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RESEARCH ARTICLE

CHARACTERIZATION OF EPS PRODUCED FROM STEM AND ROOT NODULATING *RHIZOBIUM MAYENSE*

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ABSTRACT

Exopolysaccharides (EPS) are high molecular weight polymers with long chain composed of sugar residues and secreted by microorganisms into the surrounding environment. Bacterial EPS has a complex mixture of macro molecular poly electrolytes including carbohydrates, proteins and sometimes nucleic acids, each comprising variable molecular mass and structural properties. Stem and root nodulating isolate of *Aeschynomene indica* plant was identified by 16S rRNA sequencing as *Rhizobium mayense*. Efficiency of *Rhizobium mayense* was studied using Yeast Extract Agar as a basal medium with different carbon sources and the incubation was done at 30° for 48 hours. The dry weight of EPS was found to be 1.4119 gm /100 ml in Yeast Extract Mannitol Agar. Surface topology of EPS was found smooth by Scanning Electron Microscopy. Alcohol precipitated EPS was further purified using Sephadex G200 and Sephadex G100 gels. The physicochemical characterization of purified EPS was studied and the structural characterization was confirmed by FT-IR, 2D NMR, HPLC and GC-MS analysis.

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INTRODUCTION

Exopolysaccharides are the organic macromolecules often found in the surrounding as the outer most structures of both prokaryotic and eukaryotic cells. They may be closely associated with the cell in the form of discrete capsules or else excreted as a slime layer unattached to the cell surface (Vijayabaskar *et al.*, 2011). Bacterial exopolymers are important in the interaction between bacteria and their environment and are chemically diverse (Nielson and Jahn, 1999). *Rhizobium* species can secrete outside their cell wall the large amount of long chain, high molecular weight exopolysaccharides (EPS). It is well known that acidic EPS provides the bacteria with hydrophilic and negatively charged coating or network surrounding the cells which in turn provides the protection and mechanical stability to vegetative cells against attacks, lethal drying and other adverse environmental conditions. EPS exists in a wide variety of unique and often complex structures and they are believed to provide self protection against anti microbial substances (Kumon *et al.*, 1994), starvation conditions and extreme pH and temperature (Kim *et al*., 2000). The production of EPS is dependent on the temperature and pH of the medium as well as composition of the culture medium in terms of carbon and nitrogen source and minerals content , fermentation conditions (Gorret *et al*., 2001, Saba Shamim *et al*., ?). The carbon source used for growth determines both quality and quantity of polysaccharide formation (Cerning *et al*., 1994, Nourani *et al*., 1994).

The composition and structure of EPS is varied and consisting of either homopolymers (1,2- beta –glucans or cellulose) or heteropolymers. Heteropolysaccharides are acidic polymers composed of linear arrangement of repeating units containing neutral sugars and Uronic acid as well as non carbohydrate substitutes such as acetate, pyruvate, hydroxybutyrate and succinate. The advantages of microbial polysaccharides over plant or marine macroalgal polymers are their novel functionality, easily reproducible chemical and physical properties and stable cost and supply (MacCormick *et al*., 1996). The microbial EPS due to their health benefits have been treated as highly potential molecules (Patricia Ruas-Madiedo *et al*., 2002, De Vuyst *et al*., 1999). Many interesting physical and chemical properties (e.g. stabilizing, suspending, thickening, gelling, coagulating capability) have found wide range of applications in the fields of textiles, adhesives, paper, paint, food, oil recovery, mining industry.

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Bacterial growth is often accomplished by production of EPS, that have relevant ecological and physiological functions. The nutrient status and growth phase of surface associated bacteria may influence the quality and the composition of the EPS produced (Decho, 1990). During the past fifty years a considerable number of bacterial EPS have been described, but very few have achieved great commercial success due to either to their being unable to offer better properties than those already in market or to difficulties in findings new practical applications (Sutherland, 2002).

MATERIALS AND METHODS

Identification of the isolate

Efficiency of exopolysaccharide production

Stem and root nodulating isolate of *Aeschynomene indica* plant was identified by 16S rRNA sequencing as *Rhizobium mayense*. The cultivation of *Rhizobium mayense* was done on Yeast Extract Agar as a basal medium containing 2% Glucose, 2% Xylose, 2% Arabinose, 2% Mannose, 2% Sucrose, 2% Inositol, 2% Sorbitol, 2% Mannitol and Nitrate Minimal Agar to study the efficiency of exopolysaccharide production.

