European Journal of Biological Sciences 15 (1): 08-13, 2023
ISSN 2079-2085

DOI: 10.5829/idosi.ejbs.2023.08.13

Antibacterial Activity of Exopolysaccharide Produced from *Halobacillus* sp.

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Abstract: The aim of this research is to extract exopolysaccharide (EPS) from halophilic bacteria. An exopolysaccharide producing bacteria was isolated from salt-pan sample collected from Thoothukudi. The sample was spread on zobell marine agar and mucous colonies were obtained which is indication of exopolysaccharide production. The mucous strains SP1, SP2, SP3, SP4 and SP7 were cultured on zobell marine broth and exopolysaccharide was extracted by centrifugation. The production was more in the strain SP1, the dry weight of exopolysaccharide from this strain was 1.0mg/100ml. The exopolysaccharide was estimated using phenol sulphuric acid method with glucose as a standard. The OD value at 490nm was found to be 2.11nm.The functional groups in EPS were evaluated using FT-IR. The presence of carbonyl compounds, aromatic nitrocompounds and hydroxyl group was found. *Invitro* total antioxidant activity analysed with ascorbic acid as standard for EPS crude extract. Antibacterial activity of exopolysaccharide was performed. Halophilic exopolysaccharide displayed an inhibitory zone of 15mm against *Klebsiella pneumonia*, 12mm against *Escherichia coli* and 3.0mm against *Staphylococcus aureus.* The results of antimicrobial activity have revealed that the exopolysaccharide has shown maximum inhibition against *Klebsiella pneumonia*. This work has proved that the halophilic exopolysaccharide has the capability to inhibit the pathogens and serve as an antioxidant.

Key words: Exopolysaccharide · Halophilic · Antioxidant and Antibacterial

INTRODUCTION

Exopolysaccharide is known as an extracellular compound. It is a macromolecule which is a loosely bound slime layer or a capsule that is bound strongly in microorganisms. They are complex carbohydrates. Many microbes are known to produce exopolysaccharide. Exopolysaccharide are called as by-products of microbial metabolism [1]. The exopolysaccharide are eco-friendly and biocompatible in nature.

Exopolysaccharide secreted by bacteria has many biological roles. They protect the bacteria from extreme environmental condition, surface adherence and cellular interactions[2].Bacterial exopolysaccharide are less hazardous to environment and they are biodegradable than synthetic polymers. Many bacteria isolated from extreme environments like hypothermal vents of deep sea, high salt environment and Antarctic ecosystems are the best sources of valuable exopolysaccharide.

Halophilic environment refers to the environment with high salt concentrations, it is tedious to survive in that salty ecosystem. To tolerate that environmental condition the halophilic bacteria produce exopolysaccharide to adapt that environment. In saline environment these exopolysaccharide are produced by the bacteria to maintain the osmotic environment in their cells and to make them survive in that condition [3].

In each microorganism the composition, structure and yield of exopolysaccharide may vary with conditions of fermentation. Production of exopolysaccharide required any carbon sources, nitrogen, hydrogen ion concentration, temperature and minerals [4].

Exopolysaccharide have used in industries for their significant value. They have been used in pharmaceutical, paper, food and in textile industry. Halophiles are known to produce exopolysaccharide with antibacterial, antiviral and antioxidant properties [5]. The objective of the present research is to produce

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exopolysaccharide from halophilic bacteria and to check for antibacterial and antioxidant activity.

MATERIALS AND METHODS

Isolation of EPS Producing Bacteria: For the isolation of exopolysaacharide producing halophilic bacteria, the salt span samples were collected from Thoothukudi district, Tamil Nadu. 1g gram of the sample was taken and dissolved in 100ml of sterile water. Serial dilution of the sample was made and it was spread on zobell marine agar. The plates were incubated at 37°C for 48 hours. After 48 hours it was observed for growth with mucous colonies [6].

Identification of EPS Producing Bacteria: Gram staining &Biochemical tests like as indole production, methyl red, Voges Proskauer, citrate utilization, assay for catalase, oxidase, urease, gelatin hydrolysis, caesin hydrolysis, starch hydrolysis, lipid hydrolysis and carbohydrate utilization were carried out [7].

Production of Exopolysaccharide: The mucous colonies were isolated and inoculated on Zobel marine broth and incubated in a rotator shaker at 37°C for 3 days. After three days the broth with organism was centrifuged at 6000 rpm for 10 minutes. The supernatant was collected equal amount of ethanol was added to the supernatant and after overnight incubation it was centrifuged at 6000rpm for 10 minutes. Pellet was collectedand it was dried and dry weight was checked [8].

