Biosynthesis of bacterial Exo Polysaccharides from *Pseudomonas sp. Bf1* and its characterization

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Abstract
Extracellular Polymeric Substances (EPS) are finding increasing industrial and medical applications in diverse areas. In the present work, a microbe from the bacterial grown biomass on the surface of the aged wall was isolated and identified as Pseudomonas sp BF1 (NCBI accession No: MT192327) by the 16s rRNA method. This strain was used for biosynthesis of EPS in suitable bacteriological media. The EPS thus synthesized was deproteinized by the Sevag method and the resulting solution was evaporated under vacuum at 45^{o}C to get the EPS. Qualitative carbohydrate analysis such as Fehling's solution and phenol-sulphuric acid tests on the EPS thus separated indicated the presence of carbohydrate (polysaccharide). The yield of carbohydrate present in EPS was calculated as glucose equivalent by phenol-sulphuric acid test and it was 32 percent (w/v). The EPS thus obtained was also analyzed by simultaneous Thermo Gravimetry (TG)-Differential Thermal Analysis (DTA) and FT-IR spectra.

FT-IR spectra indicated the presence of glycosidic (C-O-C), C-H, C-OH and O-H linkages supporting the presence of polysaccharide in the EPS. Comparative analysis of TG traces of EPS and the background residues of broth indicated the presence of additional new substances in the EPS not present in the broth (blank) supporting the formation of polymeric substances. The observed results conveyed that the isolated microbe may be exploited for the commercial biosynthesis of EPS.

Keywords: *Pseudomonas sp BF1,* Biosynthesis, 16S rRNA gene sequencing, Extracellular polysaccharide, Thermogravimetry, Differential thermal analysis, FT-IR spectrum.

Introduction

Bacteria produce diverse structural, functional and valuable biopolymers such as polysaccharides, inorganic polyanhydrides (such as polyphosphates), polyesters, polyamides, slime etc. with varied physico-chemical properties using simple to complex substrates. With reference to their cellular location, these biopolymeric substances could be either intracellular or extracellular[27]. Many microorganisms (gram-positive and gram-negative bacteria, archaea, fungi and some algae) are known to produce extracellular polysaccharides (xanthan, dextran, alginate, polluan, curdulan) and secret into the environment as a strategy for growing, adhering to solid surfaces and surviving adverse conditions and are termed as extracellular polymeric substances (EPS).

Unlike the intracellular biopolymers which are few with very limited applications, the range of the extracellular biopolymers is vast. These EPS also comprise a wide number of low molecular weight oligomer proteins, glycoproteins and glycolipids and surprising amounts of extracellular DNA (e-DNA). Some of these biopolymers provide the same function and others are specific for certain taxa and give out different biological functions. Generally, these molecules are released in response to the physiological stress encountered in the natural environment. They are the structural components of the extracellular matrix in which cells are embedded during biofilm development.

EPS synthesized by bacteria of all taxa and secreted into the external environment are the most abundant and rich in polysaccharides termed as exopolysaccharides. EPSs are highly heterogeneous polymers containing hetero or homopolysaccharides of a number of distinct monosaccharides and noncarbohydrate substituents with high molecular weights ranging from 10 to 1000 kDa[23,31].

The material properties of exopolysaccharides have revolutionized the industrial and medical sectors due to their biocompatibility and retinue of functional applications in diverse areas such as pharmacological, nutraceutical, functional food, cosmeceutical, herbicides, insecticides and others. They are also used as anticoagulant, antithrombin, immunomodulatory, anticancer and bioflocculants. Bacterial EPS can be used as adsorbents in bioremediation of toxic heavy metals. The inherent biocompatibility and apparent non-toxic nature of some of these bacterial exopolysaccharides have prompted their uses as scaffolds or matrices in tissue engineering, drug delivery and wound dressing and these made them more attractive as compared to polysaccharides obtained from plants and microalgae[12,27].