Extraction and quantification of Exopolysaccharide

The extraction of exopolysaccharide was done by addition of three volumes of chilled absolute alcohol. The mixture was agitated during addition of alcohol to prevent local high concentration of precipitate and left over night at 4ºC before centrifugation at7000 rpm for 20 minutes. After centrifugation, the precipitate was collected and dried at 60ºC (Congregado *et al*., 1985, Sutherland and Wilkinson – 1971, Ohno *et al*, 2000). The weight of EPS was expressed as mg/100 ml and recorded. The Exopolysaccharide mixture was deproteinized following the Sevag method. The total carbohydrate content was estimated by phenol sulphuric acid method proposed by Dubois *et al*., 1956. The amount of polysaccharides in EPS were determined and expressed as amounts of glucose by using constructed standard curves of each standard sugar. The amount of protein present in EPS was estimated by Lowry's (Lowry, 1951) method. Bovine serum albumin (BSA) protein was used as a standard protein. Amount of protein contamination in purified polysaccharides extracts were determined. The purified polysaccharide yield (%) was then calculated using the following equation:-

$$\text{Yield of polysaccharide (\%)} = \frac{\text{Weight of polysaccharides (g)}}{\text{Weight of raw material (g)}} \times 100$$

Scanning Electron Microscopy

Topology that is surface properties of EPS produced by *Rhizobium mayense* was done by Microscopy reported by Sundaram *et al*., 2002 using Scanning Electron Microscope (Model Philips XL 30 SEM) at 12-15 kV with a tilt angle of 45º. The exopolysachride was mounted on a stub, coated with gold using sputter coater and the surface topography was observed under Scanning Electron Microscope and the features were studied

Purification of EPS

Sephadex G200 was allowed to swell in 0.01M phosphate buffer pH 7.0. A 25.0 cm X 1.0 cm glass column was loaded with pre swollen gel and was allowed to settle under gravity to form packed bed column. The gel was equilibrated with 0.01M phosphate buffer pH 7.0. The partially ethanol purified EPS fraction was layered on the top of Sephadex G200 column. The column was eluted with same buffer at a flow rate of 0.1 ml/min.The purification of EPS fraction was done using Sephadex G 100 column (Gel Filtration Chromatographic Technique). The polysaccharides purification process was performed by using the method suggested by Yoon *et al*. (2003) with some modification until purified EPS obtained. Purified fractions were collected and again analyzed for carbohydrate and protein contents and stored in freeze-dried conditions. The lyophilized EPS was stored at room temperature until analysis could be performed.

Characterization of Purified Polysaccharide

Gel Permeation Chromatography

Shimadzu LC-10 A HPLC/ GPC System (Shimadzu Corp. Japan) was used to analyze the molecular weight of obtained dextran (PL aquagel-OH-Mixed 8 μM 300 x 7.5 mm, column temperature 35°C). HPLC grade water with flow rate 1.0 ml/min was used as a mobile phase. Standard Dextran of different molecular weights was used as standards. Empower TM 2 chromatography data software used for the analysis.

Fourier- Transform Infra Red Spectroscopy (FTIR) Analysis

The major structural groups of the purified EPS were detected using Fourier- Transform Infra Red Spectroscopy (FTIR) spectroscopy (Abu *et al*., 1991) and the spectrum of the polysaccharide was obtained using a Fourier Transform Infra-Red spectrophotometer (Perkin Elmer Instruments, Spectrum One FT-IR Spectrometer). One part of dried polysaccharide was ground with ninety nine parts of dried potassium bromide (KBr) powder and then compressed into salt discs of size 3mm. These discs were subjected to Ir- spectra measurement in the frequency range of 400-4000 cm^{-1}.

Nuclear Magnetic Resonance (NMR) Analysis

^{1}H-NMR and ^{13}C-NMR spectra for the polysaccharide were obtained at 25°C with a NMR spectrometer (Varian, Model AS400) 400 MHz equipped with VnmrX for Sun Microsystems Ver. 6.1 software (operating frequency 400 MHz for ^{1}H-NMR and ^{13}C- NMR). The samples were dissolved in D_2O at concentration of 10 mg/ml for ^{1}H-NMR and 40 mg/ml for ^{13}C-NMR analysis. ^{1}H chemical shifts were referenced to internal D_2O (4.79 ppm at 25°C). The purified EPS sample was hydrolyzed to monomeric units and transformed in their alditol acetates to study the chemical composition using GC- 2010 (GCM-QP 2010) SHIMADZU

chromatography equipment with a flame-ionization detector and a split injector (Jian *et al*., 2003).

