Characterization of Exopolysaccharide from *Bacillus amyloliquefaciens* BPRGS for its Bioflocculant Activity

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Abstract

Extracellular polymeric substances, also known as exopolysaccharide, or EPS, are high-molecular weight macromolecular compounds secreted by microorganisms into their environment. These extracellular polysaccharides have distinguished properties like cell adhesion, floc formation and stability. In this work, different microbial communities producing extracellular polysaccharide were isolated from marine slimy sediments from Kovalam beach, Chennai- India. The microorganisms were screened for their optimal EPS production. Among the consortia, maximum EPS producing organism was identified as *Bacillus amyloliquefaciens* BPRGS .The organism was confirmed by microscopic, biochemical and DNA sequence analysis. The effect of medium components (carbon and nitrogen sources) and environmental factors (initial pH and temperature) for biomass growth and exopolysaccharide (EPS) production in *Bacillus amyloliquefaciens* BPRGS was investigated. Among the tested carbon and nitrogen source Sucrose and Yeast extract have produced maximum EPS yields. The optimal pH and temperature was found to be at pH 9.0 and at 37°C for 72 hours. *Bacillus amyloliquefaciens* BPRGS EPS showed a high flocculation activity at 96% in kaolin suspension with EPS concentration 8 mg/100 ml. The Thermo gravimetric analysis and Differential Scanning Calorimetry results confirm that the EPS shows high thermal stability.

Key words: Extracellular polysaccharides, flocculation, *Bacillus amyloliquefaciens* BPRGS, Biopolymer and Bioflocculant.

Abbreviations: Extracellular polysaccharides (EPS).

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1. Introduction

Extracellular polymer (EPS) is a rich matrix of polymers, including polysaccharides, proteins, glycoprotein, nucleic acids, phospholipids, and humic acids. Carbohydrate was identified as the major constituent in the EPS. Exopolysaccharides (EPS) are formed by polymerization of similar or identical building blocks, which may be arranged as repeated units within the polymer. Most of these polymers are generally of high molecular weight polysaccharides (10 to 30 kDa) with variable compositions. This chain might be unbranched or branched with side chains of other compounds attached to the polymer chain (13). Based upon the composition of the chain, the polymer is classified as homopolysaccharides (having the same monomer) or heteropolysaccharides

(Different repeating monomer); for example: bacterial cellulose, dextran, levans etc. are some of the major homopolymers having glucose as the repeating unit. On the other hand, EPS like bacterial alginate and most other polymers are heteropolymers with more than two monosaccharides and other additional groups like uronic acid (13).

Different carbon sources and the carbon/nitrogen ratio (C/N) can also influence the production and composition of EPS [38]. All EPS biopolymers are highly hydrated and form a matrix, which keeps the biofilm cells together and retains water. This matrix interacts with the environment, e.g., by attaching biofilms to surfaces and through its sorption properties, which allow for the sequestering of dissolved and particulate substances from the environment, providing nutrients for biofilm organisms (16).

The EPS is the construction material of the biological active biofilm matrix having a multifunctional role in gel formation, flocculation, emulsification, absorption, film formation, and protection. Due to their many interesting physical-chemical and rheological properties with novel functionality, the microbial polysaccharides (EPS) act as new biomaterials and find a wide range of applications in many industrial sectors like textiles, detergents, adhesives, microbial enhanced oil recovery (MEOR), wastewater treatment, dredging, brewing, downstream processing, cosmetology, pharmacology, and as food additives (4).

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Bacillus amyloliquefaciens are known for their catabolic properties and degradation of complex macromolecules. *B. Amyloliquefaciens* is Gram-positive, catalase positive, aerobic, rod-shaped and motile. This particular organism is found in the soil in nature. As in the case of the family Bacillaceae, it forms a strong endospore when conditions are not favorable and it is dispersed in this form into dust and later into water supplies reaching finally plants and animals. Extracellular polysaccharide produced by the bacillus bacteria has physiochemical feature and uncomplicated biodegradability in the natural environment.

Our study reports here for the first time, the isolation and the identification of the polymer-producing microorganism as Bacillus amyloliquefaciens BPRGS and optimization of environmental conditions for the high yield of EPS and flocculating activity.

