

Characterization of Exopolysaccharides Produced by Marine *Bacillus Megaterium*

P. Ramya, D, Sangeetha

Abstract: Exopolysaccharides (EPSs) are polymers with large molecular weight that consist of various residues of sugar. These are desired because the substitution of synthetic polymers is degradable and non-toxic. Most microorganisms have the ability to synthesize and excrete exo polysaccharides with new chemical compositions, characteristics and structures in order to have important applications in various areas. The current study based on marine bacterial isolates screening the production of exo polysaccharide. Among the four exopolysaccharide producing isolates, the isolate PMSS12 had the highest production of exo polysaccharides. The efficient marine bacterial isolate PMSS12 was further identified by sequence of 16S rRNA. The PMSS12 isolate was confirmed as *Bacillus megaterium*. Then the exopolysaccharide produced by *Bacillus megaterium* was characterized by using FTIR, NMR and SEM.

Keywords: Exopolysaccharides, 16S rRNA, *Bacillus megaterium*

I. INTRODUCTION

Biologically active natural molecules trigger a great deal of interest in finding new pharmaceutical medications [1,2]. The marine environment protects a wide range of natural products and can fulfill industrial and clinical applications [3,4]. The EPS is ubiquitous in the marine ecosystem owing to its survivability and competition of marine bacteria in minimal nutrients and unfavorable environments. [5,6].

Microorganisms have an original chemical composition of glycol polymers and exciting biological processes in addition to various aquatic sources (seaweeds, animals, invertebrates). [7,8,9,10]. Marine biotechnology has still not attained an economically important area but it is a successful sector for the maintenance of useful macro molecules [11,12,13]. EPS from extremophilic microorganisms especially halophiles are comparatively less reported [14,15,16,17,18]. Production of EPS from halophilic bacteria within extreme marine habitat along with its biological activities was reviewed [19,20,21,22,23].

In certain scenarios, the nutritional conditions can also influence the molecular weight and the EPS osidic

composition. It has been shown that a novel *Alteromonas macleodii* strain produces 23.4 g/ L exo polysaccharide when grown on 15% of lactose, the highest yield for marine EPS [24,25,26,27,28,29] .

II. MATERIALS AND METHODS

A. Collection of samples

Marine sediment sample was collected from coastal area of Pichavaram, Tamilnadu, India.

B. Isolation of marine bacterial cultures

The collected sediment sample was diluted up to 10^{-7} by serial dilution technique. The 10^{-6} was inoculated by spread plate technique on Zobell Marine Agar plates. The plates were marked properly and kept in the incubator at 30°C for 48 hours. Morphologically different colonies with mucoid surface were picked and sub cultured for future use.

C. Screening of polysaccharide production

All the isolated mucoid colonies of marine bacteria were subjected to screening for EPS production. The screening was achieved by improving the Sayyed et al protocol. Zobell medium of Marine Ager was prepared by dissolving 0.75gm of yeast extract, 3gm of malt extract, 1.25gm of peptone, 0.25gm of monosodium glutamate and 7.5gm of sucrose. The water pH was set to 7.0 as well as 125mL of marine water and 125mL of distilled water and placed into petriplates after sterilization. On the solidified medium are streaked the bacterial strains to be tested for the production of EPS. At room temperature, the plates were incubated for 3 days, the production of EPS was indicated by the oozing of gum substances on the periphery of the bacterial colonies.

D. Exopolysaccharide Production and Recovery

For exo polysaccharide production the screened isolates were further evaluated. The inoculum was prepared by passing bacterial colony to 250 ml conical flask containing 50 ml Zobell Marine Broth and 5% NaCl. Inoculated flasks are incubated at a temperature of 37° C at 100 rpm for 96 hours. The Zobell marine broth was cell-free at 10,000 rpm for 20 minutes by centrifugation. Cold absolute ethanol was added to the supernatant in the ratio of 1:3 (v / v) and kept at 4 oC for 24 hours (Kim and Yim, 2007) for precipitation of exo polysaccharide.

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P. Ramya, Department of Microbiology, Faculty of Science, Annamalai University, Annamalainagar- 608002, Tamilnadu, India.
D Sangeetha*, Department of Microbiology, Faculty of Science, Annamalai University, Annamalainagar- 608002, Tamilnadu, India.
*Corresponding author E-Mail: sangeethadau@gmail.com

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The precipitates were recovered by centrifugation and purified by washing with Milli Q water. Finally, the exopolysaccharide was again precipitated by 1:3 volume of cold absolute alcohol and exopolysaccharide pellet were dried at 60° C.

E. Molecular Identification Of Efficient Exopolysaccharide Producing Isolate

Extraction of bacterial genomic DNA

Bacteria are grown at 37° C for 24 hours in Zobell marine broth. Activated cultures were collected in 2 ml microfuge tube and centrifuged at 10,000 rpm at 4° C for 10 minutes. Further DNA extraction, precipitation and purification were carried out as per manufacture protocol of HiPer Bacterial Genomic DNA Extraction Kit (Hi-media Laboratory Pvt. Ltd. Mumbai). DNA from isolate was electrophoresed in 0.8 % Agarose gel and visualized on a Gel Documentation System (Genie, Bangalore).

