Characterization of Exopolysaccharide (EPS) produced by Root Nodule isolate *Enterobacter cloacae*, isolated from *Phaseolus vulgaris*

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Abstract : Exopolysaccharides are high molecular weight compounds secreted by the organisms into their surroundings. These exopolysaccharides have found a wide applicability as food stabilizers, gelling agents, emulsifiers, flocculants, anti-oxidants, immune-modulators etc. The EPSs secreted by the organisms vary in their monosaccharide composition, solubility, surface structures, thermal stability, molecular weight, and presence or absence of certain functional groups. The present study deals with the physical and chemical characterization of the EPS produced by a root nodule isolate *Enterobacter cloacae*. The thin layer chromatography (TLC) revealed that the test EPS is composed of glucose and mannose residues. The differential scanning colorimetry (DSC) analysis revealed that the EPS was stable upto a temperature of 106ºC. Scanning electron microscopy (SEM) was done to determine the surface structure which revealed that the test EPS has a rough granular surface. It was also found that the EPS was soluble only in water and insoluble in organic solvents. Among the molecular parameters, Fourier Transfrom Infra-red (FT-IR) revealed the presence of carboxylic stretch, C-OH bend, CH_3 bend, alcohol stretch, alkynyl stretch, and aromatic stretch in the test EPS. The X-ray diffraction (XRD) profile revealed that the test EPS is partially amorphous and partially crystalline in nature. Two distinct peaks with different molecular weight were observed in the MALDI-TOF MS analysis.

IndexTerms – Enterobacter cloacae, EPS, Phaseolus vulgaris, TLC, DSC, FTIR

I. INTRODUCTION

Exopolysaccharides (EPSs) are generally high molecular weight polymers which are composed of sugar residues and are secreted by the microorganism into the surrounding environment. The production of EPS is a general property of microorganisms and occurs both in prokaryotic (bacteria, archea) and eukaryotic (algae, fungi) microbes. The organisms synthesize mainly two types of EPSs capsular and slimy and these polysaccharides can either be homo-polysaccharide which contain only one type of monosaccharide or hetero-polysaccharide containing repeating units ranging between disaccharides to heptasaccharides with some non-carbohydrate substituents such as phosphate, acetyl, acetate, succinate, pyruvate, uronic acid, sulphate esters, hexosamines and glycerol [1]. The EPS secreted by the microbes act as protective barrier for microbes against extreme dry conditions, predation, antibiotics and helps in maintaining the architecture and morphology of the matrix in which the cells reside. Several microbial polysaccharides are now produced commercially from many species of bacteria, as well as from marine algae, fungi and plants. These exopolysaccharides have found applications in food, pharmaceutical and chemical industries, and may function as bioflocculants, bioabsorbents, heavy metal removal agents, drug delivery agents etc. In the field of medicine, polysaccharides from microorganism have proven to have effective, non-toxic, immunomodulatory, antitumor, antioxidant activity [2]. Polysaccharides may also be used for encapsulating drugs for their gradual delivery and they may be used to immobilise enzymes employed for diagnosis. The most important microbial polysaccharides include xanthan, the commercial polymer produced by the bacteria *Xanthomonas campestris*, Gellan (*Sphingomonas paucimobilis*), Cellulose (*Acetobacter xylinum)*, Dextran (*Leuconostoc mesenteroides*), Spirulan (*Arthospira pltensis)*, Alginate (*Pseudomonas aeuroginosa*), Levan (*Bacillus subtilis)*, Hyaluronan (*Pasteurella multocida*) and Curdlan (*Alcaligenes* species). EPSs of microbial origin thus can be used as an alternative to toxic chemicals since the microbial products have unusual molecular structures and peculiar conformations thus conferring unique and potentially interesting properties. Also these polysaccharides are biodegradable, nontoxic, and free of secondary pollution risk and can replace currently used organic synthetic chemicals which possess inherent drawbacks of being a source of carcinogenic monomers, and are often non-biodegradable. The present study deals with the characterization of EPS produced by *Enterobacter cloacae*, a rod-shaped (0.3-0.6 x 0.8-2.0 μm), gram-negative, gamma-proteobacter mesophilic organism.