2. Materials and Methods

2.1. Maintenance of culture and preparation of inoculum

Serial dilution and pour plate method was used for the isolation of EPS producing bacteria from the rocks at the Kovalam Beach, Chennai- India. *Bacillus amyloliquefaciens BPRGS* was isolated, based on their slime colony growth on the Zobell marine agar plates were separated and sequenced. The pure culture was maintained on a nutrient agar slant supplemented with glucose (2%, w/v) and subcultured at monthly intervals with overnight incubation at the 37 OC followed by storage at 4°C. Inocula (200 ml) were prepared from a single slant in a chemically defined modified STN medium (31) containing the components (g/L) Sucrose 20; Tryptone 10; K_2HPO_4 0.067; KH_2PO_4 0.083; $MgSO_4.7H_2O$ 5; NaCl 10; $CaCl_2$ 1; $FeCl_3$ 0.005; $MnCl_2$ 0.001; Na_2MO_4 0.001; $ZnCl_2$ 0.001; distilled water 1000 ml; pH 7.0 was sterilized at 121°C for 20 min. The flasks were incubated at 37± 2°C for four days.

2.2. Extraction of Extracellular polysaccharides and Biomass estimation

The *Bacillus amyloliquefaciens BPRGS Sp.* grown culture flask were autoclaved at 121°C for 20 min and centrifuged at 10,000 rpm for 20 min the supernatant were mixed with 3 volumes of ice cold ethanol and held at 4°C for 12h to precipitate the extracellular polysaccharide. The precipitate was collected and dried at 80 °C, diluted with deionized water and precipitated again with ice cold ethanol in the ratio (1:3) and dried to a constant weight at 80°C. Biomass were separated in a centrifuge at 10,000 rpm for 15 minutes. The supernatant was decanted; pellet was washed in deionized water and dried to a constant weight at 80-100°C.

2.4. Carbohydrate and Uronic acid and Protein estimation

The total neutral sugar content was determined by the phenol sulfuric acid method (10) with glucose as the standard and Uronic acids were measured using M-hydroxydiphenyl acid method, using D-glucuronic acid as the standard (7). Protein concentration estimation was performed by Lowry et al (21). The color developed during the assay procedure was read at 650nm. Using the standard curve of bovine serum albumin.

3.0. Optimization studies

3.1. Effect of Carbon and Nitrogen, pH, Temperature on the production of EPS

STN medium (31) yeast extract 0.5; K_2HPO_4 0.067; KH2PO4 0.083; $MgSO_4.7H_2O$ 5; NaCl 10; $CaCl_2$ 1; $FeCl_3$ 0.005; $MnCl_2$ 0.001; Na_2MoO_4 0.001; $ZnCl_2$ 0.001; distilled water 1000 ml; pH 7.0 was sterilized at 121°C for 20 min. To the prepared STN medium four percentages of each glucose, sucrose, maltose, lactose and fructose were added to find the effect of carbon sources on the EPS production. Sources such as ammonium chloride, urea, yeast extract and phenyl alanine added at 1% to find the effect of nitrogen. Initial pH 5, 7, 9 and 11 was adjusted using 0.1 N HCL and 0.1 N NaOH were performed to find the effect of pH and different temperature studies at 20°C, 28°C, 37°C and 50°C were also performed to identify the effect of temperature for the production of EPS to the above contained STN medium. The flasks were incubated at 37± 2°C for 4 days and every 12 hours the culture supernatant was precipitated with ice cold ethanol (1:3) ratio, dried and the biomass were weighed and expressed in (g/L).

3.5. Instrumental analysis

The biomass and EPS were fixed in 2% glutaraldehyde (W/V) for 2 h separately after washing with saline solutions; they were dehydrated in 30–100% water–ethanol series. The air-dried particles were coated with gold in argon medium. Scanning electron microscopy (SEM) observations were performed on a scanning device attached to a JEOL JM-5600 electron microscope at 20 kV accelerating with an electron beam of voltage 5–6 nm.

Fourier transform infrared spectroscopy (FTIR) was used to determine the functional groups and the chemical bonds present in the biologically active fraction of the EPS and

thus determine its chemical nature. The FTIR analysis was performed by using a Perkin-Elmer Co., a USA model spectrum with samples dispersed in the pellets of KBr. The dried active fraction of EPS samples (0.3-0.5 mg) was grounded in about 80mg of spectra-grade KBr (sigma) and pressed into pellets under about 5-6 tons cm^{-2} pressure with the help of a hydraulic press. The spectral measurements were carried out in the absorbance mode. The measurements were carried out in the mid-infrared range from 4000 to 400 cm–1 with plain KBr pellet was used as the background reference.

EPS sample (6-7mg) was loaded in platinum TGA pan and gravimetric analysis made under pure nitrogen atmosphere, from 0°C to 800°C using a temperature gradient of 10°C/min. Scans are routinely recorded as duplicates using TGA Q50 (V20.6 Build 31). DSC analysis, required quantity of EPS samples (3-4mg) was loaded in aluminum DSC pan and gravimetric analysis made under reduced nitrogen atmosphere, from 0°C to 200°C using a temperature gradient of 10°C/ min. Scans are routinely recorded as duplicates using DSC Q200 (V23.10 Build 79).

