

Original Research Article

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Isolation and Characterization of Exopolysaccharide producing *Bacillus cereus* from Brown Seaweed- *Sargassum wightii*

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ABSTRACT

The bacterial extracellular polymeric substances (EPS) have huge applications in biotechnological and industrial sector. Biochemical composition determines its role in biofilm formation and pathogenicity as well as beneficial applications in various industries as thickeners, stabilizers, gelling agents etc. In the present study, aimed at screening the EPS producing bacteria from three species of brown seaweed and the EPS producing bacteria was isolated from *Sargassium wightii*. The EPS extraction was optimized and maximum production was recorded in BHI broth (1.65 mg ml^{-1}) and media supplemented with glucose (1.56 mg ml^{-1}). The extracted EPS contained 69.9% carbohydrate. Structural analysis by FTIR revealed the presence of carbohydrate (peak at 3292.93 cm^{-1} , & $1200-1000 \text{ cm}^{-1}$) and S-S stretch (peak at 600 cm^{-1} & 492 cm^{-1}). The isolate was identified as *Bacillus cereus* by 16S rRNA sequencing and species specific PCR targeting *bla* gene. Complete study on biochemical composition and structure of EPS will facilitate the employment of this novel biopolymer in food and pharmaceutical industry.

Keywords

Exopolysaccharides,
brown seaweeds,
Bacillus cereus,
FTIR

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Introduction

Biopolymers are the polymers obtained from various biological organisms containing covalently bonded monomers and are classified into polysaccharides, polypeptides and polynucleotides (Ates, 2013). The main source of biopolymers from marine environment includes macro algae, micro

algae, bacteria, and fungi. Among the microorganisms, bacteria are widely accepted as the source of exopolysaccharide with different functional properties and can be exploited for novel industrial and biotechnological applications. Exopolysaccharides (EPS) are high molecular weight polymers secreted by bacteria, consisting of different functional groups such

as acetyl, succinyl or pyruvyl, sulfate etc (Tang *et al.*, 2012). Traditional polysaccharide sources include both plant and algae which includes functional starch, galactomannan, pectin, carrageenan, pectin, and alginate (Vroman, and Tighzert, 2009). Microbial sources include xanthan gum, gellan, alginate, glucans, cellulose, hyaluronan, succinoglycan and levan (Zhao *et al.*, 2017). Biodegradation ability of EPS from bacterial origin, can replace the traditional polysaccharides sources to a larger extent. However, the high cost of production and low yield from bacterial sources may limit the use in industrial scale.

Microbial cells generally contain extracellular biopolymeric structures which aids in rigidity as well as functional attributes to overcome adverse conditions. In general microbial cells produces two types of EPS, namely capsule EPS which produces during the log phase of bacterial growth and slime EPS which are produced mainly during the stationary phase (Plante and Shriver, 1998). The polysaccharide fraction of bacterial EPS mainly comprises of either homo polysaccharides or heteropolysaccharides (Kumar *et al.*, 2007). The studies have been shown that the extracted extracellular materials (polysaccharides, lipids, glycoproteins and lipopolysaccharides) have wide applications in textile and food industries as stabilizers, gelling agents, adhesives, thickening agents, emulsifying agents, flocculants and flushing agents (Becker *et al.*, 1998).

In the recent years, researchers are focusing on the bacterial communities and its interaction with seaweeds. Among all the microbes associated with seaweeds, bacteria can be seen either on seaweed surface or in cell cytosol and even determine the life cycle of eukaryotic organisms (Delbridge *et al.*, 2004). Nicholas *et al.*, (2005) reported better seaweed survival because of nutrient enhancement by

bacterial EPS. In marine environment, EPS helps to increase the elemental uptake and dissolution of organic compounds which inturn make them available for microbial growth and other surrounding communities (Logan and Hunt, 1987). The bacterial associates with *Laminaria japonica* was reported to have plantlet growth promoting effect (Dimitrieva *et al.*, 2006).

