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Isolation and Characterization of Exopolysaccharide Producing Bacteria from Pak Bay (Mandapam)

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ABSTRACT

Bacterial exopolysaccharides possess a wide variety of properties that may not be found in more traditional polymers of plant or algal or animal origin. In the present study, the exopolysaccharide producing bacteria was isolated and characterized from coastal (Mandapam) area of Pak bay, Tamilnadu, India. Soil samples were collected and the exopolysaccharide producing strains were screened and characterized by 16s rDNA sequencing method. The effect of different carbon sources on exopolysaccharide production was examined. Then the exopolysaccharide was synthesized and characterized by calorimetric, IR and HPLC method. Calorimetric analysis of exopolysaccharide reveals the composition of exopolysaccharide and in the IR analysis the band at 1385 to 1380 cm⁻¹ indicates the presence of C-H stretching. An absorbance at 1730 cm⁻¹ indicating the presence of carboxyl group. In addition, a small absorption at 1550 cm⁻¹ indicating the presence of either amino sugars or proteins were present in exopolysaccharide. *Exiguobacterium* sp. was isolated and it produces considerable amount of exopolysaccharide when the medium was supplied with sucrose.

Key words: Exopolysaccharide, 16S rDNA Sequence, *Exiguobacterium* sp., bacterial exopolysaccharides, IR spectrum, HPLC analysis

INTRODUCTION

Bacterial exopolysaccharides are ubiquitous in marine resources and are distributed in the form of free living or associated forms, such as biofilm, microbial mats etc. Microbial exopolymeric substances are produced by both prokaryotes and eukaryotes. The sea has a plentiful source of biological and chemical diversity. The ocean appropriately contains nearly 3 million described species, but nearly about 99% of bacteria cannot be cultured (Lee *et al.*, 2007) and few mariner microbes are currently cultivated.

The fluctuation in the pressure, nutrients, salinity and pH in the marine leads to the production of exopolysaccharide by the bacteria (Decho, 1990). Exopolysaccharide have a wide range of applications in ecological, physiological and industrial fields. Exopolysaccharide protects the bacterial cell from harsh environment such as desiccation (Passow, 2000), involve in the biofilm formation (Rodriguez-Valera *et al.*, 1981) and bioremediation activity (Allison, 1998). Therefore, the present study was conducted to isolate and characterize the exopolysaccharide produced by marine bacteria.

MATERIALS AND METHODS

Isolation of bacteria: Soil samples were collected from (Mandapam area) Pak bay. The soil samples were serially diluted and a known aliquot was plated on to Zobell agar media incubated at 30°C for 7 days. Some strains exhibited mucoid surface on the growth media. It indicates the production of exopolysaccharide by bacteria (Ng and Hu, 1989). The strains were routinely subcultured and maintained in Zobell agar slants as stock culture for further analysis.

Characterization of bacterial isolate: The 16S rDNA was amplified by polymerase chain reaction (PCR) using the primers F-5' AGA GTT TGA TCC TGG CTC AG 3' and R-5' GGT TAC CTT GTT ACG ACT T 3'. The cycle sequencing reaction was performed using BigDye terminator V3.1 cycle sequencing Kit containing AmpliTac DNA polymerase (from Applied Biosystems, P/N: 4337457). The sequencing reaction - mix was prepared by adding 1 µL of BigDye v3.1, 2 µL of 5x sequencing buffer and 1 µL of 50% DMSO. To 4 µL of Sequencing reaction-mix was added 4 Pico moles of primer (2 µL) and sufficient amount of plasmid. The constituted reaction was denatured at 95°C for 5 min. Cycling began with denaturing at 95°C for 30 sec, annealing at 52°C for 30 sec and extension for 4 min at 60°C and cycle repeated for a total 30 cycles in a MWG thermocycler. The reaction was then purified on sephadex plate (Edge Biosystems) by centrifugation to remove unbound labelled and unlabelled nucleotides and salts. The purified reaction was loaded on to the 96 capillary tubes on ABI 3700 DNA analyzer and electrophoresis was carried out for 4 h. DNA sequence was obtained using DNA sequencer (ABI 310). The PCR product was sequenced using the same products were primers and other internal primers to confirm the sequence. Blast program (www.ncbi.nlm.nih.gov/blast) was used to assess the DNA similarities (Al-Nahas *et al.*, 2011).