3.9. Molecular studies

The 96 hrs grown cultures were centrifuged at 10,000rpm for 10 minutes and cell biomass is collected and DNA was extracted by the CTAB NaCl method (28). The extracted DNA was re-suspended in appropriate volume of Tris-EDTA (TE) buffer and stored at -20°C. Agarose gel (0.7%) was made in 0.5 x Tris Borate EDTA (TBE) buffer and run at 60V for 1 h at 10°C in an electrophoretic apparatus. The DNA bands were visualized under UV transilluminator (Biotech R & D Laboratories, India) and compared with known DNA molecular weight markers.

Protein molecular weight was determined the bacterial cells was lysed by heating at 100°C in 125 µl of 60 mM Tris HCl (w/v), SDS/1 mM EDTA (pH 6.8) for 5 min and then diluted to 1 ml with the same buffer without SDS. SDS PAGE was performed on 16% (W/V) acrylamide slab gels with a discontinuous buffer system (30). EPS samples were prepared according to gels were fixed using Alcian Blue in acetic acid (25) and stained by the silver method (36).

3.11. Bioflocculating activity

The flocculating activity was measured according to the method of Kurane et al (19) using a suspension of kaolin clay as test material with minor modifications. Kaolin was suspended in distilled water at a concentration of 5000 mg l^{-1} (kaolin suspension). In a test tube, 4.5 ml of kaolin suspension was added and mixed with 0.25 ml of $CaCl_2$ solution (90 mM). To this mixture, 100µl of 10 mg/L concentration of the EPS of the test bioflocculant was added and vortexed for 30s and left for 5 min at room temperature. The absorbance of the upper phase at 550 nm (A) was measured with a spectrophotometer (The raw and the oxidized samples were scanned using UV–Visible spectrophotometer (Varian, Cary 100 Concentration). A control experiment in which 100µl of distilled water, instead of the bioflocculant, added to the suspension was performed in the same manner, and the absorbance was measured (B). Flocculating activity (%) was defined and calculated as:

$$Flocculating\ activity\ (\%) = \frac{(B-A)}{B} \times 100$$

The activity was expressed as the mean value from duplicate determinations. To determine the exact role of cations, different cations at different concentration and at a range of pH was analyzed against commercially available Xanthan gum from Loba chemicals, India it's an extracellular polysaccharide used in the food as additives. Different cations used were $CaCl_2$, $FeCl_3$, $AlCl_2$, KCl and $CaCO_3$ in 1 mmol/L concentration, different concentration of cation 0.1 mmol/L, 1 mmol/L, and biopolymer concentration from 0.5 to 4.5 mg/L and different pH range from 2 to 8 were performed to analyze the flocculating activity.

4. Results and discussions

4.1. Effect of Carbon and Nitrogen, pH, Temperature on the production of EPS

The carbon source for the EPS production, five different carbon sources were used at 4% concentration glucose, fructose, lactose, sucrose and maltose.

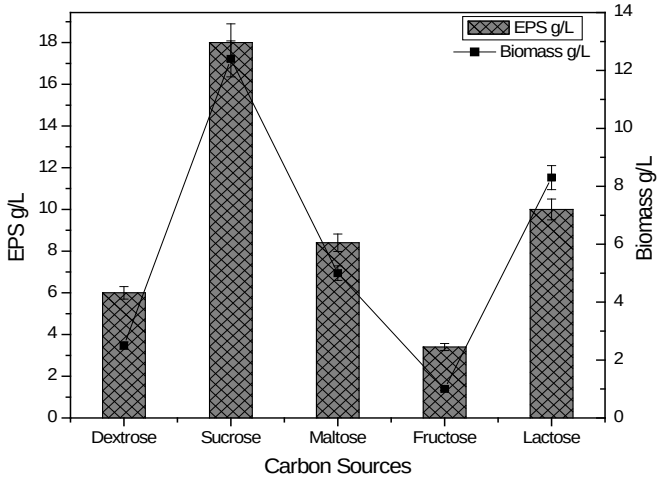

Fig. 1. Effect of carbon sources on the production of EPS

The highest yield of EPS from the strain *Bacillus amyloliquefaciens BPRGS sp.* was observed when sucrose was supplied as the carbon source with a yield of 18g/L EPS (Fig.1).