Their functions include adherence of cells to surfaces, migration of prokaryotes in groundwater, protection from engulfment by predatory protozoa and white blood cells (phagocytes), protection from perennial effects of drying or desiccation in certain soil bacteria or from attack by antimicrobial agents of plant or animal origin and the communal life of biofilm. Their functions also include

cryoprotection for growth at low temperatures and high salinity in marine environments. At the cell wall, they serve for structural and protective purposes and they may take the form of a covalently bound cohesive layer outside the cell wall.[2,4,6,9,10,24]

The EPSs synthesized by microbial cells vary greatly in their composition and hence in their chemical and physical properties[6,9,12]. They usually contain pentoses, hexoses, amino sugars and uronic acid which are linked most commonly by 1,4- or 1,3- glycosidic linkages in the backbone which impart strong rigidity and also by 1,2- or 1,6- linkages which also give rigidity to the polymer. They also contain non carbohydrate moieties such as sulphate, phosphate, acetic acid, succinic acid and pyruvic acid. The EPSs may be neutral (e.g. dextran, scleroglucan) or acidic (xanthan, gellan) in nature and are used in the stabilization of foods, production of several industrial and pharmaceutical compounds. They are also used as thickening and gelling agents to modify the rheological properties of solution.

Biosynthesis of polysaccharides favorably occurs in a growth medium by batch culture fermentation with carbon/nitrogen ratio of 10: 1. By manipulating the nutrient supply, differential synthesis of polysaccharides can be achieved. By limiting nitrogen and metal ions in the medium neutral and acidic polysaccharide respectively are mainly synthesized. 90% oxygen saturation is ideal for good growth and polysaccharide synthesis. It was predicted that more than 100 enzymatic reactions are involved directly or indirectly in biosynthesis of polysaccharides[25,30].

Hence, the present objective of the work was to investigate the production of extracellular polymeric substances (biofilm strain) from *Pseudomonas taiwanesis* and subsequent extraction, purification and characterization of the bio formed bacterial polysaccharides by FTIR-ATR, TGA, DTA and qualitative carbohydrate test.

Material and Methods

Materials: Nutrient medium (HIMEDIA GRMT 477-500G)[11]of pH 7.3 having the broth ingredients (wt(g) / 50 ml) peptone (0.25), sodium chloride (0.25), HM peptoneB (0.075) and yeast extract (0.075) was used.

Methods

Isolation and identification of Biofilmforming *Pseudomonas sp. BF1:* The bacteria grown contaminants were collected in a sterile container from the wet surface of aged walls by scrapping inoculated at 37º C in the sterile water for 2 hr. Then plated in nutrient agar plate by serial dilution from 10^{-3} to 10^{-6} and incubated at 37˚C for 18hr. After the incubation the eight morphologically different strains (S1-S8) were picked up and inoculated in the nutrient broth and incubated for 18hr at 37˚C. Then the ability to form biofilm by the isolates was screened using microtiter plate assay[15]and from this the potential strain that forms the biofilm was identified.

16SrRNA gene sequencing and phylogenic tree construction: The higher biofilm forming strain was subjected to biochemical characteristics according to Bergey's Manual of Determinative Bacteriology and confirmed through molecular characterization by 16S rRNA gene sequencing[7]. This was done at Molecular biology lab, Pondicherry Central University, India through an automated DNA sequence (MEgabace, GE). Briefly, bacterial genomic DNA was extracted by phenol-chloroform method[14]and the 16S rRNA gene was amplified by using primers 8F(5'-AGAGTTTGATCCTGGCTCAAG-3') and 1492(5'- 18 GGGCGGTGTACAAGGC-3'). PCR was run with 35 cycles consisting of initial denaturation at 95°C for 1 min, annealing at 55°C for 1 min and extension of 1.5 min at 72°C followed by final extension of 5 min at 72°C. Then the phylogenetic tree was constructed using neighbor-joining method[26].

Biosynthesis and Extraction of EPS: The inoculum was prepared by transferring a loopful of identified potential strain namely *Pseudomonas sp. BF1* into 50 ml nutrient medium [HIMEDIA GRMT 477-500G][11] and incubated under shaking for 48 hrs at 37℃. The fermented cultural broth was then harvested and the cell suspension was removed through centrifugation [REMI (RM7)] at 4100 x g for 20 min to separate the biomass. The crude supernatant was collected separately and passed through 60-120 mesh silica gel column (3 cm) and eluted using Millipore water. The broth solution thus obtained was taken in a glass beaker and evaporated at 55ºC in a hot air oven for 5 days. Final traces of water were removed by drying under vacuum in a desiccator for 24 hrs. The solid polymeric substance thus obtained was used for its characterization.