II. RESEARCH METHODOLOGY

Production and Extraction of EPS

The EPS production and extraction was done as per the method of Sayyed [3]. A 24 hour old culture of *Enterobacter cloacae* was inoculated in 50ml sterilized yeast extract mannitol broth (YEMB) containing gl^{-1} , Yeast extract,01; Mannitol,10; Calcium carbonate, 01; Magnesium sulphate, 0.0177; K_2HPO_4, 0.1, pH; 7. The flask was incubated at 28ºC for three days. After that the broth was centrifuged at 10,000 rpm for 20 minutes at 4°C to remove the cells as pellet. Supernatant was separated and to it equal volume of chilled acetone was added to extract the EPS. The extracted EPS was lyophilized and the pellet (biomass) was dried till constant weight was achieved.

Partial purification of EPS

The crude lyophilized EPS obtained was subjected to dialysis using dialysis membrane and was then concentrated on polyethylene glycol (PEG).

Physical characterization

The characterization of the test EPS was done for the following:-

Determination of the monosaccharide composition by thin layer chromatography (TLC)

The monosaccharide composition of the test EPS was determined as per the method described by Song [4]. In this 10mg of the test EPS was dissolved in 2N TFA (triflouro acetic acid) and was incubated at 100°C for 6-8hrs. The resulting hydrolysate was diluted with the same volume of distilled water and neutralized with 1 N NaOH solution after cooling at room temperature. The remaining solution was filtered before analysis. For saturating the tank the developing solvent containing n-butanol, 2-propanol, water and acetic acid in the ratio 7:5:4:2 was poured and the tank was covered with the lid for 25-30 minutes. Spotting of the samples was done on silica gel 60 plates to determine the monosaccharide component of the hydrolysate. The samples (10 microlitre) were spotted 2 cm from the bottom of the plate with at least 1cm distance from each other. After spotting, the spots were dried using the hair dryer and the plate was placed in the saturated tank and the jar was sealed. The plate was taken out of the jar and was air dried as soon as the developing reagent reached the top of the jar. After the development process the plates were sprayed with a 5% sulphuric acid solution in ethanol and were heated at 100°C until the spots appeared. After the development of the spots Rf was calculated as the ratio of the distance moved by the solute and the distance moved by the solvent (solvent front).

Differential scanning colorimetry analysis (DSC)

DSC can be used to measure the crystalline phase, transition temperature, glass transition temperature, heat of fusion or latent heat of melting of a compound. In this 3-4 mg of the test EPS was loaded in aluminum DSC pan and gravimetric analysis was done under reduced nitrogen atmosphere, from 0°C to 300°C using a temperature gradient of 10°C/ min.

Solubility

The solubility of the test EPS was determined in various organic (acetone, chloroform, dimethyl sulphoxide, ethanol, ethyl acetate, methanol and xylene) and inorganic (water) solvents as per Patil [5]. For this, the 5gm of the test EPS was immersed in 2 ml of the solvent, was stirred and observed for pellet formation.

Scanning electron microscopy (SEM)

Scanning electron microscopy was performed to investigate the surface texture of the test EPS. 1mg of the test EPS was mounted on the metal bead, coated with gold for 10-15 minutes and was observed for surface structure under scanning electron microscope.

Molecular characterization

FTIR analysis

EPS was analyzed by FTIR spectroscopy to detect the functional groups present in the structure. The FTIR of the test EPS was recorded with a Perkin-Elmer 1720 spectrometer over KBr pellet at CIL, Panjab University, Chandigarh. In this method, 5mg of sample was manually blended with 100mg of KBr powder and dessicated overnight at 50°C under reduced pressure prior to analysis.

X-ray powder diffraction (XRD)

To determine the atomic and molecular structure of the test EPS, X-ray crystallography was done. The samples were examined in powder form maintaining the operating 2ɵ range between 5-60º at a scanning speed of 2º/min using Cu Kα radiations.

MALDI-TOF

MALDI-TOF of the test EPS was performed at PGIMER, Chandigarh for the structural analysis of the oligosaccharides. The EPS was partially degraded with 100mM trifloroacetic acid. The digest was diluted with 80% aqueous ethanol and was concentrated under N_2. 1mg of the sample was dissolved with 1ml of water and was mixed with equal volume of 2, 5-dihydroxybenzoic acid (10mg/ml) prior to analysis.

III. RESULTS AND DISCUSSION

Monosaccharide composition

The monosaccharide composition of the purified test EPS produced by *Enterobacter cloacae* was determined by TLC. Based on the results of TLC analysis, the trifloroacetic acid hydrolysate of the EPS showed a single apparent spot on TLC (Rf = 0.497), indicating the identical Rf value to glucose (Rf = 0.491) and mannose (Rf=0.49) (**Figure 1**). From the results it can be interpreted that the EPS produced by *Enterobacter cloacae* is composed of glucose and mannose.

