosity of EPS/BE1
Small molecular weight co-solutes can induce a variety of effects in aqueous polysaccharide solutions, ranging from simple changes in the polymer conformational shape to alterations in gelation and solubility and transitions in ordered solution structure (Yalpani, 1988). The binding of cations to polycarboxylic acid derivatives (anionic polysaccharides) is inversely proportional to the ionic radius of the cation. In a polymer-cation system, about 80 to 90% of the cations remain closely (~0.5 to 1.0 nm) bound to the polymer, while the remainder being distributed in the double layer.

Conditions that govern reactivity of a polysaccharide with metallic ions are mainly pH, temperature and the soluble salt concentration in the solution. Under highly alkaline conditions, polyvalent metal salts may cause gelation or precipitation. Hence, in the present study, the effect of various salts on the stability of viscosity EPS/BE1 was tested at both pH 7.0 and 9.0 with or without aging at both 30°C and 75°C as shown in table 3.

Addition of NaCl, CaCl₂.2H₂O, Na₂CO₃, ZnSO₄.7H₂O or CoCl₂.6H₂O (50.0 g/l) to the EPS/BE1 solution (7.5 g/l) caused an increase in the viscosity of the solution. The highest increase in viscosity (1.5 times) of the EPS solution was observed when ZnSO₄.7H₂O was added indicating a strong interaction of Zn²⁺ ions with the EPS/BE1 chains. Upon aging at 30°C for 90 days, although in general a marginal decrease in the viscosity of the solutions was observed, in case of Na₂SO₃, MgCl₂ and CuSO₄, an increase in the viscosity was noted. An increase in the viscosity and formation of gels in the presence of added salts was reported for a bacterial EPS (Boyle and Reade, 1983). In case of succinoglycan, H⁺ and Ca⁺² were found to give high viscosities in the solution, whereas, the Na⁺ form had extremely low viscosity.
Table 3. Effect of various salts on the viscosity of EPS/BE1

<table>
<thead>
<tr>
<th>Salt</th>
<th>pH 7.0 Before aging</th>
<th>pH 7.0 After aging* (30°C)</th>
<th>pH 9.0 Before aging</th>
<th>pH 9.0 After aging (75°C)</th>
</tr>
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<tbody>
<tr>
<td>Control</td>
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<td>5100</td>
<td>4872</td>
<td>4812</td>
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<tr>
<td>NaCl</td>
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<td>4806</td>
<td>5394</td>
<td>5148</td>
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<td>Di ammonium phosphate</td>
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<td>5040</td>
<td>60</td>
</tr>
<tr>
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<td>2952</td>
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<tr>
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<td>4950</td>
<td>5484</td>
<td>3816</td>
</tr>
<tr>
<td>Na₂SO₄</td>
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<td>5112</td>
<td>5076</td>
<td>4464</td>
</tr>
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<td>5124</td>
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<td>5460</td>
<td>7440</td>
<td>5100</td>
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</table>

Viscosity of the EPS solutions were measured (at a shear rate of 2.9 sec⁻¹) using Brookfield LV DV II+ viscometer equipped with a small sample adapter (SSA-8R) and spindle number 16 at 30±1°C. To the EPS/BE1 solution (final concentration of 7.5 g/l) various salts were added to a final concentration of 50.0 g/l. The pH of the solution was adjusted to 7.0 and incubation was carried out at 30±1°C for 90 days. The pH of the solution was adjusted to 9.0±0.2 with NaOH (1.0 N) and aging was carried out at 75°C for 20 h. The solutions after aging at 75°C and pH 9.0±0.2 were cooled and further aged at 30±1°C for 90 days.

The addition of various salts to aqueous solutions *Rahnella aquatilis* gum led to a decrease in its viscosity (Pintado et al., 1998). In fact, scleroglucan got precipitated when aluminum sulfate and magnesium sulfate were added each at a concentration of 10% (w/v) (Kang and Cottrel, 1989).
At pH 9.0, in majority of the cases, an increase in viscosity was observed after addition of the respective salts, which was more pronounced in case of $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ and $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$. Contrary to the increase in viscosity of the EPS/BE1 solution observed after the addition of $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ at pH 7.0, no increase in viscosity was noted at pH 9.0. After aging of the solutions at pH 9.0 for 20 h at 75°C, a decrease in viscosity of the EPS/BE1 solution was observed in majority of the cases, the most drastic reduction in viscosity being observed in case of diammonium phosphate, whereas no such reduction was observed when another phosphate group containing salt $\text{Na}_2\text{HPO}_4$ was added. Hence, it could not be concluded that the solution viscosity of EPS/BE1 was sensitive to phosphate salts. In fact, the viscosity of xanthan gum solution has been reported to be incompatible with phosphate (Yalpani, 1988). Overall, at both pH 7.0 and pH 9.0, irrespective of the temperature of aging, divalent metal ions showed increase in viscosity of EPS/BE1 solution probably due to weak gel formation.

When the EPS/BE1 solutions aged at 75°C (pH 9.0) for 20 h in presence of were further incubated at 30°C for 90 days, in majority of the cases, an increase in the viscosity was observed suggesting that the reduction in viscosity during aging 75°C (pH 9.0) was not due to depolymerization of the EPS chains. In case of EPS/BE1 solution containing $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ after aging at pH 9.0 and 75°C a loss of viscosity to a tune of 40% was obtained. However, upon further incubation at 30°C for 90 days, a nearly 1.5 times increase in viscosity of the EPS/BE1 solution was obtained compared to control (without $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$). At already mentioned, at temperatures beyond 70°C, in solution, EPS/BE1 chains are more likely to exist in random coil conformation and show increased thermal motion. As a consequence, due to reduced entanglements of the EPS chains, the solutions of EPS/BE1 show less viscosity with increase in temperature. In presence of various cations and in particular divalent cations, it is probable that the EPS chains might have been immobilized in random coil conformation. Upon cooling also, entanglement of EPS chains might have been prevented resulting in lower viscosity. However, during incubation for a period of 90 days, the polymer chains might have slowly assumed extended and more ordered...
conformation and simultaneous interaction with cations had resulted in an increase in viscosity (figure 19).

Figure 19. A proposed mechanism for increase in viscosity of EPS/BE1 during incubation in presence of salts

(A) Binding of cations to the EPS resulting in increase of viscosity at 30°C before aging, (B) Decrease in viscosity due to assumption of random coil conformation resulting in insufficient binding of cations with the EPS chains, and (C) Regaining extended conformation and binding of cations and high viscosity

3.9.11 Effect of acids on viscosity of EPS/BE1 solutions

When the effect of various acids was tested on the viscosity of EPS/BE1 solution, in case of phosphoric acid, around 50% decrease in viscosity was observed (figure 20). In the presence of citric acid, acetic acid and tartaric acid, a moderate decrease in viscosity (not exceeding 30%) was observed indicating that EPS/BE1 can withstand acidic conditions when incorporated into a formulation having acidic pH. The decrease in viscosity of the EPS/BE1 solution observed the presence of formic acid might be due to the partial hydrolysis of EPS/BE1 chains. It has been reported earlier that xanthan gum solutions also lost viscosity in presence of either 5% acetic acid or 25% phosphoric acid (Kang and Cottrell, 1989).

CONCLUSION

EPS/BE1 showed high viscosity at low concentration, good thermal, pH and salt stability. It also exhibited high pseudoplasticity, stability to acids and various salts. The utility of the EPS for various functions is given in figure 21. The appreciable yield stress exhibited by the EPS, in other words, the existence of solid like structure in the absence of shear, suggests that it can be used as a stabilizer for suspension, emulsions and foams, which is stable in presence of salt and acid for food, pharmaceutical, cosmetic, paint, explosives and fertilizer applications. The anionic
nature coupled with high pseudoplasticity and stability at high pH and temperature, can be of use in textile printing, paper coating and welding electrode manufacturing.

**Figure 20. Effect of acids on the viscosity of EPS/BE1**

![Figure 20](image)

Viscosity of the EPS solutions were measured (at a shear rate of 2.9 sec⁻¹) using Brookfield LV DV II+ viscometer equipped with a small sample adapter (SSA-8R) and spindle number 16 at 30±1°C. EPS/BE1 and respective acids were used at a final concentration of 4.0 g/l (w/v) and 100.0 g/l (w/v), respectively.