Gas Chromatography- Mass Spectroscopy (GC- MS) Analysis

The purified EPS sample was hydrolyzed to monomeric units and transformed in their alditol acetates. 0.1g of crude sample was mixed with 1.25ml of 72% sulfuric acid with a glass stick and incubated for 60min at 30°C. The mixtures were diluted with 13.5ml of distilled water and incubated were cooled and 3.1ml of 32% NaOH (w/v) was added. At the end of hydrolysis, 0.2ml of sample was taken separately and 2ml of 2% sodium borohydride in dimethyl sulfoxide was added. The mixtures were then shaken well at 40°C for 90min. after which 0.2ml of glacial acetic acid was added to decompose excess of sodium borohydride. After cooling, 4 ml of acetic anhydride and 0.4 ml of 1-methylimidazole were added to the solution. The mixtures were then incubated for 10 min at room temperature and then 20 ml of distilled water was added 8ml of dichloromethane was added and the mixture was shaken vigorously for total alditol acetate extraction. The upper layer was removed and the lower phase was washed three times with 20ml of distilled water. The dichloromethane was evaporated at 40°C under vacuum and final alditol acetate residues were dissolved in 1ml of Alditol acetates were separated on a 30 m x 0.25mm ID x 0.25µm film thickness column DB 5ms (Agilent) attached to the GC- 2010 (GCM-QP 2010) SHIMADZU chromatography equipment with a flame-ionization detector and a split injector. High purity hydrogen was used as the carrier gas at a flow rate of 1.40 ml/min. The column temperature was maintained at 200°C and 240°C respectively and 1µl sample in dichloromethane was injected through a glass-lined splitter, set at 1/90 ratio. The absorption was read between 40m/z and 800m/z. in boiling water bath for 4 hrs. After incubation, mixtures to decompose excess of acetic anhydride. After cooling, dichloromethane and stored at -20°C.

HPLC and Liquid Chromatography-Mass Spectroscopy (LC-MS) Analysis

The mixture was separated by reversed phase HPLC on an Adsorbosphere column-NH2, (250 x 4.6 mm column) using both isocratic and gradient elution with acetonitrile/water and detected using Waters ELSD 2420. In ELSD, the mobile phase is first evaporated. Solid particles remaining from the sample are then carried in the form of a mist into a cell where they are detected by a laser. The separated fractions were subjected to UV analysis using Agilent 8453 coupled with Diode array detector. HPLC–MS analysis was performed with LCMSD/Trap System (Agilent Technologies, 1200 Series) equipped with an electro spray interface. The MS spectra were acquired in positive ion mode. The mobile phase consisted of 0.10% formic acid in HPLC grade deionized water (A) (milli-q-water (subjected to IR radiation under 3.5 micron filters) and Methanol (B) taken in the stationary phase of Atlantis dc 18 column (50 x 4.6mm - 5µm). The gradient program was as follows: 10% B to 95% B in 4 min, 95% B to 95% B in 1 min, 95% B to 10% B in 0.5 min followed by 10% B in 1.5 min at a flow rate of 1.2 ml per minute. The column oven temperature was kept at 40°C and the injection volume was 2.0 µL. Product mass spectra were recorded in the range of m/z 150-1000. The instrumental parameters were optimized before the run.

RESULTS AND DISCUSSION

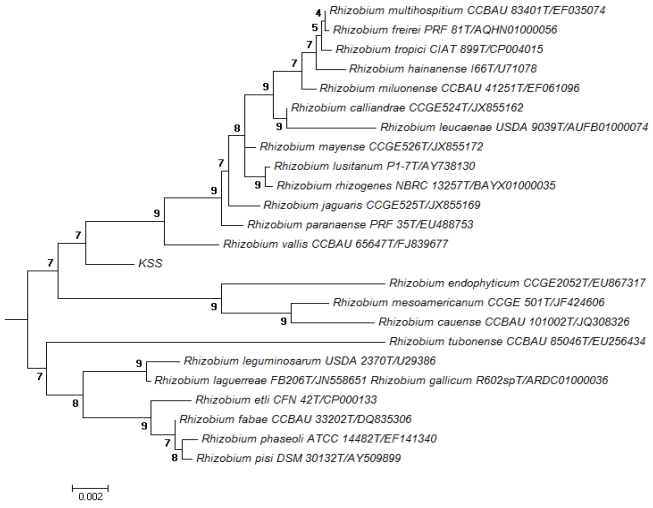
The stem nodulating isolate obtained from *Aeschynomene indica* plant is identified by 16S rRNA sequencing technique. The DNA sequence of AIRS-I isolate was compared with Gene Bank Data Base using BLAST algorithm available from NCBI (www.ncbi.nlm.nih.gov). It showed a 98.64% query coverage as *Rhizobium mayense* by 16S rRNA with as Accession number of JX855172 in Gene Bank Data Base. The isolate was named *Rhizobium mayense.* The Sequences of the primer pair used for amplification –