Sevag Method for Deproteination of EPS: The protein present in EPS was removed from the Sevag method[3]. A known weight of biosynthesized EPS was dissolved in Millipore water to get a 9% solution (w/v). Two ml of this solution was added to 2ml of a mixture of analytical grade chloroform and n-butanol prepared in the volume ratio of 5:1. This was shaken well and incubated at 37ºC for 30 minutes and this showed two distinct layers. The top turbid layer was separated and centrifuged at 7000 rpm for ten minutes and the chloroform layer was discarded. The pellet obtained was removed after decanting the solvent. The pellet was washed with AR acetone three times and dried under vacuum and used for further characterization.

Carbohydrate Analysis

Fehling's test: The crude EPS was analyzed qualitatively for the presence of polysaccharide using Fehling's solution test. A known volume of EPS solution was heated at 70˚C with few drops of conc. HCl solution for 10mins and cooled. To this, Fehling's solution was added and heated slightly and the color change was observed.

Phenol-sulphuric Acid Method: Phenol-sulphuric acid method was used[5,16,22] to quantify the unknown carbohydrate

content in the biosynthesized EPS through the standard glucose curve (absorbance at 490 nm Vs glucose concentration (mg/ml)). In this a known volume of the solution of biosynthesized EPS and standard glucose solutions of various concentrations were mixed with a known volume of 80% (w/v) phenol in different test tubes and vortexed.

To each of the solutions a known volume of concentrated sulphuric acid was added and incubated at 37°C for 10mins and the absorbance values at 490 nm were measured and standard graph (Absorbance Vs Glucose concentration) was constructed. The carbohydrate content in EPS in terms of glucose equivalent was obtained from the measured absorbance value of the EPS solution at 490 nm through intrapolation in the standard glucose curve. An average of five measurements was taken as the carbohydrate content in EPS.

Characterization of Extracellular Polymeric Substance

Thermal Analysis: Simultaneous TG/DTA analysis of biosynthesized EPS sample was carried out both in air (N_2 /O_2 , 80/20) and nitrogen for the temperature range from room temperature to 550°C at a heating rate of 10°C per min on NETZSCH STA 2500 regulus (German) for the sample size 5 to 10mg using alumina crucibles as sample holder.

FT-IR Analysis: The FT-IR spectra were recorded in ATR mode with a resolution of 4cm^{-1} on SHIMADZU FT-IR spectrophotometer to identify the functional groups present in EPS.

Results and Discussion

16S rRNA Gene Sequencing of Potential Strain: The microtiter assay of 8 strains (S1 to S8) picked-up from the inoculated wall contaminants as discussed earlier revealed that strain S2 has more potential to form biofilm on any matrix. 16S rRNA gene sequencing study and the phylogenic tree analysis of the isolated potential strain identified and confirmed the strain as *Pseudomonas sp. BF1*. The 16S rRNA gene sequence obtained from this study was also deposited in NCBI GenBank with an accession number MT192327[18]. This strain was used for the successful biosynthesis of ECP. The constructed phylogenic tree is shown in figure 1.

Carbohydrate Analysis by Fehling's and Phenol-Sulphuric Acid Test: Fehling solution[13,19] and phenol-sulphuric tests on the HCl treated EPS were positive indicating the presence of reducing sugars. Fehling solution which contains alkaline cupric hydroxide was reduced to red cuprous oxide precipitate when it is added to the HCl hydrolysed EPS solution and heated. This indicated the presence of reducing sugars in EPS[13,19]. The nutrient broth (blank) gave a negative test with Fehling's solution which corroborated that the carbohydrate was formed during biosynthesis using the *Pseudomonas sp. BF1*. The phenol-sulphuric acid test of EPS solution gave a yellowish brown coloured product with an absorption maximum at 490 nm similar to glucose[5,16].

Phenol-sulfuric acid method is the most reliable method for the quantitative estimation of total carbohydrate among quantitative assays used for carbohydrate. In hot acidic medium, glucose is dehydrated to hydroxymethyl furfural which reacted with phenol to give yellowish-brown product. The absorbance value(at 490 nm) of the yellow-brown-colored product of acid hydrolysed EPS with phenol is a measure of reducing sugar content[5,16] in EPS. The amount of total carbohydrate present in EPS calculated in terms of glucose equivalent was 32 % (w/v, weight per unit volume of brothsolution). That is one litter of broth solution will have 320 g of reducing sugar which is a good yield.