PHYLIP version (3.57) was used to assess the sequence data. Phylogenetic tree was constructed by the neighbor joining method.

Isolation and purification of exopolysaccharide: For the isolation exopolysaccharide producing strain was grown for 7 days at 32°C in a Zobell marine broth (Raguenes *et al.*, 2003). The culture was centrifuged at 10000 rpm for 15 minutes and the supernatant was precipitated with 3 volumes of ice cold ethanol and stored at 4°C overnight, before being centrifuged. The exopolysaccharide was resuspended in distilled water and centrifuged. To remove excess salt from exopolysaccharide the pellet was dissolved in distilled water and dialyzed (mol wt cutoff 8000 dalton) against distilled water for 2 days. It was concentrated and stored at room temperature until analysis.

Characterization of exopolysaccharide: The Total Protein content in the sample was determined (Lowry *et al.*, 1951) with bovine serum albumin as a standard. The neutral carbohydrate content was determined by the orcinol sulphuric acid (Titus *et al.*, 1995) and meta-hydroxydiphenyl method (Filisetti-Cozzi and Carpita, 1991) was used to detect the uronic acid level in exopolysaccharide.

Effect of carbon source on growth and production of exopolysaccharide: In order to determine the effect of different carbon sources (sucrose, glucose, galactose and lactose) on the growth and exopolysaccharide production, the exopolysaccharide producing strain was cultivated in Zobell marine broth for 24 h. From the 24 h culture 5 mL of culture aliquots was inoculated in 100 mL of basal medium (peptone 5 g, yeast extract 3 g, malt extract 3 g, distilled water 500 mL and sea water 500 mL pH 7.0) supplemented with different carbon sources (sucrose, glucose.

galactose, lactose) in different flasks. Then the growth was determined by measuring the O.D value at 520 nm at regular time intervals and the exopolysaccharide was extracted as mentioned above and the dry weight was measured (Lijour *et al.*, 1994).

FT-IR spectroscopy: Pellets for infra red analysis were obtained by grinding a mixture of 2 g of exopolysaccharide with 200 g of potassium bromide. FT-IR spectra was recorded with a resolution of 4 cm⁻¹ in the 4000-400 cm⁻¹ region (Omoike and Chorover, 2004).

HPLC Analysis of Basal exopolysaccharide: The exopolysaccharide (0.1 g) was hydrolyzed by treating with 1.25 mL of 72% sulphuric acid and was incubated for 60 min at 30°C. Then 13.5 mL of distilled water was added and placed it in a water bath for 4 h. After 4 h the mixture was cooled and 3.1 mL of 32% sodium hydroxide was added. Then the hydrolyzed sample was dissolved in methanol. The acid hydrolyzed exopolysaccharide sample was analyzed with a High Performance Liquid Chromatography (HPLC) system (SHIMADZU LC 10 AT VP) equipped with Aqueous GPC start up Kit column and eluted with distilled water at a flow rate of 1.0 mL min⁻¹ at 20°C. (Vijayabaskar *et al.*, 2011).

RESULTS

Exopolysaccharide producing bacterial strain (S9) was isolated from marine soil sample. After 7 days of incubation at 32°C on zobell agar medium a circular convex colony with a mucoid tenure and orange colored colony was observed.

The most promising strain S9 was characterized as G⁺ve, rod shaped motile bacteria. The 16S rDNA sequence analysis showed high percentage of similarity to the genus *Exiguobacterium* sp. (Genbank Accession Number JF830805). Figure 1 shows the phylogenetic relationship of *Exiguobacterium* sp. based on 16S rDNA sequences. Phylogenetic analyses of the strain S9 showed it was belonged to the Phylum Firmicutes and Bacillales Family XII and it was closely related to the genus *Exiguobacterium*.