**Figure 21. Viscosity characteristics of EPS/BE1 for various applications**

![Figure 21](image)


CHAPTER 3

CHARACTERIZATION OF EXOPOLYSACCHARIDE OF RHIZOBIUM RADIOBACTER BE1

PART C

MACROMOLECULAR AND SUPRAMOLECULAR CHARACTERIZATION
CHAPTER SUMMARY

There exists a close relationship between the shape and biological functions of polysaccharides. Exploration of the overall shapes of polysaccharide chains starts from the knowledge regarding the conformations of its repeat unit structure.

Towards obtaining a reliable 3D model for EPS/BE1, a conformational analysis of the trisaccharide repeat unit (α-D-GlcpA-β-D-Glcp-α-D-Glcp) was carried out using the SWEET-II program. The trisaccharide could be dissected into two overlapping disaccharide segments. The conformational energy map of disaccharide fragment α-D-GlcpA-β-D-Glcp indicated the absolute energy minimum at $\phi = -26.67^\circ$ and $\psi = -20^\circ$, while for the disaccharide fragment α-D-Glcp-β-D-Glcp, the energy minimum was found at $\phi = -40^\circ$ and $\psi = -17.78^\circ$ indicating the most thermodynamically favoured conformations of the above disaccharide segments.

In case of carbohydrates, the glycosidic torsions $\phi$, $\psi$, ($\omega$) are the main determinants of its 3D structure. Recently, similar to proteins, in case of polysaccharides also, Ramachandran type profiles have been used to describe the effects of rotation around the glycosidic bond $\phi, \psi$ coordinates and called as Carbohydrate Ramachandran Plots. In the carbohydrate Ramachandran plots of both oligosaccharide fragments although two troughs (of $\phi, \psi$ dihedral angles) of favoured conformational energy were obtained, only one of them was found to be populated indicating it to be the predominant conformation. The highly populated $\phi, \psi$ dihedral angles obtained for the disaccharide fragments using carbohydrate Ramachandran plots were: ($\phi = -26.0^\circ$, $\psi = -19.0^\circ$) for α-D-GlcpA-β-D-Glcp and ($\phi = -39.0^\circ$ and $\psi = -16.9^\circ$) for α-D-Glcp-β-D-Glcp. On the basis of the most thermodynamically favourable conformation, the SWEET-II program provided a 3D model for the repeat unit of EPS/BE1.

The X-ray powder diffractogram of EPS/BE1 did not reveal any sharp diffraction peak indicating the absence of crystallinity. Small Angle X-ray Scattering (SAXS) is a well established technique to determine the supramolecular structure of soft-matters such as proteins and polysaccharides at the nano-scale. In the present study, the EPS/BE1 molecules in highly viscous solutions (8.0%, w/v) were initially...
assumed to form gel-like polymeric network consisting of mutually interpenetrable coils of EPS chains. The SAXS data of EPS/BE1 fitted satisfactorily to the mathematical model corroborating the assumptions used for the supramolecular structure of viscous solutions of EPS/BE1. The existence of the SAXS scattering suggested that the heterogeneities present in the viscous EPS/BE1 solution had sizes in a nanoscale. For EPS/BE1, the dynamic correlation length, $\xi$ (the distance between two polymeric chain in the network) and the static correlation length, $\xi'$ (the average size of heterogeneous domains in polymeric network) were found to be 0.97 nm and 6.306 nm, respectively. The above observations indicated that in the viscous EPS/BE1 solution, the denser domains were distributed in a matrix of rod-like EPS chains. Compared to carrageenan, EPS/BE1 network (at 8.0% concentration) was less swollen and more condensed. Further, the presence of two different slopes in Guinier plot indicated that there was either an aggregation of EPS chains or formation of larger EPS structures.

The double logarithmic plot indicated the presence of three approximately linear regions a, b and c having slopes -2.08, -0.97, -2.66 respectively. The presence of a linear region with slope around -1 (slope b) suggested that the EPS/BE1 solution followed various fundamental models for scattering of coiled polymeric chains. The slope of 2.07 and 0.97 indicated the presence of both disc shaped and rod shaped structures in EPS/BE1 solution. In the Kratky plot of EPS/BE1 also, a sharp upturn or peak was observed at $-2.0 \text{ nm}^{-1}$, suggesting the presence of an ordered structure.

Phases more ordered than liquids but less ordered than that of crystals are called liquid crystals or mesophases, which share properties normally associated with both liquids and crystals. The lyotropic liquid crystals are formed by molecules in presence of a solvent. In the nematic phase the molecules maintain a preferred direction as they diffuse throughout the sample. A chiral nematic or cholesteric phase consists of helical structures of molecules arranged in a nematic fashion. EPS/BE1 solution (8.0% w/v) when observed under polarizing microscope, showed birefringence indicating (i) the existence of liquid crystalline mesophases due to anisotropic nature of the EPS/BE1 molecules, and (ii) the meso-structural symmetry belonged to the non-cubic subgroup. The formation of mesophases by EPS/BE1
solution was found to depend mainly upon the concentration of the EPS/BE1 solution and temperature. For EPS/BE1, stable liquid crystalline phases were seen up to a temperature of 80°C, beyond which the solution became isotropic and the mesophase disappeared. Similarly, below a concentration of 1.0% (w/v) no mesophase was seen, while at a concentration of 1.0% (w/v), mesophase was observed in a very small area of sample. Although at a concentration of 2.0% (w/v) and 4.0% (w/v) mesophase could be observed, more distinct mesophase and phase transition could be observed at a concentration of 8.0% (w/v) only. In mesophase of EPS/BE1, both rod-like (small and medium molecular weight chains) and disk-like (high molecular weight chains) molecules were aligned either homeotropically or homogenously in different regions exhibiting distinct textures characteristic of such an arrangement. On the basis of optical textures, supported by the data obtained from SAXS, the mesophase of EPS/BE1 was determined to be chiral nematic or cholesteric.
I. MACROMOLECULAR STRUCTURE

3.11 Introduction

Polysaccharides exhibit a great number of shapes and arrangements. They may either (i) form random coils in the amorphous solid state or in molecularly dispersed solutions, or (ii) may possess quite regular and even symmetric shapes in the crystalline state e.g. extended helices and sheet like structures found in polysaccharides. The helices may consist of single, double or triple strands and these conformational arrangements may remain in solution when the solid is dissolved. Amylose and xanthan are double stranded, while curdlan, laminaran, schizophyllan, scleroglucon, and lentilinan have triple helical organization (Zugenmaier, 1998; Kajiwara and Miyamoto, 1998). Rees (1977) classified polysaccharides into four conformational classes: (i) extended ribbons found in α-(1,3) and β-(1,4) glucans such as cellulose, chitin, mannan, polymannuronic acid, polyglucuronic acid and xylan, (ii) coiled springs having β-(1,3) or α-(1,4) linkages exemplified by amylose, (iii) crumpled ribbon, and (iv) flexible coils observed in case of polysaccharides having α- or β- (1,6) linkages such as dextran.

3.11.1 Conformation of polysaccharides

The multiple hydroxyl functionality of the monomeric sugar units confer unique and diverse properties to the polysaccharides compared to other biopolymers. Thus, aldohexose sugars may be linked (1, 2), (1, 3) or (1, 4) in a polysaccharide chain through either equatorial or axial disposition with respect to the sugar ring. When two aldohexopyranose sugars are linked (1, 6), the conformational freedom of the glycosidic linkage is increased as the two sugars are separated by a greater mean distance. Together the regio- and stereochemistry of the linkage govern the inherent conformational freedom of a given linkage, the polysaccharide chain trajectory, the equilibrium spatial distribution of the chain and the rates of transformation between conformations characteristic of that distribution. Because of the above factors, polysaccharides exhibit more conformational diversity than the other polymers of natural origin. Conformational analysis of polysaccharides provides information about their spatial organization, which is responsible for recognition processes in living systems (Aspinall, 1982; Yalpani, 1988; Brant, 1997; Gerbst et al., 2007).
3.11.2 Conformational analysis of polysaccharides

Exploration of the overall shapes of carbohydrate chains usually starts from the knowledge or assumptions about the conformations of the component sugar rings. In general, the shape of naturally occurring pyranose rings in carbohydrate chains may be regarded as fixed in a chair conformation in which C-6 is equatorial i.e. $^4C_1$ for D-sugars. For glycosidic bonds in which the linkage is through an oxygen atom attached to a ring carbon, the relative orientations of the participating residues can be defined completely by the two dihedral angles $\phi$ and $\psi$. When the connecting linkage is between C-1 of one residue and C-6 of its neighbour, there is an extra covalent bond with a torsion angle ($\omega$). The term linkage conformation is used to define a distinct set of values for these angles ($\phi$, $\psi$) or ($\phi$, $\psi$, $\omega$). In ordered conformations the values of torsion angles are fixed (by cooperative interactions between residues), whereas in disordered conformations (random coils) continuous fluctuation occurs. Chain flexibility provides a strong entropic drive (conformational entropy), which generally overcomes energy considerations and induces the chain to adopt disordered random coiled states in solutions (Aspinall, 1982; Yalpani, 1988; Imberty and Perez, 2000).