The exploitation of biopolymers with novel functionalities from marine environments has been considerably increased. In this study, an attempt was made to isolate and extract EPS produced from seaweed associated bacteria. Further, the chemical and structural properties of EPS produced from seaweed associated bacterium in pure cultures lead to unravel the constituents which will help to increase our understanding of the seaweed-bacterial association in the marine environment.

Materials and Methods

Isolation and purification of bacteria producing exopolysaccharide (EPS)

Three species of seaweed viz *Sargassum wightii*, *Turbinaria conoides*, *Padina gymnocephalus* were collected from Gulf of Mannar, Mandapam coast, Tamil Nadu (India) and screened for exopolysaccharide producing bacteria. A total of 25g was dried seaweed aseptically suspended in phosphate buffered saline (1X) and serial dilutions were made. From each dilution, one ml was then inoculated into tubes of 9 ml EPS culture medium containing 0.2 g KH_2PO_4 ; 1.5 g K_2HPO_4 ; 0.2 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$; 0.1 g $\text{CaSO}_4 \cdot 2\text{H}_2\text{O}$; 2.0 mg FeCl_3 ; 0.5 g yeast extract and 20 g sucrose (per liter). Then the samples were spread plated in duplicate onto trypticase soy agar (BD, Mumbai, India) and incubated at 37°C for 48 hrs. Bacteria that produce EPS characterized by colonies of bacteria that form thick slime (mucoid) was

subsequently selected (Tallgren *et al.*, 1999) and purified by streaking the four quadrants to obtain single colonies.

Extraction of exopolysaccharide from bacteria

EPS was extracted following the method of Berekaa and Ezzeldin (2018). The bacterial isolates were inoculated to the EPS culture medium and incubated at a temperature of 37°C for 10 days in the shaker incubator (MaxQ 6000, Thermoscientific, USA) with 200 rpm rotation. At the end of incubation, the cultures were centrifuged (Centrifuge 5804R, Eppendorf, India) at 9000 rpm for 20 min. The supernatant was collected and mixed with cold alcohol in 1:2 ratio. Deposition of biomass in the form of exopolysaccharide was washed with distilled water and dried at 60°C for 2-4 hours. The isolate which yielded maximum production of EPS was selected for further studies.

Effect of different culture media and carbon sources on Exopolysaccharide production

The liquid medium showing maximum yield was optimized by inoculating the bacterial culture in to three different liquid media *i.e.*, luria bertani broth, trypticase soy broth, brain heart infusion broth supplemented with 2 % glucose and incubated at a temperature of 37 °C for 10 days in shaker incubator at 200 rpm. Similarly, the effect of different carbon sources (2%) *viz.*, glucose, maltose, fructose, cellobiose and trehalose on EPS production were also studied as per Sonawdekar and Gupte (2016).

Biochemical characterization of crude EPS

Chemical analysis of EPS was performed by determining the carbohydrate (Dubois *et al.*, 1956), protein (Lowry *et al.*, 1951), uronic

(Blumenkrantz, and Asboe-Hansen, 1973) and sulphate content (Cha *et al.*, 1999).

Structural characterization of crude EPS

Structural analysis of crude EPS was carried out using FTIR (Fourier-transform infrared spectroscopy). Infrared spectra of the purified EPSs fractions were recorded in the 4000–400 cm⁻¹ region using a FT-IR system (Nicolet iS 10, Thermo Fisher Scientific, USA)