RPP2 – CCAAGCTTCTAGACGGITACCTTGTTACGACTT
FDD2 – CCGGATCCGTCGACAGAGTTTGATCITGGCTCAG

The obtained DNA sequence

CGTGGGTATTTGGCAATGGGCGCTAGCCTGATCCAGC
CATGCCGCGTGAGTGATGAAGGCCCTAGGGTTGTAA
AGCTCTTTCACCGGAGAAGATAATGACGGTATCCGG
AGAAGAAGCCCCGGCTAACTTCGTGCCAGCAGCCGC
GGTAATACGAAGGGGGCTAGCGTTGTTCGGAATTACT
GGGCGTAAAGCGCACGTAGGCGGATCGATCAGTCAG
GGGTGAAATCCCAGGGCTCAACCCTGGAACTGCCTTT
GATACTGTCGATCTGGAGTATGGAAGAGGTGAGTGG
AATTCCGAGTGTAGAGGTGAAATTCGTAGATATTCGG
AGGAACACCAGTGGCGAAGGCGGCTCACTGGTCCAT
TACTGACGCTGAGGTGCGAAAGCGTGGGGAGCAAAC
AGGATTAGATACCCTGGTAGTCCACGCCGTAAACGAT
GAATGTTAGCCGTCGGGCAGTATACTGTTCGGTGGCG
CAGCTAACGCATTAAACATTCCGCCTGGGGAGTACG
GTC

Phylogenetic tree of *Rhizobium mayense* based on 16S rRNA sequence



The cultivation of stem and root nodulating *Rhizobium mayense* was done on Yeast Extract Agar as a basal medium with different carbon sources and the incubation was done at

30° for 48 hours. The dry weight of EPS was found to be 1.4119 gm /100 ml in Yeast Extract Mannitol Agar. A better precipitation and recovery of the biopolymer was done by ice cold absolute alcohol. Alcohol precipitation method gave maximum yield of 1.4119 gm % exoploysaccharide with 2% Mannitol as a carbon source as shown in following Figure 1.

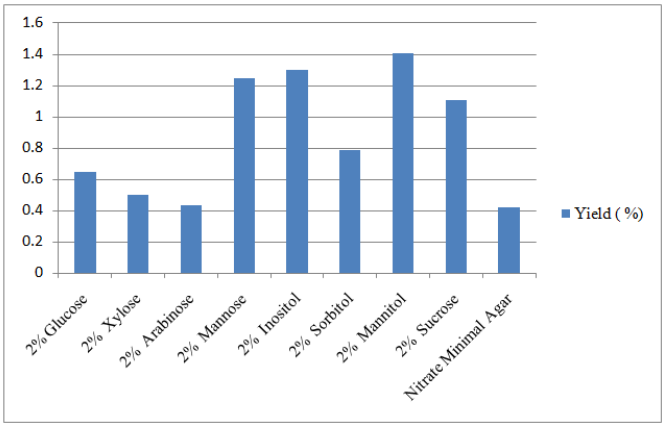


Figure 1. Effect of carbon source on EPS production

Figure 2. EPS Extraction by Alcohol Precipitation

Figure 3. Extracted Crude EPS

Figure 4. Partially Purified EPS

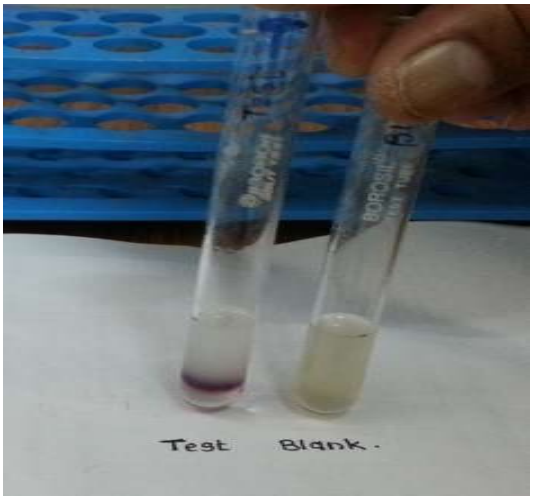


Figure 5. Molish's Test for EPS

The qualitative detection of carbohydrate content was done by Molisch's test. The total carbohydrate content of the deproteinized EPS was determined by Dubois method and found to be 179µg/ml. The reducing sugar content was detected by Dinitro Salicylic Acid method and was found to be 510 µg/ml. The protein content was detected by Folin Cialcaltue method and was found to be 115 µg/ml.