3.11.3 Molecular mechanics

In theoretical conformational analysis molecules can be represented as mechanical systems in which atoms are regarded as point masses. The chemical bonds are characterized by some natural values of lengths and valent angles. The conformations of real molecules reach such values that allow the conservation of the natural values as far as possible. Steric effects are modelled by van der Waals interactions. If the system is stressed, the molecule deforms in the predictable direction according to the energy gradient of the stress. The method for calculation of this deformation is named molecular mechanics (Gerbst et al., 2007).

3.11.4 Force fields

Molecular mechanics uses force field concept, which is represented by a set of equations with their parameters that describe the energy variations of a molecule due to deviation of its geometry and parameters from the ideal values. A molecule is considered as a collection of atoms held together by the harmonic forces. These forces can be described by the potential functions of structural features like bond lengths,
bond angles etc. A combinations of these potential functions is called the \textit{force field} (Tvaroska, 1989). Among various force fields, for conformational analysis of complex carbohydrates, MM3 has been proven to be well suited (Bohne et al., 1999). The force field MM3 uses bond dipole approximation for modelling of electrostatic interactions and accounts for the influence of exo-anomeric effect. Recently, the use of MM3 force field has been reported for conformational studies of pentasaccharide repeat units of an EPS of \textit{Lactobacillus} sake 0-1 and rhamnogalacturonan (Gerbst, 2007).

3.11.5 \textbf{Computational methods for studying conformation of oligosaccharides and molecular modelling of polysaccharides}

Carbohydrates are difficult to model because of their highly polar functionality and the differences in electronic arrangements such as the anomic, exo-anomeric and gauche effects that occur during the conformational and configurational changes. Computational methods for molecular modelling involving molecular mechanics approach have become indispensable for exploration of the conformational space of oligosaccharides. \textbf{The conformational analysis of polysaccharides is usually based on structural information obtained from low molecular weight analogues, such as its disaccharide segments} (Yalpani, 1988; Grachev et al., 2007).

The determination of conformational preferences of oligosaccharides is first performed by describing their preferred conformations on potential energy surfaces as a function of their glycosidic torsional angles: $\phi$ and $\psi$. It is assumed that each glycosidic linkage displays a conformational behaviour that is independent of the other structural features in the molecule. Both torsions are sequentially rotated in small increments over the full 360° range. At each point of the grid the energy according to the force field in use is calculated. It is then represented by the energies of all the conformations available as a contour map in $\phi$ and $\psi$ space. These contour maps enable graphical description of energy changes as a function of the relative orientation of the monosaccharides. They indicate the shape and position of the energy minima, the routes for interconversion between conformers and the heights of the transitional barriers. The most likely conformation is expected to have the lowest potential energy. The \textit{different orientations of the pendant groups} have a \textit{limited}
influence on the conformational space of the disaccharide (Imberty and Perez, 2000).

3.11.6 SWEET-II program
The SWEET program available at http://www.glycosciences.de, rapidly converts the commonly used sequence information of a complex carbohydrate directly into a preliminary but reliable 3-D model. In the SWEET-II program, 3D structure of the repeat unit of a polysaccharide is determined using the rigid rotation approach, in which the sugar rings themselves and the exocyclic groups are kept fixed in their appropriate conformation. The COC bond angles of the glycoside linkages are fixed to a value of 117°. The software program initially performs the conformational energy calculations using the MM3 force field. The essential steps for construction of a 3D model using SWEET-II program involves the following steps: (i) oligomer is constructed using a library of monosaccharides which are then linked according to the linkage information provided in the sequence definition, (ii) pre-optimized conformations are generated to judge the results, and the (iii) preliminary conformations are optimized using the molecular mechanics force field MM3 by software package TINKER that has been interfaced to SWEET-II. Currently, SWEET-II is able to deal with all sorts of linear and branched oligo- and polysaccharides (Bohne et al., 1999).

Similar to proteins, for carbohydrate structures, linkage torsions can be evaluated using 'Carbohydrate Ramachandran Plot' where backbone torsional angles $\phi$ and $\psi$ are plotted against each other. The frequency of populated torsion angles depends upon the monosaccharide residues involved and the kind of linkage. The Carp program (available at http://www.glycosciences.de) (i) analyses the carbohydrate data given in PDB files using the pdb2limacs algorithm and compares them to the data available in GlyTorsionDB, and (ii) generates carbohydrate Ramachandran plots for the optimized conformation(s) of oligosaccharide (Lutteke et al., 2005).

3.11.7 Random coil conformation of polysaccharides
If a polymer is regarded as a string of segments (whose length is called Kuhn length or steps) in which each unit is joined to the next by a perfectly flexible joint, the
polymer chain is extremely unlikely to be stretched out straight and assumes a conformation known as *random coil* which can be envisaged as a summation of a large number of disordered shapes (Yalpani, 1988). A *fractal object* is an assembly of particles distributed in space in such a way that the average number of particles \( n(R) \) within a sphere of radius \( R \) surrounding an arbitrary particle varies as \( R \) to some fixed power \( D \) called the *fractal dimension* (Beucage, 1996). There are several types of fractal structures that impart special properties to liquids: random walk, rigid polymers, branched polymers and colloidal aggregates. Particles can also be described in terms of their dimension in the sense that a rod is 1D, a disc is 2D and a sphere is a 3D object. For a mass fractal object such as a polymer coil the mass is given by the size raised to the dimension and in this sense the *Gaussian (random) polymer coil is a 2D object*. Fractal concepts are indispensable in the characterization of stochastic processes such as 'aggregation' and the subsequent 'transformation' during aging (Santen et al., 1995).

### 3.11.8 SMALL ANGLE X-RAY SCATTERING (SAXS)

X-rays incident upon a particle get scattered in all directions due to the differences (contrast) in electron density between a given region and its neighbour. The angle \( \theta \) is defined as the one-half of the angle of deflection of the scattered radiation, \( q \), relative to the incident radiation \( q_0 \) impinged upon a particle. All scattering patterns in small angle regime reflect a decay of intensity in \( q \) due to the fact that at decreasing size scales the number of electrons in a particle is proportional to the decreasing volume. Hence, there exists an inverse relationship between particle size and scattering angle (Stivala and Patel, 1987; Kajiwara and Miyamoto, 1998; Svergun and Koch, 2003).

A scattering process obeys a reciprocal law which relates the distance \( r \) in an ordinary (real space) with the scattering vector \( q \) in Fourier (scattering) space by the phase factors defined by \( \exp(-iqr) \) i.e. the scattered intensity \( I(q) \) is given by the Fourier transformation of the electron density distribution in the object:

\[
I(q) = V \int_0^\infty 4\pi r^2 \gamma(r) \exp(-iqr)dr
\]
Here the magnitude of the scattering vector is given by \((4\pi/\lambda) \sin (\theta/2)\) with \(\lambda\) and \(\theta\) being the wavelength and the scattering angle, respectively. \(\gamma(r)\) is a correlation function representing the average of the product of two electron density fluctuations at a distance \(r\). \(V\) is the volume of the particle. The distance distribution function \(P(r)\) is defined as,

\[
P(r) = V r^2 \gamma(r)
\]

which is characteristic of the shape of the scattering object.

3.11.8.1 Radius of gyration \((R_g)\)

The radius of gyration \((R_g)\) represents the root mean square distances of the elements of a polymer chain from its centre of gravity, which is a measure of its spatial extension. The radius of gyration is obtained from the Guinier equation.

\[
\ln I = \ln I_0 - KR_g^2 (2\theta)^2 \quad \text{or} \quad \ln I = \ln I_0 - R_g^2 Q^2/3
\]

Where, \(K = (2\pi/\lambda)^2/3\), \(Q = (4\pi/\lambda) \sin \theta\), and \(2\theta\) is the scattering angle in radians. Since the decay of the intensity at very small angles (inner portion of the scattering curve) is a Gaussian function of \(2\theta\). In the Guinier plot \([\ln I(q)\) versus \(q^2\)], the slope of the straight line obtained in the range of \(q<1.3/R_g\) gives the value to \(R_g\).