Biochemical and molecular identification of EPS producing bacteria

Biochemical identification of the bacterial isolate was carried by tests such as Gram staining, oxidase, catalase, citrate utilization and starch hydrolysis (Tallent *et al.*, 2012). The biochemically confirmed isolate was further confirmed by 16S rRNA sequencing employing universal primers 27F and 1492R (27F 5'-AGAGTTTGA TCCTGGCTCAG-3' and 1492R (5'-GGTTACCTTGTTACGAC TT-3')) as well as *Bacillus cereus* specific PCR targeting *bla* gene (Das *et al.*, 2009). Briefly, the crude DNA was extracted from the overnight culture by boiling method (Oliwa Stasiak *et al.*, 2010). For 16S rRNA sequencing, amplification was performed using PCR mixture made to a final volume of 30µl containing 1X PCR buffer, 1.5 mM MgCl₂, 2.5 µl of each forward and reverse primer, 100µM of dNTPs mixed with 1U Taq DNA polymerase, 2 µl of DNA template and rest by adding sterile Millipore water (Sanchez *et al.*, 2011). The reaction was carried out in a thermocycler (Applied biosystem, USA) with following steps-an initial denaturation at 94°C for 2min, followed by 30 cycles of denaturation at 94° C for 30 sec, annealing at 52°C for 60 sec, extension at 68° C for 90 sec and a final extension at 68° C for 7 min. PCR reaction condition for the primer *bla* consisted of 30 cycles of 94°C for 45 sec (denaturation), 55°C for 45 sec (annealing) and 72°C for 45

sec (extension). After amplification, the PCR products were analyzed by electrophoresis with 1.5% agarose gel. The partial bacterial 16S rRNA gene sequences were submitted to NCBI (National centre for biotechnology Information) BLAST search on the gene bank nucleotide database to identify the sequences with higher similarity.

Results and Discussion

Screening of bacteria from seaweed for production of exopolysaccharides

The exploitation of bacterial metabolites with bioactive potential from marine environment become a major area of research due to its biocompatible, non toxic properties which opt out the role of synthetic polymers to a large extent (Mano *et al.*, 2007). Seaweeds are rich in bioactive polymers having commercial significance and major source of hydrocolloids such as alginate, agar and carrageenan. In the present study, brown seaweeds *viz.*, *Sargassum wightii*, *Padina gymnocephalus*, and *Turbinaria connoides* were screened for EPS producing bacteria. Initial screening for EPS production by the bacterial isolates was carried out on the basis of appearance of colony on the TSA plate. A total of five distinct morphological isolates (B1, B2, C1, W1 and W2) from *Sargassum wightii* and *Padina gymnocephalus* were selected. A comparative study on the potential of these isolates for maximal production of exopolysaccharide was carried out by using liquid broth media. The colony characteristics of the selected isolates and EPS yield in EPS culture media are presented in Table 1. Out of five isolates, the isolate B1 from *Sargassum wightii* showed maximum EPS production (1.06 mg ml^{-1}) and was selected for further studies (Fig 1). The EPS production is often accompanied with aging of the culture and exhaustion of available nutrients in the media. The appearance of thick slime/mucoid

colonies on the solid media was taken to be the initial screening criteria for polysaccharide producing bacteria (Hereher *et al.*, 2018). According to Mostefaoui *et al.*, (2014) the probable easiest method for screening of EPS producing bacterial colonies is visual inspection even though it is insensitive.

The results of biochemical characterization of the isolates are depicted in Table 2. The isolate was identified as *Bacillus cereus* by 16S rRNA sequencing and the sequences was submitted in the public domain with accession number MK595701.1. Polymerase chain reaction targeting species specific *bla* gene showed amplification of 533bp (Fig 2). Li *et al.*, (2016) reported an extracellular matrix containing an unusual polysaccharide, in the dormant spores of *B. cereus* and investigated its key role in the adaptation and fitness to the environment. Sonawdekar and Gupte (2016) have isolated EPS producing *B. cereus* from oil contaminated sites from in and around Navi Mumbai and Thane districts of Maharashtra. Similarly, EPS producing *Bacillus cereus* GU 812900 was isolated from the stainless steel test panel and it contained 54% sugar and 1.85% protein (Bragadeeswaran *et al.*, 2011). Singh and others (2011) identified the major bacteria associated with seaweeds *Ulva* and *Gracilaria* a *Marinomonas* spp. and *Bacillus* spp.

Production of exopolysaccharides by *Bacillus cereus*

Effect of media

The bacterial production of EPS depends highly on composition of substrate and environmental conditions (Rabha *et al.*, 2012). Among different media tested for EPS production, the dry weight of EPS was recorded maximum in BHI (1.65 mg ml^{-1}) followed by LB broth (1.12 mg ml^{-1}) and TSB (0.62 mg ml^{-1}), which indicate BHI medium

provides suitable nutrients for the production of EPS. BHI is more nutritious than LB and TSB in terms of nitrogenous source and hence the bacteria get sufficient time to produce the polysaccharide to protect the deleterious effects of slow exhaustion of nutrients from the media. Pal and Paul (2013) reported that EPS production in *Cupriavidus pauculus* KPS 201 is positively influenced with the increase of nitrogen and phosphate in the growth medium.