Characterization of EPS

TGA/DTA Analysis: The thermal degradation patterns of EPS and that of the broth(blank)(TGA curve) and the associated enthalpy change (DTA trace) will furnish information about the formation of any new material in the broth during bacterial growth. The TGA/ DTG and DTA thermograms of EPS and that of the background components of broth recorded in air and nitrogen atmospheres are shown in figures 2 to 4.

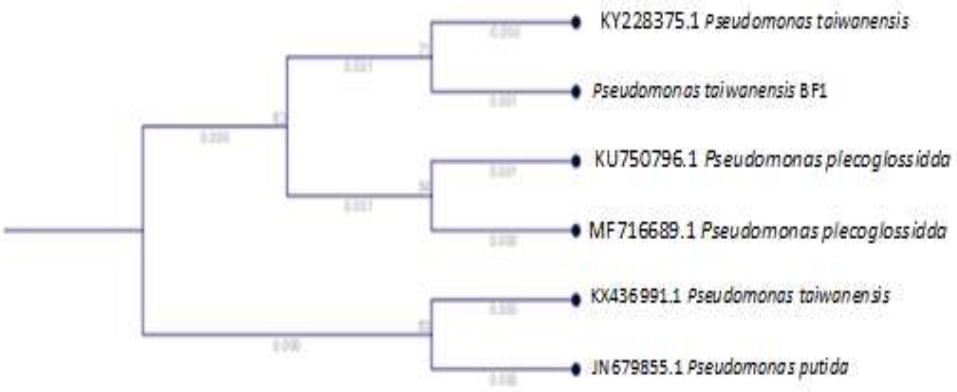

Fig. 1: Phylogenetic tree of *Pseudomonas sp. BF1* (NCBI accession No.: MT192327)