Estimation of Carbohydrates: Phenol sulphuric acid method was performed to estimate the carbohydrates. 5% phenol, stock standard glucose solution, working standard glucose solution and working standard for the sample were prepared. Seven dry test tubes were taken, dilutions were prepared using glucose standards at concentrations 40, 80,120,160,200µl and the final volume was made upto 200µl by adding distilled water. 0.2ml of 5% phenol was added in each tube then 1ml of sulphuric acid was added and mixed well. The tubes were kept in water bath at 30°C for 20 minutes [9]. OD value was read using spectrophotometer at wavelength 490nm.

Fourier Transform Infrared Spectroscopy Analysis of EPS: The bacterial exopolysaccharide was examined using a Fourier transform infrared spectrophotometer. A Perkin-Elmer FT-IR equipment was used to examine the IR spectroscopies of bacterial EPS and dextran sulphate, aiding in the identification of the different sulphate, carboxyl and hydroxyl groups of these sample molecules [10]. Separately, a 3mm diameter salt disc was created by mixing one part extract with 90 parts dried potassium bromide (KBr). These discs were examined using an IR spectrophotometer. The absorbance was calculated between 400 and 4000 cm^{-1}.

***In vitro* Total Antioxidant Activity of Exopolysaccharide:** The tubes with various concentration of EPS 100µl, 250µl, 500µl, 750µl and 1000µl and reagent solution (0.6Msulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate)were incubated at 95°Cfor90min.After the mixture was brought to room temperature, the absorbance of each solution was measured at 695 nm against a blank. Ascorbic acid was used as control for this process. Then the total antioxidant activity was calculated using the formula [11].

Total antioxidant activity = Abs control - Abs Sample/Abs control × 100

Antibacterial Activity of Exopolysaccharide: The antibacterial activity of crude exopolysaccharides from Halophilic bacteria was assessed against five distinct bacterial test species *Escherichia coli, Staphylococcus aureus, Klebsiela pneumonia* using the paper disc assay technique [8]. A 6 mm diameter Whatman No. 1 filter paper disc was sterilized by autoclaving it for 20 minutes at 121°C. Different extracts were infused into the sterile discs at concentrations of 0.5, 1, 1.5 and 2 mg/ml. The 24-hour-old cultures were aseptically swabbed on Muller Hinton agar (microbiological grade) plates using a sterile cotton swab. On the discs with the appropriate labels, the standards of tetracycline (1 mg/ml dissolved in distilled water) were added. At 35°C, the plates were incubated for 24 hours. The diameter of the inhibitory zone for each well was measured and represented in millimeters to obtain the results.

RESULTS

Isolation & Identification of EPS Producing Bacteria: After 72 hours of incubation, mucous colonies were observed which an indication of exopolysaccharide producing bacteria. The colonies were white, slimy and ropy. The selected mucous colonies were named as SP1 to SP7. Those strains were maintained for EPS production.

Fig. 1: Ethanol precipitation of the substrate

Table 1: Morphological and biochemical characteristics of *Bacillus* sp

S. No	Biochemical tests	Results
1.	Catalase test	Positive
2.	Oxidase	Negative
2.	Indole test	Negative
3.	Methyl Red test	Negative
4.	Voges-Proskauer test	Positive
5.	Citrate Utilization test	Positive
6.	Gelatin	Positive
7.	Caesin	Positive
8.	Starch	Positive

Table 1a: Estimation of carbohydrate by phenol sulphuric acid method

Sample	Concentration	OD value at 490nm
Glucose	0.2	1.33
Glucose	0.4	1.46
Glucose	0.6	2.09
Glucose	0.8	2.35
Glucose	1	2.48
Exopolysaccharide	0.6	2.11

Table 2: Total antioxidant activity

Concentration	Ascorbic acid	Exopolysaccharide
100µl	52.55	46.23
250µl	61.45	57.18
500µl	69.87	65.75
750µl	83.14	78.21
1000µl	94.23	87.24

Identification of EPS Producing Bacteria: The selected strain was identified by various morphological and biochemical characteristics. Strain SP1 shows maximum EPS production in zobell marine broth than other strains.

Morphological and Biochemical Characteristics: This strain SP1 was identified as Gram positive, rod shaped bacilli. According to Bergey's manual of Determinative Bacteriology, the selected strain was identified as *Halobacillus* sp. Biochemical studies were performed and results were presented in Table. 1.