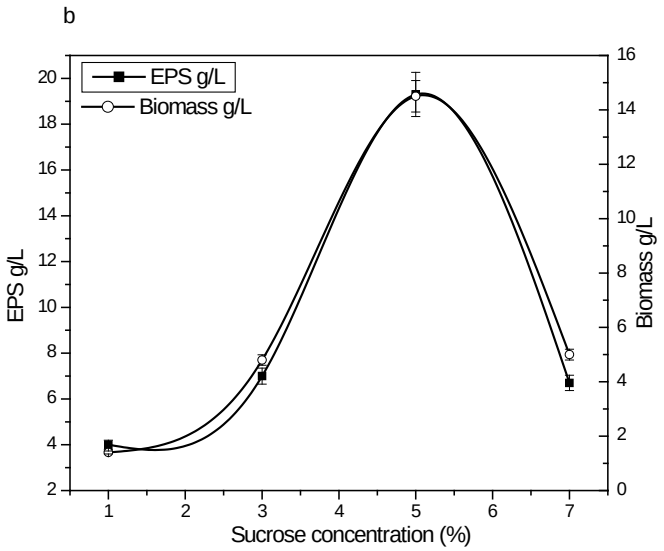

Fig. 2. Effect of sucrose concentration on the production of EPS

Optimization of different concentration of sucrose study at 96 hours 5% sucrose concentration was produced 19.3 g/L of EPS and 15g/L biomass. In other reports maltose as the carbon source gave maximum EPS 10.45 g/L (12). An EPS yield of 0.065 ± 0.013 and of 0.297 g ± 0.054 g/L substrate after 72 h was obtained for Glucose and jute, respectively (37). *Pseudomonas* sp shows production of 7–18 g/L of EPS using glycerol as the sole carbon source (20). The 5% sucrose concentration gave 19.3 g/L EPS and 15.0 g/L Biomass. The growth exponential phase increased with the gradual increase of EPS with cell weight from 48 to 96h. There is a sudden decline in the growth curve after 96 hours, which shows a decreased production of EPS of 19.3g/L to 7g/L (Fig.2). The optimal sucrose concentration in EPS production from E. *Cloacae* is 3% which gave 2.72 g/L (17). 10% for Lactobacillus strain LB 80 gave 21g/L(2).

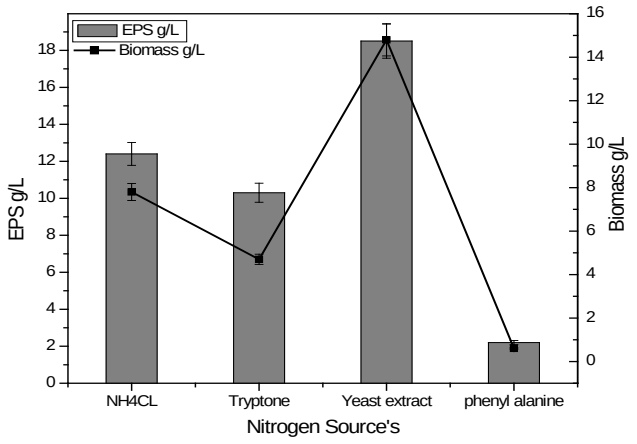

Fig. 3. Effect of nitrogen sources on the production of EPS

To find out the optimal nitrogen source for the production of EPS nitrogen supplements ammonium chloride, tryptone, yeast extract and phenyl alkaline were used at 0.5% concentration. The highest yield of EPS from the strain *Bacillus amyloliquefaciens BPRGS* was observed when yeast extract was supplied as the nitrogen source with a yield of EPS at 18.5 g/L (Fig.3).

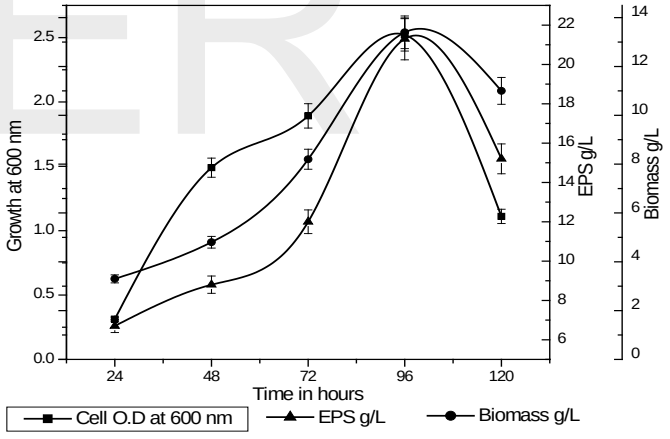

Fig. 4. Effect of Yeast extract on the production of EPS

Upon different concentrations of yeast extract study, 1% concentration shows the increase in EPS yield 21.3 g/L. The exponential growth rate from 72 to 96 hours shows increased EPS and biomass. The EPS yield decreased as yeast extract concentrations increased more than 1%. Cell growth, however, increased as the concentrations of yeast extract increased. 0.05% of yeast extract gave 2.71 g/L (17). Growth and EPS

production by individual strain was also influenced by nitrogen sources (39, 29).