3.11.8.2 Porod’s Law

A sphere having smooth surface can be decomposed into several spherical scattering elements that bisect the particle/matrix intervene. The number of such spheres is proportional to the surface area for the particles divided by the area per scattering element \(r^2\) or \(1/q^2\). In the asymptotic limit, \(qR_g >> 1\), the scattering probes the interface of such particles. In the case of homogenous particles with average surface area \(S\), this leads to the Porod behaviour, Porod’s law can be used to measure the surface area of domains in the nano scale.

\[
I(q) = 2\pi N \Delta \rho^2 S q^{-4}
\]
3.11.8.3 Variations in Porod’s Law

Power law variation of \( I(q) \) is very commonly observed in SAXS from particle systems composed of both compact and fractal morphologies as summarized below.

\[
I(q) \propto q^{-p}
\]

Where, if \( p = 4 \Rightarrow \text{sharp interface}, \) \( 3 \leq p < 4 \Rightarrow \text{surface fractal}, \) \( p < 3 \Rightarrow \text{mass fractal}, \) \( p = 2 \Rightarrow \text{Gaussian polymer chain}. \) Many polydisperse systems consisting of multiple structural levels such as primary particles, aggregates and their agglomerates, display structurally limited power law regions with intervening Guinier regions.

3.11.9 Liquid crystals or mesophases

Phases more ordered than liquids but less ordered than that of crystals are called liquid crystals or mesophase which share properties normally associated with both liquids and crystals. On the contrary, in the isotropic phase, no long-range orientational and positional order exists (figure 15). In the liquid crystals, as the molecules undergo diffusion (without losing orientational and/or positional order), one molecular axis (known as director) tends to point along a preferred direction (Collings and Hird, 1997). To display mesophases a molecule should possess (i) rigid zone(s) corresponding to the molecular long axis, (ii) polar groups and/or weak dipolar groups at one of the ends and (iii) be anisotropic i.e. either their shape is such that one molecular axis is different from the other two or different parts of the molecules have very different solubility properties (Burducea, 2004). Of the two types of liquid crystals, thermotropic liquid crystals are obtained by heating a solid substance and lyotropic liquid crystals are formed by molecules in presence of a solvent (Neto, 1992).

3.11.10 Lyotropic mesophases of polysaccharides

Many amphiphilic sugar derivatives can have mesogenic phases where the sugar molecules contact each other and form intermolecular hydrogen bonds, resulting in a two dimensional ordering (Van Doren et al., 2000). Rod-like macromolecules in solution undergo transition from an isotropic state to a state of partial long range orientational order (lyotropic liquid crystal) at a critical concentration. Lyotropic mesophases include lamellar \( L \), cubic \( I \) or \( V \), hexagonal \( H \), nematic \( N \), cholesteric \( Ch \) and some intermediate ones. In solution flexible polymers assume a more random coil
conformation, while the rigid polymers exist in rod-like shapes. These structures form domains that are anisotropic and within which there is a nematic order of the chains. In the nematic phase (figure 15) the molecules maintain a preferred direction as they diffuse throughout the sample (Collings and Hird, 1997; Gray and Winsor, 1974). Although in the nematic phase, the molecules are distributed at random, due to the forces arising from mutual attraction, the molecules adopt a common mean parallel orientation.

Figure 15 Various phases of matter

(A) crystalline solid (long range ordering) (B) Isotropic liquid (random ordering) (C) nematic phase (orientational ordering) (D) nematic discotic phase (orientational ordering)

3.11.11 Chiral nematic phases

Molecules that form nematic phases are long and rod-like in shape (Van Doren et al., 2000). Chiral centres in a monomeric unit of such molecules lead to a chiral chain conformation (helix) and may lead further to clusters which then form the basis for a supramolecular helicoidal structure. In nematic liquid crystals, dipole-dipole interactions and mutual polarisability are the forces that hold the arrangement together. On macroscopic scale a swirling can occur whereby macroscopic helicity i.e. helical structures consisting of many non covalently bonded molecules is formed resulting into chiral nematic or cholesteric phase as shown in figure 16. Highly concentrated hydroxypropyl cellulose forms chiral nematic liquid crystals showing iridescent colours caused by a supramolecular helicoidal arrangement of nematic sheets (Collings and Hird, 1997; Gray and Winsor, 1974). Glucuroxyxans possess surface charges and molecular structure such as flexible side chains, both of which favour the formation of cholesteric assembly (Reis et al., 1991).
3.11.12 Homeotropic and homogeneous alignment of molecules

In **homeotropic** alignment the molecules that constitute the phase structure are oriented such that their long axes are normal to the supporting matrix (figure 17). The polarized light in the above case is unaffected by the material and so light cannot pass through the analyzer and complete blackness appears. In **homogenous** (planar) arrangement the constituent molecules of the liquid crystal phase are oriented parallel to the supporting substrates and exhibit birefringence and coloured textures. However, when the long axes are in line with either polarizer, light is extinguished. Where chiral molecules constitute certain liquid crystal phases, the helical axis is the optical axis so alignment of the helical pitch is selectively reflected. Pitch is the distance along the helical axis over which the director does a full rotation of $2\pi$ (Collings and Hird, 1997). If the pitch length is comparable to the wavelength of visible light, then iridescent colours are seen in reflection and the complementary colours are seen on transmission (iridescent texture). If the pitch length of the helix is not comparable to that of the wavelength of the visible light, the texture obtained will be optically extinct (black) and is called pseudohomeotropic.

Figure 17. Homeotropic and homogenous alignment of nematic liquid crystals
3.11.13 Examples of lyotropic liquid crystalline phases of polysaccharides

Lyotropic liquid crystalline phases have been reported for natural polysaccharides such as xanthan, schizophyllan, scleroglucan, konjac glucomannan and levan in aqueous solutions, chitin in dimethyl acetate+LiCl, amylose ether in chloroform and various chitosan derivatives in a number of organic solvents including dimethyl sulphoxide (DMSO) and dioxane (Oertel and Kulicke, 1991; Schorsch et al., 1995; Zugenmaier, 1998). The supramolecular structure of xanthan was investigated and the cholesteric spherulites observed were modelled for xanthan (Oertel and Kulicke, 1991). Typical schlieren texture has been reported for levan suggesting the presence of either a nematic or chiral nematic order with a large pitch. Similarly, fingerprint textures reported for aqueous liquid crystalline systems of xanthan, schizophyllan, and scleroglucan indicated large sizes of pitch. Chiral nematic liquid crystal behaviour of aqueous schizophyllan and scleroglucan were identical as the two polysaccharides had essentially the same triple helical structure. Chitin also showed chiral nematic structure (Zugenmaier, 1998).

3.11.14 Applications of lyotropic liquid crystals

In biomedical area, liquid crystals have uses in controlled drug delivery, protein binding and phospholipids labeling. Liquid crystals have different applications such as temperature sensing devices, cosmetics, solvents in chemical reactions, in chromatographic media, in spectroscopy, in holography and in electro-optical display devices (Kumar, 2006). Compared to starch, the starch ether derivatives acquired liquid crystallinity which allowed them to be processed by a novel orientation technique designed especially for such main chain lyotropic liquid crystalline polymers (Zhao et al., 1998). Recently, lyotropic liquid crystal behaviour was used for detection of microbial immune complexes in real time (Helfinstine et al., 2006). Liquid crystalline molecules are considered as models for biological membranes. Moreover, the implication that liquid crystal phase transitions play important role in the organization and functioning of cells and tissues is long standing (Kotz and Kosmella, 1999).
3.12 THE PRESENT STUDY

Determination of the three-dimensional structure of oligosaccharides and understanding of the molecular basis of their recognition by receptors represent the main challenges in structural glycobiology (Imberty and Perez, 2000). There exists a close relationship between the shape and biological functions of polysaccharides and it is assumed that knowledge of the conformational behaviour of the bacterial surface polysaccharide in solution may help in 'mimic' approach (Clement et al., 2003). As discussed later in this chapter, the viscous aqueous solutions of EPS/BE1 exhibited liquid crystalline behaviour, which was a consequence of its supramolecular structure in solution. In order to explain the formation of liquid crystalline mesophases and ultimately to delineate the supramolecular organization of the EPS in solution, small angle x-ray scattering (SAXS) analysis of the viscous solution of EPS/BE1 was carried out. SAXS is a powerful method for study of nano-order structures of polymers in a solution or in gel state (Svergun and Koch, 2003). It allows defining exactly the liquid crystalline phase boundaries as well as the region of coexistence of different phases (Mezzenga et al., 2005).