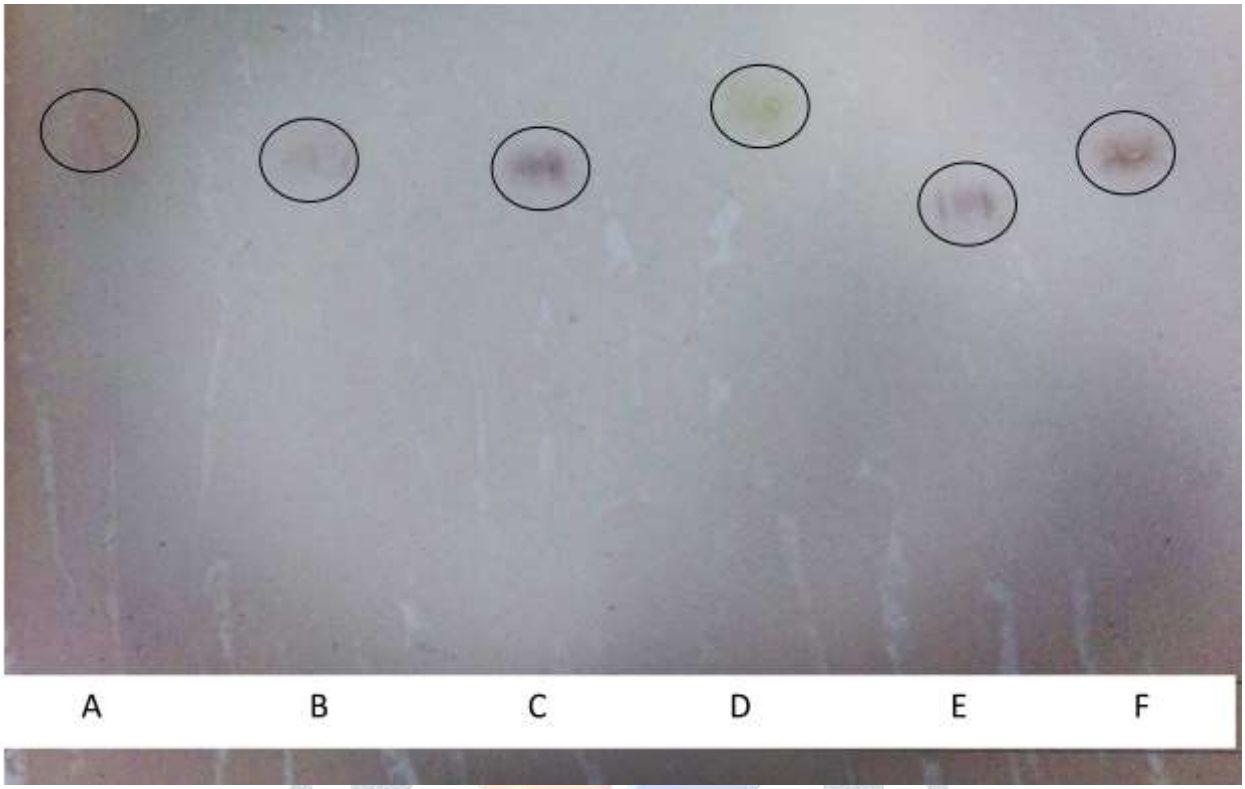

Figure 1: Determination of monosaccharide composition by thin layer Chromatography; A and B): test EPS; C): Glucose; D): Xylose; E): Galactose; F): Mannose

Differential scanning calorimetric analysis (DSC)

Commercial applications of an EPS is crucially dependent on its thermal and rheological behavior. To check the effect of temperature on EPS produced by *Enterobacter cloacea*, DSC analysis of the test EPS was carried out. The results obtained revealed that the test EPS was stable upto 106 ºC. The transition temperature and the temperature of crystallization of the test EPS was found to lie in a range 106.73 - 109.73 ºC and 107.03 – 107.66 ºC, respectively. From the results obtained it can be concluded that the test EPS produced by *Enterobacter cloacae* is a thermostable and its degradation starts at a temperature greater than 228ºC (**Figure 2**).