F. 16S rRNA SEQUENCING FOR IDENTIFICATION OF ISOLATE:

The identification of isolate was made by evaluating similarity index, total score, query coverage and E-value. The sequence of 16S rRNA achieved was submitted to the National Center for Biotechnology Information through the NCBI Sequence Submission Wizard. (<https://submit.ncbi.nlm.nih.gov/subs/genbank>).

Phylogenetic tree using neighbor joining algorithm was constructed to determine taxonomic position of the isolate.

G. EXOPOLYSACCHARIDES PRODUCED BY BACILLUS MEGATERIUM CHARACTERIZATION

Fourier transform-infrared (FTIR) analysis

The Exo polysaccharides functional groups analysis was determined using FTIR spectrophotometer. A sample of EPS was mixed with KBr in the ratio of 1:100 to form a KBr pellet with a dimension of 1 mm in thickness. The absorbance spectrum of EPS was determined in the wavelength range of 4000-5000cm⁻¹. The FTIR analysis was performed using Bruker FTIR instrument at IIT (Indian Institute of Technology, Chennai).

H. 1H AND 13C NMR ANALYSIS

EPS structural elucidation was calculated using ¹H and ¹³C NMR spectrum analysis. The NMR spectra measurements were performed using JEOL JNMECA 500 NMR spectrophotometer at IIT (Indian Institute of Technology, Chennai). The sample was dissolved in D2O. The sample spectrum was recorded at 500 MHz in the ¹H NMR and 125.7 MHz in the ¹³C NMR. The delay time was 5 s for ¹H NMR and 2 s for ¹³C NMR.

I. SCANNING ELECTRON MICROSCOPIC ANALYSIS

Scanning electron microscopic analysis (SEM) determined the morphology of dried EPSs. EPS were coated on gold

particles during the scanning of the electron micrograph at 3000X and micrograph was visualized. EPSs were taken in full and the view and structure of the surface were studied.

III. RESULTS AND DISCUSSION

A. Exopolysaccharide production

A total of 14 morphologically different marine bacterial cultures were isolated and screening was done for the efficiency to produce extracellular polysaccharides. Out of 14, only four bacterial isolates were produced EPS. Further the four bacterial isolates were analyzed to check the polysaccharide content. The marine isolate PMSS12 exhibited maximum yield of exopolysaccharides (4.3 g/l). Selim *et al.*, 2018 reported that 18 isolates were isolated from the Ageeba beach sediment (MarsaMatrooh Governorate) and 9 isolates only produced EPS. Due to its maximum EPS production (8.25 g L⁻¹), strain number five was chosen for further analysis.

Table.1 Exopolysaccharide production of the marine bacterial isolates

S.No	Marine Isolates	Exopolysaccharide Yield (g/l)
1.	PMSS4	2.3
2.	PMSS5	2.5
3.	PMSS7	2.1
4.	PMSS12	4.3

B. Phylogenetic analysis of Efficient Marine Isolate (PMSS12)

The strain producing highest amount of EPS was characterized by phylogenetic analysis. Phylogenetic analysis revealed that the marine bacterium PMSS12 was *Bacillus Megaterium*.

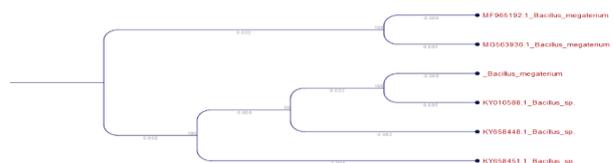


Fig.2 Phylogenetic tree of the Marine Isolate PMSS12

Description	NCBI Blast contig					
	Max score	Total score	Query cover	E value	Ident	Accession
Bacillus flexus strain AHBR10 16S ribosomal RNA gene, partial sequence	1035	1035	100%	0.0	100%	KF241523.1
Bacillus flexus strain G55 16S ribosomal RNA gene, partial sequence	1035	1035	100%	0.0	100%	KC608847.1
Bacillus sp. GUS gene for 16S ribosomal RNA, partial sequence, isolate: GUS	1035	1035	100%	0.0	100%	AB175615.1
Bacillus sp. sgn11 16S ribosomal RNA gene, partial sequence	1035	1035	100%	0.0	100%	JX456249.1
Bacillus aryabhattai strain OW18 16S ribosomal RNA gene, partial sequence	1035	1035	100%	0.0	100%	JG265668.1
Bacillus megaterium strain p59_E11 16S ribosomal RNA gene, partial sequence	1035	1035	100%	0.0	100%	JQ833967.1
Bacillus megaterium strain p59_ED8 16S ribosomal RNA gene, partial sequence	1035	1035	100%	0.0	100%	JQ833964.1
Bacillus megaterium strain p59_E03 16S ribosomal RNA gene, partial sequence	1035	1035	100%	0.0	100%	JQ833959.1
Bacillus megaterium strain p58_H03 16S ribosomal RNA gene, partial sequence	1035	1035	100%	0.0	100%	JQ833962.1
Bacillus megaterium strain p55_ED4 16S ribosomal RNA gene, partial sequence	1035	1035	100%	0.0	100%	JQ833968.1
Bacillus megaterium strain p58_C01 16S ribosomal RNA gene, partial sequence	1035	1035	100%	0.0	100%	JQ835843.1
Bacillus megaterium strain p29_H06 16S ribosomal RNA gene, partial sequence	1035	1035	100%	0.0	100%	JQ835831.1

Fig.316S rRNA Sequence of Marine Isolate PMSS12 submitted to NCBI.