In past, SAXS has been used to investigate the structure of starch and starch products (Pikus, 2005), cross-linked hyaluronan (Gamini et al., 2002), dextran (Hirata et al., 2003), temperature transitions and gelation mechanism of gellan, carrageenan, and curdlan and the impact of inorganic salts on carrageenan and gellan (Mischenko et al., 1996; Yuguchi et al., 2002; Yuguchi et al., 2003). Recently, structure of marine bacterial EPS at different pH values was studied by Doga et al. (2005).

From the literature, it appears that the information on liquid crystalline properties of the exopolysaccharides is lacking, although as part of characterization, extensive physical, chemical and rheological characterization of several exopolysaccharides have been often reported. Since liquid crystalline behaviour of a polysaccharide is as a consequence of its supra molecular organization, studies were undertaken to understand the supramolecular organization of viscous solution of EPS/BE1.
3.13 MATERIALS AND METHODS

3.13.1 X-ray diffraction
The X-ray diffraction pattern of EPS/BE1 powder was recorded using a Philips X-ray diffractometer with CuKα radiation. The samples were scanned from 0 to 100 (2θ).

3.13.2 Conformational analysis
To generate a 3D model of the trisaccharide repeat unit of EPS/BE1, initially, the oligosaccharide repeat unit structure of EPS/BE1 was fed into the SWEET program (available at http://www.heidelberg.de). The 3D model of the polysaccharide generated by the SWEET-II program was displayed using the TINKER program suite interfaced with SWEET-II program. Conformational maps for each of the disaccharide unit of the EPS/BE1 were generated using Carp program.

3.13.3 Small angle X-ray scattering (SAXS)
SAXS analysis of EPS/BE1 solution (8.0% w/v) was carried out on an Anton Paar Analytical SAXSess instrument equipped with PW 3830 X-ray generator at 25°C. As the x-ray beam was point collimated, no slit desmearing was required for the analysis.

3.13.4 Polarization microscopy
The mesophases of the polysaccharide solutions were observed with a Leitz Laborlux 12 POL polarizing microscope equipped with a heating stage. The polymer samples (8.0 % w/v) either in distilled water or in 0.13 M KCl were placed between a slide and a coverslip, sealed to prevent fluid evaporation and kept on a heating stage. The samples were subjected to various temperatures and the extent of birefringence was observed after 5 min.

Chemicals
All the chemicals used were of analytical grade and was obtained locally.

Reproducibility
All the experiments have been carried out in duplicates and have been performed three times.
3.14 RESULTS AND DISCUSSION

3.14.1 Conformational analysis

Unlike proteins, carbohydrates cannot yet be described in terms of their three-dimensional or secondary structural motifs. Therefore, currently no knowledge based methods can be applied for modelling of 3-D structures of oligosaccharides. Nevertheless, as a first approximation one can make the assumption that each glycosidic linkage shows conformational behaviour which is independent from structural features of the rest of the molecules (Bohne et al., 1999). Such an approach was supported by Imberty et al. (1990; 1991) who claimed that any occurrence of interaction between different residues could only result in a reduction of the available conformational space around the disaccharides. The conformational analysis of polysaccharides is usually based on structural information obtained from disaccharide segments (Yaipani, 1988; Grachev, 2007). The configurational energy $V(\phi, \psi)$ of a polysaccharide chain may be considered to be the sum of the conformational energies of each of its constituent residues over a range of $\phi, \psi$ pairs. The probability $P(\phi, \psi)$ for a particular chain conformation with energy $V(\phi, \psi)$ to occur is inversely related to the conformational energy above the least energetic conformation, $V_0(\phi, \psi)$. Thus, the overall coil dimensions can be derived from the probability distribution for local geometries. The probability of a polysaccharide segment to assume a conformation with energy within 2 kcal/mol of $V_0(\phi, \psi)$ is 0.95-0.99 (Yaipani, 1988). In the conformational analysis, the non-bonded interaction, especially the van der Waals interactions have a dominating influence on the conformations of many oligosaccharides and it is easy to rank conformations in their order of increasing or decreasing van der Waals repulsion. A given grid point on the map is assigned to be a minimum if all adjacent energy grid points exhibited higher energy (Tvaroska, 1989).

For obtaining a reliable 3D model of repeat unit of EPS/BE1, initially, the conformational analysis of its trisaccharide repeat unit ($\alpha$-D-Glc\textsubscript{p}A-$\beta$-D-Glc\textsubscript{p}-$\alpha$-D-Glc\textsubscript{p}) was carried out. The trisaccharide repeat unit was comprised of two disaccharide segments i.e. $\alpha$-D-Glc\textsubscript{p}A-$\beta$-D-Glc\textsubscript{p} and $\beta$-D-Glc\textsubscript{p}-$\alpha$-D-Glc\textsubscript{p}. In the software program, the monosaccharide residues and the linkages present in the
trisaccharide repeat unit of EPS/BE1 were fed into the input boxes. Towards calculation for the conformational energy, in the first iteration which provided a global energy map, a complete rotation around the glycosidic bond (from 180° to 180°) at 20° increments was carried out and the van der Waals interaction energies based on the MM3 parameterization at each step was calculated. In the second iteration, using a step size of 1.8, a fine conformational map with 324 grid points around the minimum of global energy map was calculated. Although the conformational energy maps for each of the two disaccharide segments were obtained after both first and second iteration, only the conformational maps obtained after the second iteration is presented here. **Figure 1** shows the mesh plot and **figure 2 and 3** gives values of energy minimum and conformational energies for the most thermodynamically favoured conformation for each of the two disaccharide units. The interaction energies (Kcal/mole) between the two sugar moieties were divided into 10 intervals between minimal and maximal energy value indicated by numbers and colours as given in the legend. **The absolute minimum of the grid that was found is indicated by a cross (+) and its values are given at the bottom of the legend.** The conformational maps obtained for each disaccharide unit of the oligosaccharides showed the dependence of energy of a molecule on the φ and ψ angles.

**Figure 1.** Population maps for both glycosidic linkages of the trisaccharide repeat units of EPS/BE1

The conformational energy map of the oligosaccharide fragments (A) α-D-GlcpA-β-D-GlcP and (B) α-D-Glcp-β-D-GlcP calculated through rigid rotation approach after second iteration (step size of 1.8°). The van der Waals interaction energies were calculated for all grid points. Grid points with high energies are neglected. The conformational map is calculated from the generated energy hyper surface by projecting iso-energy contours around the minima found into the φ, ψ plane.
In case of disaccharide fragment α-D-GlcPA-β-D-Glcp the absolute energy minimum was found to be at $\phi = -26.67^\circ$ and $\psi = -20^\circ$ and for the disaccharide fragment α-D-Glcp-β-D-GlcP the minimum was found at $\phi = -40^\circ$ and $\psi = -17.78^\circ$ indicating the most favoured conformation of these disaccharide segments. The above mentioned values for $\phi, \psi$ angles exhibiting the most favoured conformation for respective disaccharides was used by the SWEET-II to generate the 3D model of the repeat unit of EPS/BE1.

3.14.2 Carbohydrate Ramachandran plot

In case of carbohydrates, the glycosidic torsions $\phi, \psi, (\omega)$ are the main determinants of its 3D structure. Hence, for conformational analysis of individual glycosidic linkages, most often, Ramachandran-type profiles have been used to describe the effects of rotation around the glycosidic bond $\phi, \psi$ coordinates (Frank et al., 2007). Since, complex carbohydrates are rather flexible molecules, several conformations for each glycosidic linkage can be populated (Bohne and von der Lieth, 2002). GlycoMapsDB (available at www.heidelberg.de) is a database containing more than 2500 calculated conformational maps for a variety of di- to pentasaccharide fragments contained in N- and O-glycans. As oligosaccharides representing the branch points of the N-glycans are also included in the set of fragments, the influence of neighbouring residues is also reflected in the conformational maps (Frank et al., 2007). Carp program generates carbohydrate Ramachandran plots on the basis of the structural information provided about the $\phi, \psi$ dihedral angles of the oligosaccharides. Each disaccharide substructures in PDB file format were uploaded on to the Carp software and the carbohydrate Ramachandran plots obtained were as shown in figures 4 and 5.