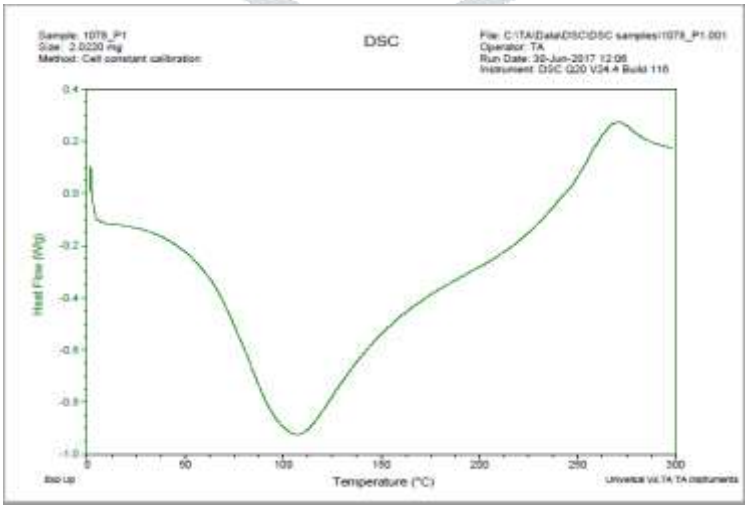

Figure 2: The DSC analysis of the test EPS produced by *Enterobacter cloacae*. W/g: Watt per gram

Solubility

The evaluation of the solubility of the EPS was done by dissolving it in various inorganic and organic solvents, and it was observed that the test EPS was soluble in water only, and insoluble in all other solvents. (**Table 1**). The results are in accordance with Parstersan [6] who also reported the EPS produced by *Enterobacter cloacae* WD7 was soluble only in water.

Table 1: Solubility of test EPS in different solvents

S.No.	Solvent	Test EPS
1.	Acetone	Non-soluble
2.	Chloroform	Non-soluble
3.	Dimethyl sulphoxide	Non-soluble
4.	Ethanol	Non-soluble
5.	Ethyl acetate	Non-soluble
6.	Methanol	Non-soluble
7.	Tetrahydrofuran	Non-soluble
8.	Water	Soluble
9.	Xylene	Non-soluble

Scanning or Surface electron microscope (SEM)

The scanning or surface electron microscopy of the test EPS was performed to analyze its surface structure. The results revealed the EPS had a rough granular surface (**Figure 3 a**) with small pores, which were generally responsible for the solubility of the EPS in water (**Figure 3 b**).

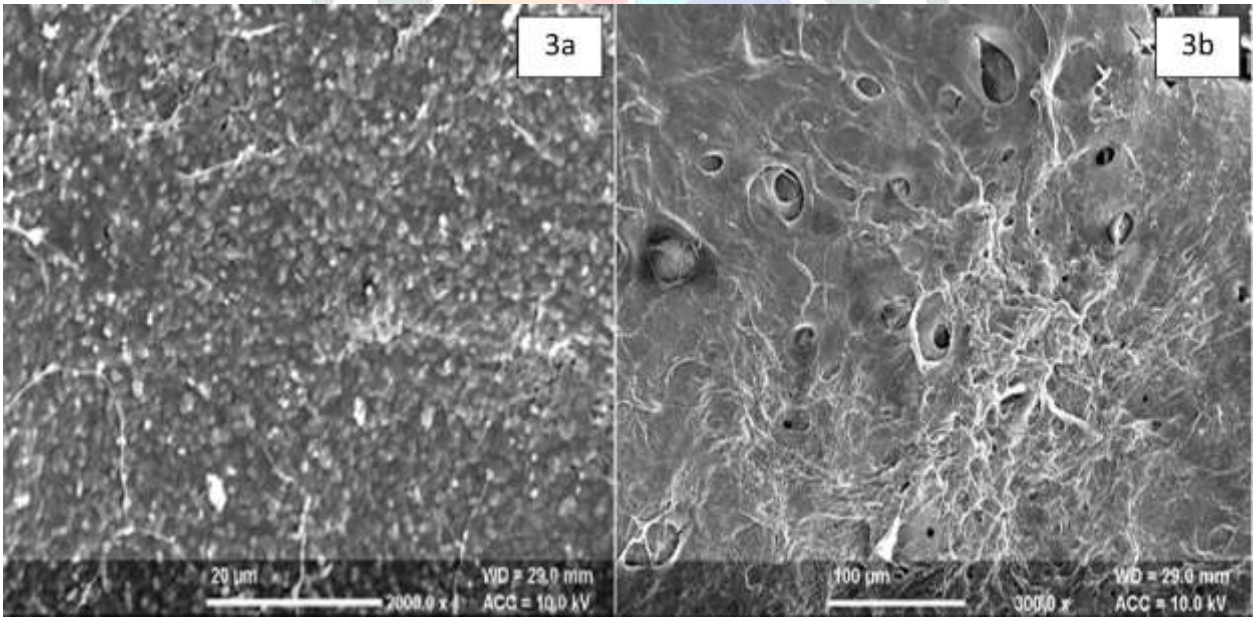

Figure 3a and 3b: Scanning electron microscopy showing the rough granular and porous surface of the test EPS produced by *Enterobacter cloacae*.