Table 4: Antibacterial activity of crude exopolysaccharide against pathogens

			Halophilic crude exopolysaccharide (mm)		
S. No	Test organisms	Standard	10µl	15µl	20µl
1.	*E. Coli*	22	8.2	10	12
2.	*Staphylococcus aureus*	28	1.5	2.4	3.0
3.	*Klebsiella pneumonia*	27	13.4	14.13	15

Production of Exopolysaccharide: The broth culture was centrifuged and supernatant was collected. After the collection of supernatant the equal volume of ethanol was added and incubated overnight at 4°C and after incubation the precipitation was observed. The precipitation of crude extract is centrifuged and pellet is collected and dried. The dry weight of the exopolysaccharide was found to be 1.0mg/100ml in SP1. The production of EPS was more in this strain hence this strain was used for further studies (Fig. 1).

Estimation of Carbohydrates: The carbohydrate in exopolysaccharide was estimated using phenol sulphuric acid method with glucose as standard. At 0.6mg of expolysaccharide concentration in 490nm the OD value of the EPS sample was found to be 2.11nm (Table - 1a).

Fourier Transform Infrared Spectroscopy Analysis of EPS: In the present study the bacterial exopolysaccharide was observed to have peak 650cm^{-1} represents the presence of represents C-Br stretch (alkyl halides), 754.17 cm^{-1} represents the presence of C-H (aromatics), 1010.70 cm^{-1} and 1141.86 cm^{-1} denotes the presence of C-N stretch (aliphatic amines), 1543.05 cm^{-1} indicates the presence of N-O asymmetric stretch (nitro compounds), 2958.80 cm^{-1} represents the presence of C-H stretch (alkanes) and 3444.87cm-1 represents the presence of hydroxyl group.

***Invitro*total Antioxidant Activity of Exopolysaccharide:** The phospho-molybdenum method was carried out to check the total antioxidant activity of sulfated polysaccharides (Fig. 3). The maximum antioxidant activity was observed in 1000 µg/ml of exopolysaccharide and it was 87.24± 0.19% as compared with the standard ascorbic acid calibration curve (Table 2).

Antimicrobial Activity of Exopolysaccharide: Halophilic exopolysaccharide displayed an inhibitory zone of 15mm against *Klebsiella pneumonia*, 12mm against *Escherichia coli*, 3.0mm against *Staphylococcus aureus*. The result of antimicrobial activity has revealed that the exopolysaccharide has shown maximum inhibition against *Klebsiella pneumonia.*