To find out the optimal temperature for the production of EPS, various temperatures ranging from 20°C, 28°C, 37°C and 50°C, the culture flasks were incubated. EPS produced 23.5 g/L and 16.7 g/L of biomass at 37°C (Fig.3) optimal production of EPS the temperature was 37°C for 96h shows increased growth rate, EPS and cell weight. Other reports states EPS yield was 42% higher at 25°C Instead of 37 °C 51.3 mg g^{-1} compared with 21.3 mg g^{-1} (15).

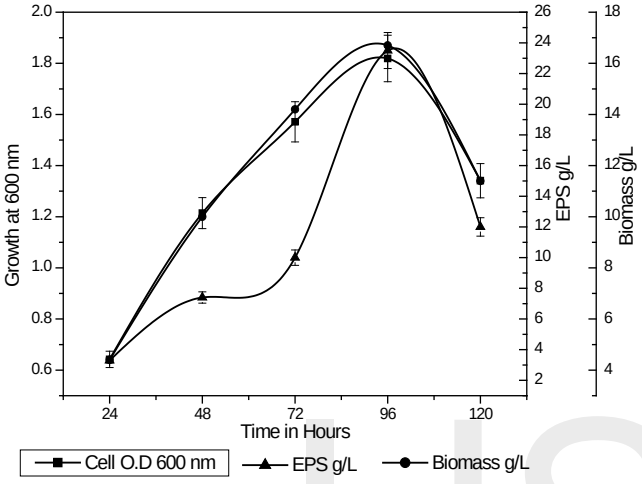

Fig. 5. Effect of Temperature at 37°C on the production of EPS

To find out the optimal initial pH for the production of EPS collected from *Bacillus amyloliquefaciens BPRGS.* with different initial pH 5, 7, 9, 11 conditions, EPS 26.5 g/L was observed at pH 9, 23 g/L at pH 7 and 16.4 g/L at pH 11 (Fig.6).

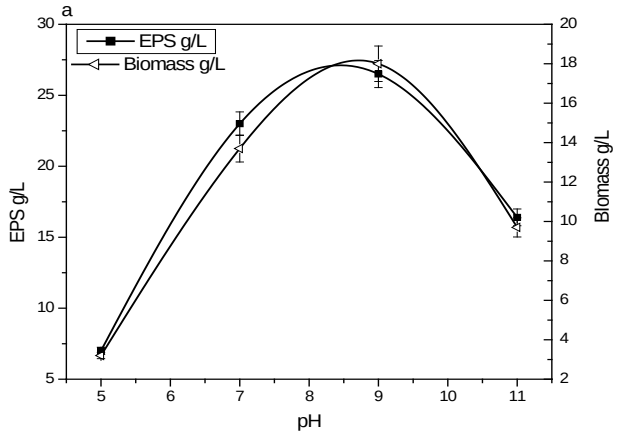

Fig. 6. Effect of pH on the production of EPS

The growth rate increases from 48 to 96 hours with increase in biomass at 18 g/L, and after 96 hours the yield was decreased .

Optimum EPS production (14.96 g/l) was obtained in the neutral pH range (12). The viscous produced in the initial pH 7.5 in other reports was as low as 2 g/L (3). Previous studies with glucose-grown viscosus extracellular polysaccharides have shown that maximum culture growth occurred at pH 6.1 (14).

4.5. Carbohydrate, Uronic acid and Protein content of (µg/mg EPS)

Carbohydrate 242.76 µg/mg EPS, Uronic acid content shows 443.2 µg/mg EPS, and protein 240.2 µg/mg EPS respectively.

4.6. Instrumental analysis

Scanning Electron Microscopy of EPS from *Bacillus amyloliquefaciens BPRGS*

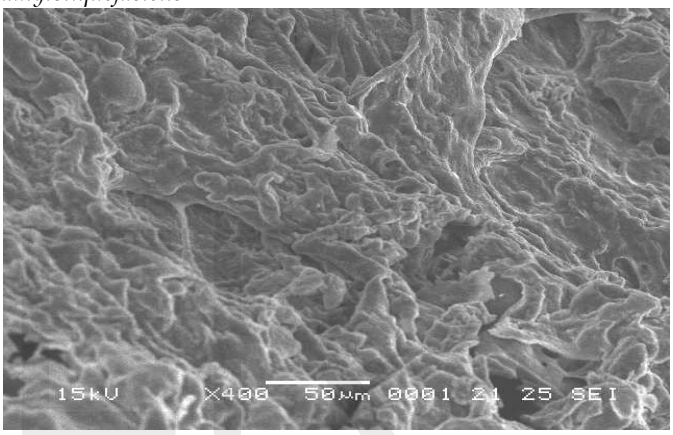

Fig.7. SEM image of EPS

(Fig.7) revealed that the EPS extracted with ice cold ethanol shows porous nature. The small pore distribution and the porous indicated thin mycelia structure confirm the capillary forces to retain water.