Molecular characterization

Fourier transform infrared analysis of EPS (FTIR)

FTIR analysis was used to identify the molecules, proteins and functional groups found in the exopolysacharides produced from *Enterobacter cloacae* (**Figure 4**). The FTIR analysis obtained for the EPS showed that the absorption peaks located at 3325.54 corresponds to alcohol/phenol O-H stretch, 2939.663 to carboxylic acid O-H stretch, 2153.82 to alkynyl C=C stretch, 1723.70 to aromatic C=C stretch, 1372.71 to CH3 bend , 1253.72 to C-O-C stretch, and 1056.28 to C-OH. Thus, the FTIR spectrum revealed that the EPS produced by *Enterobacter cloacae* is a complex polysaccharide containing a range of functional group.

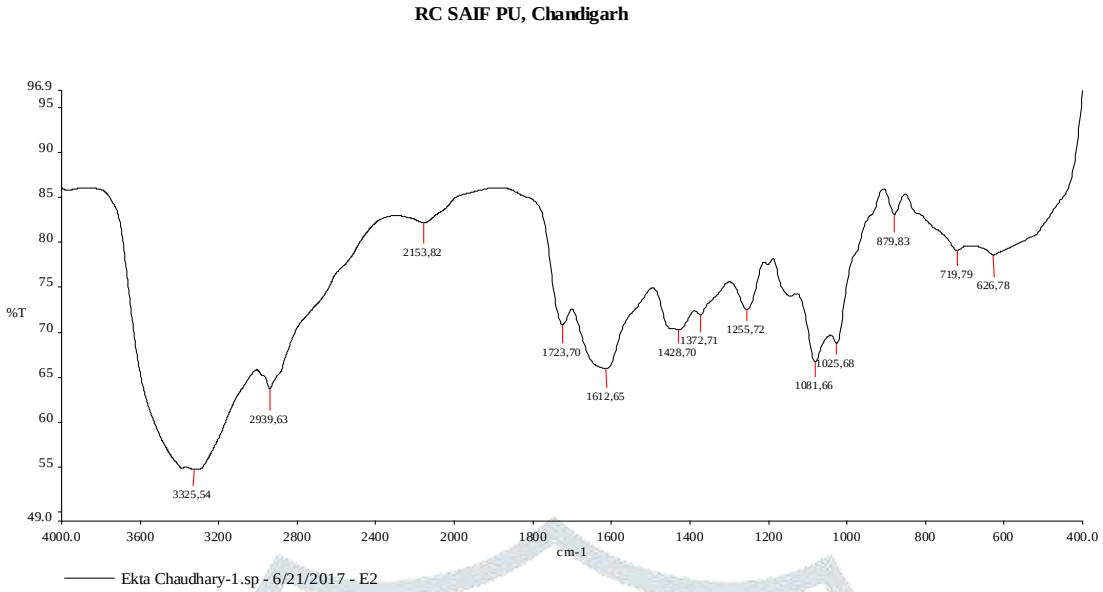

Figure 4: FTIR analysis of the test EPS produced by *Enterobacter cloacae* in the range 400-4000 cm^{-1}; %T: percentage transmission

X-ray diffraction analysis

The XRD profile and interplanar spacing (*d*-spacing) are the basic characteristics of a polysaccharide and are useful for comparing or studying the nature of EPS isolated from different sources. The polysaccharide produced by the organism can either be partly crystalline or partly amorphous. X-ray powder diffraction (XRD) is a rapid analytical technique most widely used for phase identification of the material. The XRD profile of the EPS produced by *Enterobacter cloacae* exhibited characteristic diffraction peaks at 9.7418º, 17.3659 º, 19.8772 º and 20.4708, with interplanar spacing (d-spacing) 9.0793, 5.10668, 4.4668 and 4.3385, respectively (**Figure 5**). Crystalline parts give sharp narrow diffraction peaks while amorphous component gives a broad peak. Thus from the XRD pattern it can be concluded that the test EPS consists of both amorphous and crystalline region with a crystallinity index of 0.33.