The $\phi, \psi$ dihedral angle for the most favoured conformation for both disaccharide fragments of the trisaccharide repeat unit was almost identical when the carbohydrate Ramachandran plots obtained using Carp program and the fine conformational map calculated by SWEET-II were compared. Although the carbohydrate Ramachandran plots of both oligosaccharide fragments showed two troughs of favoured conformational energy, only one of these was populated indicating it to be the predominant conformation (figures 4 and 5).
The $\phi, \psi$ dihedral angles obtained for the disaccharide fragments using carbohydrate Ramachandran plots were: ($\phi = -26.0^\circ$, $\psi = -19.0^\circ$) for $\alpha$-D-GlcPA-$\beta$-D-Glc$\beta$ and ($\phi = -39.0^\circ$ and $\psi = -16.9^\circ$) for $\alpha$-D-GlcP-$\beta$-D-GlcP.

Figure. 2. Fine conformational map of $\alpha$-D-GlcPA-$\beta$-D-GlcP

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<th>Psi ($\psi$)</th>
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The torsion angles are over $\phi = \text{H1-C1-O-C4}$ and $\psi = \text{C1-O-C4-H4}$. The interaction energies (kcal/mol) between the two sugar moieties are divided into 10 intervals between minimal and maximal energy values indicated by numbers and colors are:

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</tbody>
</table>

The absolute minimum energy of the grid (step size 1.8°) is indicated by (+) at torsion angles $\phi = -26.67^\circ$ and $\psi = -20.00^\circ$.
The torsion angles are over: $\phi = H2-C2-O-C1$ and $\psi = C2-O-C1-H1$. The interaction energies (kcal/mol) between the two sugar moieties are divided into 10 intervals between minimal and maximal energy value indicated by numbers and colors are:

1. $[0.0-0.8]$
2. $[0.8-1.6]$
3. $[1.6-2.3]$
4. $[2.3-3.1]$
5. $[3.1-3.9]$
6. $[3.9-4.7]$
7. $[4.7-5.4]$
8. $[5.4-6.2]$
9. $[6.2-7.0]$
10. $[7.0-7.8]$

The absolute minimum energy of the grid is indicated by (+) at torsion angles $\phi = -40.00^\circ$ and $\psi = -17.78^\circ$. 
The observation of two different low energy values can be attributed to the fact that the linkages with α- orientation are quite restricted for the rotations about the φ torsion angle, whereas the ψ torsion angles are much more flexible. A similar observation was obtained in case of pentasaccharide fragments of an O-specific polysaccharide of *Shigella flexneri* 5a (Clement et al., 2003). Recently, using SWEET II platform, the role of the length of oligosaccharide models used in predicting polysaccharide conformational and spectral characteristics has been studied (Grachev et al., 2007).
14.3 3D structure of the trisaccharide repeat unit of EPS/BE1

From the conformational energy calculations, the energetically most favoured 3D structure of the repeat unit of EPS/BE1 is as shown in figure 6. The current version of the SWEET-II program the 3D structure for generates only the most favoured conformation out of a manifold. However, it has been reported that the reliability of the generated model is very high as in most of the cases studied by the authors of the program, the selected examples of the \( \phi, \psi \) values of the minimum found by SWEET procedure were quite similar to values found using other methods (Bohne et al., 1998).

3.14.4 X-ray powder diffraction

Determination of the crystallinity in a polymer using of powder diffraction method is based on the assumption that the experimental intensity curve obtained is a linear combination of the crystalline and the amorphous component present in that polymer.
Usually in polysaccharides, the crystallinity if present is only partial. Powder (unoriented sample) pattern provides information about the interplanar spacings in a definite unit cell (Danishefsky et al., 1970). The X-ray powder diffractogram of EPS/BE1 (Figure 7) did not reveal any sharp diffraction peak indicating the absence of crystallinity. However, two broad amorphous peaks were observed. In a previous report also, although the chitosan powder showed peaks indicating crystallinity, the films formed from chitosan were amorphous and hence no peaks were detected in the x-ray diffractogram (Cervera et al., 2004).

Figure 7. X-ray diffractogram of EPS/BE1

The x-ray diffraction pattern of EPS/BE1 was recorded using a Philips X-ray diffractometer with CuKα radiation. The samples were scanned from 0 to 100 (2θ).

3.14.5 SMALL ANGLE X-RAY SCATTERING (SAXS)

Small angle x-ray analysis of EPS/BE1 solution was carried out to understand the structure of EPS/BE1 molecules in viscous solution. In previous study, Dogsa et al. (2005) had proposed a mathematical model to describe the behaviour of exopolysaccharide solutions when analysed by SAXS. The above model for scattering intensity of gels has been already successfully applied for studying the solution structure of PDMS gels (Geissler et al., 1997), polymer gels (Horkay et al., 1996, 2000; Hecht et al., 2001) and for gels formed by alginate (Draget et al., 2003). Following the model of Dogsa et al. (2005), in the present study also, the EPS/BE1
molecules in highly viscous solutions were assumed to form gel-like polymeric network consisting of mutually interpenetrable coils. The network was neither symmetrical nor entirely homogenous in terms of local polymer chain density. There were regions with higher density of polymer with an average diameter $\Xi_j$ (a \textit{static correlation length}). These denser domains were considered static in comparison to the regions of the network with lower density. The average distance $\bar{\xi}$ between polymer chains in the lower density regions is the \textit{dynamic correlation length}. The mathematical expression used by Dogsa et al. (2005) for scattering intensity of EPS system is as given below:

$$I(q) = \Delta \rho^2 \left( \frac{kT\phi^2}{M_{os}} \frac{1}{1+q^2\bar{\xi}^2} + \sum_j \frac{8\pi\langle \delta\phi^2 \rangle_j \Xi_j^3}{(1+q^2\Xi_j^2)} \right)$$

$I(q)$ is scattering intensity normalized to scattering volume; $T$ is the absolute temperature of the polymer, $k$ is the Boltzmann constant, $\phi$ is the polymer volume fraction; $\langle \delta\phi^2 \rangle_j$ is the mean-square amplitude of polymer volume fraction fluctuations due to the presence of $j^{th}$ inhomogeneity (denser domain); $\Xi_j$ is the static correlation length, $\Delta \rho^2$ is the scattering contrast factor; and $M_{os}$ is the longitudinal osmotic modulus defined by,

$$M_{os} = \phi (M\pi/M\phi) + 4G/3$$

Here $\pi$ is the osmotic pressure, and $G$ is the osmotic modulus, which was introduced due to elasticity arising from cross links. In the above model the presence of cross-links was not considered, however, the polymeric coils were assumed to at least partially interpenetrate each other, and therefore elasticity was present. The scattering data obtained in case of EPS/BE1 fitted to the mathematical model of Dogsa et al. (2005) satisfactorily indicating that the assumptions used for structure of EPS/BE1 solution were true.
The existence of the SAXS scattering suggested that the heterogeneities present in the EPS/BE1 (8.0%, w/v) solution had sizes in a nanoscale. The SAXS scattering curve where the intensity $I(q)$ was plotted as a function of absolute value of scattering vector, $q = 4 \pi \sin \theta / \lambda$ (where $2\theta$ is scattering angle, and $\lambda$ is wavelength), is as shown in figure 8.

Figure 8. Small angle X-ray scattering from EPS/BE1 in water

Scattering was obtained from 8.0 % (w/v) solution of EPS/BE1 using Anton Paar Analytical instrument at 25°C.

An inverse relationship existed between particle size and scattering angle. Hence, the most intense scattering that occurred at small $q$ values suggested the presence of large structures in the EPS/BE1 solution. In general, presence of a peak in the SAXS curve has been taken as an indication for the presence of crystalline order in the polysaccharide. For example, in a previous study on starch products, as the semi-crystalline structure of amylopectin was destroyed, no visible peak was detected in the SAXS curves (Pikus, 2005). Similarly, the SAXS curves for EPS/BE1 also, did not exhibit any peak indicating the absence of any crystalline order. In SAXS, spherically symmetrical objects give scattering patterns with distinct minima, while very anisometric particles yield featureless scattering curves, which decay much slowly than those of globular particles. **The absence of a distinct minima and a slowly**
decaying scattering curve in case of EPS/BE1 indicated that the EPS molecules did not have spherical shape and were anisometric.

In case of EPS/BE1 solutions also, the SAXS curves obeyed Porod's law. Using Porod's law, the specific surface area and the size distribution function for the heterogeneities present in the solution can be obtained. The static correlation length $\xi_j$, which was the average size of heterogeneous domains in polymeric networks, remained larger than the dynamic correlation length, $\xi$, which is a measure for the distances between polymeric chains in the network. For EPS/BE1, the dynamic correlation length, $\xi$ and the static correlation length, $\xi_j$ were found to be 0.97 nm and 6.306 nm respectively. The above observations indicated that in the viscous EPS/BE1 solution, the denser domains were distributed in the matrix of rod like EPS chains. The denser regions of EPS/BE1 might have arisen due to aggregation or H-bond formation.