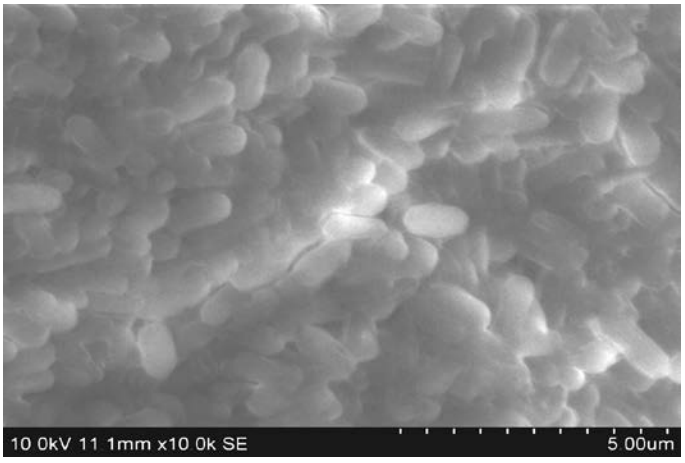

Fig. 8. SEM image of Biomass

(Fig. 8) Shows the cocci spherical structure adhered to each other with mucilaginous gel.

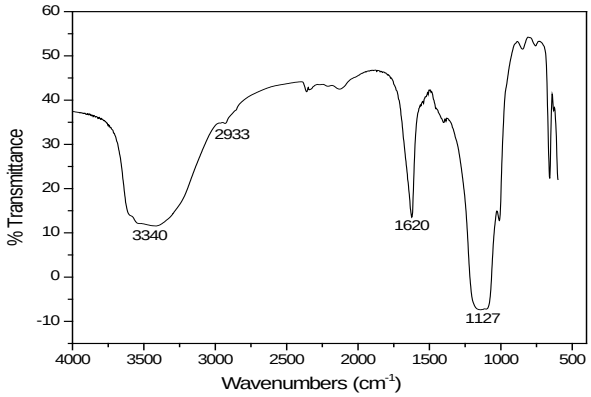

Fig. 9. FTIR of EPS

The IR shows (Fig. 9) a broad intense peak at 3340 cm^{-1} which is characteristic for hydroxyl and amine group, absorption at 2933 cm^{-1} stretch of $–CH_2$ groups, 1147-1380 cm^{-1} suggest the presence of carboxyl group. Protein related bonds at 1650 $cm^{-1.}$ The absorption peaks at 1250 and 1064 cm^{-1} indicate the C–O stretching in ether or alcohol groups. In other articles also corresponds our results that the four bands appeared in 3409.14 cm^{-1}, 2932.47 cm^{-1}, 1725.84 cm^{-1} and 1379.63 cm^{-1} corresponded to the stretching vibrations of hydroxyl, C-H, carboxyl, and C–H bending of aliphatic CH_2 and an infrared band at 1643.05 cm^{-1} (Amide I) and 1554.37 cm^{-1} (9).

Thermo gravimetric analysis measures the weight loss of a material as a function of temperature. The samples were analyzed with a heating rate of 10° C min^{-1} under nitrogen atmosphere.

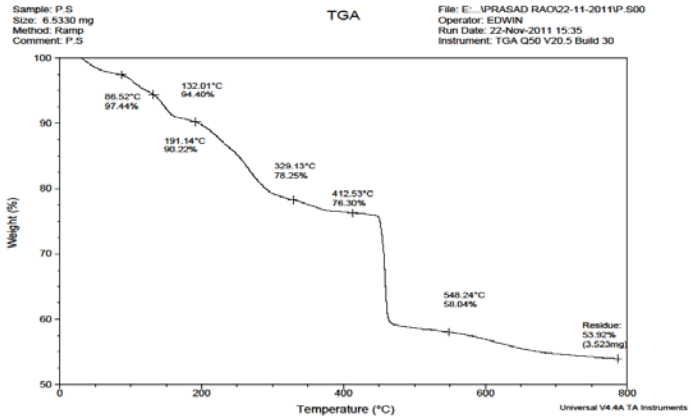

Fig. 10. TGA analysis of EPS

TGA (Fig. 10) Showed that degradation of the first phase from 0-180°C shows 10% weight loss for water, second phase 250°C-550°C shows 20.21% weight loss, depolymerization started after this temperature and continued till 550°C resulting in a major weight loss of about 21%. The next phase of degradation from 550-800°C was very minor weight loss and remains 3.523 mg out of 6.5330 mg. The remaining 50% of the sample in this experiment proves that this EPS has good thermal stability. This result also corresponds fifteen percent of total EPSs weight loss from 30 to 124°C was recorded for phase 1 degradation, thereafter second phase of degradation (54.6%) was observed with maximum loss at ≥ 240°C temperature (32). Phase 1 degradation may be due to water evaporation during the heating process while the second phase of degradation is attributable to thermal decomposition same as other study (34).