C.CHARACTERIZATION OF EXOPOLYSACCHARIDES

Fourier transform-infrared (FTIR) analysis of Exopolysaccharide

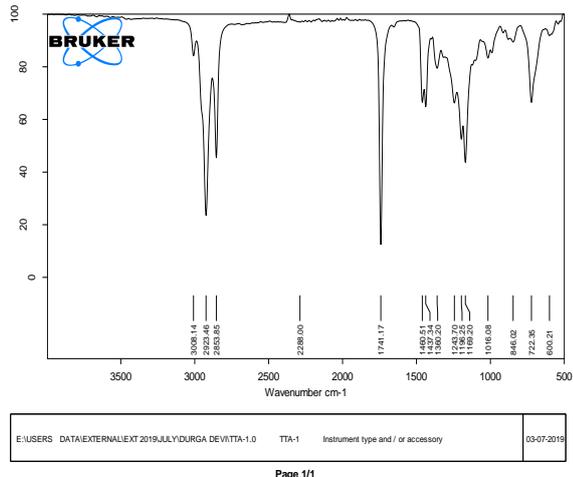


Fig. 4 FTIR Spectrum of Exopolysaccharides produced by *Bacillus megaterium* (PMSS12)

NMR analysis of Exopolysaccharides

EPS sample ¹H NMR spectrum revealed signals between the 3,659 to 4,932 ppm ranges suggesting protons of anomeric carbons indicating the sample as heptasaccharide. The 3,361 ppm signal is consistent with the existence of protonated carbon adjacent to the electronegative group. The signal was applicable to protonated carbon adjacent to less electronegative groups at 2,506 ppm (Siddharth *et al.*, 2017).

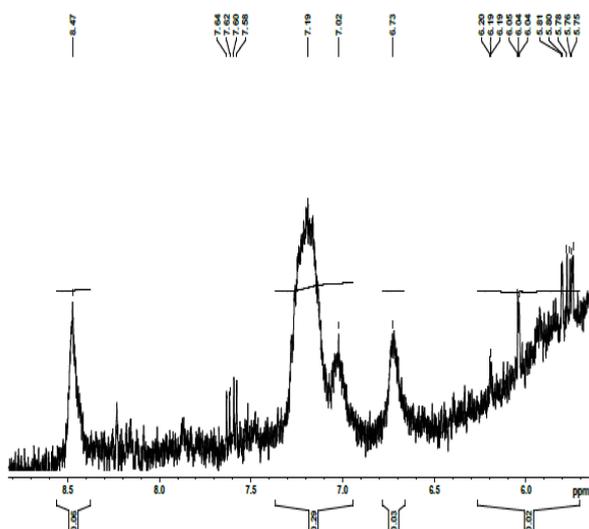


Fig. 5 ¹H NMR spectrum of Exopolysaccharide in D₂O at 90°C

Scanning Electron Microscopic analysis of EPSs

The Micro structure of Exopolysaccharide shows numerous pores and few small inter granular or intra granular pores. Particles are Agglutinating and present in the spherical shape in the sample.

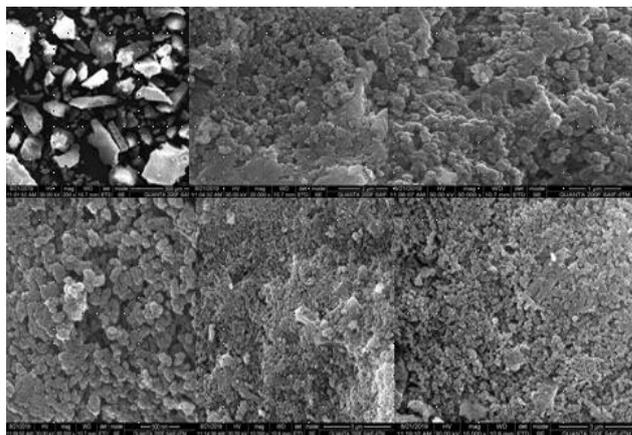


Fig.6 Scanning Electron Micrograph showing the surface of *Bacillus megaterium* PMSS12 exopolysaccharide at 3000X

IV. CONCLUSION

It can be concluded that isolation of marine bacterial samples can provides variety of microbial strains for sources of new marine bio molecules. This research found that certain marine bacterial strains could be modified to produce exo polysaccharides of high molecular weight. that can be used as bioactive molecules.

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