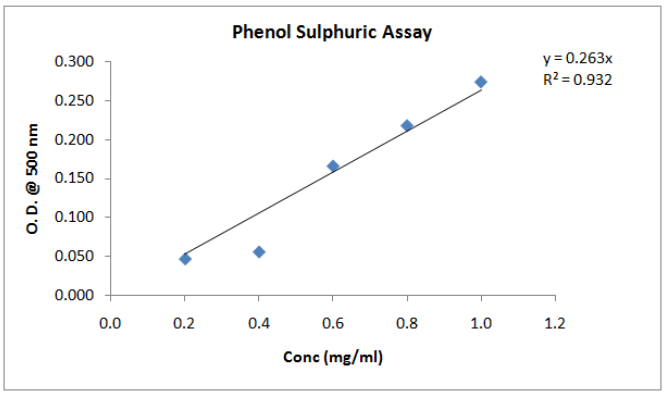


Figure 6. Standard Graph of Total Carbohydrates of EPS

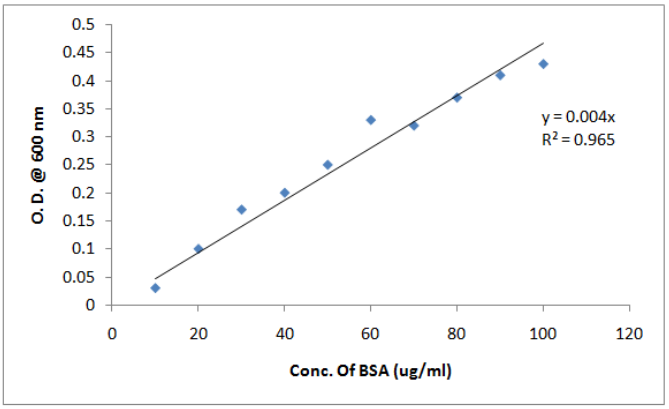


Figure 7. Standard Graph of Total Proteins of EPS

Topology that is surface properties of EPS produced by *Rhizobium mayense* was done by using Scanning Electron Microscopy and smooth surface of EPS was obtained.

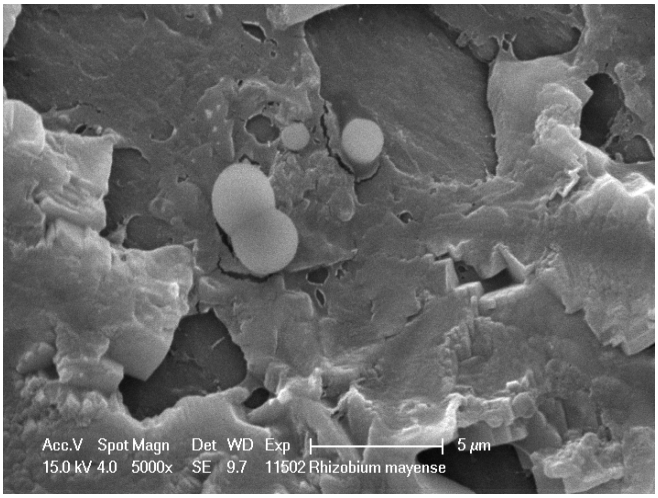


Figure 8. SEM View by 5000X

The partially purified EPS was further purified by Gel Permeation Chromatography using Sephadex G 200 gel. The elution profile showed that the polysaccharide was separated from its protein content. The purified EPS was obtained as single peak during initial elution with phosphate buffer. Overall 96.14% recovery with 1.31% fold purity of purified EPS was obtained.