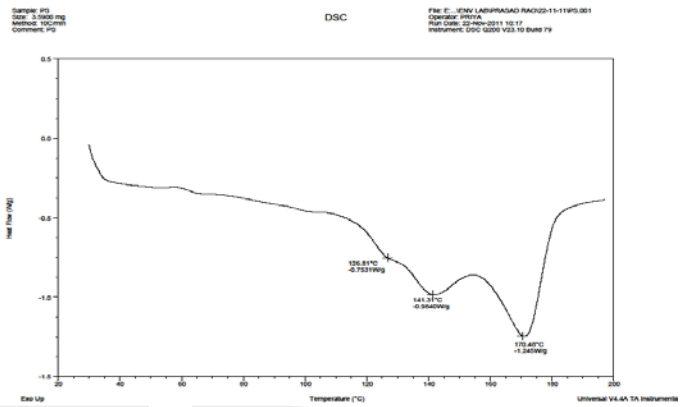

Fig. 11. DSC analysis of EPS

The differential Scanning Calorimetry (DSC) thermal analysis heating rate of 10°C min^{-1} under nitrogen atmosphere DSC (Fig. 11) Shows characteristic endothermic transition of EPS at 170.48°C. Other references the thermal event registered in this sample were a sharp endothermic peak centered between 70 and 72 °C with an onset at 60–65 °C (26).

4.9. Molecular studies

EPS from *Bacillus amyloliquefaciens* BPRGS

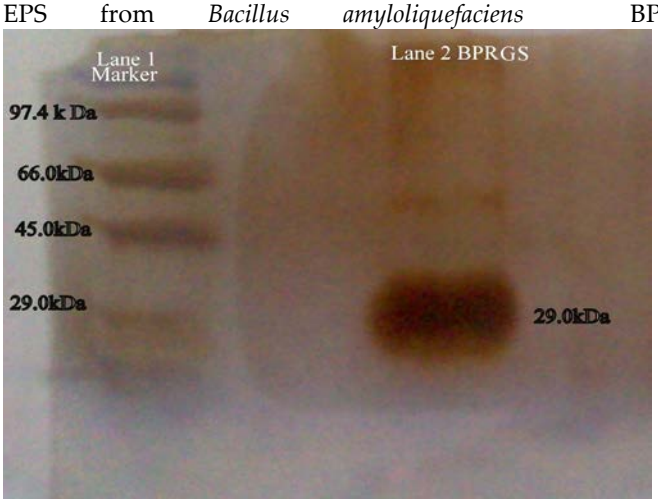

Fig. 12. SDS-PAGE

In the (Fig. 12) shows a clear band of 29 kDa was visible in the lane 2. Previous reports states the medium and low molecular weight fractions were stained by alcian blue/silver stain of cell wall extracts from *Sinorhizobium fredii* (33).

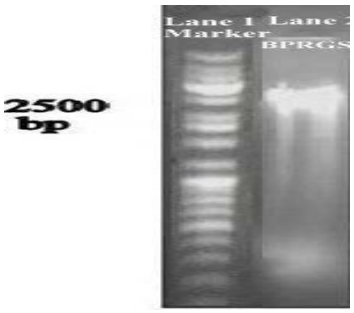

Fig. 13. DNA Gel-Electrophoresis

DNA-Gel Electrophoresis (Fig.13) shows the DNA along with other bacillus sp. The lane 1 confirms the DNA of 2500bp from *Bacillus amyloliquefaciens* BPRGS. The DNA sequencing is performed using the 16s RNA sequencing method. The sequence is submitted to NCBI (GENBANK NO: JN104601.1).

4.12. Bio-flocculating activity

The flocculating activity of the EPS was measured by using a kaolin suspension. In order to neutralize the charge, 1ml of 90 mM $CaCl_2$ was added in 50 ml of 4.0 g/l kaolin suspension. The optical density (OD) of the clarifying solution was measured with a spectrophotometer at 550 nm. After calculation, the flocculating activity of *Bacillus amyloliquefaciens BPRGS* was 75.0%. A slight modification was done by depriving the calcium cations.