Effect of carbon source

The effect of carbon source on EPS production by *B. cereus* was studied with both monosaccharides (glucose and fructose) and disaccharides (maltose, cellobiose and trehalose). Among the different sugars supplemented in media, glucose (1.56 mg ml^{-1}) was most efficient for EPS production (Fig 3). It has been reported that the EPS production is directly correlated with the amount of carbohydrate present in the medium and its optimum concentration varies depending upon the individual microorganism (Ergene and Avcı, 2017). Glucose was the most efficient carbon source for *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophiles* (Yuksekdag and Aslim, 2008). Grobber *et al.*, (1997) reported that three times more production of EPS with glucose than fructose as sugar source and the type of EPS also varies with carbon source. These results are in contrary to Hereher *et al.*, (2018) who reported that due to the relative ease of polymerization, disaccharides help in EPS production than monosaccharides. Further, Lee *et al.* (1997) observed that sucrose was efficient for the production of EPS from *Bacillus polymyxa*. The polysaccharide, starch was more effective in enhancing the EPS production by *Pseudomonas stutzeri* AS22 than glucose (Maalej *et al.*, 2014).

Chemical and structural analysis of EPS

Fazio *et al.*, (1982) have shown that EPS from marine bacterium is rich in galacturonic acids. During chemical analysis of the EPS from *B. cereus*, it was observed that EPS contained 69.9% carbohydrate, 8.1% protein, 3.2% total uronic content and 1.5% sulphate content.

This is in accordance with observation of Singh *et al.* (2011) who reported that the quantity of carbohydrates, protein and sulfate was 343.14, 107.68 and 50.28 mg l^{-1} , respectively in EPS produced by *Bacillus licheniformis*.

Generally, the bacterial EPSs have higher carbohydrate content than sulphate and protein (Zhenming and Yan, 2005).

Structural analysis of EPS was carried out by FT-IR analysis (Fig. 4) which showed a characteristic N-H and OH stretch at around 3292.93 cm^{-1} and a C-H stretching vibration at around 2925 cm^{-1} (Deepika *et al.*, 2016).

The absorption peaks within $1650\text{-}1540 \text{ cm}^{-1}$ attributed to vibrations of a CO, NH and CN bending of protein and peptides.

The absorption peaks within $1200\text{-}1000 \text{ cm}^{-1}$ attributed to vibrations of a broad stretch of C O and C O C glycosidic bands, which revealed the presence of carbohydrates (Zhang *et al.*, 2013) that would be sugar monomers in the EPS.

The absorption peak at 600 cm^{-1} and 492 cm^{-1} could be attributed to the S-S stretch. The absorption observed at $1500\text{-}1600 \text{ cm}^{-1}$ could be attributed to the stretching vibration of C=C and C-N groups. Peaks at 884 cm^{-1} ascertain the presence of glycosidic linkage bonds. The composition and components of exopolymeric substance of bacteria have large implications in their bioactive properties.

Fig.1A) colony morphology of exopolysaccharide producing bacterial isolates on Trypticase soya agar and **(B)** crude extract of exopolysaccharide produced by *Bacillus cereus* in Luria bertani broth