Although, the dynamic correlation length of EPS/BE1 was less than that observed for carrageenan (3 to 4 nm) (Evemenenko et al., 2001), the static correlation length was found to be nearly comparable (8 to 10 nm). Data for different polymeric gels suggested that the dynamic correlation length $\xi$ correlated well with osmotic susceptibility $\phi^2/M\infty$. The increased $\xi$ meant that the average distance between chains increased and this could be interpreted as swelling of the network also. The ratio of two main parameters i.e. $\xi_j$ and $\xi$ for EPS/BE1, was similar to that reported earlier for a marine bacterial EPS (Dogsa et al., 2005), suggesting that compared to carrageenan, EPS/BE1 network (at 8.0% concentration) was less swollen and more condensed.

Similar to the present study, in a previous report, SAXS revealed a heterogeneous structure that could be described by three characteristic correlation lengths. Large globular particles with a $R_g$ of 308±10 nm appear embedded in a disordered medium with a correlation length of $\xi\approx 120$ nm and a fractal dimension of $df = 3.2±0.3$ (Coviello et al., 1997). Moreover, in case of curdlan gels, both the presence of liquid like (composed of flexible chains) and solid like (completely associated chains)

domains were suggested. Interestingly, cyclic β-(1,2) glucan chain assumes a shape of a flat disc and a linear homolog the shape of a cylinder (Kajiwara and Miyamoto, 1998).

For ideal monodisperse systems, the Guinier plot i.e. ln $I(q)$ versus $q^2$ should be a linear function whose intercept gives $I(0)$. Guinier plot can be considered as a test of the sample homogeneity and deviations indicate either attractive or repulsive interparticle interactions leading to interference effects. The value of $I(0)$ is proportional to the squared contrast of the particle, the number of particles in the illuminated volume and the intensity of the transmitted beam. Further, the presence of two different slopes in Guinier plot indicated that there was either an aggregation of EPS chains or formation of larger EPS structures (figure 9).

Figure 9. Guinier plot for SAXS from EPS/BE1

Scattering was obtained from 8.0% w/v solution of EPS/BE1 using Anton Paar Analytical instrument at 25°C.

As reported by Dogsa et al. (2005), the experimental scattering curve obtained for EPS/BE1 was fitted only up to 1.4 to avoid the noise at higher angle. The double logarithmic plot in figure 10 indicated the presence of three approximately linear regions a, b and c having slopes -2.08, -0.97, and -2.66 respectively. The presence of a linear region with slope around -1 (slope b) suggested that the EPS/BE1 solution followed various fundamental models for scattering of coiled polymeric chains. The
slope of 2.07 and 0.97 indicated the presence of both disc shaped and rod shaped structures in EPS/BE1 solution.

Essentially, a strongly self attracting chain would pack itself into a ball, this would make a sphere (3D; slope= 4), a strongly self repelling chain would stretch out into a completely extended rod (1D; slope=1). Polymer chains that neither attract nor repel themselves show slope=2. This corresponds to a “random walk” or “Gaussian Chain”. Since the linear region with slope c exhibited high amount of noise, it could be concluded that scattering curve indicated the presence of two different size-scales in EPS/BE1. In a previous report also, in case of SAXS studies on a marine EPS isolated from a marine bacterium from Northern Adriatic Sea, log-log plot indicated two different slopes in the q region up to 1.4 (Dogsa et al., 2005). Horkay et al. (1996; 2000) found a two step behaviour in polymeric gels and Coviello et al. (1997) observed a three-step heterogeneity for the EPS from Rhizobium leguminosarum 8002. The diversity of EPS/BE1 structure was more evident from Kratky-Porod plots (figure 11).

Figure 10. Log-log plot of scattering intensity of EPS/BE1

Scattering was obtained from 8.0 % w/v solution of EPS/BE1 using Anton Paar Analytical instrument at 25°C. Scattering up to q =1.4 was only considered for analysis. a, b, and c are the slopes of respective linear regions.
Scattering was obtained from 8.0% w/v solution of EPS/BE1 using Anton Paar Analytical instrument at 25°C.

The Kratky-Porod or worm-like chain model describes polymers as a thin, inextensible rod made up of continuous and elastic material. The high scattering intensity observed for EPS/BE1 at low $q$ was more similar to the earlier report on a marine EPS, where there was a steep decrease in $I \times q$ intensity with increase in $q$ followed by an approximately constant $I \times q$ region (Dogsa et al., 2005).

Interestingly, 3D model of the repeat unit structure of the EPS/BE1 pointed to a zigzag flat ribbon like structure for EPS/BE1 chains. For these structures the bonds connecting each sugar residues to its two glycosidic oxygens are parallel or subtend obtuse angle mediated by the presence of O-C-4 and C-1-O bonds across the $\beta$-D-glucose residue in cellulose (Aspinall, 1982). These ribbon polymers has been reported to possess an unusual rigidity: they rather freely bend inside one plane (the plane of main flexibility), but their out of plane rigidity is extremely high. Anisotropy of the rigidity induces a shape anisotropy of the chains, which tend to adopt a disc-like conformation (figure 12). The shape anisotropy in turn can cause an orientational ordering of the coils and in particular lead to the formation of nematic liquid crystalline phases with various symmetries (Nyrkova, 1997). Persistence length and
flexibility of a polymer chain i.e. the extent of random walk behaviour can be studied from Kratky plot \([q^2 I(q) \text{ versus } q]\).

Figure 12. Disk shaped conformation of EPS/BE1 in random coil state.

At higher \(q\), the Kratky-Porod worm like chain acted more like a rigid rod (i.e. self repelling). Moreover, the folded EPS structures or aggregates are self attracting at a short length scale, which in the Kratky plot after exhibiting a low \(q\) region show a maximum, and then slowly decay at higher \(q\) region (Glatter and Kratky, 1982). In case of amylose, in Kratky plot, an upturn was observed at low \(q\) region indicating its double stranded structure. Similarly, the cyclic-\(\beta\)-glucan also showed a peak in the Kratky plot (Kajiwara and Miyamoto, 1998). In the Kratky plot of EPS/BE1 (figure 13) also, a sharp upturn or peak was observed at \(\sim 2.0 \text{ nm}^{-1}\), indicated a more ordered structure either due to aggregation of EPS chains, formation of cross-linking regions or a random coil conformation having a two dimensional structure where the due to folding, the Kuhn segments within an EPS chain forming an ordered structure.

Figure 13. Kratky plot for SAXS from EPS/BE1

Scattering was obtained from 8.0 % (w/v) solution of EPS/BE1 using Anton Paar Analytical instrument at 25°C.
Thus, the Kratky plot of EPS/BE1 at the lower $q$ region showed similarity towards cyclic $\beta$-D-Glucan as well as amylose thereby suggesting that the denser EPS/BE1 domains formed a disc like structure involving sporadic or extended double helical regions. Apart from the two-dimensional order, the Kratky plot for EPS/BE1 also indicated the presence of semi-rigid chains in of EPS/BE1. The above observation was quite significant because in case of cellulose derivatives, the semirigidity of the chains was responsible for forming liquid crystalline mesophases. Using a Kratky plot, in previous report, when the gel structure of xyloglucan in various kinds of mono- or polyhydric alcohol/water systems was studied by SAXS, it was found that the presence of alcohols caused random aggregation of xyloglucan chains (Yuguchi et al., 2004).

The above results unambiguously indicated that the EPS/BE1 molecules are present as entanglement of both random coil and rod shaped forms in solution, although the former dominated the conformation. The EPS molecules existed as a network of more condensed and dynamic domains forming a gel like network. It also exhibited worm like chain characteristics exhibiting semiflexible nature. Further, the formation of disk shaped random coil structure suggested the existence of extended ribbon like chain conformation. A schematic depiction of proposed structure of EPS/BE1 in viscous solutions is shown in figure 14.