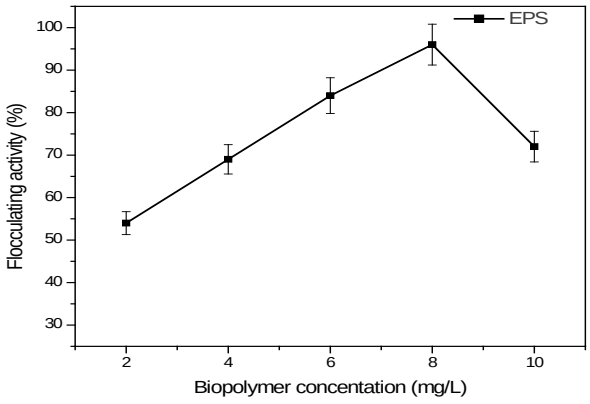

Fig. 14. Effect of biopolymer concentration on flocculation

Results showed even in the absence of calcium cations EPS showed the maximum activity of 70% at 100 mg/L. The effect of different cation study showed the results that $CaCl_2$ cation gives 94% of flocculating activity form cations as in (Fig. 15).

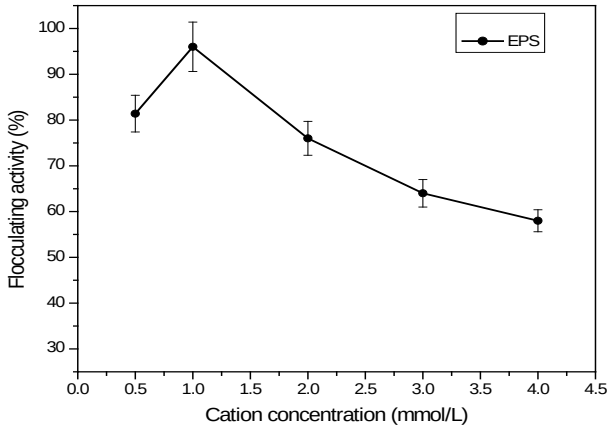

Fig. 15. Effect of different concentration of cation ($CaCl_2$) on flocculating activity

The effect of different concentration of selected $CaCl_2$ cations showed 96% of the flocculating activity of EPS from *Bacillus amyloliquefaciens BPRGS* (Fig. 15), xanthan showed 87% flocculating activity at 1 mmol concentration of $CaCl_2$ cation (Fig. 16).

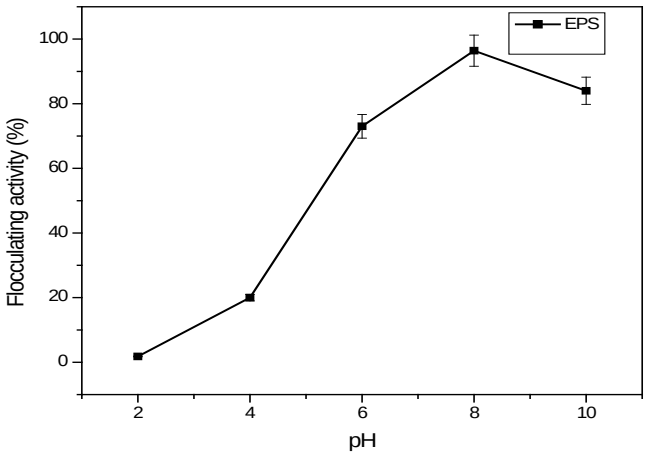

Fig. 16. Effect of different pH on flocculating activity

The effect of pH study showed pH 8 with 96% of flocculating activity. The effect biopolymers such as EPS form *Bacillus amyloliquefaciens BPRGS* and xanthan gum concentration 8 mg/L showed 96% of the flocculant activity in (Fig. 16).

Previous article shows 92.4% flocculating activity at 1mmol/L of different cations (8).

5. Conclusion and future challenges

Bacillus amyloliquefaciens BPRGS was isolated from marine sediments. Our study concentrated on maximum EPS production with effect of duration, carbon and nitrogen source, with their concentration, pH and temperature. *Bacillus amyloliquefaciens BPRGS* produced maximum EPS production at 37°C and alkaline pH 9 at static condition. Incubation period required for maximum EPS production was 96h, 5% sucrose concentration and 1% yeast extract concentration were favorable for maximum production of EPS 26.5 g/L. The characterization results of EPS from *Bacillus amyloliquefaciens BPRGS* proved that the EPS is porous and have high thermal stability and insoluble in non-polar solvents. The produced EPS shows high flocculating activity of 96 % when compared with commercial xanthan. This shows EPS from *Bacillus amyloliquefaciens BPRGS* can be employed in the treatment of waste water.

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