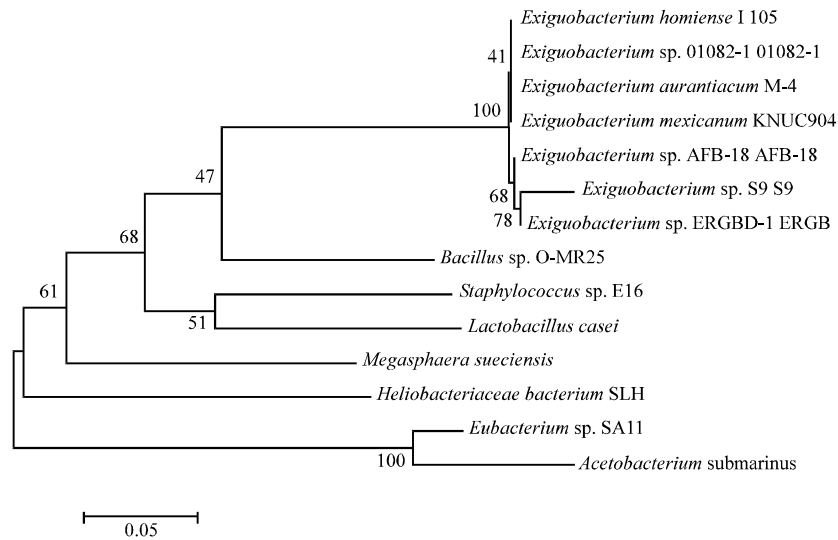


Fig. 1: Phylogenetic analysis of *Exiguobacterium* sp., s9. Phylogenetic relationship among *Exiguobacterium* sp., S9 and selected marine bacteria. The percentage number at the nodes indicates the level of bootstrap support for the branch point in topology

The chemical composition of bacterial exopolysaccharide is presented in Fig. 2. The protein content is very low level (4%) and the amount of neutral sugar is about 78% and contains uronic acids. The bacterial exopolysaccharide contains protein and small amount of uronic acids which cannot be removed by purification process.

When compared with other sugars the highest yield of exopolysaccharide and tremendous growth was observed, when sucrose was supplied as a whole source of carbon (Fig. 3, 4). The exopolysaccharide production was significantly influenced by the type of sugars as a carbon source. The amount of exopolysaccharide production was not only determined by the Carbon: Nitrogen ratio and concentration of sugars. It also influenced by the type of sugars used as carbon source.

FT-IR spectra of the *Exiguobacterium sp.* (Fig. 5) displayed a broad O-H stretching band above 3000 cm^{-1} and intense absorptions between 1650 and 1050 cm^{-1} characteristic of polysaccharides. The band at 835 to 805 cm^{-1} indicates the presence of substitution. The band at 1385 to 1380 cm^{-1} indicates the presence of C-H vibration. An absorbance at 1730 cm^{-1} indicated the presence of carboxyl groups (Lijour *et al.*, 1994). In addition, a small absorption at 1550 cm^{-1} indicating the presence of either amino sugars or proteins were present in exopolysaccharide.

The exopolysaccharide after being hydrolyzed and dissolved with methanol was analyzed for its sugar composition by HPLC. By comparing the retention time found on with the

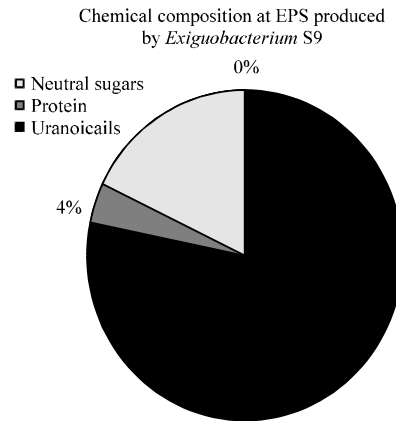


Fig. 2: Chemical composition of bacterial exopolysaccharide. Chemical composition of bacterial exopolysaccharide, contains 78% sugars 18% of proteins and 4% of uranic acids