Figure 14. Schematic depiction of EPS/BE1 structure in viscous solution

$E_j$ is an average size of denser domains in polymeric networks. Dynamic correlation length $\xi$ is a measure of distances between polymeric chains in the network.
3.14.6 Liquid crystalline mesophases of EPS/BE1

The mesophases of EPS/BE1 solution (8.0% w/v) either in water or in 0.1M KCl was investigated by optical polarizing microscopy. In the optical polarizing microscopy, as a rule, an isotropic solution is indicated by a black field, whereas an anisotropic medium illuminates the field due to birefringence and exhibits distinct optical textures. EPS/BE1 solution (8.0% w/v) when observed under polarizing microscope, showed birefringence (figure 18 and 19) indicating (i) the existence of liquid crystalline mesophases due to anisotropic nature of the EPS/BE1 molecules, and (ii) the meso-structural symmetry belonged to the non-cubic subgroup.

The formation of mesophases by EPS/BE1 solution was found to depend mainly upon the concentration of the EPS/BE1 solution and temperature at which the mesophases were observed. Stable liquid crystalline phases were seen up to a temperature of 80°C, beyond which the solution became isotropic and the mesophase disappeared. Similarly, below a concentration of 1.0% (w/v) no mesophase was seen, while at a concentration of 1.0% (w/v), mesophase was observed in a very small area. Although at a concentration of 2.0% (w/v) and 4.0% (w/v) mesophase was observed, more distinct mesophase and phase transition could be observed at a concentration of 8.0% (w/v). Hence, further observations were carried out only using 8.0% (w/v) solution of EPS/BE1. In previous reports, the mesophases of polysaccharides or their derivatives were studied in water as well as in NaCl or KCl solutions (Oertel and Kulicke, 1991; Schorsch et al., 1995). In the present study, the mesophases of EPS/BE1 solution (8.0% w/v) were also investigated both in distilled water and in 0.13 M KCl solution in order to study the effect of the presence of a salt on mesophase formation.

When the aqueous solution of EPS/BE1 (8.0% w/v) was observed under the polarizers at 30°C, more bright areas were present indicating a high amount of anisotropy in the solution, although the texture observed could not be assigned to any specific pattern of molecular arrangements (figure 18A). In contrast, at 30°C, in presence of KCl, although strong birefringence was seen at the edges, more proportion of dark areas were seen at the centre of the field indicating that at this particular temperature homeotropic arrangement of the EPS/BE1 molecules was preferred (figure 19A). When the sample was sheared, although a flow birefringence was observed, no
definite optical texture was seen. At 35°C, in presence of KCl (0.13 M) iridescent colours were observed indicating a chiral nematic phase with helicoidal organization of the molecules (figure 19B). The selective reflection of light was due to the twisted structure of the molecules and the variation in the colour observed suggested a variation in the pitch.

Figure 18. Polarizing microscopic textures observed for EPS/BE1 liquid crystal

Textures were EPS/BE1 solution (8.0% w/v) observed in a Zeiss polarizing microscope at various temperatures (A) 30°C, (B) 45°C, (C) 75°C, and (D) 80°C.

The colour observed in the sample changed from yellow to red with increasing thickness of the sample and each step back to yellow. These changes were more pronounced in the thinner part of the sample, because of the stronger relative change of pitch.
Figure 19. Polarizing microscopic textures observed for EPS/BE1 in presence of KCl

Textures were EPS/BE1 solution (8.0% w/v 0.13M KCl) observed in a Zeiss polarizing microscope at various temperatures (A) 30°, (B) 35°, (C) and (D) 45°, and (E) 75°, (F) 80° C.

At 45°C, EPS/BE1 solution in water exhibited more abundant and intense bright areas although the distribution of bright areas was not uniform (figure 18B). Also, faint maltese crosses were also observed. In fact, the molecules could form aggregates through end-to-end alignment leading to more anisotropic regions. These aggregates
lie perpendicular to the path of light and parallel to the supporting substrate. SAXS analysis indicated that in viscous solution of EPS/BE1, the EPS chains existed in both disk-like random coil and rigid rod-like shapes. Hence, it was possible that in case of EPS/BE1, at 45°C in the absence of KCl, the random coil form predominated giving a more entangled network instead of uniformly aligned rods.

In the presence of KCl at 45°C, both bright and dark regions could be observed as shown in figure 19 C and D. The pattern of birefringence suggested that the disk shaped random coil molecules and the rod shaped must have stacked in a nematic fashion. Few of the molecules were oriented in the direction of light and many of them remained parallel to the direction of light resulting in high degree of anisotropy. Further, Maltese crosses were clearly visible at certain areas of the microscopic field suggesting homeotropic alignment of molecules. In the above arrangement, the molecules remained normal to the substrate due the extended conformation conferred by the presence of KCl. In presence of KCl, due to charge screening, the EPS/BE1 chains got elongated and formed rod-like structures resulting in more uniform and oriented structure. Further, when the coverglass was shifted, reflection of the colours was observed indicating a planar texture. In this texture the sample was uniformly aligned with the twist axis perpendicular to the plane of the structure. Moreover, presence of crack like patterns observed at certain areas in the mesophase indicated alignment discontinuities within the supramolecular arrangement of EPS/BE1 molecules.

At 75°C, in case of solution of EPS/BE1 in water, the mesophase structure was becoming more anisotropic. Coloured texture was observed at the edges, indicated the twisting arrangement of molecules (figure 18C). Twisting of the molecules might have been mediated by high mobility of the molecules at high temperature. At 75°C, in the presence of KCl, flow birefringence was observed (figure 19E). Due to high mobility of the molecules, extended rod-like conformation and anisotropy, large bright areas having a streaming appearance were seen. Within the dark areas bright fingerprint and within bright areas dark fingerprint textures were seen confirming the presence of cholesteric phase whose helical axis remained parallel to the supporting surface. Fingerprint textures arise due to the periodic extinction of light caused by the
change in the molecular direction along with the helical structure (Collings and Hird, 1997).

Fingerprint textures were observed in earlier studies also for hydroxypropyl chitosan and chitin (Dong et al., 2001; Belamie et al., 2006). At the edges of the microscopic field, large bright regions were visible exhibiting iridescent colours. Overall, the presence of more dark regions within bright regions indicated that the molecular order was progressively getting diminished as the temperature of the sample was increased. Nevertheless, the mesophase structure observed in presence of KCl was found to be more ordered than that observed at 75°C in the absence of KCl. At 80°C, in the absence of KCl, although bright regions were seen due to anisotropic nature of the solution, with no definite textures was visible, indicating that the solution was becoming more isotropic with increase in the temperature (figure 18D). In contrast, at 80°C, in presence of 0.13 M KCl, fingerprint texture and coloured texture was still visible indicating that the molecules were present in the helicoidal or nematic cholesteric order (figure 19F). In a previous report, for xanthan gum (1% w/v) solution, in the absence of salt, the anisotropy disappeared at approximately 55°C. However, in the presence of KCl, the birefringence exhibited by xanthan molecules persisted even at 80°C, although the birefringence decreased slightly between 25 to 80°C (Schorch et al., 1995). In case of EPS/BE1, irrespective of the presence or absence of salt the anisotropy persisted even up to 85°C.

3.14.9 Supramolecular ordering of EPS/BE1 is cholesteric or chiral nematic
The formation of liquid crystalline mesophases by EPS/BE1 could be explained using the Doi-Edwards model (Zhao et al., 1998). At low concentration of the EPS, when water was in excess, each rod-like molecule was confined within a tube-like region composed of the neighbouring molecules where it could translate along its own axis with negligible interference. On the whole the distribution of molecule orientations remained isotropic and no mesophases were observed. Raising the volume fraction of EPS/BE1 caused an increasing interference of rod like macromolecules, so that rotation of a rod was blocked by the surrounding rods resulting in a nematic liquid crystalline phase. Also, formation of mesophase by EPS/BE1 might partly be
attributed to the high yield stress exhibited by its solutions as observed also in case of xanthan, schizophyllan or scleroglucan (Lapasin and Pricl, 1999; Belamie et al., 2006). Similarly, the disc like random coils of EPS/BE1 were stacked in a less orderly way leading to a nematic order.

3.15 Conclusion

Overall, in the liquid crystalline mesophase of EPS/BE1, which was nematic in nature, both the rod-like (small and medium molecular weight molecules) and the disk-like (high molecular weight) molecules were aligned either homeotropically or homogenously in different regions exhibiting distinct textures characteristic of such an arrangement. Among them the twisting of rod shaped molecules formed helicoidal arrangement ultimately resulting in a chiral nematic or cholesteric phase shown in figure 20.

Figure 20. A schematic depiction of probable arrangement of EPS/BE1 in the cholesteric phase

![Schematic depiction of probable arrangement of EPS/BE1 in the cholesteric phase](image)

(A) Nematic phase  B) Chiral nematic or cholesteric phase

BIBLIOGRAPHY