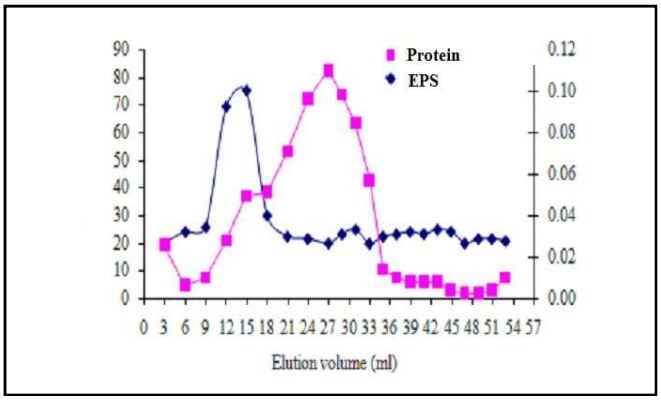


Figure 9. Elution Profile of EPS purification

Figure 10. Purified EPS

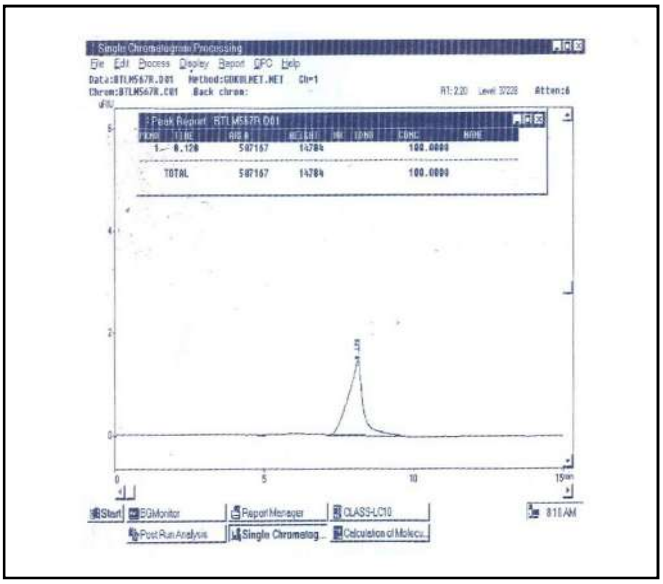


Figure 11. Chromatogram of Purified EPS

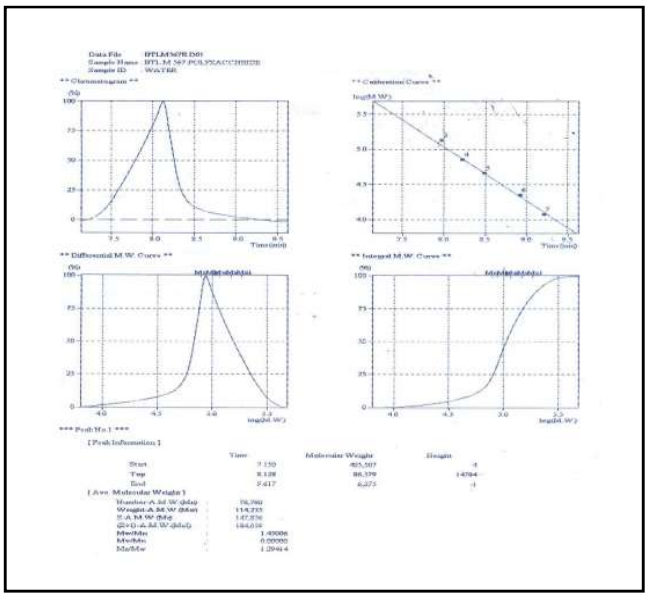


Figure 12. Standard Curves of various Dextran samples

The eluted fractions were concentrated and dried in freeze dryer. The yield of purified polysaccharides was 20% (w/w) with around 80% polysaccharides purity (1.31% fold purity expressed as glucose equivalence). The molecular weight of EPS was calculated by constructing a calibration curve, in which the logarithm of the molecular weight of the Dextran standards ranging from 620 to 9,05,000 Da was plotted as a function of the retention time using ChemStation GPC Data Analysis Software. The molecular weight (Mw) of purified EPS was found to be 1,14,235 Da.

FTIR

FTIR studies of purified EPS revealed high absorbance in the finger-print region i.e. 1,200 - 950 cm^{-1}. A strong peak at 3,362 cm^{-1} showed - OH stretching vibration with aliphatic –CH stretching vibration peak at 2,926 cm^{-1}. Peak at 1,638 cm^{-1} shows presence of bound water. Absorption peaks at 870 cm^{-1} and 922 cm^{-1} showed the presence of alpha configuration of sugar units. The characteristic peaks at 1,155 cm^{-1}, 1,124 cm^{-1}, 1,015 cm^{-1} indicated glucose residue having alpha-pyranose. Glycosidic bridge and -C-O-C- a broad stretching peak at 1,155 cm^{-1} was observed. The characteristic peaks at 1,124 cm^{-1} and 1,015 cm^{-1}, of the polysaccharide were belonged to α-(1→6) and α-(1→4) linkages respectively.