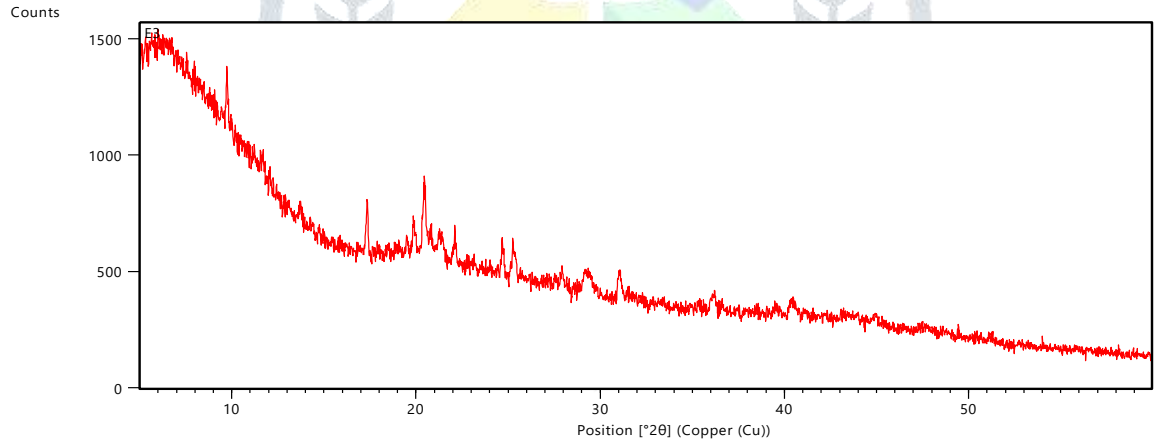

Figure 5: X-ray diffraction pattern of the test EPS

MALDI-TOF analysis

MALDI-TOF analysis of the test EPS was done to determine its molecular weight. The resultant mass spectrum contained an x-axis representing m/z (mass divided by charge) and a y-axis representing absolute intensity (the number of ions of each species that reach the detector). The MALDI-TOF MS of the test EPS revealed two mass peaks with molecular weight of 7.7 x 10^5 and 15.1 x 10^5 Da, respectively (**Figure 6**).

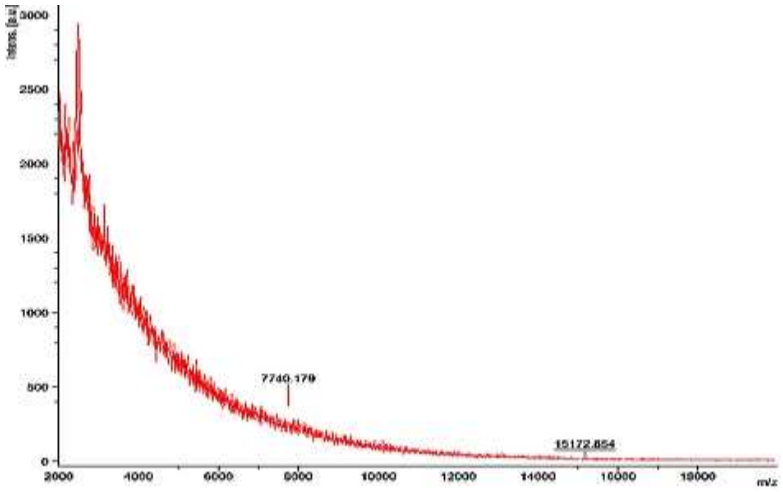

Figure 6: MALDI-TOF analysis of the test EPS. m/z: mass by charge ratio; Intensity(au): arbitrary units

III. CONCLUSION

The EPS produced by root nodule isolate *Enterobacter cloacae* was observed to be a high molecular weight, water soluble, heteropolymer having a rough granular surface. The DSC analysis revealed the stability of the test EPS upto 106º C. The transition temperature and the temperature of crystallization was found to lie in a range 106.73-109.73ºC and 107.03-107.66ºC, respectively. The FTIR analysis concluded that the test EPS is composed of a range of functional groups. The XRD profile exhibited different diffraction patterns indicating its amorphous and crystalline nature. Two distinct mass peaks with molecular weight of 7.7 x 10^5 and 15.1 x 10^5 Da, respectively were seen in MALDI-TOF. Attributing to the above mentioned characteristics its applicability as a thermostable food stabilizer, gelling agent, emulsifier, or a flocculant can be explored.

IV. Acknowledgment

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