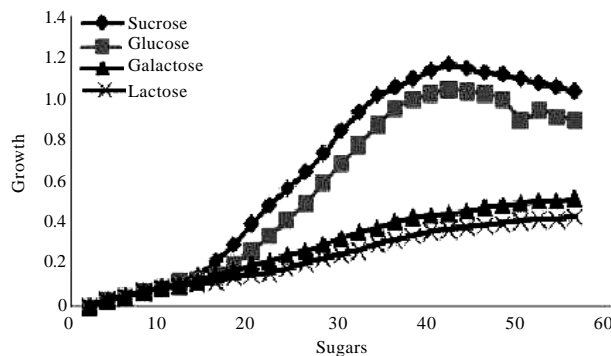


Fig. 3: Effect of different sugars on the growth of *Exiguobacterium sp.*, S9. The sugar sucrose influences the better growth and yield of exopolysaccharide

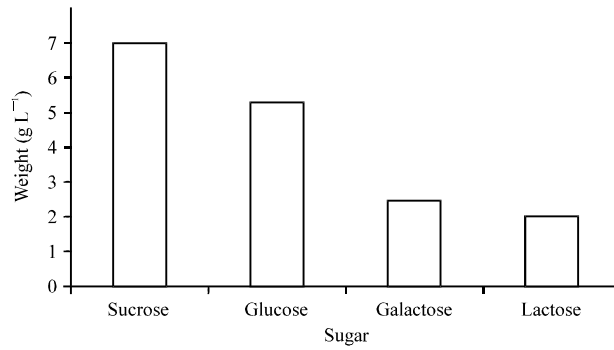


Fig. 4: Dry weight of exopolysaccharide on using different carbon source. The isolate S9 produced maximum amount of exopolysaccharide when the media is supplied with sucrose on compared with other sugars

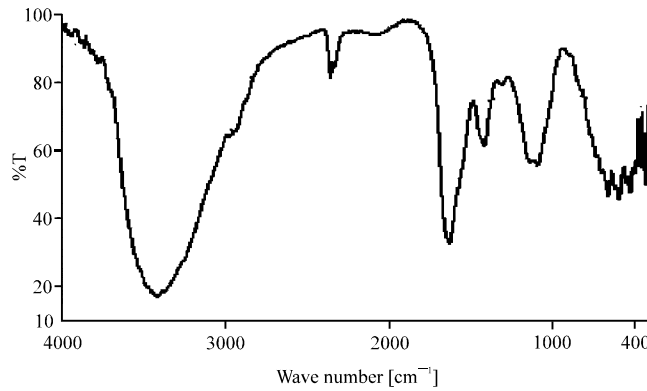


Fig. 5: FT-IR spectrum of exopolysaccharide. In the IR spectrum of exopolysaccharide the band at above 3000 cm⁻¹ and intense absorption between 1650 and 1050 cm⁻¹ are characteristic of polysaccharides

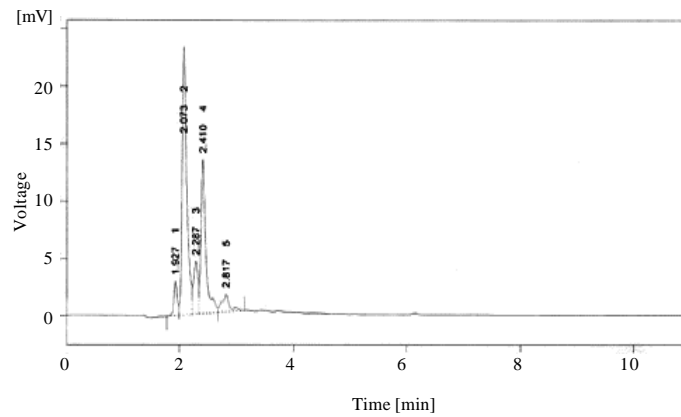


Fig. 6: HPLC results bacterial exopolysaccharide. The HPLC spectrum for exopolysaccharide shows the retention time obtained was found to be 1.927 as myo-inositol, 2.073 as glucose, 2.410 as galactose and 2.817 as fructose

standard retention time of carbohydrates, the distinct peaks obtained were found to be 1.927 as myo-inositol, 2.073 as glucose, 2.410 as galactose and 2.817 as fructose (Fig. 6).