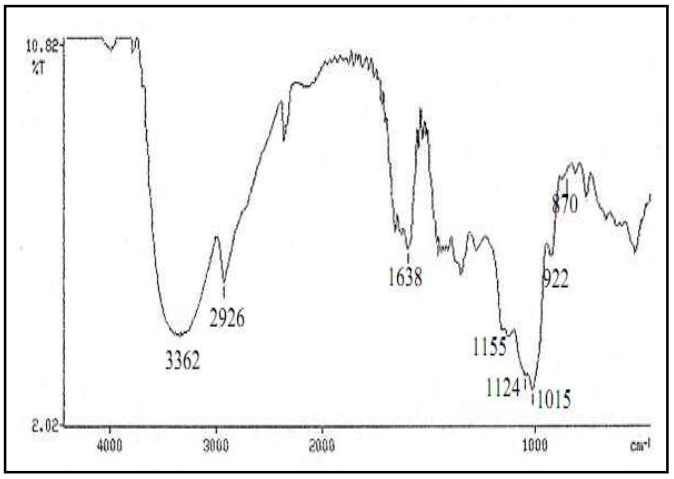


Figure 13. FT-IR Spectra of Purified EPS by *Rhizobium mayense*

NMR

The bacterial EPS extracts gave characteristics bands for EPS. In our studies, it was observed that, carbonyl (C=O) stretching peak and OH stretching peak was at broad and the maximum peak and the band at 1000-1500 showed the presence of polysaccharide. On the basis of literature values, it can be predicted that resonance at 104.88 ppm and 98.41 ppm corresponds to C-1 of α-(1→4) and α-(1→6) Glc*p* residues respectively. The major resonances of anomeric region occur at 98.41 ppm indicates linkage of C-1 as well as the signal at 66.25 ppm indicate linkage of C-6. According to FT-IR and ^{13}C-NMR data presence of α-(1→6) and α-(1→4) linkages were observed in the polysaccharide. On the basis of peak intensities abundance of α-(1→6) found to be major contributor with respect to α-(1→4), linkage in the polysaccharide.

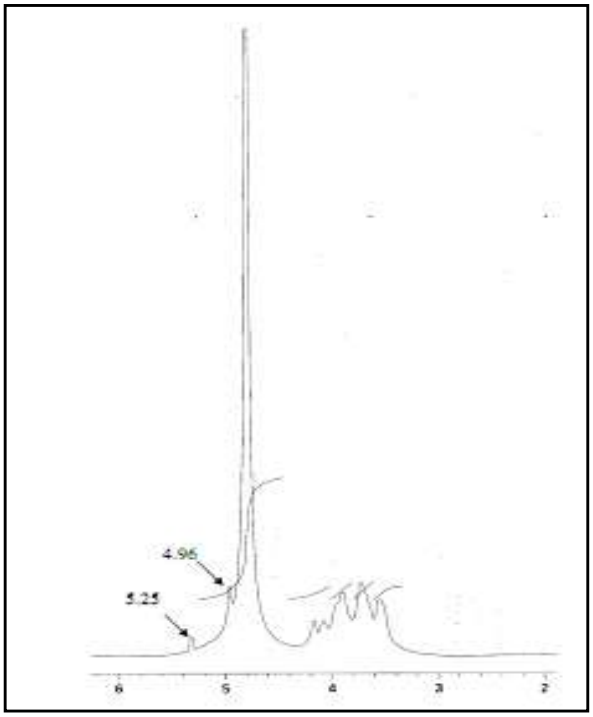


Figure 14. ^{1}H Chromatogram Spectrum

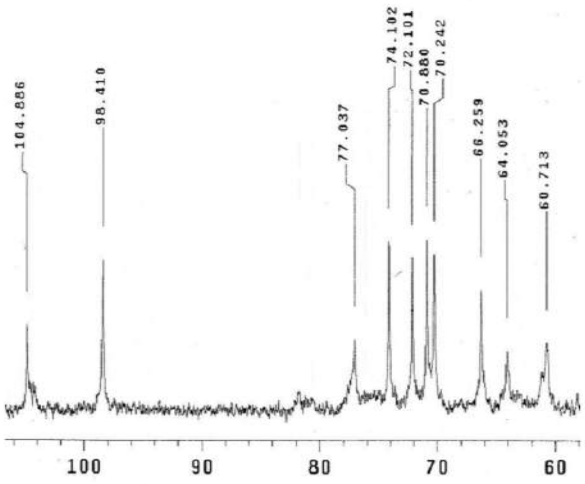


Figure 15. ^{13}C NMR Spectrum of purified EPS

HPLC

HPLC was applied in which different independent peaks were identified and molecular mass was determined with retention time. Further detailed GS- MS studies have showed the presence of two major peaks that corresponds to 1,1, Ethanediol, diacetates, 1-Tri decenal dicholorobenzne, hexoses, deoxyhexoses for bacterial EPS. HPLC-RID chromatogram showed purified EPS-I was composed of two monosaccharides – Arabinose and Glucose – in a molar ratio of 1:3.45; EPS-II was composed of three monosaccharides – Rhamnose, Arabinose and Glucose – in a molar ratio of 1:6.25:17.86.