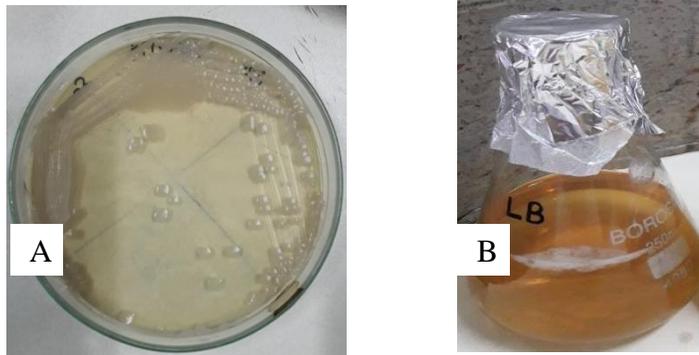
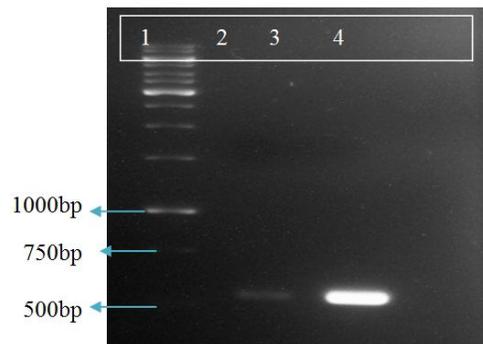


Fig.2 *B. cereus* group specific PCR.



Lane 1: 1kb ladder, Lane 2: *B. cereus* isolates recovered from *S. wightii*. Lane 3: Positive control, Lane 4: Negative control

Fig.3 Effect of different sugars on EPS production by *Bacillus cereus*

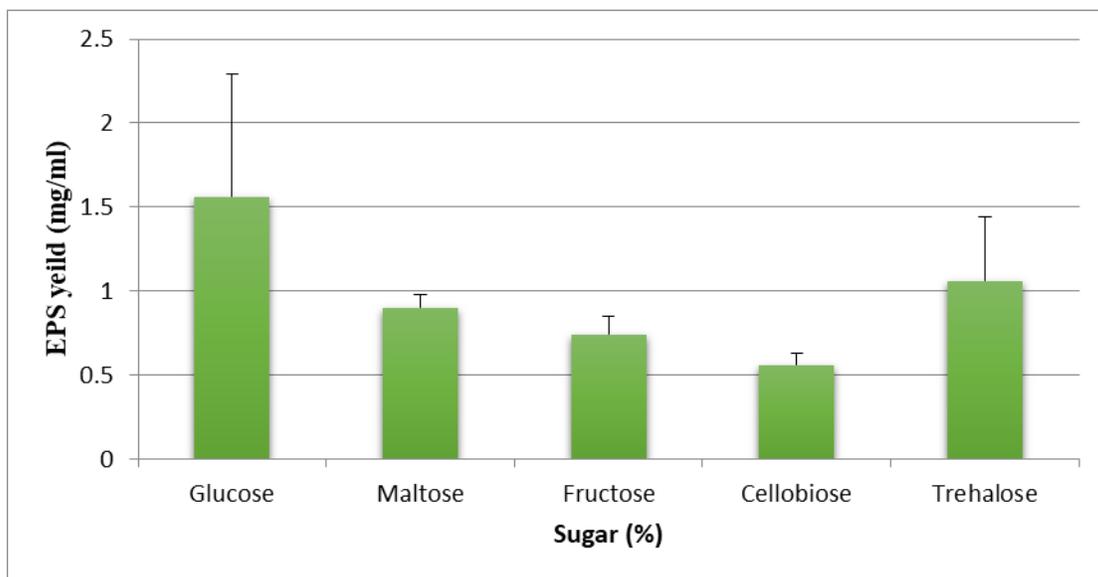
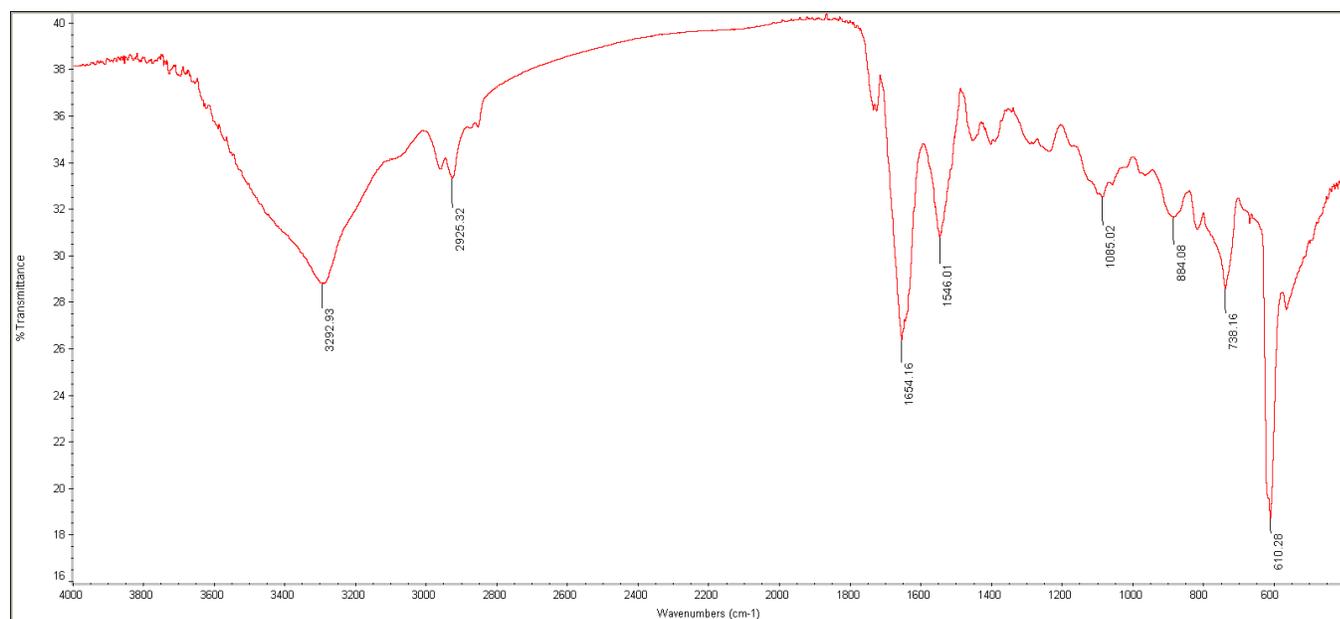