DISCUSSION

According to Cambon-Bonavita *et al.* (2002) and Junkins and Doyle (1992) the exopolysaccharide producing strains are generally developed mucoid colonies. During the screening process the presence of pigmentation in all strains was noticed. It provides resistant to the bacteria (Rimington, 1931; Brown and Lester, 1982). Bacterial strains isolated from marine soil produce extra cellular polymer with an enhanced mucoid morphology. Results from 16s rDNA sequencing indicates the strain was closely related and belongs to the genus *Exiguobacterium* sp.

The concentration and the type of carbon source determined the exopolysaccharide production by the bacteria. The production may be influenced by the metabolic process of the bacteria to utilize different carbon sources.

Exopolysaccharide produced by *Exiguobacterium* sp. was analyzed calorimetrically and FT-IR spectroscopy. Based on the IR spectrum of absorbance have been assigned to different functional groups such as ether, sulphate, carboxylic etc. It confirms the polysaccharides and low amount of ester sulphate group. Similar results were observed previous studies of exopolysaccharide from *Alteromonas* and *Pseudoalteromonas* by Passow (2000), Brown and Lester (1982) and Pal *et al.* (1999). The exopolysaccharide produced at different carbon sources are primarily composed of carbohydrates. Some other organic compounds such as uronic acids, sulfates, proteins may be found in bacterial exopolysaccharide (Quesada *et al.*, 2004; Sutherland, 2001). Production of exopolysaccharide by a bacterial cell plays an important role in the aggregation (Bejar *et al.*, 1996; Chan *et al.*, 1984). When released into the water, a combination of biological, chemical and physical forces causes this colloidal material to form aggregates (Alldredge and Jackson.,1995; Passow, 2000; Kiorboe, 2001) and protect the cells from environment stress such as osmotic pressure, pH variation etc. The stickiness is an important in terms of the affinity of these exopolysaccharide for binding to other cations such as dissolved metals (Biddanda, 1986). Increased knowledge of the role of bacterial exopolysaccharide will also provide insight into possible commercial uses for these novel polymers.

The FTIR spectrum of exopolysaccharide revealed characteristic functional groups, such as stretching C-H at 3313.48, 2975.96 and 2935.46 cm^{-1} and a weak COOH stretching peak at 3195.48 cm^{-1} . Further, stretching peak was noticed at 1284.50 cm^{-1} which corresponds to amide. A broad stretching of C-O-C, C-O at 1000-1200 cm^{-1} corresponds to the presence of carbohydrates specifically, the peaks at 1114.78 cm^{-1} range ascertain the presence of uronic acid, o-acetyl ester linkage bonds (Fig. 6). A comparison of functional groups presents that exopolysaccharide having a higher number of variable functional groups was more complex than the other exopolysaccharide reported previously (Rougeaux *et al.*, 1996).

CONCLUSION

The exopolysaccharide are widely distributed in marine an environment. It helps the bacterial communities to survive in extreme environment and act as an anchor between the bacterial cell and its immediate environment. Several exopolysaccharide produced by bacteria in extreme marine habitats have a potential role.

The study is a first step towards understanding the sole of these exopolysaccharide in the area of Pak bay. Complete study on the chemistry and the structure of bacterial exopolysaccharide will provide the positive approach to employ this novel biopolymer in several industries like pharmaceutical, food-processing fields and environment protection.

Considering that the most of the marine bacteria and their metabolites were unexplored, it is reasonable to state that the isolation and identification of new microorganisms will provide wide opportunities in forth coming years.

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