GC-MS- GS- MS studies have showed the presence of two major peaks that corresponds to 1, 1, Ethanediol, diacetates, 1-

Tri decenal dicholorobenzne, hexoses, deoxyhexoses for bacterial EPS. A number of small peaks are also present, but these do not necessary correspond to alditol acetate.

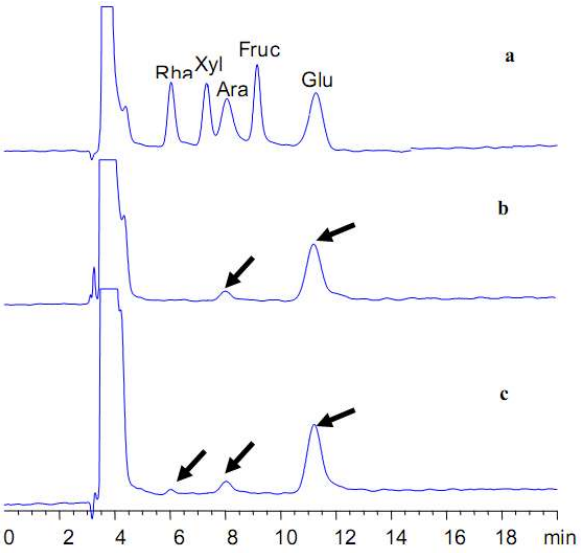


Figure 16. HPLC-RID Analysis of EPS

DISCUSSION

The organism used in the present study is a root and stem nodulating Rhizobial strain obtained from *Aeschynomene indica* plant which was identified as *Rhizobium mayense* based on 16 S rRNA sequencing. High level of EPS production was achieved by *Rhizobium mayense* in the basal medium of Yeast Extract containing 2% Mannitol as a carbon source. The usage of EPS compounds in the food industry have been intensively investigated. The novel properties of microbial EPS such as xanthan, alginate and curdlan may improve the food viscosity. It is also considered to apply the microbial EPS for food edible coating production that effectively would protect products from spoilage (Becker *et al*., 1998). The EPS obtained from the medium contains water soluble EPS that can be recovered by precipitation with three volumes of ice cold absolute alcohol. Lee *et al*., 1995 has also reported the use of two volumes of acetone. Surface properties of EPS produced by *Rhizobium mayense* was found smooth.

Eight different bacterial cultures for EPS production were studied by Indraneel *et al*., 1999 for their EPS production and surface properties. The partially purified EPS was further purified by Gel Permeation Chromatography using Sephadex G 200 gel and Sephadex G 100. About 96.14% recovery with 1.31% fold purity of purified EPS was obtained. The molecular weight of EPS was calculated as 9,05,000 Da using ChemStation GPC Data Analysis Software. FTIR spectrum of purified EPS proved the presence of free hydroxyl, aliphatic and -C-O-C- a broad stretching. Here NMR was applied in which EPS extracts gave characteristics bands for EPS. The resonance at 104.88 ppm and 98.41 ppm corresponds to C-1 of α-(1→4) and α-(1→6) Glc*p* residues respectively. HPLC was applied in which independent peaks were identified and the molecular mass was determined with retention time. GS- MS studies have showed that In case of complex EPS, neutral sugars are identified by their derivatives, alditol acetate (Swardekar *et al*., 1965) by GC-MS., which was proposed by Hoebler *et al*. (1989) the presence of two major peaks that corresponds to 1, 1, Ethanediol, diacetates, 1-Tri decenal dicholorobenzne, hexoses, deoxyhexoses for bacterial EPS. A number of small peaks are also present, but these do not necessary correspond to alditol acetate.

Conclusion

The organism used in the present study is a root and stem nodulating Rhizobial strain obtained from *Aeschynomene indica* plant which was identified as *Rhizobium mayense* based on 16 S rRNA sequencing. Exopolysaccharide production by *Rhizobium mayense* depends on the carbon source in the medium and was found maximum in the Yeast Extract Mannitol Agar. The exopolysaccharide produced by *Rhizobium mayense* is high molecular weight and biopolymer contains hydroxyl and methyl groups mainly. The results obtained from the physicochemical and structural characterization of exopolysaccharides revealed that it has ide applications in various fields as a viscosifier.

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