Table.1 Morphological and biochemical characteristics of the isolates and the EPS yield

Isolates	BI	B2	C1	WI	W2
Sample source	<i>Sargassum wightii</i>	<i>Sargassum wightii</i>	<i>Sargassum wightii</i>	<i>Padina gymnocephalus</i>	<i>Padina gymnocephalus</i>
Colony morphology	Light brown, mucoid, large 2-3mm size, smooth	Light brown, mucoid, large 2-3mm size, smooth	Creamy, mucoid, small size, smooth	Whitish, Mucoid large 2-3mm size, irregular edge	Whitish, Mucoid large 2-3mm size, irregular edge
Gram reaction	Gram positive, spore forming, rod	Gram positive spore forming, rod	Gram negative non spore forming, cocci	Gram negative, non spore forming, rod shaped	Gram negative
Oxidase reaction	Positive	Positive	Negative,	Positive	Positive
Motility	Motile	Motile	Non motile	Non motile	Non motile
Indole	Negative	Negative	Negative	Positive	Positive
Catalase	Positive	Positive	Positive	Positive	Positive
Glucose fermentation	Positive	Positive	Positive	Positive	Positive
Lactose fermentation	Negative	Negative	Positive	Negative	Negative
Starch hydrolysis	Positive	Positive	Negative	Negative	Negative
Protease activity	Positive	Positive	Negative	Negative	Negative
Citrate utilization	Positive	Positive	Negative	Positive	Positive
EPS yield (mg ml ⁻¹)	0.82	1.06	Nil	0.3	Nil

Fig.4 FT-IR Spectrum of crude EPS from *Bacillus cereus*



The present study gives an insight to the production of EPS by bacteria associated with *Sargassum wightii*, *Turbinaria connoides*, *Padina gymnocephalus*. *Bacillus cereus* was the potential EPS producing bacterium isolated and the EPS production was enhanced in the presence of glucose. The extracted exopolysaccharide from *Bacillus cereus* may be applied in many fields such as textiles, pharmaceuticals and food industry after carrying out further studies pertaining to its unique properties.

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