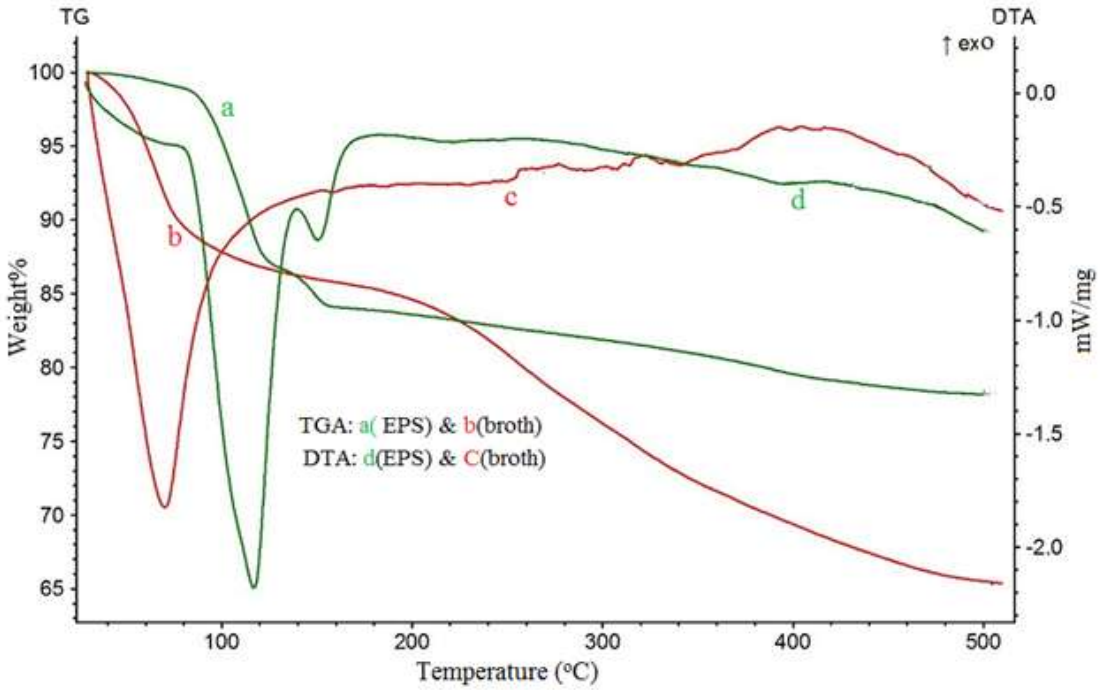

Fig. 2: TG/DTA thermograms of EPS and broth components in nitrogen

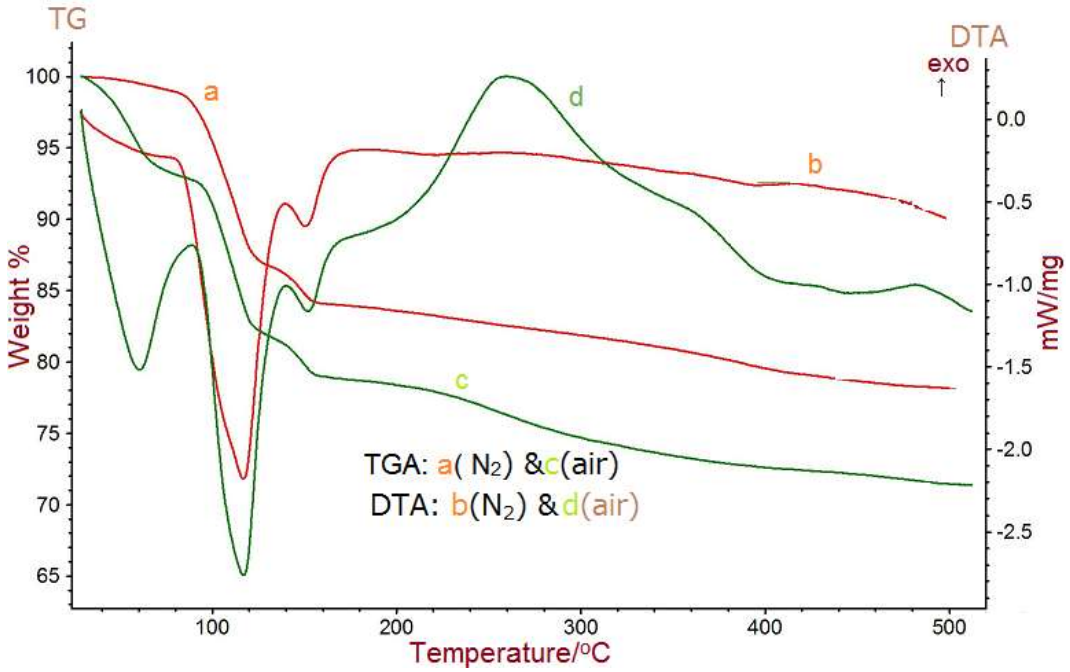

Fig. 3:TG/DTA thermograms of EPS in air and nitrogen

TGA of EPS revealed a multistep degradation unlike that of broth which showed a broad single step degradation after moisture /volatile impurity (if any) loss. DTA revealed that the degradation steps of EPS were endothermic in nitrogen. But the degradation in air was endothermic up to 160°C and exothermic after 160°C (Figures 2 and 3).

Comparative analysis of TG/DTA thermograms of broth residue (background components in biosynthesis medium) and that of biosynthesized EPS (Fig. 2) showed that the former showed a more and sharp weight loss at 60°C exothermically perhaps due to loss of moisture and other volatile impurities. But EPS showed sharp weight loss at an elevated temperature around 125°C. Unlike broth residue which showed single step degradation upto 300°C, the biosynthesized EPS displayed two-step degradation (Fig. 2). The residual mass in EPS and background component in nutrient medium (in N_2) were 78.51% and 65.07% respectively at 550°C (Fig. 2).

The higher % reside at 550°C for EPS was more likely due to the oligomeric substances of polysaccharides. The

endothermic mass loss from 0 to 80˚C in EPS was due to moisture loss, whereas the endothermic weight loss from 80-175°C may be due to volatile impurities and low molecular weight polysaccharides and glycoprotein/glycolipids if any etc. But the endothermic weight loss observed in background components of broth was > 20% unlike in EPS which was around 1%.