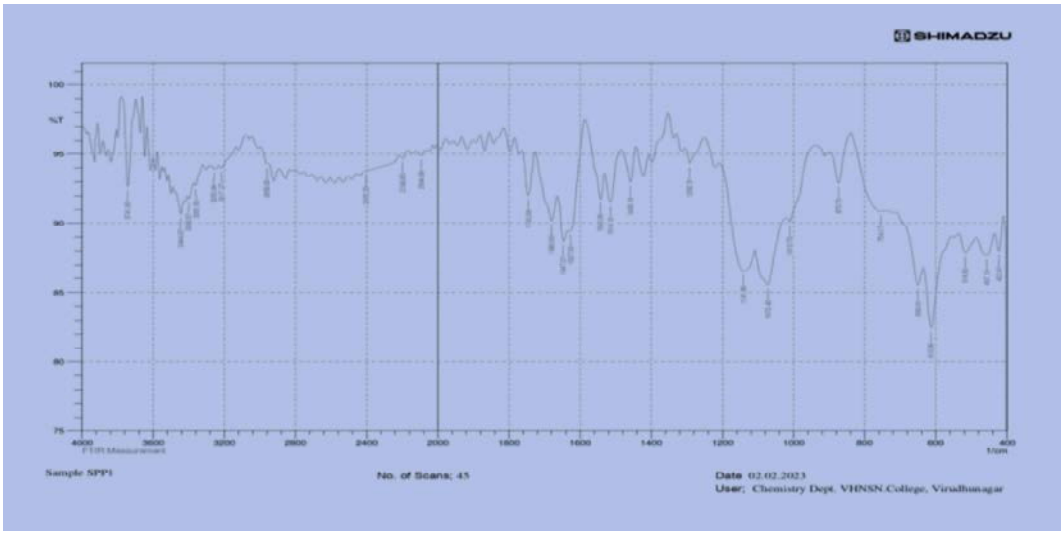

Fig. 2: Fourier Transform Infrared Spectroscopy

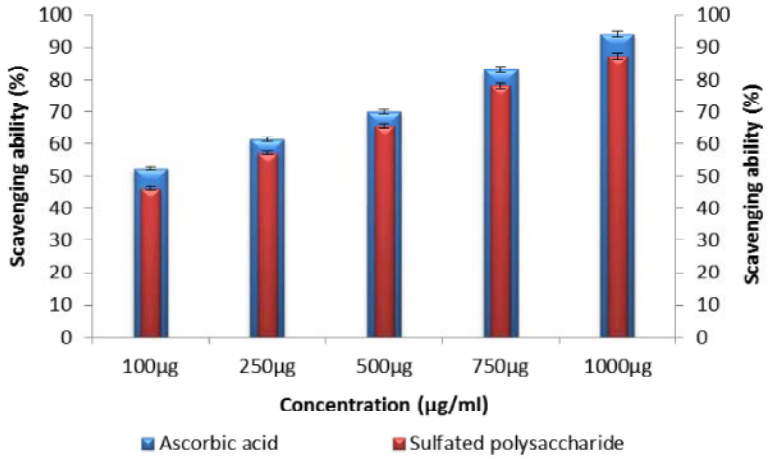

Fig. 3: Total antioxidant activity

DISCUSSION

A heterogeneous matrix of polymers made up of proteins, nucleic acids, phospholipids and polysaccharides are called exopolysaccharides. Exopolysaccharide are often found in bacteria and cyanobacteria but they have also been found in marine microalgae including *Chroomonas sp*., *Dunaliella salina* and the mushroom *Phellinus linteus*[12].

Different natural sources of both aquatic and terrestrial environments, including freshwater, marine water, wastewater, soils, biofilms, as well as extreme niches like hot springs, cold environments, hypersaline and halophilic environments, salt lakes and salterns, have yielded EPS-producing microorganisms [13].

To sustain the high saline conditions, halophilic microorganisms use biochemical strategies, this include solute synthesis inorder to maintain the structure of the cell and its function. These solutes include compounds like bacteriorhodopsins, exopolysaccharides, hydrolases, biosurfactants [14]. A number of environmental variables, including as light and nutritional stress, influence the synthesis and release of EPS. Marine microbes are prolific exopolysaccharide producers and various marine microorganisms have been isolated from the water for EPS synthesis. A Mediterranean Sea Archaea *Haloferax mediterranei* that produces EPS has been isolated.

In the isolation of exopolysaccharide producing bacteria we have observed the mucous colonies the same result was reported by Amal *et al*. [14] by visual

appearance the mucoid colonies were determined as EPS producing bacteria. In the present study the production of EPS was carried out by centrifugation and ethanol precipitation. Similarly, Mei *et al*. [15]. The broth for fermentation was centrifuged at 10,000 rpm for 25 minutes and 1:2 volume ethanol was added.

In the current study the FT-IR analysis showed the presence of 650cm^{-1} represents the presence of represents C-Br stretch (alkyl halides), 754.17 cm^{-1} represents the presence of C-H (aromatics), 1010.70 cm^{-1} and 1141.86 cm^{-1} denotes the presence of C-N stretch (aliphatic amines), 1543.05 cm^{-1} indicates the presence of N-O asymmetric stretch (nitro compounds), 2958.80 cm^{-1} represents the presence of C-H stretch (alkanes) and 3444.87cm^{-1} represents the presence of hydroxyl group. The FT-IR analysis of exopolysaccharide produced from *P. rutenica* shown the absorption at 3543.89 and 2928.52 cm^{-1} are representing the presence of hydroxyl groups, the peak at 1628.7 show the presence of carboxylate group and 1119.22 cm^{-1} represents CH_3 bending *Saravanan et al*. [16]. In this present work *Invitro*total antioxidant acitivity of halophilic exopolysaccharide was checked, it was observed that total antioxidant activityof EPS was found to be 46.23 to 87.24%. Likewise, Jeganathan *et al*. [17] observed that the hydroxyl radical scavenging activity of exopolysaccharide from *H.miurensis* was 61% and DPPH scavenging activity was 84%. Similarly, the DPPH scavenging activity of exopolysaccharide of marine bacterium *Microbacterium aurantiacum* was 80% and hydroxyl radical scavenging activity was 90% it was reported by Kulwinder *et al*. [10].

In this current research antimicrobial activity of crude exopolysaccharide was observed the diameter of inhibitory zone range between 1.0 to 15mm. The maximum inhibition was against *Klebsiella pneumonia*. Bachir *et al*. [8], reported that the antimicrobial activity of exopolysaccharides from yoghurt culture was found to be the diameter of inhibition zones ranged between 9 and 13 mm. The large zone of inhibition was observed against *E. coli*, while the lowest was recorded against the *S. aureus*.

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