Analysis of TG /DTG traces in air and nitrogen of EPS (Fig. 4) indicated that the weight loss occurred in 4 steps up to 550°C in air and by 3 steps in nitrogen. The more number of steps in oxidative degradation compared to pure thermal degradation may be attributed to complex thermal reactions involving degradation, depolymerization, fragmentation, cyclisation, oxidation, oxidation of initially formed thermal degradation products etc. Reactions were compared to 3 in pure thermal degradation where there is no oxidative degradation[17,20,21,29]. The degradation at temperature >250°C in air is exothermic due to oxidative degradation (Fig. 4).

The residual mass of deproteinized EPS at 550 C in air was 71.3% which was less than that in nitrogen (78%) (Fig. 2) The residual mass of broth components in nitrogen at 550°C was 72%. Hence the 6% extra residue of EPS in N_2 originated from its polysaccharide components.

FTIR-ATR Spectrum of EPS: The FT-IR spectrum of EPS recorded under ATR mode is shown in figure 5. The spectrum showed prominent absorption peaks at the frequencies 3280 (broad), 2900, 2345, 1431, 1363, 1068 and 862 cm^{-1} in the functional group and finger print regions indicating the presence of various functional groups of organic molecules formed in the biosynthetic product (EPS). The absorption peaks at 3280 cm^{-1} (broad) and 2900 cm^{-1} in the FT-IR spectrum (Fig 5) of EPS were attributed to the O-H and C-H stretching vibrations[1].

The absorption frequencies at 1068 and 1431 cm^{-1} were due to the C-O-C stretching and symmetric bending vibration of CH_2. The absorption at 1431 cm^{-1} may also arise due to the amide – III present in the traces of unremoved protein if any in EPS[1]. The absorption frequencies at 2345 and 1363cm^{-1} were most likely due to asymmetric stretching of C-H and C-O bending respectively. The characteristic peak around 862 cm^{-1} implied the presence of *β*-D-glycosidic linkages in the polysaccharides[1, 8]. The finger print region (1000-1200 cm^{-1}) is dominated by ring vibrations overlapping with stretching vibrations of C-OH and glycosidic (C-O-C) bond vibrations.

Conclusion

The results of the present study clearly implied that the bacterial strain *Pseudomonas taiwanes* is identified by 16SrRNA has the potential to biosynthesis polysaccharide rich extracellular polymeric substances in suitable broth media. The FT-IR spectrum and carbohydrate analysis indicated the presence of polysaccharides to the extent of 32% (w/w) in EPS. TGA/DTA studies on EPS and the background components of the media indicated the presence of additional substances (polysaccharides) that were not present in the background matrix.

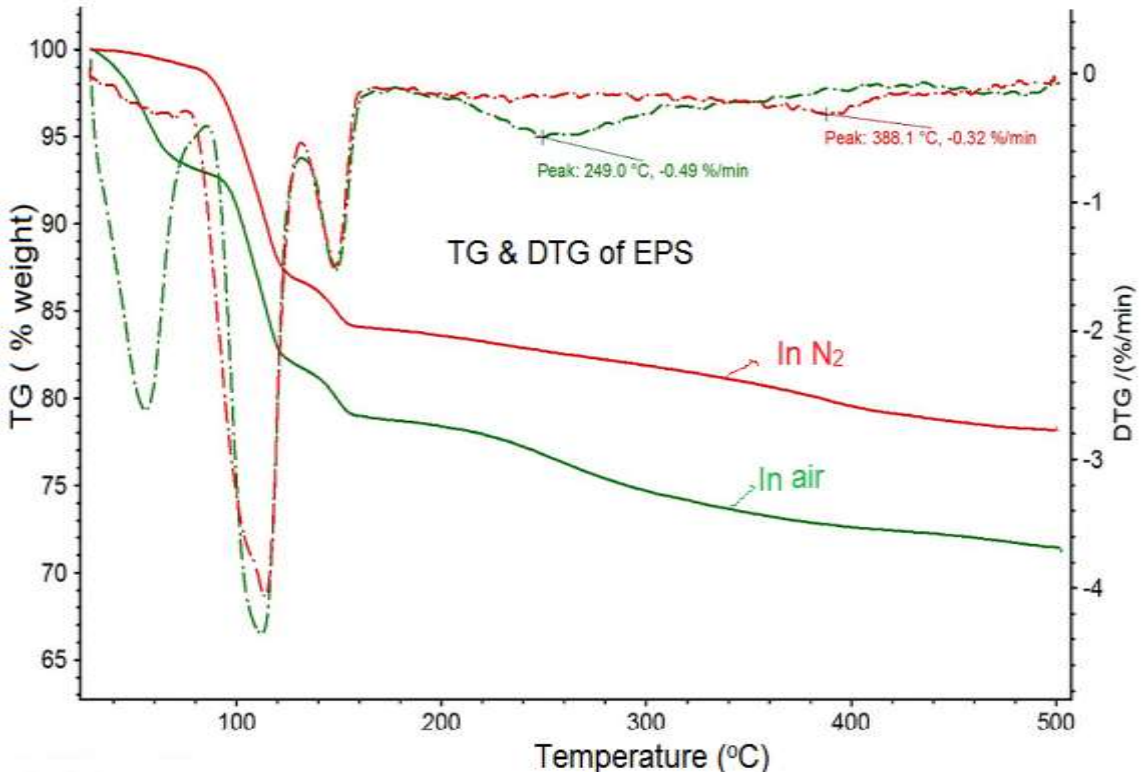

Fig. 4: TG/DTG traces of EPS in air and nitrogen

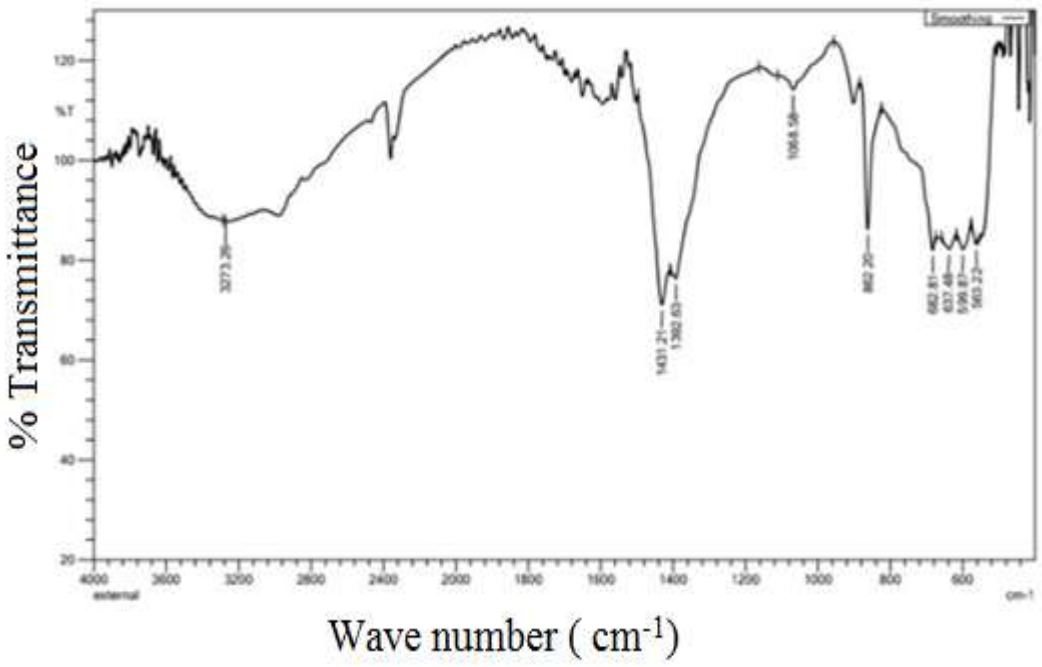

Fig. 5: FTIR-ATR Spectrum of EPS

The multistep degradation of EPS in air was attributed to the complex reactions of the formed bio-molecules mainly polysaccharides and other biomolecule if any as impurities. The biosynthesized polysaccharides may be used for the production of bio-surfactant, flocculating agent in wastewater treatment, nano-particles for the degradation of industrial dyes, superabsorbent polymers, drug carriers for drug delivery etc. They may also find uses in pharmacological, nutraceutical, functional food, cosmeceutical etc. areas. Further prospects may also include their uses in antithrombotic and anticancer therapy, immunomodulation etc.

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